MATING-TYPE GENES AND SEXUAL POTENTIAL IN THE ASCOMYCETE GENERA *ASPERGILLUS* AND *PENICILLIUM*

Carly E Eagle, BSc

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Abstract

Mating-type and other 'sex-related' genes in the filamentous ascomcyete genera *Aspergillus* and *Penicillium*, were examined to investigate the potential sexual capacity of supposedly asexual species and also the possible evolutionary route and ancestry of mating strategy and mating-type genes.

Two heterothallic and one homothallic sexual species were screened to determine the presence and genomic organisation of mating-type genes. An additional gene has previously been detected in *Neosartorya fischeri, N. fumigata* and *Penicillium marneffei*. This gene was also detected and sequenced in the heterothallic species, *Emericella heterothallica* and the homothallic species, *Eurotium repens*. The expression of this gene was investigated under conditions that cause expression of mating-type genes in these species.

Mating-type and other 'sex-related' genes were investigated in asexual Aspergilli that have been genome sequenced. Expression of mating-type, α-factor pheromone precursor, pheromone receptor and two transcription factor encoding genes were also investigated. Gene expression varied between species, but no genes displayed mating type-dependent expression.

Previous studies had developed a degenerate PCR diagnostic approach to identify putative *MAT1-1-1* and *MAT1-2-1* gene fragments. This degenerate PCR diagnostic was performed on *Penicillium* species in the subgenus *Penicillium* to determine the presence or absence of mating-type genes. Mating-type gene fragments or whole open reading frames were sequenced from four of these *Penicillium* species. RT-PCR analyses were also performed on these species, and *MAT1-1-1* and *MAT1-2-1* gene expression was confirmed in three of the four *Penicillium* species.

The overall structure of the mating-type loci and idiomorphs of the *Aspergillus* and *Penicillium* species revealed certain common features. The ancestral mating strategy of the Eurotiomycetes has been suggested to be homothallism. Whilst this remains possible, alternative evolutionary scenarios are suggested from this investigation.

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Contents

Abbreviations

ACM	*Aspergillus* complete media
ADP	adenose diphosphate
ATP	adenose triphosphate
BLAST	basic local alignment search tool
bp	base pair
cAMP	cyclic adenose monophosphate
cm	centimetre
°C	degrees Celcius
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetra-acetic acid
g	gram or gravitations
ITS	internal transcribed spacer
Kb	kilobase
L	litre
LBA	Luria Bertani agar
LBB	Luria Bertani broth
MEA	malt extract agar
MEM	malt extract media
mg	milligram
min	minute

mM	millimolar
mtDNA	mitochondrial DNA
ng	nanogram
OA	oatmeal agar
ORF	open reading frame
PCR	polymerase chain reaction
RAPD	ramdon amplified polymorphic DNA
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphisms
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
sec	second
TAIL-PCR	thermal asymmetric interlaced polymerase chain reaction
TBE	tris borate EDTA buffer
µg	microgram
µl	microlitre
mM	micromolar
w/v	weight per volume

Chapter 1 Introduction to Asexual and Sexual Reproduction in Fungi

1.1 General Background

The study of fungal sexuality commenced over a century ago (Blakeslee 1904). The vast majority of fungal species are able to reproduce by sexual and asexual means. However, currently, around one fifth of fungi are thought to reproduce via asexual means alone and have traditionally been classified in the phylum Deuteromycota (Hawksworth *et al.* 1995).

Deuteromycota is an artificial group containing species that may be more closely related to sexual species than asexual species; it is therefore largely a redundant classification (Chang *et al.* 1991; Taylor 1995; Turgeon 1998). The majority of species formerly classified as Deuteromycota show taxonomic affinity to the phylum Ascomycota, which is now used for both sexual and asexual species.

The number of truly asexual species is decreasing as sexual cycles are discovered or induced in species where the mating-type (*MAT*) genes have been found (Horn *et al.* 2009a; Horn *et al.* 2009b; Houbraken *et al.* 2008; Kerényi *et al.* 2004; O'Gorman *et al.* 2009; Ware *et al.* 2007). These genes are essential during the sexual cycle in fungi and yeasts (Coppin *et al.* 1997; Keszthelyi *et al.* 2007; Nelson 1996; Turgeon 1998). By convention, sexually reproducing species have two Latin names. Firstly, the anamorphic (asexual state) name, this normally derives from the morphology of the asexual spores e.g. *Aspergillus*. Secondly, the teleomorphic (sexual state) name, which is chosen on the basis of morphology of the sexual fruiting body e.g. *Emericella* species (teleomorph of certain *Aspergillus* anamorphs) characteristically produce Hülle Cells, which may surround developing cleistothecia (fruiting bodies), whereas *Petromyces* species (another teleomorphic genus with an *Aspergillus* anamorph) develop cleistothecia within compact hardened masses of hyphae (Malloch and Cain 1972b; Malloch and Cain 1972a; Raper and Fennell 1965). In this thesis, when both an anamorphic and teleomorphic name exists, the teleomorphic name will be used in accordance with the Botanic code.

Most sexually reproducing species retain the ability to reproduce asexually. The switch from asexual to sexual reproduction is dependent on various, often species-specific, environmental cues. In the fission yeast *Schizosaccharomyces pombe*, nutrient (typically nitrogen) starvation has been shown to be a major cue (Leslie and Klein 1996; Pöggeler 2001), whereas in the filamentous ascomycete *Emericella nidulans* (anamorph: *Aspergillus nidulans*), carbon and oxygen limitation appear to be the dominant cues (Champe *et al.* 1994; Dyer 2007).

The focus of this thesis will be on sexual reproduction and asexuality in filamentous ascomycete fungi, therefore these topics will be described in more detail in the following sections.

1.2 Asexual Reproduction in Fungi

Asexual reproduction involves the production of asexual spores (conidia) via mitotic duplication. As a result, progeny are genetically identical to their parents, except for occasional mutations, and are therefore known as clones (Milgroom 1996). Clones are identified as "repeatedly sampled, multilocus genotypes that are unlikely to arise by chance in sexual recombination" (Anderson and Kohn 1998). Asexual reproduction demands less metabolic energy compared to sexual reproduction, because specialised mating and fruiting structures do not need to be produced. However, asexual organisms can accumulate deleterious mutations in their genome and, as explained by the concept of 'Muller's Ratchet', these can be difficult to remove once they become established in populations (Muller 1964). This may be why many asexual lineages seem to be short-lived compared to sexual lineages (Berbee and Taylor 1993). One notable exception is the 360 species belonging to the Rotifer phylum, within the class Bdelloidea (bdelloid rotifers). This class has existed for 35-40 million years, with no known sexual cycle (Welch and Meselson 2000). There has been recent evidence for widespread horizontal gene transfer within bdelloid rotifers, which might explain the evolutionary persistence of this particular group of asexual organisms (Gladyshev *et al.* 2008).

Apart from energy conservation, there may also be other benefits to purely asexual reproduction. Beneficial gene combinations are not broken up via recombination as might be the case in sexual species (Milgroom 1996). If a species is well adapted to an ecological niche that is unchanging, sexual reproduction, which produces new gene combinations may be unnecessary and possibly detrimental. Therefore, well adapted

asexual species may propagate rapidly, efficiently and at low energy cost compared to sexual reproduction (Houbraken *et al.* 2008; Nelson *et al.* 1997a; Wu and Miller 1997). This may be the reason why many species retain their asexual cycle while having their sexual cycle as a 'back up' to increase variation when necessary. O'Donnell *et al*. (2004) genotyped over 100 isolates of *Fusarium oxysporum* collected over four continents from hospitals and the general environment. Over 70% of these isolates were found to have arisen from a single clonal lineage and were therefore very closely related. Some isolates examined had either diverged from the main lineage or had arisen from a different lineage(s) (O'Donnell *et al.* 2004), suggesting that mechanisms to generate variation have been retained in this asexual species.

1.3 Sexual Reproduction in Ascomycete Fungi

1.3.1 Introduction to Ascomycota

Ninety eight percent of fungal species are contained within the Dikarya, this includes both the Ascomycota and Basidiomycota phyla, which are believed to have diverged from each other 400 ± 125 million years ago (Berbee and Taylor 1993; James *et al.* 2006a).

The sexually reproducing Ascomycota contains approximately 30,000 species and is the largest group containing 58 orders and 17 classes of fungi (Hawksworth *et al.* 1995; Hibbett *et al.* 2007). It is divided into three subphyla, these are; Taphrinomycotina (including *Schizosaccharomyces* and *Pneumocystis* species), Sacchromycotina (including *Saccharomyces*, *Candida*, *Kluyveromyces* and *Yarrowia* species) and Pezizomycotina (including *Aspergillus*, *Cochliobolus*, *Histoplasma*, *Penicillium*, *Fusarium*, *Sordaria*, *Neurospora*, *Podospora* and *Magnaporthe* species). Each subphylum is monophyletic, and the Pezizomycotina is the largest subphylum (James *et al.* 2006a) and the main focus of this thesis.

There are two main types of sexual breeding system in fungi, heterothallism and homothallism. These will be described in sections 1.3.2.1 and 1.3.2.2, respectively.

Regardless of the sexual breeding system employed by a species, fundamental aspects of the sexual cycle are relatively universal in the Pezizomycotina.

For a diagrammatic representation of the following events see Figure 1.1. Female reproductive structures, ascogonia, are the first visible structures in the sexual cycle. The ascogonia (2) are coiled hyphae and in many species have a receptive hyphal tip, called the trichogyne (3). Prior to fertilisation, some species may envelope the ascogonia with surrounding hyphae, this is known as a protoperithecium (3), in other species the ascogonia remains unenclosed. The male/donor element, the antheridium (2), which is, a microconidium, a macroconidium or a hypha. In outcrossing species, the antheridium is from the individual of the opposite mating type, in selfing species this is from the same individual (Gwynne-Vaughan and Williamson 1932). Fertilisation is said to occur when the antheridium enters the ascogonium (3) however, karyogamy (fusion of nuclei) is postponed in most ascomycetes (7) (Nelson 1996). In some filamentous ascomycete, and most yeast, species karyogamy occurs immediately after fertilisation of the ascogonium (Nelson 1996; Shiu and Glass 2000). Fertilisation initiates the development of the fruiting body (4), which will contain the sexual spores. Traditionally, fruiting body morphology has been used to classify the sexual filamentous ascomycetes. The fruiting bodies and groups are; perithecia – pyrenomycetes e.g. *Neurospora crassa*; apothecia – discomycetes e.g. *Sclerotinia sclerotiorum*; cleistothecia – plectomycetes e.g. *Eurotium repens* (anamorph: *Aspergillus reptans* or *Aspergillus repens*); and pseudothecia – loculoascomycetes e.g. *Cochliobolus heterostrophus* (Clarkson *et al.* 2003; Nelson 1996). Molecular data has provided support for some of these groupings, but shown some, for example the discomycetes and plectomycetes, to be artificial taxa (James *et al.* 2006a). Regardless of fruiting body morphology, the following events seem relatively universal throughout the filamentous ascomycetes. After fertilisation, but before karyogamy, each nucleus multiplies, producing numerous multinucleate cells. In the case of heterothallic species, nuclei of opposite mating type, recognise each other and migrate to specialised cells, known as the ascogeneous hyphae (4). This is achieved via internuclear recognition pathways, which may involve the *MAT* genes (Shiu and Glass 2000). These then give rise to crosiers (5), in which the two nuclei undergo coordinated mitosis, septae then form, producing uninucleate cells and a binucleate cell, which differentiates into the ascus mother cell (6). It is in this cell that karyogamy can take place (7). The diploid nucleus undergoes recombination (8), then meiosis (9), followed by one (or possibly several, depending on the species) post meiotic mitosis (10). These haploid cells then form ascospores (sexual spores) (11) (Bistis 1998; Coppin *et al.* 1997; Nelson 1996).

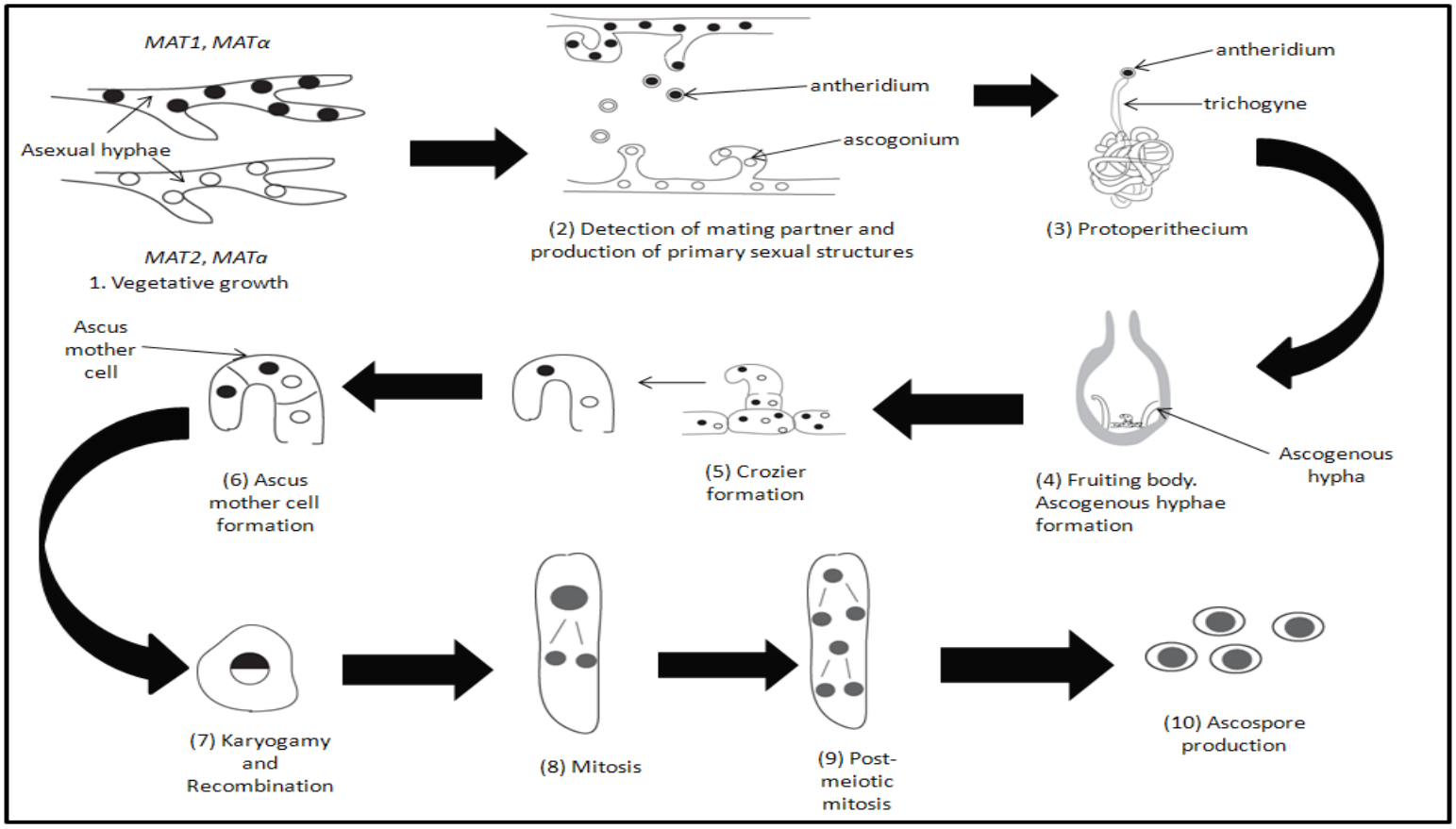


Figure 1.1:Sexual cycle of a 'typical' Filamentous Ascomycete (Pezizomyctoina) species. [Adapted from Coppin *et al*. (1997), Metzenberg and Glass (1990), Nelson (1996) and Raju (1992).]

1.3.2 Sexual Reproductive Strategies in Ascomycete Fungi

1.3.2.1 Heterothallism

By definition, heterothallic species are unable to undergo sexual self-fertilisation and instead are obligated to outcross with individuals of the opposite mating type. By convention, the two complementary mating types found in filamentous ascomycete fungi are termed MAT1-1 and MAT1-2 (Turgeon and Yoder 2000) unless there are previous long standing terminologies e.g. MATa and MATA (*Neurospora crassa*), MAT+ and MAT- (*Podospora anserina*). Outcrossing always results in recombination, due to the involvement of two, distinct nuclei and genomes (Glass and Kuldau 1992).

Sexual reproduction by heterothallic species has many benefits over purely asexual reproduction, as follows. First, ascospores have been shown to be more resistant to temperature extremes (Baggerman and Samson 1988; Dijksterhuis and Teunissen 2004; Eicher and Ludwig 2002; King and Halbrook 1987; Obeta and Ugwuanyi 1997), they may also be used as more effective agents to disseminate and possibly spread disease (Barve *et al.* 2003; Clarkson *et al.* 2003; Cozijnsen and Howlett 2003; Nelson 1996) and could have greater longevity when compared to asexual spores (Anderson and Kohn 1998; Metzenberg and Glass 1990).

Secondly, favourable mutations can be brought together efficiently to create favourable gene combinations via recombination, in contrast to asexuality in which mutations have to arise in one individual sequentially and spread via this individual. Recombination during the parasexual cycle is rare, therefore recombination is most often associated with sexual reproduction (Barton and Charlesworth 1998; Felsenstein 1974). Consequently, a recombining population can evolve faster, via accelerated natural selection, than a clonal population in a changing environment (Felsenstein 1974; Goddard 2007; Goddard *et al.* 2005; Hurst and Peck 1996; Zeyl and Bell 1997). Faster evolution may lead to faster acquisition of drug resistance as well as other characteristics that can promote infection. This has been shown in *Gibberella zeae* (anamorph: *Fusarium graminearum*), where an increased infectivity and adaptability to change was shown in sexual populations compared to asexual populations (Jenczmionka *et al.* 2003).

Thirdly, if an unfavourable mutation becomes widespread in a population within one generation, a sexual population has the opportunity to remove this via recombination. In contrast, an asexual population is unable to remove this mutation and 1 mutation per individual becomes the 'basal mutation load' of the population. If another mutation arises then the 'basal mutation load' becomes two and so forth. This is the essence of the concept of Muller's Ratchet. Once a critical number of mutations is reached the population may become unfit and extinct, which may be why most asexual species seem to be relatively short-lived (Barton and Charlesworth 1998; Berbee and Taylor 1993; Felsenstein 1974; Muller 1964). Furthermore, accumulation of mutations may result in a negative epistatic association whereby a synergistic effect is seen between multiple mutations. For example, four mutations have a greater effect on fitness than would be predicted from the sum of the four single mutations alone. This can be highly detrimental if they are disadvantageous mutations or highly beneficial if the mutations are advantageous (Goddard 2007). Indeed, sexual populations may be relatively free from the effects of Muller's Ratchet even if sexual reproduction is rare and/or inbreeding is common (Charlesworth *et al.* 1993; Hurst and Peck 1996). It has been suggested that in a constant environment sexual reproduction may not increase a population's fitness *per se*, but it may help purge the population of deleterious mutations (Goddard 2007; Hurst and Peck 1996; Zeyl and Bell 1997).

Finally, stochastic formation of a similar adaptive mutation (e.g. utilisation of a new carbon source) in several individuals within the same population in one generation can lead to clonal interference between successive generations, via competition for the carbon source. This is not the case in a sexual population, where both mutations can be brought together fairly rapidly, before competition can arise (Goddard 2007).

For sex and recombination to be beneficial for a population, recombination must act to create advantageous gene combinations rather than break them apart i.e. linkage disequilibria between adaptive genes. This is most likely to occur in a changing environment (e.g. nutrient source, temperature, parasite load, introduction of a fungistatic or fungicidal drug). A changing environment may make previously advantageous gene combinations disadvantageous or neutral, and thus recombination is desirable. In a stable environment once advantageous gene combinations have been established, asexual reproduction may be most desirable (Goddard 2007; Hurst and Peck 1996; White *et al.* 1998).

Interestingly, in the basidiomycete plant pathogen *Ustilago maydis*, the intial stages of the sexual cycle involving dikaryon formation are necessary to allow infection of corn, the plant host, without the formation of a dikaryon cell, infection cannot proceed (Kämper *et al.* 1994). Therefore, the sexual cycle may have other specialised benefits for fungal species.

1.3.2.2 Homothallism

Homothallic species are, by definition, able to self-fertilise and complete the sexual cycle without the need for a partner. Homothallic species are therefore able to form ascospores, with the associated benefits listed above (section 1.3.2.1) compared to purely asexual reproduction. Homothallic species are also able to develop ascospores with a compatible mating partner under appropriate environmental cues i.e. they are not restricted to self-fertilisation (Burnett 2003). They can therefore benefit from the advantages of recombination also.

Self-fertilisation does not result in recombination as only one genome is involved. This means that from a genetics perspective, homothallic sexual reproduction by self-fertilisation is equivalent to asexual reproduction and effectively clonal (see below) (Nauta and Hoekstra 1992b).

Emericella nidulans is a homothallic filamentous ascomycete (Pontecorvo 1953; Schwarz 1928), which has a mainly clonal population structure, indicating reproduction by predominantly self-fertilisation and/or asexual means. However, outcrossing does occur, possibly to retain or increase the genetic variability of the species (Croft and Jinks 1977; Geiser *et al.* 1994). A similar pattern is seen in the homothallic plant pathogen *Sclerotinia sclerotiorum* (Anderson and Kohn 1998). Outcrossing in homothallic species also results in recombination. Therefore, the particular advantages of heterothallism apply to homothallism but only when outcrossing is involved.

1.3.2.3 Pseudohomothallism

Pseudohomothallic species resemble homothallic species in that they produce progeny capable of selfing. Pseudohomothallism is also known as secondary homothallism. Genetic investigations have shown a sexual heterokaryon containing genetically different nuclei (within a common cytoplasm) is formed, which can undergo recombination (Tinline and Macneill 1969). There are two types of ascospore produced

by these species. The more common contains two compartmentalised nuclei, one from each parent. These are diploid and self-fertile i.e. are functionally homothallic (Coppin *et al.* 1997; Metzenberg and Glass 1990). The other type of ascospore is smaller and less common. When produced these are haploid, self-sterile and contain a single nucleus (Bistis 1998; Mathieson 1952; Nelson 1996). The haploid progeny must find a compatible mating partner i.e. are heterothallic. As pseudohomothallic reproduction leads to ascospore formation, species using this breeding system benefit from the same advantages of ascospore production as those produced by heterothallic or homothallic reproduction.

1.3.2.4 Parasexuality

Hyphal fusion is a common phenomenon in ascomycetes during the sexual cycle. Vegetative hyphal fusion is rarer, but can lead to genetic exchange and recombination (at a very low rate) in the so-called 'parasexual cycle' (Anderson and Kohn 1998). Parasexuality is a process that occurs in asexual species, which is different from sexual reproduction, but one that allows fusion and recombination of distinct nuclei, albeit at a very low rate (Anderson and Kohn 1998; Jinks 1952b; Tinline and Macneill 1969). Unlike pseudohomothallism, the heterokaryon formed in parasexuality is a result of vegetative hyphal fusion, with no ascospores being produced and therefore none of the advantages they endow (Anderson and Kohn 1998). The fusion of nuclei through parasexuality is thought to be a rare event (Anderson and Kohn 1998; Pontecorvo 1956), yet it has been shown in the laboratory for several species including; *Eurotium amstelodami* (anamorph: *Aspergillus vitis*) (Lewis and Barron 1964); *Fusarium oxysporum*; *Penicillium chrysogenum*; *Penicillium expansum*; and *E. nidulans* (Pontecorvo 1956; Pontecorvo and Sermonti 1954). Its abundance and relevance in natural populations remains to be shown conclusively. Parasexuality has been shown to occur in nature for *Penicillium cyclopium*, although nuclei fusion was not shown (Jinks 1952a). Parasexuality may be a rare event, and recombination is less frequent when compared to sexual reproduction, but this method of genetic recombination does seem to be more evolutionarily stable and able to proceed faster than the sexual cycle (Pontecorvo 1956). During parasexuality anastomosis (hyphal fusion) occurs, creating a vegetative heterokaryon. The two nuclei may then fuse and form a diploid nucleus, which can multiply mitotically. During multiplication, mitotic crossing over of chromosomes can occur at low rates. After crossing over, the diploid nucleus undergoes haploidization to produce haploid progeny

(McGuire *et al.* 2005; Pontecorvo 1956). It has been shown that even if nuclei fusion does not occur, nuclei contained within the same cytoplasm are able to cooperate i.e. a heterokaryon may be able to survive in an environment where the parental homokaryons cannot (Jinks 1952a; Jinks 1952b).

However, the parasexual cycle requires the presence of genetically compatible isolates of the same heterokaryon group (VCG). Also, unlike ascospores that are resistant to adverse environmental conditions, the mycelium of vegetative heterokaryons is subject to a range of environmental stressors which might cause damage. Therefore the formation of vegetative heterokaryons may not be a very practical survival strategy under adverse conditions (Jinks 1952b).

1.3.2.5 Sexual and Vegetative Incompatibility

Pseudohomothallism and parasexuality rely on the formation of sexual and vegetative heterokaryons, respectively. To form heterokaryons, it is necessary that compatible loci or so-called 'incompatibility complexes' (het, vic or vcg) must be present (Leslie 1993; McGuire *et al.* 2005). If heterokaryons form between individuals which differ in a HET locus, the resulting heterokaryon is not viable. Incompatibility complexes have been shown in various species, including *Neosartorya fumigata* (anamorph: *A. fumigatus*) (Debeaupuis *et al.* 1997) and *E. nidulans* (Pontecorvo 1953). In *E. nidulans* 8 HET loci are present and multiallelism is known in at least two of these. A difference in one of these HET loci is sufficient to prevent the formation of vegetative heterokaryons. In heterothallic species the MAT loci themselves may act as HET loci, but they are not sexual incompatibility loci (see section 1.4.1). In an asexually reproducing species or population, subpopulations may exist due to vegetative incompatibility between groups of isolates and, due to genetic hitchhiking, predictions can be made about other genes that will be present in these groups. In a sexually reproducing population, recombination between HET loci and other genes in the genomes would prevent these associations (Geiser *et al.* 1998b; Leslie 1993; Pel *et al.* 2007; Ramirez-Prado *et al.* 2008).

1.3.3 Population Genetics

Asexual reproduction and self-fertilisation produce progeny that are clones of their parent. Consequently, all the genes in the genome of an individual have the same evolutionary history as all the other genes leading to the phenomenon of gene

'hitchhiking' (Anderson and Kohn 1998; Thomson 1977). Indicators of clonal reproduction include significant linkage disequilibria, congruent phylogenies between genes (due to common evolutionary history) and the existence of subpopulations within species (due to vegetative incompatibility) (Xu 2007). However, in sexual, recombining species there is virtually independent reassortment and recombination of genes. Consequently, all genes in a genome may have their own, distinct evolutionary history. This also means that many genes in the genome are unlinked, and finding a particular gene e.g. a growth requirement, gives no indication about the presence or absence of another gene e.g. one associated with virulence. Indicators of recombination include linkage equilibrium (as gene associations are disrupted), incongruent phylogenies (when constructed using different genes) and no subpopulations within species (Geiser *et al.* 1998b; Xu 2007). Sexual species tend to be more genetically diverse than asexual species and are also expected to have mating-type genes in an equal proportion in the population (Milgroom 1996). There are notable exceptions to the *MAT* gene ratio expectation (Lengeler *et al.* 2000b). These may be due to alternate and compounding selection forces on the *MAT* genes.

A complicating factor when utilising population genetics to determine if a species appears to be clonal (i.e. asexual) or recombining (i.e. sexual) is the potential occurrence of the parasexual cycle and of self-fertilisation. Rare parasexuality may be indistinguishable from rare sexual recombination, and cannot be ruled out as a source of recombination until a sexual cycle has been seen and the frequency of occurrence ascertained. As already stated, homothallic reproduction involving self-fertilisation produces clones of the parent and is therefore indistinguishable from asexual reproduction. Most species observed appear to be on a continuum of clonal to recombining population structure. Finding no, or 100% recombination remains elusive even in known asexual and sexual species (Croft and Jinks 1977; Fisher *et al.* 2005; Linde *et al.* 2003; Paoletti *et al.* 2005).

The population genetics of various sexual and supposedly 'asexual' species have been studied in order to determine the extent to which sexual reproduction is occurring in these species. Varying levels of sexual reproduction have been reported. As expected a heterothallic sexual species such as *Ajellomyces capsulatus* (anamorph: *Histoplama capsulatum*) displays a recombining population structure (Bubnick and Smulian, 2007), and an asexual species such as *Penicillium marneffei* displays a clonal population

structure (Fisher *et al.* 2005). However, there are sexual species that seem to be mainly clonal and/or have disequilibrium in their *MAT* gene ratio. Examples include *E. nidulans* (Geiser *et al.* 1994), *Didymella rabiei* (anamorph: *Ascohyta rabiei*) (Barve *et al.* 2003) and *Magnaporthe grisea* (anamorph: *Pyricularia grisea*) (Consolo *et al.* 2005). There are also asexual species that have a recombining population structure and/or a 1:1 distribution of *MAT* genes, these include *Coccidioides immitis* (Mandel *et al.* 2007) and *Rynchosporium secalis* (Linde *et al.* 2003). Asexual species with recombining population structures are possibly sexual species with a currently unidentified (either cryptic or latent) sexual stage. Such a stage has recently been discovered for *N. fumigata* (O'Gorman *et al.* 2009). Therefore, to identify potentially sexual species in supposed asexual populations it is prudent to study *MAT* gene ratios.

1.4 Molecular-Genetic Basis of Sexual Reproduction in Ascomycete Fungi

Much is known about the molecular genetic basis of sexual development in ascomycete fungi, with a number of key genes identified as being involved with sex, as will be described in the following sections. The key mating-type genes will be described in detail (sections 1.4.1 to 1.4.3), and then other genes will be described thereafter (sections 1.4.4 to 1.4.8). Much of the previous work in the Pezizomycotina has focussed on sexual development in *Aspergillus* species, so many examples will be cited from literature concerning *Aspergillus*.

It has been suggested that between 200 and 400 genes are involved in the sexual cycle of fungi (Dyer *et al.* 1992). Figure 1.2 shows some of the genes involved in the sexual cycle of *E. nidulans*. Antheridial development cannot be seen in *E. nidulans*, therefore production of cleisothecia and Hülle cells are the first visible signs of sexual reproduction (Champe *et al.* 1994). For the sexual cycle to proceed, certain environmental cues must be received by the mycelia, these are processed by various proteins to either promote or repress the sexual cycle.

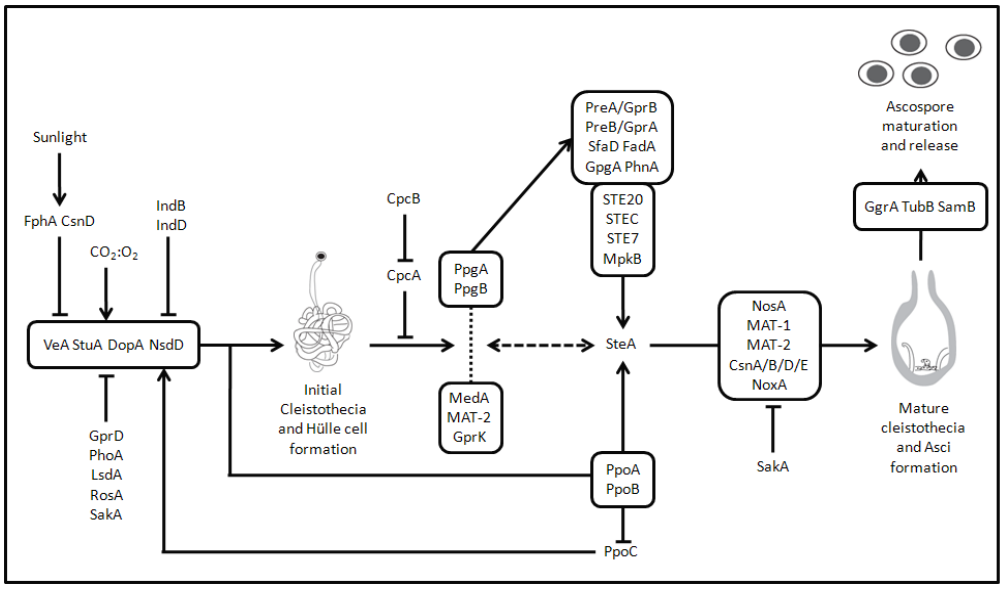


Figure 1.2: Genes involved in the sexual cycle of *E. nidulans*. [Adapted from Dyer (2007).]

1.4.1 Mating-Type Genes in Filamentous Ascomycetes (Pezizomyotina)

Sexual reproduction in heterothallic filamentous ascomycetes is mediated by genes present at the mating-type (MAT) locus (Coppin *et al.* 1997; Nelson 1996; Turgeon 1998) and can only occur when compatible 'mating types' are present (Dyer *et al.* 1992). Mating type is determined by the presence or absence of *MAT* genes (Shiu and Glass 2000). Filamentous ascomycetes have a single-locus, two-allele mating system (Kronstad and Staben 1997). This biallelic mating system is also seen in yeast species e.g. *Saccharomyces cerevisiae* (Mayrhofer and Pöggeler 2005). However, in basidiomycetes (e.g. *Coprinopsis disseminatus*) the situation is more complicated. The *MAT* genes may be bi- or multiallelic and there is another multiallelic locus, which must be different for sexual compatibility (James *et al.* 2006b). For more details on yeast and basidiomycete *MAT* genes see sections 1.4.1.2 and 1.4.1.3, respectively.

In filamentous ascomycetes (Pezizomycotina) there are two key types of *MAT* gene. First the *MAT1-1* type (abbreviated to *MAT-1*), which is characterised by the presence of DNA sequence encoding an alpha-domain protein (termed MAT1-1-1). Second, the *MAT1-2* type (abbreviated to *MAT-2*), which is characterised by the presence of sequence encoding a high mobility group (HMG) domain protein (termed MAT1-2-1) (Coppin *et al.* 1997; Turgeon and Yoder 2000). These characteristic *MAT* genes do not seem to be related by structure or descent (Metzenberg and Glass 1990). Consequently, the regions that contain the *MAT* genes are termed 'idiomorphs' in heterothallic species

(Metzenberg and Glass 1990). Idiomorph regions have more recently been found to share some regions of sequence similarity, leading to speculation that they may share a common evolutionary origin (Debuchy and Turgeon 2006). In homothallic species regions containing *MAT* genes are known simply as MAT loci rather than idiomorphs. Throughout this thesis the terms MAT idiomorph and MAT locus will be used interchangeably.

The MAT idiomorphs have flanking regions that show DNA sequence similarity and high amino acid conservation within protein encoding regions both within, and between, species (Barve *et al.* 2003; Rydholm *et al.* 2006; Sharon *et al.* 1996; Turgeon 1998). This sequence homology decreases to random levels in the idiomorphic region, apart from partial conservation between species of homology in the *MAT* gene encoding regions. The basic idiomorph structure is shown in Figure 1.3.

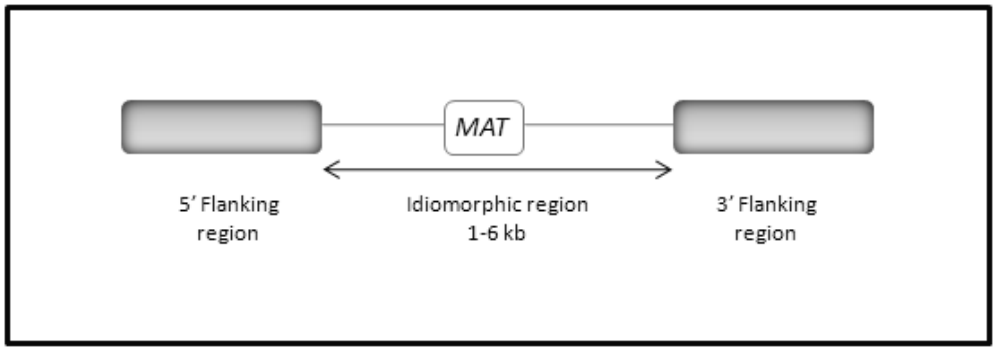


Figure 1.3: Overview of idiomorph organisation in Ascomycete species.

The flanking regions can contain a gene encoding a GTPase activating protein (Cozijnsen and Howlett 2003), a β-glucosidase encoding gene (Yun *et al.* 1999), a cytoskeletal protein encoding gene, *SLA2* (Melms *et al.* 1999) and/or a DNA lyase encoding gene, *APN2* (Johnson *et al.* 1998). The positions of the *SLA2* and *APN2* genes are particularly highly conserved across euascomycete fungal species (Debuchy and Turgeon 2006). The *SLA2* and *APN2* genes are only found bordering the MAT idiomorph region (Mandel *et al.* 2007; Melms *et al.* 1999), suggesting that they were closely linked to the MAT region progenitor (Rydholm *et al.* 2007). It has been suggested that suppression of recombination in the MAT idiomorph may have accelerated their divergence until the arrangement seen now (Bistis 1998; Fraser *et al.* 2004). Genes other than *MAT1-1-1* and *MAT1-2-1* may also be present in the idiomorphic region depending on taxonomic grouping, as well as various arrangements of the flanking genes, as illustrated in Figures 1.4 to 1.7. Thus, up to three *MAT* genes may be present in the MAT-1 idiomorph of

Sordariomycete fungi (*MAT1-1-1*, *MAT1-1-2*, *MAT1-1-3*), compared to other taxonomic groupings such as the genus *Cochliobolus* where species contain only one *MAT* gene at the MAT locus (Yun *et al.* 1999). Of particular relevance to the present study has been the discovery of a putative *MAT1-2-4* gene, which has been detected in the MAT-2 locus of various Eurotiomycete fungi (see Chapter 3) (Bubnick and Smulian 2007; Fraser *et al.* 2007b; Mandel *et al.* 2007; Paoletti *et al.* 2005; Rydholm *et al.* 2007; Woo *et al.* 2006). Most recently a novel class of *MAT* genes has been found at the MAT-1 idiomorph of Leotiomycete fungi (Eyres 2008).

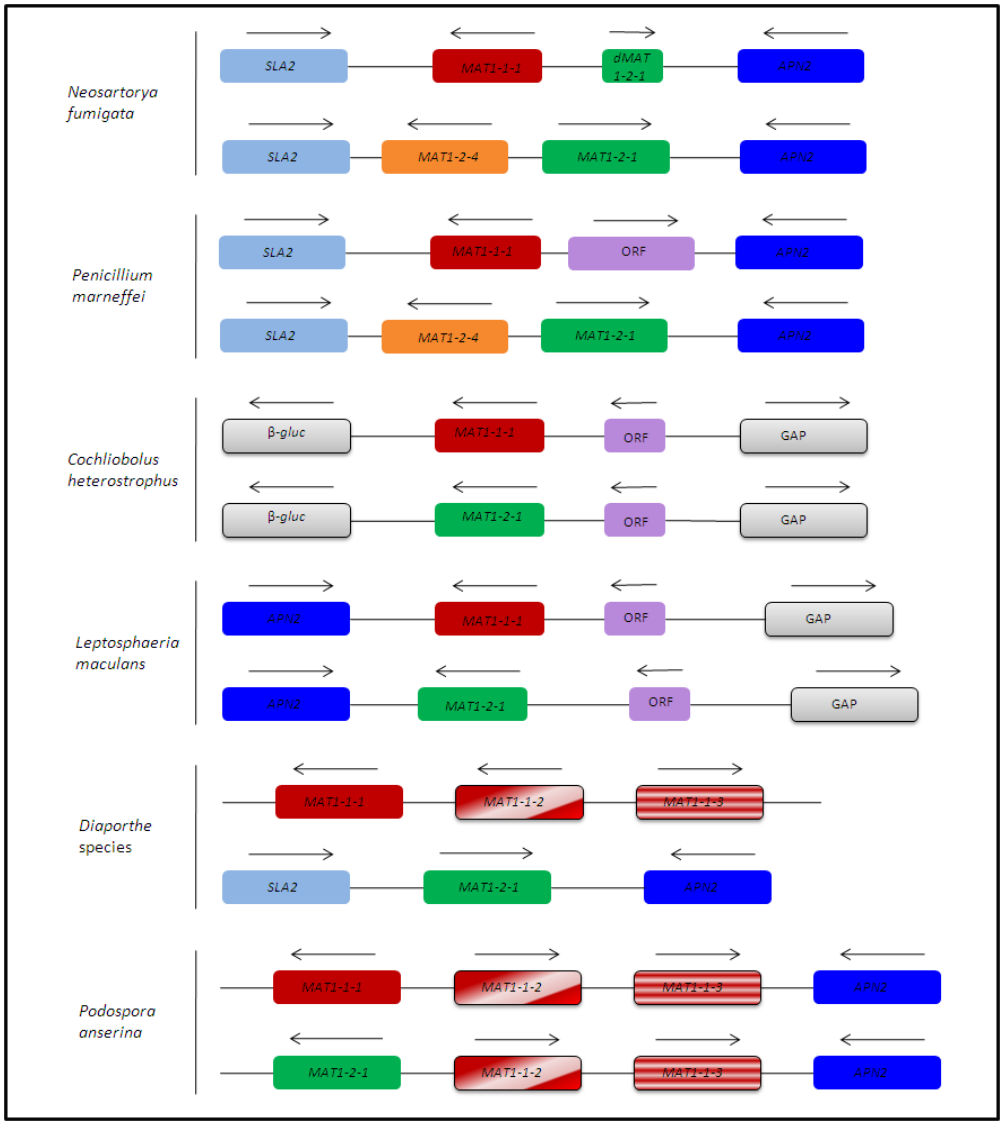


Figure 1.4: Organisation of *MAT* genes in the MAT idiomorph of heterothallic Pezizomycete species. [Adapted from Cozijnsen and Howlett (2003), Debuchy *et al*. (1993), Debuchy and Coppin (1992), Kanematsu *et al*. (2007), Paoletti *et al*. (2005), Pel *et al*. (2007), Woo *et al*. (2006) and Yun *et al*. (1999).] Arrows indicate gene orientation. Prefix 'd' refers to a mutated partial ORF.

The *MAT* genes are believed to be necessary for the induction of the sexual cycle and production of sexual structures (Hiscock and Kües 1999). The *MAT* genes themselves contain sequences that encode DNA binding domains (Coppin *et al.* 1997) and may be transcriptional regulators (Jacobsen *et al.* 2002). The alpha-domain MAT1-1-1 protein is a transcriptional activator that cooperates with other proteins to promote the expression of specific genes involved in sexual reproduction (Coppin *et al.* 1997; Dyer 2007). The HMG-domain of the MAT1-2-1 protein contains an acidic, proline-rich, C-terminal which is similar to regions found in transcriptional activators (Coppin *et al.* 1997). The HMG-box alone has been shown to be sufficient for DNA binding *in vitro* (Kronstad and Staben 1997).

The *MAT* genes may be viewed as the master regulators of pathways involved in the expression of pheromones and pheromone receptors (Pöggeler 2002), as well as post-fertilization events (Dyer 2007; Shiu and Glass 2000), such as cell cycle arrest (Banuett 1998). The products of the complimentary *MAT* genes have been shown to interact with each other in *Sordaria macrospora* (Jacobsen *et al.* 2002). It may be this interaction that stimulates the various response pathways (Jacobsen *et al.* 2002).

1.4.1.1 Mating-Type Genes in Homothallic and Asexual Ascomycetes

Many homothallic ascomycete species have been shown to possess and express *MAT* genes (see Figures 1.5 and 1.6). As these species are able to self-fertilize, their need for *MAT* genes, pheromone precursor and receptor genes etc. is perhaps not obvious. However, progression through the sexual cycle is stalled or prevented without them (Mayrhofer *et al.* 2006; Paoletti *et al.* 2007; Pöggeler *et al.* 2006). The *MAT* genes also seem to be required for events downstream in fruiting body production (Figure 1.2). Given that homothallic species retain their ability to outcross, it has been suggested that self-fertilisation does not by-pass the normal requirements for outcrossing sex, but instead requires activation of these pathways within a single individual (Paoletti *et al.* 2007).

The arrangement of the *MAT* genes in different homothallic species is more diverse than that seen in heterothallic species, with some almost unique arrangements observed in the former (Figures 1.4 to 1.7). The majority of homothallic species contain both *MAT1-1-1* alpha-domain and *MAT1-2-1* HMG-domain encoding genes. These *MAT* genes may be unlinked with the *SLA2* and *MAT1-1-1* on one chromosome and the *APN2* and *MAT1-*

2-1 on another chromosome, as seen in *E. nidulans* (Figure 1.5) (Paoletti *et al.* 2007). A similar arrangement of unlinked *MAT* gene is seen in *N. fischeri*. However, here functional *SLA2* and *APN2* genes are found flanking the *MAT1-1-1* gene, whereas degenerated *SLA2* and *APN2* genes are observed flanking the *MAT1-2-1* gene on a different chromosome (Figure 1.5) (Rydholm *et al.* 2007). In *E. crustaceum* (anamorph: *P. crustaceum*) functional *SLA2* and *APN2* genes flank the *MAT1-1-1* gene, with the degenerated *SLA2* (and possibly *APN2*) flanking the *MAT1-2-1* gene (Figure 1.5) (Hoff personal communication). In *Petromyces alliaceus* (anamorph: *Aspergillus alliaceus*) the *MAT1-1-1* and *MAT1-2-1* genes are linked with functional *SLA2* and *APN2* genes flanking them (Figure 1.5) (Ramirez-Prado *et al.* 2008).

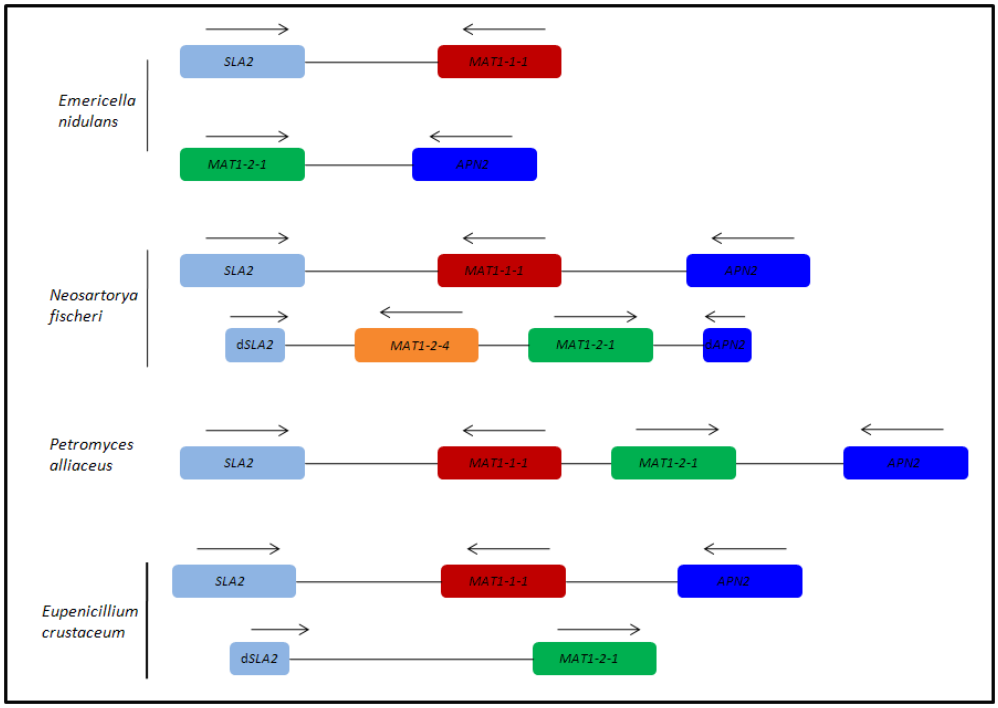


Figure 1.5: Organisation of *MAT* genes in the MAT loci of homothallic Pezizomycete species. *E. nidulans*, *N. fischeri* and *E. crustaceum* have unlinked MAT loci, with the *MAT* genes appearing on different chromosomes, whereas *P. alliaceus* has a single MAT locus. [Adapted from Rydholm *et al*. (2007), Paoletti *et al*. (2007), Ramirez-Prado *et al*. (2008) and Hoff (personal communication).] Arrows indicate gene orientation. Prefix 'd' refers to a mutated partial ORF.

Cochliobolus lutrellii and *C. homomorphus* have fused *MAT1-1-1* and *MAT1-2-1* genes, however they are inverted between these species (Figure 1.6) (Yun *et al.* 1999). In *C. kusanoi* the *MAT* genes are not fused but are linked in the genome (Figure 1.6) (Yun *et al.* 1999). In *C. cympogonis* the *MAT* genes are both present in the genome, but they appear to be unlinked (Figure 1.6). However, in *G. zeae* and *S. macrospora* the *MAT* genes are unfused but linked within the genome (Figure 1.7) (Pöggeler *et al.* 1997; Yun

et al. 2000). There are seven known homothallic *Neurospora* species. Of these, *N. terricola*, *N. sublineota* and *N. pannonica* have been definitively shown to possess both *MATA* and *MATa* gene sequences in their haploid genomes. However, the other four species *N. Africana, N. galapogensis, N. dodgei* and *N. lineolata* only hybridise to the *MATA* gene which encodes the alpha domain protein with the apparent absence of MATa hybridising sequence. The method of homothallism and the trigger of the sexual cycle in these species is unknown (Glass *et al.* 1990b).

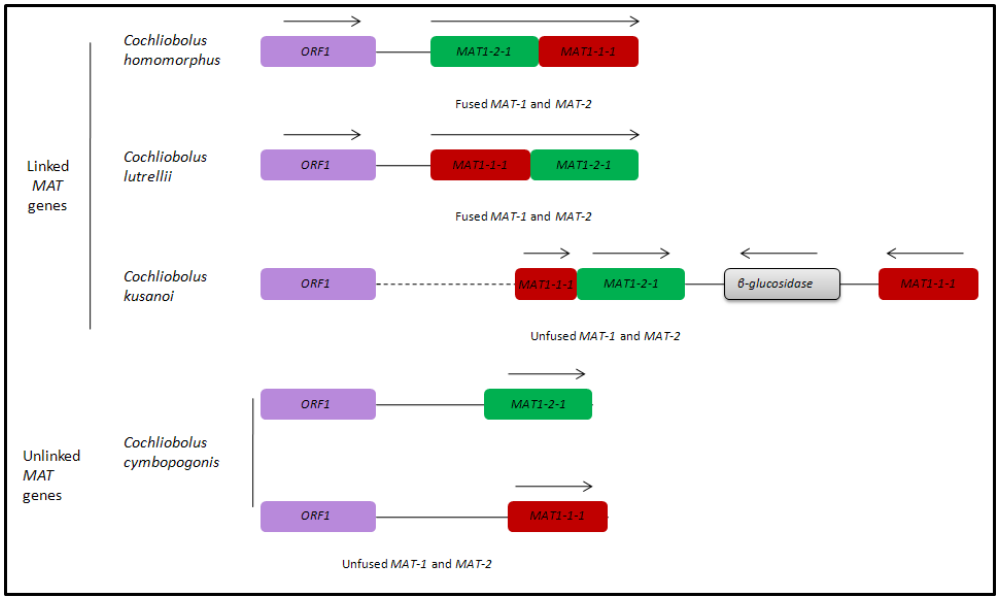


Figure 1.6: Organisation of *MAT* genes in the MAT loci of homothallic *Cochliobolus* species. *C. homomorphus* and *C. lutrellii* have fused, linked *MAT* genes. *C. kusanoi* has unfused, but linked *MAT* genes within the same locus. *C. cympogonis* has unlinked *MAT* genes, in an arrangement similar to *E. nidulans*. [Adapted from Yun *et al*. (1999).] Arrows indicate gene orientation.

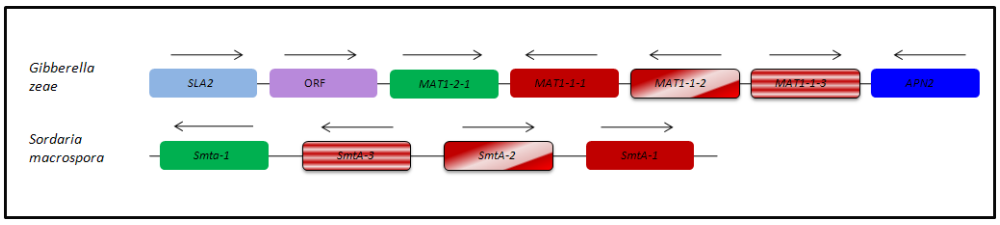


Figure 1.7: Organisation of *MAT* genes in the MAT loci of homothallic species, *Gibberella zeae* and *Sordaria macrospora*. Both species contain a single linked MAT locus. [Adapted from Pöggeler *et al*. (1997) and Yun *et al*. (2000).] Arrows indicate gene orientation.

MAT genes have also been found, and their expression shown in many supposedly asexual species (Galagan *et al.* 2005; Kerényi *et al.* 2004; Sharon *et al.* 1996; Woo *et al.* 2006; Yun *et al.* 2000). Using transformation approaches, certain *MAT* genes have been found to be functional when inserted into sexual species (Arnaise *et al.* 1993; Coppin *et al.* 1997; Pöggeler *et al.* 1997; Pyrzak *et al.* 2008; Sharon *et al.* 1996). This may suggest a

'cryptic' sexual cycle in these 'asexual' species, where the sexual structures have not yet been seen. Possession of *MAT* genes may also be due to previous sexual reproduction which has subsequently been lost in the recent evolutionary history of these species. However, it may be that the *MAT* genes have additional functions in these species and their sequence homology is maintained as a consequence, for example in *N. crassa* the *MAT* genes have been shown to have additional roles in fertilization, and also in vegetative incompatibility, a function which is independent of sexual reproduction (Glass *et al.* 1990a; Staben and Yanofsky 1990).

Sequencing of MAT idiomorphs has so far shown a general conservation of gene order, but not always gene orientation, in heterothallic ascomycetes and yeasts (Figure 1.4) (Bubnick and Smulian 2007; Cozijnsen and Howlett 2003; Galagan *et al.* 2005; Mandel *et al.* 2007; Pöggeler 2001; Ramirez-Prado *et al.* 2008; Rydholm *et al.* 2007; Schirawski *et al.* 2005; Singh *et al.* 1999; Yokoyama *et al.* 2003; Yun *et al.* 1999). This implies that *MAT* gene placement within the genome is important for expression and function (Kronstad and Staben 1997). Ectopic integration of *MAT* genes was unable to confer fertility in *N. crassa* (Kronstad and Staben 1997) and *E. nidulans* (Pyrzak *et al.* 2008). While, in *P. anserina* (Arnaise *et al.* 1993) and *C. heterostrophus* (Sharon *et al.* 1996) fertility was only partially restored.

1.4.1.2 Mating-Type Genes in Yeasts

The MAT loci in many budding and fission yeasts contain additional genes compared to filamentous ascomycetes, although there are parallels. The budding yeast *Saccharomyces cerevisiae* is the most extensively studied, and *MAT* genes have also been found in various other *Saccharomyces, Candida*, *Schizosaccharomyces* and *Kluyveromyces* species (Figure 1.8). Both α and **a**-type mating-type-like (MTL) loci have been found in the supposedly 'asexual' species *Candida glabrata* and the once considered asexual species *C. albicans*. A *MTL**a*** locus has also been found in asexual *Candida parapsilosis*, although no functional MAT**a**1 protein was found. The *MTLα* locus could not be found within the genome of *C. parapsilosis*, although Southern blotting suggested that *MTLα*-like sequences were present but their location was not ascertained (Logue *et al.* 2005). It may be that, like *C. glabrata* and *K. lactis* the MAT loci are on different chromosomes and will be found after further genome sequencing (Figure 1.8) (Aström *et al.* 2000; Muller *et al.* 2008).

In *S. cerevisiae* the MATα locus encodes two proteins. The *MATα1* gene, which encodes an alpha-domain protein, and the *MATα2* gene, which encodes a homeodomain protein (Astell *et al.* 1981; Butler 2007). *K. lactis* also possesses a *MATα3* gene at the MATα locus, encoding the MATα3 protein. The MATα3 protein is required for optimal mating proficiency in MATα cells, although its true function has not been determined (Aström *et al.* 2000; Butler 2007). The MAT-1 locus in filamentous ascomycetes is equivalent to the MATα locus in budding yeast. The MAT**a** locus (equivalent to the MAT-2 locus in filamentous ascomycetes) in yeast encodes one confirmed and one putative protein. The *MAT**a**1* gene encodes a putative homeodomain protein. The MAT**a**2 is not a confirmed protein, and seems to have resulted from a recombination event as this seems to be a truncated MATα2 protein (Astell *et al.* 1981). MAT**a**2 protein appears to have been lost in *S. cerevisiae* and *C. glabrata* (Astell *et al.* 1981; Herskowitz 1989; Muller *et al.* 2008).

The MATα1 protein controls the expression of α-specific genes necessary for mating. The MATα2 protein represses transcription of *MAT**a*** genes which are otherwise constitutively expressed (Astell *et al.* 1981; Haber 1998; Herschbach *et al.* 1994; Rine *et al.* 1979; Strathern *et al.* 1981). The MAT**a**1 protein itself is not required for mating, but when produced it acts in concert with the MATα2 protein to repress haploid genes and express α/**a** diploid genes, including those involved in sporulation (Astell *et al.* 1981; Aström *et al.* 2000; Butler 2007; Haber and George 1979; Strathern *et al.* 1981).

Schizosaccharomyces pombe also possess two alternate mating-type loci, termed the minus (m) and plus (p) loci (Figure 1.8). These loci each encode two proteins MAT1-Mm and MAT1-Pm, which are required for entry into meiosis. MAT1-Mc and MAT1-Pc proteins determine the mating type of cells (Nielsen and Egel 2007)

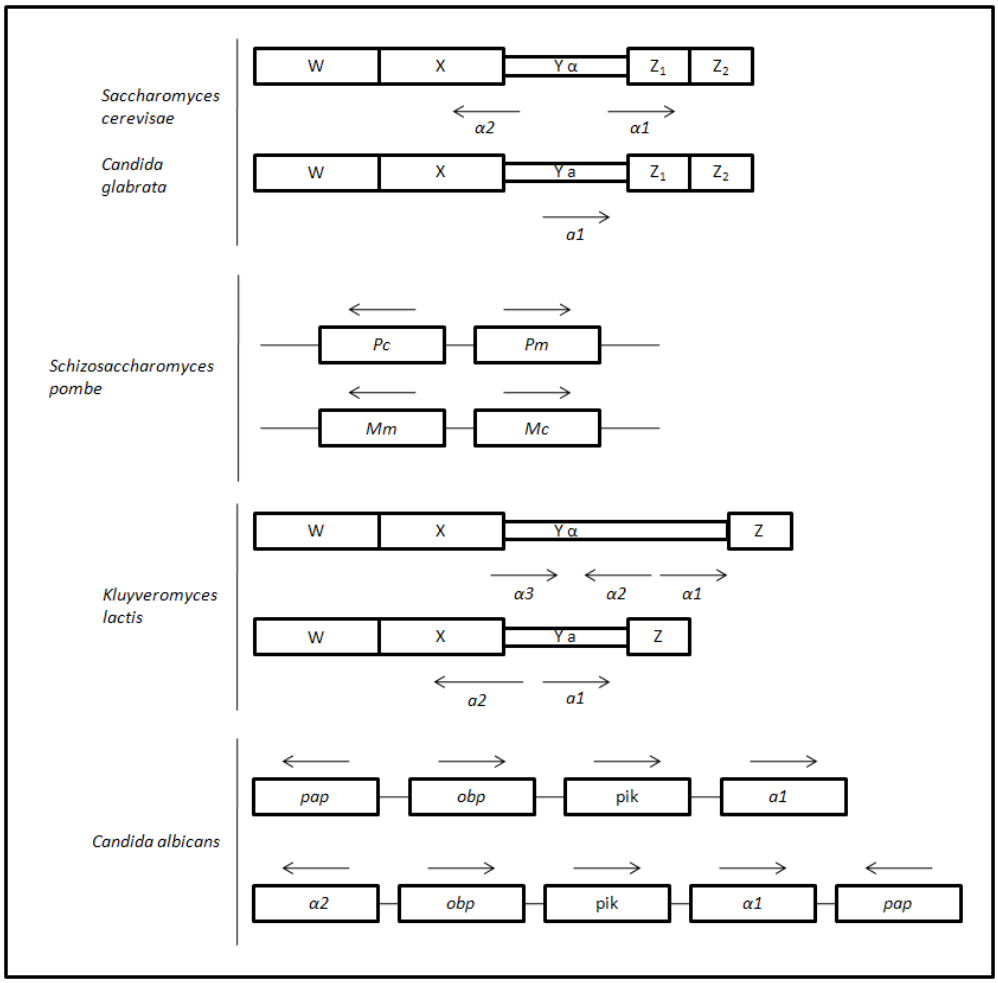


Figure 1.8: Arrangement of MAT loci in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis, Candida glabrata* and *Candida albicans*. [Adapted from Astell *et al*. (1981), Fabre *et al*. (2005), Haber (1998), Hull and Johnson (1999), Muller *et al*. (2008), Nielsen and Egel (2007) and Schmidt and Gutz (1994).] Arrows indicate gene orientation. pap = poly(A) polymerase, obp = oxysterol binding protein-like protein, pik = phosphatidylinositol kinase.

1.4.1.3 Mating-Type Genes in Basidiomycetes

Mating-type genes have also been found in a variety of basidiomycete species. The arrangement of mating-type genes is markedly different to yeast and filamentous ascomycetes, as will be described in the following section.

Unlike Ascomycota species, which produce an ascus as the sexual structure and ascospores, Basidiomycota species produce a basidium and basidiospores (Fraser *et al.* 2004). The basidiomycetes are divided into two groups. Homobasidiomycetes, that produce unicellular basidia and heterobasidiomycetes, that produce septate basidia (Kämper *et al.* 1994). Most basidiomycetes have a tetrapolar mating-type system. This involves the presence of two, unlinked, biallelic or multiallelic loci, which control mating in heterothallic species. The resulting haploid progeny may be one of four permutations

of the alleles present in the parental isolates (Casselton and Kües 1994; Kämper *et al.* 1994). Homobasidiomycetes e.g. *Schizophyllum commune* and *Coprinopsis cinerea* possess unlinked A and B loci, each containing tightly linked α and β subloci (Casselton and Kües 1994; Casselton and Kües 2007; Koltin and Raper 1967; Papazian 1951; Stankis and Specht 2007). In *S. commune* there are 9 different 'Aα', 32 different 'Aβ', 9 different 'Bα' and 9 different 'Bβ' alleles. This leads to 288 'A' locus combinations and 81 'B' locus combinations, making 20,000 theoretical mating types in *S. commune*, with 12,000 theoretical mating types being produced by *C. cinerea* (Casselton and Kües 1994).

The Aα locus encodes two genes, *Y* and *Z*. Gene *Y* encodes a homeodomain protein, HD2, which has amino acid homology with the *Saccharomyces cerevisiae* MAT**a**1 protein. Gene *Z* encodes another homeodomain protein, HD1, that shares amino acid homology with the *S. cerevisiae* MATα2 protein. The Aβ locus encodes the *Y* gene, encoding HD2, however no other genes have been found in this locus. The Aα and Aβ loci are functionally redundant as they regulate identical developmental pathways (Stankis and Specht 2007). The Bα and Bβ loci encode cell signalling components, pheromones and pheromone receptors (Casselton and Kües 1994).

Heterobasidiomycetes e.g. *Ustilago maydis* are biallelic at the 'a' locus and multiallelic at the 'b' locus. The a locus contains the *mfa-1* or *mfa-2* genes, which encode pheromones with a CAAX motif, like the **a**-factor pheromone produced by *S. cerevisiae*. The a locus also contains *pra-1* or *pra-2* genes which encode pheromone receptors similar to the STE3 receptor in *S. cerevisiae* that binds **a**-factor pheromone (see section 1.4.6) (Bakkeren and Kronstad 2007; Banuett 2007; Banuett *et al.* 2008; Kämper *et al.* 1994). The b locus encodes the *bE* and *bW* genes, both encode homeodomain proteins. Gene *bE* encodes the HD1 protein with homology to the *S. cerevisiae* MATα2 protein. Gene *bW* encodes the HD2 protein with homology to the *S. cerevisiae* MAT**a**1 protein. When HD1 and HD2 are combined from different lineages they control sexual development after cell fusion and dikaryon production, including meiosis and sporulation (Bakkeren and Kronstad 2007; Banuett 2007; Banuett *et al.* 2008; Kämper *et al.* 1994; O'Shea *et al.* 1998).

The tetrapolar mating system is believed to have evolved once, compared to the bipolar mating system which may have evolved numerous times. The tetrapolar mating system results in increased outcrossing and a decreased possibility of selfing (Casselton and Kües 1994; James *et al.* 2006b; Kämper *et al.* 1994; Specht *et al.* 1994; Whitehouse

1949). There has been considerable research into mating type determination in basidiomycete fungi. This is beyond the scope of the present study, but is reviewed by Banuett (2007) and Casselton and Kües (2007).

1.4.2 Mating-Type Switching

Certain fungal species exhibit the unusual property of 'mating-type switching'. This refers to the phenomenon whereby an isolate of one mating type is able to give rise to progeny of a different mating type. This has been most widely studied in ascomycetous yeasts, but has also been seen in filamentous ascomycetes, as will now be described.

1.4.2.1 Mating-Type Switching in Yeasts

Section 1.4.1.2 described the MAT loci arrangements seen in various budding and fission yeast species. The mating type of heterothallic fungal species is determined by which set of *MAT* genes are expressed at any one time. In some yeast species e.g. *Saccharomyces cerevisiae, Schizosaccharomyces pombe, K. lactis* and the asexual *C. glabrata*, there are, in addition to the *MAT* genes at the main MAT locus, transcriptionally inactive 'silent cassettes' that encode *MAT* genes elsewhere within their genome (Figure 1.9). These silent cassettes contain second copes of the *MAT* genes and were first found in *S. cerevisiae* (Hicks *et al.* 1979). The alternate *MAT* genes are termed Hidden MAT Left (HML usually 'α') and the Hidden MAT Right (HMR usually '**a**'). As the silent cassettes are transcriptionally inactive it means that both types of mating-types genes are not expressed concurrently and at a molecular level the species appear heterothallic, but at a functional level they have the capability to exhibit homothallism.

The silent cassettes contain complete 'Y' regions which encode the MAT proteins, although some of the flanking regions are missing from the cassettes. The cassette orientations are inverted in *S. cerevisiae* when compared to the other yeast species. *C. glabrata* and *K. lactis* also have their cassettes on different chromosomes (Figure 1.9).

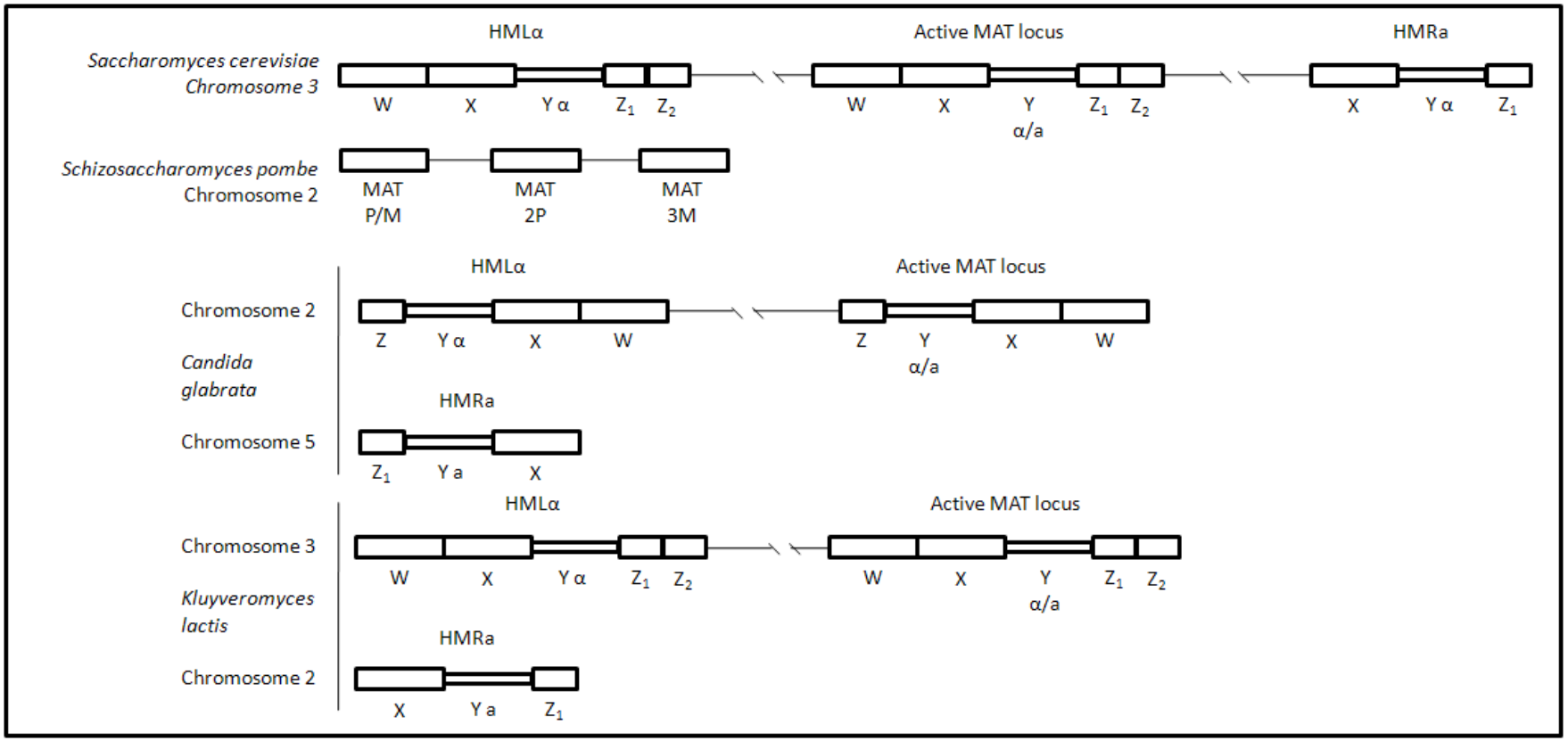


Figure 1.9: Mating-type silent cassettes and active loci in *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Candida glabrata* and *Kluyveromyces lactis*. [Adapted from Aström *et al*. (2000), Butler (2007), Muller *et al*. (2008) and Nielsen and Egel (2007).]

The silent cassettes are transposed into the MAT locus in order to switch the mating type of a cell. In *S. cerevisiae* and *C. glabrata* this is performed by a HO endonuclease enzyme. No homolog of this gene has been found in *K. lactis* or *S. pombe* although mating-type switching occurs in both these species. *K. lactis* contains a putative HO endonuclease recognition site on the Y-Z junction, but this does not resemble that of *S. cerevisiae* or *C. glabrata* (Aström *et al.* 2000; Herskowitz 1989). *K. lactis* is able to switch mating type, although at a lower rate than in *S. cerevisiae*. The ability of *K. lactis* to switch is also reduced over meiotic generations. Yeast species that are able to switch mating type and are therefore functionally homothallic may have a defective *HO* endonuclease gene, or the *HO* gene is not present. The acquisition of the *HO* gene is believed to have occurred relatively recently, along with the evolution of the silent cassettes that they transpose (Figure 1.10) (Butler *et al.* 2004; Herskowitz 1989). *Ashbya gossypii* has silent cassettes but no *HO* gene so probably cannot switch, while *K. lactis* has an alternate system enabling low-level switching. In contrast, the fission yeast *S. pombe* uses a system of cyclic switching involving replication fork stalling at DNA breaks and reannealing at the silent *MAT* cassettes [reviewed in Nielsen and Egel (2007)].

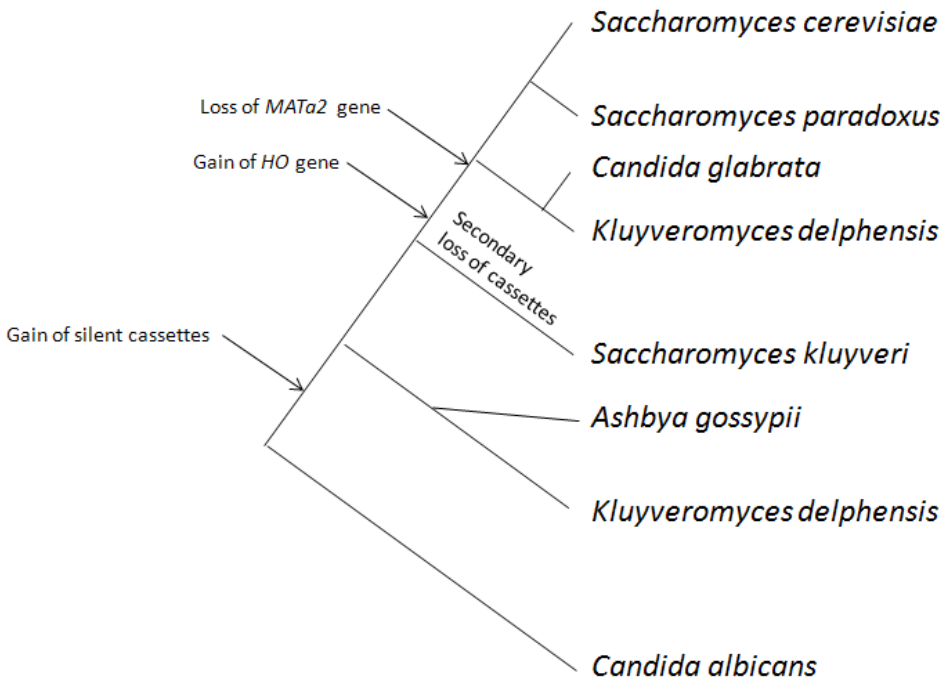


Figure 1.10: Phylogeny of the acquisition of the *HO* endonuclease gene, and silent cassettes. [Adapted from Butler (2007) and Herskowitz (1989).]

Candida albicans does not have silent cassettes as they are believed to have been acquired after this species diverged (Figure 1.10) (Hull and Johnson 1999; Hull *et al.* 2000; Tzung *et al.* 2001). It is also believed that the *HO* gene has been acquired after the evolution of silent cassettes (Figure 1.10). Expression of the *HO* gene is regulated via three different mechanisms, as follows: *HO* is only expressed in **a** or α homozygous cell not **a**/α cells, expression is only in late G_1 phase of the cell cycle, and *HO* is only expressed in mother cells i.e. those that have already divided, but not daughter cells (Butler 2007; Butler *et al.* 2004; Herskowitz 1989).

*MAT**a***/*MAT**a*** and *MATα*/*MATα* diploids cannot initiate meiosis or spore formation whereas *MAT**a***/*MATα* diploids can, due to the interaction of MAT**a**1 and MATα2 proteins. The advantage of mating-type gene switching is the maintenance of proximity to cells of opposite mating type, thereby maximising the probability of producing sexual progeny. Diploid cells are more resistant than haploid cells to environmental stresses (section 1.3.2.1) (Barbera and Petes 2006; Haber 1998; Schmidt and Gutz 1994; Valencia-Burton *et al.* 2006).

1.4.2.2 Mating-Type Switching in Pezizomycotina

Bidirectional mating-type switching appears to be commonplace in many yeasts. Yet in filamentous ascomycete fungi this has never been seen. Unidirectional mating-type switching has been confirmed in *Ceratocystis* species (Harrington and McNew 1997; Witthuhn *et al.* 2000). In these species, self-fertile parental isolates possessing both MAT-1 and MAT-2 loci produce self-fertile progeny and self-sterile progeny. The latter possess only a MAT-1 idiomorph. The MAT-2 idiomorph is deleted in self-sterile isolates and these behave as MAT1-1 heterothallic isolates. When isolates possess both MAT loci they act as homothallic or MAT1-2 heterothallic isolates. Due to the fact that in order to switch mating type the MAT-2 locus is deleted, only isolates possessing both loci can switch mating type. This gene cannot be regained by MAT1-1 heterothallic isolates making switching in these species unidirectional and irreversible (Harrington and McNew 1997; Witthuhn *et al.* 2000). Four other species of Pezizomycotina have been suggested to undergo unidirectional mating-type switching (*Chromocrea spinulosa, Sclerotinia trifoliorum* and *Glomerella cingulata*) (Coppin *et al.* 1997; Perkins 1987), although the molecular mechanism of switching in these species is unknown.

1.4.3 Evolution of Reproductive State and Mating-Type Genes

There has long been debate over whether homothallism or heterothallism is the ancestral state in ascomycete fungi. The identification of *MAT* genes over the past 15 to 20 years has added new insights into this debate and over the evolutionary origins of the *MAT* genes themselves.

Idnurm *et al*. (2008) showed that a gene encoding a HMG-domain protein, with homology to the modern day MAT1-2-1 proteins is present in an early diverged fungal lineage, the Zygomycota. However, unlike species within the Dikarya, the species studied, *Phycomyces blakesleeanus* and *Rhizopus oryzae*, do not appear to contain a *MAT1-1-1* alpha-domain encoding gene within their sex determining region. This suggests that the HMG-domain was the first, ancestral sex determining gene and that the alpha-domain was sequestered at a later point in evolution to its current purpose (Idnurm *et al.* 2008). It should be noted that the SRY-HMG-domain protein determines sexual identity in mammals, showing that this class of proteins may have ancient roles in sex determination (Dyer 2008).

The conserved heterothallic gene arrangement observed within and between species, clades and groups suggests that heterothallism is the overall ancestral sexual strategy within fungi (Butler 2007; Butler *et al.* 2004; Coppin *et al.* 1997; Fraser *et al.* 2007b; Kronstad and Staben 1997; Rydholm *et al.* 2007; Yun *et al.* 1999). However, there are studies disputing this conclusion (Galagan *et al.* 2005; Varga *et al.* 2000b). Figure 1.11 gives the possible genetic pathways of the evolution of reproductive strategy, irrespective of ancestor.

The primary evidence that heterothallism is ancestral comes from studies in *Cochliobolus* species (Yun *et al.* 1999). Here, all heterothallix species examined had the same arrangement of *MAT* genes at a single MAT locus, whereas homothallic species showed a variety of MAT loci arrangements, consistent with a state derived from heterothallism. In the case of *C. kusanoi*, two recombination events appear to have occurred, the first was the insertion of a partial *MAT1-1-1* sequence into the MAT-2 locus. However, this was not sufficient to confer homothallism, so a second event transferring the whole *MAT1-1-1* gene was necessary for homothallism (Figure 1.6 and section 1.4.1.1). Data from various other genera containing species with fused or unfused, linked or unlinked *MAT* genes also suggests a single recombination event at the

MAT locus may be sufficient to confer homothallism. For example, Inderbitzin *et al.* (2005) showed that in *Stemphylium* species, a CCAT motif is present at the fusion site of MAT-1 and MAT-2 regions. This fusion may be the result of a previous uneven crossover event, which resulted in homothallism. This motif is also present in unfused MAT-1 and MAT-2 idiomorphs of *Stemphylium* species suggesting a possible site of recombination (Inderbitzin *et al.* 2005; Shiu and Glass 2000).

It has also been suggested, on theoretical grounds, that a homothallic species is more likely to evolve in a heterothallic population compared to the reverse event, providing further evidence that heterothallism is the ancestral sexual reproductive strategy (Nauta and Hoekstra 1992b). Exchanging *MAT* genes between closely related species has been performed leading to restoration of partial fertility (Arnaise *et al.* 1993; Coppin *et al.* 1997; Pöggeler *et al.* 1997; Pyrzak *et al.* 2008; Sharon *et al.* 1996). It is therefore conceivable to imagine an exchange or acquisition of a *MAT* gene by a heterothallic species might allow it to become homothallic.

The investigation of evolutionary pathways is further confused by sample size i.e. whether a single genus, sister or multiple genera, the phylum Ascomycota, or kingdom Fungi as a whole, are examined and also the number of genes used in analysis. For example, heterothallic Aspergilli are suggested to have evolved from a homothallic ancestor, based on phylogenetic analysis of β-tubulin and hydrophobin gene sequences (Galagan *et al.* 2005; Geiser *et al.* 1998a; Varga *et al.* 2003). Further evidence from MAT locus studies supporting a homothallic ancestor in the Aspergilli is the presence of a partial *MAT1-2-1* gene sequence within the MAT-1 idiomorph in *N. fumigata* (see Figure 1.4 and section 1.4) (Paoletti *et al.* 2005). However, this partial sequence could be the result of an abberant recombination event. The reverse of this situation is seen in *Stemphylium* species (Debuchy and Turgeon 2006) and closely related genus *Cochliobolus* (Fraser *et al.* 2007b). However, when *Cochliobolus* species, *Ajellomyces capsulatum* and *Aspergillus* species are taken together a heterothallic species is believed to be ancestral. Therefore, in the genus *Aspergillus*, heterothallism may have re-evolved from a homothallic ancestor, which may explain the comparative rarity of heterothallism in this genus (Fraser *et al.* 2007b; Galagan *et al.* 2005).

The suggested ancestral homothallic gene arrangement can be seen in Figure 1.11. While this gene arrangement is seen in *S. macrospora* and *G. zeae* (Figure 1.7), it has only recently been found in the homothallic species *P. alliaceus* (Figure 1.5) (Pöggeler *et*

al. 1997; Ramirez-Prado *et al.* 2008; Yun *et al.* 2000). Various *MAT* gene arrangements have been found in other homothallic species, most of them unique to a given species (see Figures 1.5 to 1.7 and section 1.4.1.1). The lack of a conserved homothallic *MAT* gene arrangement, compared to a relatively well conserved heterothallic *MAT* gene arrangement does suggest a heterothallic ancestor or a single conversion event from homothallism to heterothallism in the evolutionary history of the Pezizomycotina. However, this hypothesis will continue to be debated, especially as more genome sequencing data becomes available, allowing genome wide phylogenies instead of single gene comparisons to be compiled.

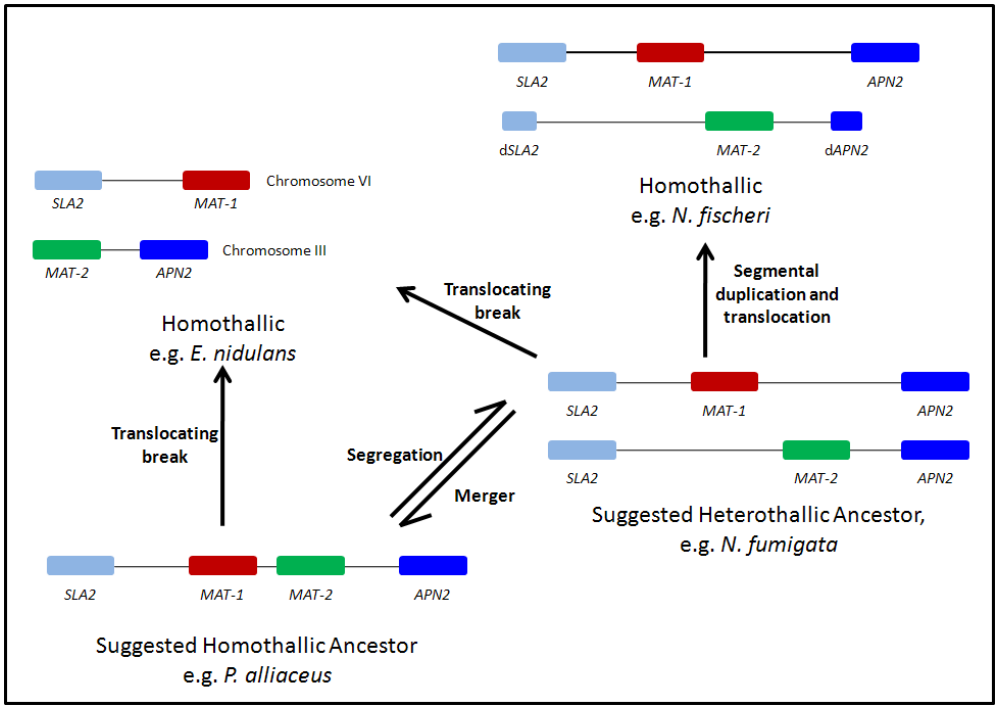


Figure 1.11: Possible evolutionary routes of the *MAT* genes and MAT loci evolution using *Aspergillus* species as examples. [Adapted from Galagan *et al*. (2005), Paoletti *et al.* (2005 and 2007), Ramirez-Prado *et al*. (2008) and Rydholm *et al*. (2007).]

MAT gene evolution in budding yeast species has been of particular interest as the acquisition of silent *MAT* cassettes and the *HO* endonuclease gene to allow switching to occur. Mating-type switching allows species to be functionally homothallic, with mother cells being compatible with daughter cells, but yeast species are heterothallic at a molecular level as each cell only expresses one mating type at any one time. Figure 1.10 gives the proposed evolutionary pathway of gene acquisition and sexual mating strategy for budding yeast species. Acquisition of silent cassettes is thought to have occurred once early in the evolution of this group but instead of making species homothallic, the

silent cassettes remained untranscribed and were not subjected to RIPing (see section 1.5) or degeneration. The *HO* endonuclease gene was acquired later, although *K. lactis* can undergo low-level switching without using HO endonuclease. From the evidence collected so far it would seem a functionally homothallic lifestyle evolved from a heterothallic ancestor with no silent cassettes (*C. albicans*) to a heterothallic with silent cassettes and low-level switching (*K. lactis*). This species then acquired the *HO* endonuclease gene, but retained low-level switching (e.g. *K. delphensis*), before switching became more efficient (e.g. *S. cerevisiae*) (Butler 2007; Butler *et al.* 2004; Herskowitz 1989). *Schizosaccharomyces pombe* is also able to switch mating type at a similar rate to *Saccharomyces cerevisiae*, however there is no *HO* endonuclease gene or recognition site found in this species and *S. pombe* uses a replication fork stalling system (section 1.4.2.1) (Nielsen and Egel 2007).

1.4.4 Transcription Factors

Other transcription factors, in addition to *MAT* genes, are also required for correct sexual development in filamentous ascomycete species.

1.4.4.1 VeA

The *veA* gene was first described over 50 years ago by Käfer (1965), and has since been found to affect sexual and asexual reproduction in various species of *Aspergillus* and also *N. crassa* (*ve1*). As shown in Figure 1.2, *veA* is affected by light: red light in *E. nidulans* and blue light in *N. crassa* (via *fphA* and *csnD*). Mutation of *veA* allows asexual conidiation in the dark, whereas conidiation is usually restricted to the light in *E. nidulans* (Bayram *et al.* 2008b; Mooney and Yager 1990). VeA has also been shown to modulate an extracellular signalling factor (FluG), which is necessary for asexual development (Bayram *et al.* 2008b; Lee and Adams 1994; Yager *et al.* 1998). Expression of *veA* has been shown to be highest in the initial stages of reproduction i.e. during mycelial fate determination (Kim *et al.* 2002). Consequently VeA is believed to repress asexual reproduction, via *fluG*, in the absence of red light in *E. nidulans* and presence of blue light in *N. crassa* and also acts to promote sexual reproduction. Mutation of *veA* also results in a block of sexual reproduction in *E. nidulans*, although not via *stuA* or *nsdD* regulation, suggesting VeA is essential for sexual reproduction (Calvo 2008).

1.4.4.2 NsdD and WC

Nsd (never in sexual development) mutants are not able to form sexual structures in *E. nidulans*. Four complementation groups have been identified, these are: *nsdA, nsdB, nsdC* and *nsdD* (Han *et al.* 2001). The most extensively studied of these gene sets, *nsdD*, and has been shown to encode a DNA-binding GATA-type transcription factor with a type IVb zinc finger DNA-binding motif (Han *et al.* 2001). Forced or over expression of *nsdD* in conditions unfavourable to sex results in the induction of the sexual cycle (Han *et al.* 2001). This suggests that NsdD is a positive regulator of the early steps of sexual reproduction and is essential for the process. Over expression of *nsdD* has also been shown to partially or fully complement mutations in *veA* and *nsdB*, suggesting it may have overlapping or downstream roles of these genes (Han *et al.* 2001). Over expression of *nsdD* results in reduced transcription of *nsdD*, suggesting NsdD acts in an auto-regulatory negative feedback loop. Other genes, such as *flbA* (a member of the 'Regulator of G protein Signalling Protein' group)*, nsdA, nsdB* and *veA* also appear to be involved in *nsdD* expression. *nsdD* gene expression is unaffected by low glucose, but was reduced in high salt conditions suggesting a possible role in this sensory pathway (Han *et al.* 2003).

White collar (*WC*) genes 1 and 2 are also believed to encode phototrophin-like GATA-transcription factors possessing zinc finger motifs. WC1 and WC2 are components of the blue light signalling in *Neurospora* species and *wc1* and *wc2* transcription is induced by blue light exposure initiating sexual development unlike *E. nidulans* where red light exposure inhibits sexual reproduction (Bayram *et al.* 2008b; Linden *et al.* 1997).

1.4.4.3 Pro, RosA and NosA

So-called '*pro*' mutants are blocked at the early stages of protoperithecium formation (Figure 1.1), and the Pro proteins are therefore essential for fruiting body formation. Pro proteins are either directly or indirectly involved in the regulation of gene expression during sexual development. Various *pro* genes have been identified in heterothallic and homothallic species, including *N. crassa*, *E. nidulans* and *S. macrospora*.

The *pro1* gene encodes a polypeptide with homology to the DNA-binding domain of a C_6 zinc finger region. Outside the C_6 zinc finger motif homology decreases, but homology is high enough for the heterothallic species, *N. crassa* and *Sordaria brevicolis pro1* genes to

complement a *pro1* defect in *S. macrospora*. Pro1 appears to be essential to promote fruiting body development in the transition from protoperithecia to perithecia (Masloff *et al.* 2002).

A homolog in *E. nidulans* of the *S. macrospora pro1* is the *rosA* gene, but their products serve very different roles in sexual development. The *rosA* gene encodes a $Zn(II)_2Cys_6$ transcription factor that is transiently upregulated during asexual development and carbon starvation. RosA, unlike Pro1, is a negative regulator of sexual development in *E. nidulans* and may act to repress *nsdD, veA* and *stuA* expression (Vienken *et al.* 2005). An additional *pro1* homolog has been detected in *E. nidulans* termed '*nosA*', which encodes another $Zn(II)_2Cys_6$ transcription factor. *nosA* is upregulated during late asexual development and during carbon starvation and repressed by RosA. NosA is required for fruiting body maturation in *E. nidulans* and is believed to act downstream of NsdD (Vienken and Fischer 2006).

The *pro11* gene encodes a protein with similarity to the vertebrate multimodular WD40 repeat proteins striatin and zinedin. The *S. macrospora* Pro11 possesses a putative calmodulin binding site and seems to be involved in Ca^{2+}- and calmodulin-dependent signalling in cells and may function as a scaffolding protein. Pro11 may act to regulate signalling by organising kinases, phosphatases and transcription factors (Pöggeler and Kück 2004).

Finally, Pro41 is an endoplasmic reticulum membrane protein encoded by *pro41*. Homologues of Pro41 have been found in *S. macrospora, M. grisea, N. crassa, E. nidulans* and *N. fumigata*. Transcript levels of *pro41* have been shown to be upregulated during sexual development, possibly via activation by Pro1. Pro41 is essential to sexual development in *S. macrospora* and is involved in Ca^{2+}-dependent signalling and lipid biosynthesis as well as processing of proteins that are secreted, including pheromones (Nowrousian *et al.* 2007; Nowrousian *et al.* 2005).

1.4.4.4 StuA, DopA and MedA

The *stuA* gene encodes a transcription factor affecting sexual morphogenesis e.g. formation of cleistothecia and Hülle cells (Champe *et al.* 1994; Clutterbuck 1969; Dutton *et al.* 1997; Wu and Miller 1997). DopA is important in the spatiotemporal organisation of multicellular structures. It may also have a role upstream in *veA* and *stuA* gene

expression (Pascon and Miller 2000). MedA is a developmental regulator also involved in sexual morphogenesis. It may promote or repress a series of genes involved in the sexual cycle (Busby *et al.* 1996; Clutterbuck 1969).

1.4.5 Environmental Sensing Genes

1.4.5.1 FphA

FphA is a phytochrome, which detects red light. Genes encoding FphA-like proteins have been found in numerous fungal species, such as *E. nidulans, N. fumigata, Giberella moniliformis* and the basidiomycetes *U. maydis* and *Cryptococcus neoformans*. However, this gene has not been found in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. FphA binds flavin chromophore as does WC1. WC1 may act as the blue light receptor in *N. crassa*. Expression of *fphA* causes repression of the sexual cycle (Blumenstein *et al.* 2005; Froehlich *et al.* 2005).

1.4.5.2 PhoA, LsdA, SakA, IndB and IndD

The *phoA* gene is a cyclin-dependent kinase that represses the sexual cycle during phosphorous limitation in *E. nidulans* (Bussink and Osmani 1998). In *E. nidulans* the *lsdA* gene product represses the sexual cycle, in conditions of high salt. It may act directly on *veA*, however this is unproven (Lee *et al.* 2001). The *sakA* gene encodes a mitogen activated protein (MAP) kinase, which represses the sexual cycle during osmotic and oxidative stress. This gene also acts later in the sexual cycle to repress cleisothecial and asci development (Kawasaki *et al.* 2002). The *indB* and *indD* genes are involved in the asexual cycle and their products interact to repress *nsdD* transcription (Kwon *et al.* 2003). However, both these genes are repressed by VeA in *E. nidulans* (Calvo 2008).

1.4.6 Pheromone Genes and Associated Signalling Cascades

In heterothallic fungal species, mating partners are able to attract and grow towards each other, as a result of the production of diffusible chemical hormones termed ‘pheromones’.

The detection of pheromones, and stimulation of the response pathway is a key first step in the sexual cycle, seemingly causing a switch from asexual to sexual reproduction

(Casselton 2002; Hiscock and Kües 1999). Until this point in the sexual cycle, environmental cues alone have stimulated much of the cellular differentiation (see Figure 1.2 and section 1.4). Pheromones are diffusible polypeptides (Bistis 1956; Bistis 1996; Bistis 1998) encoded by pheromone precursor genes, which are secreted by the classical secretory pathways (Pöggeler 2002). The pheromones interact with mating-type specific receptor proteins.

The expression of the pheromone precursor genes appears to be modulated by the *MAT* genes (Coppin *et al.* 1997; Pöggeler *et al.* 2006). It has been shown by Pöggeler *et al*. (2006) that pheromone expression is suppressed in *MAT* deletion strains of *S. macrospora*. However, in these deletion strains, pheromone receptor expression remained unaffected. Interestingly, deletion of the pheromone receptor genes caused down-regulation of the pheromone precursor genes in *S. macrospora*, suggesting that a loss of expression of receptors may repress the expression of pheromone genes (Mayrhofer *et al.* 2006). This observation supports the theory that the pheromone receptors, may be involved in a positive feedback loop via the transcription factor STE12, see Figure 1.2.

In ascomycete species there are two types of pheromone produced during mating, the **a**-factor-like and the α-factor-like (Casselton 2002; Pöggeler 2001). The **a**-factor-like lipopeptide pheromone consists of 20-24 amino acids, containing a hydrophobic CAAX motif at its C-terminus. The **a**-factor is produced by MAT**a** (*S. cerevisiae* and *N. crassa*), MAT1-2 (*C. parasitica*) and MAT+ (*P. anserina*) isolates to attract isolates of the opposite mating type (Banuett 1998; Casselton 2002). The **a**-factor is encoded by *mfa* (*S. cerevisiae* and *N. crassa*), *ppg2* (*S. macrospora*), *mf2/1* and *mf2/2* (*C. parasitica*), *mfp* (*P. anserina*) genes. No **a**-factor precursor encoding gene (*ppgb*) has so far been found in the Aspergilli or *G. zeae* (Dyer *et al.* 2003; Kim *et al.* 2008; Paoletti *et al.* 2005). In contrast, the α-factor-like group of pheromones is a 8-13 amino acid residue hydrophilic protein that results from post-translational proteolytic cleavage of a larger polypeptide by a Kex2 protease. This cleavage results in multiple (2-7) copies of the α-factor being produced by MATα (*S. cerevisiae*), MATA (*N. crassa*), MAT1-1 (*C. parasitica*, and *N. fumigata*) and MAT- (*P. anserina*). The pheromones are encoded by *mfα* (*S. cerevisiae*), *ccg4* (*N. crassa*), *ppg1* (*G. zeae* and *S. macrospora*), *mf1/1* (*C. parasitica*) and *ppgA* (*E. nidulans* and *N. fumigata*) genes. The pheromone precursor genes were first described in *S. cerevisiae* and homologs were then found in other species including homothallic

species (*E. nidulans*, *G. zeae* and *S. macrospora*) and asexual species (Bobrowicz *et al.* 2002; Coppin *et al.* 2005; Dyer and Paoletti 2005; Dyer *et al.* 2003; Galagan *et al.* 2005; Kim *et al.* 2008; Paoletti *et al.* 2005; Paoletti *et al.* 2007; Pöggeler 2000; Pöggeler 2002; Seo *et al.* 2004; Woo *et al.* 2006; Zhang *et al.* 1998). Pheromone precursor genes are important in pheromone production and secretion, however they do not seem to affect vegetative growth or other downstream sexual structures or pathways (Mayrhofer and Pöggeler 2005).

The **a**- and α-factor pheromone genes, like the *MAT* genes themselves, share little sequence homology, which implies that they also do not have a common ancestor (Pöggeler 2002; Pöggeler and Kück 2001). However, the binding regions of the receptors do show structural homology (Banuett 1998).

To initiate mating, the pheromone must bind to a G-protein-coupled pheromone receptor (GPCR) on the cell surface. This receptor is anchored to the cell via seven-transmembrane domains. The third loop is responsible for G-protein coupling, while the carboxy-terminal domain is in the cytoplasm and is believed to mediate pheromone endocytosis and breakdown (Elion 2000; Leberer *et al.* 1997).

Like the pheromones themselves, there are two classes of pheromone receptor that bind one specific type of pheromone. Pheromone receptors were first described in *S. cerevisiae* and have since been found in other ascomycete species. The **a**-factor pheromone binding receptors are encoded by *ste3* (*K. lactis, P. marneffei* and *S. cerevisiae*), *map3* (*S. pombe*), *pre1* (*N. crassa, S. macrospora* and *G. zeae*) and *preA* (*E. nidulans* and *N. fumigata*) genes. The α-factor pheromone binding receptors are encoded by *ste2* (*K. lactis, P. marneffei* and *S. cerevisiae*), *mam2* (*S. pombe*), *pre2* (*N.crassa, S. macrospora* and *G. zeae*), and *preB* (*E. nidulans,* and *N. fumigata*) genes (Bobrowicz *et al.* 2002; Coppin *et al.* 2005; Coria *et al.* 2006; Kim *et al.* 2008; Paoletti *et al.* 2005; Pöggeler 2000; Pöggeler and Kück 2001; Seo *et al.* 2004; Woo *et al.* 2006).

Genome analyses of a variety of fungal species have revealed that the two pheromone precursor and two pheromone receptor genes are all contained within the genome regardless of mating type, unlike the situation for *MAT* genes (section 1.4.1). In many heterothallic filamentous ascomycete and yeast species pheromone precursor and receptor expression is mating type-specific (Bobrowicz *et al.* 2002; Coppin *et al.* 2005; Coria *et al.* 2006; Pöggeler and Kück 2001; Pöggeler *et al.* 2006). However, in several

species, pheromone precursor and, more commonly, pheromone receptor gene expression is mating type-independent. (Paoletti *et al.* 2005; Pöggeler and Kück 2001). Furthermore, in some species, pheromone genes may only be expressed in conditions that favour sexual reproduction such as nitrogen and carbon starvation e.g. *E. nidulans* (Courtice and Ingram 1987; Paoletti *et al.* 2007), whereas in others, the pheromone genes may be constituvely expressed albeit at comparatively low levels e.g. *N. fumigata* (Paoletti *et al.* 2005). Constitutive and mating type-independent expression of the pheromone receptor genes in numerous, not closely related, species suggest possible roles in processes not related to mating (Pöggeler 2001).

Detection of pheromone by the seven-transmembrane-domain GPCR pheromone receptor causes conformational changes in this protein. This catalyses the exchange of GDP for GTP initiating a dissociation-reassociation cycle of the Gα subunit with the Gβγ subunit (Dohlman and Thorner 2001). This leads to free Gβγ, which can associate with the receptor. The Gβγ subunit is now active, and while the Gγ anchors the subunit to the membrane, Gβ binds it to the N-terminus of STE5 activating a specific mitogen activated protein kinase (MAPK) cascade (Figure 1.12) (Coria *et al.* 2006; Gustin *et al.* 1998; Leberer *et al.* 1997). The pheromone MAPK cascade was first discovered in *S. cerevisiae* and has been most extensively studied in this species, although homologs of the genes involved have been found in other yeast species and some filamentous ascomycete species. There are five different MAPK cascades in *S. cerevisiae* involving different MAP kinases (Gustin *et al.* 1998).

STE5 is a MAPK-binding, scaffold protein and acts to keep the MAP kinases in close association (Figure 1.12) (Elion 2000). Numerous genes are involved in this cascade, including a MAP kinase [termed FUS3 (*S. cerevisiae* and *K. lactis*), MpkB (*E. nidulans*), Cek1 (*C. albicans*) and Cpr1 (*C. neoformans*)], a MAP kinase kinase [termed STE7 (*S. cerevisiae*, *C. neoformans*, *K. lactis* and *E. nidulans*) and Hst7 (*C. albicans*)], a MAP kinase kinase kinase [termed STE11 (*S. cerevisiae*), STEC (*E. nidulans*) and Nrc1 (*N. crassa*)], and a MAP kinase kinase kinase kinase [termed STE20 (*S. cerevisiae*, *E. nidulans* and *C. neoformans*) Cst20 (*C. albicans*)]. STE50 is a protein of unknown function, but is required for optimal MAP kinase kinase kinase functioning (Figure 1.12) (Banuett 1998; Coria *et al.* 2006; Elion 2000; Galagan *et al.* 2005; Lengeler *et al.* 2000a; Vallim *et al.* 2000).

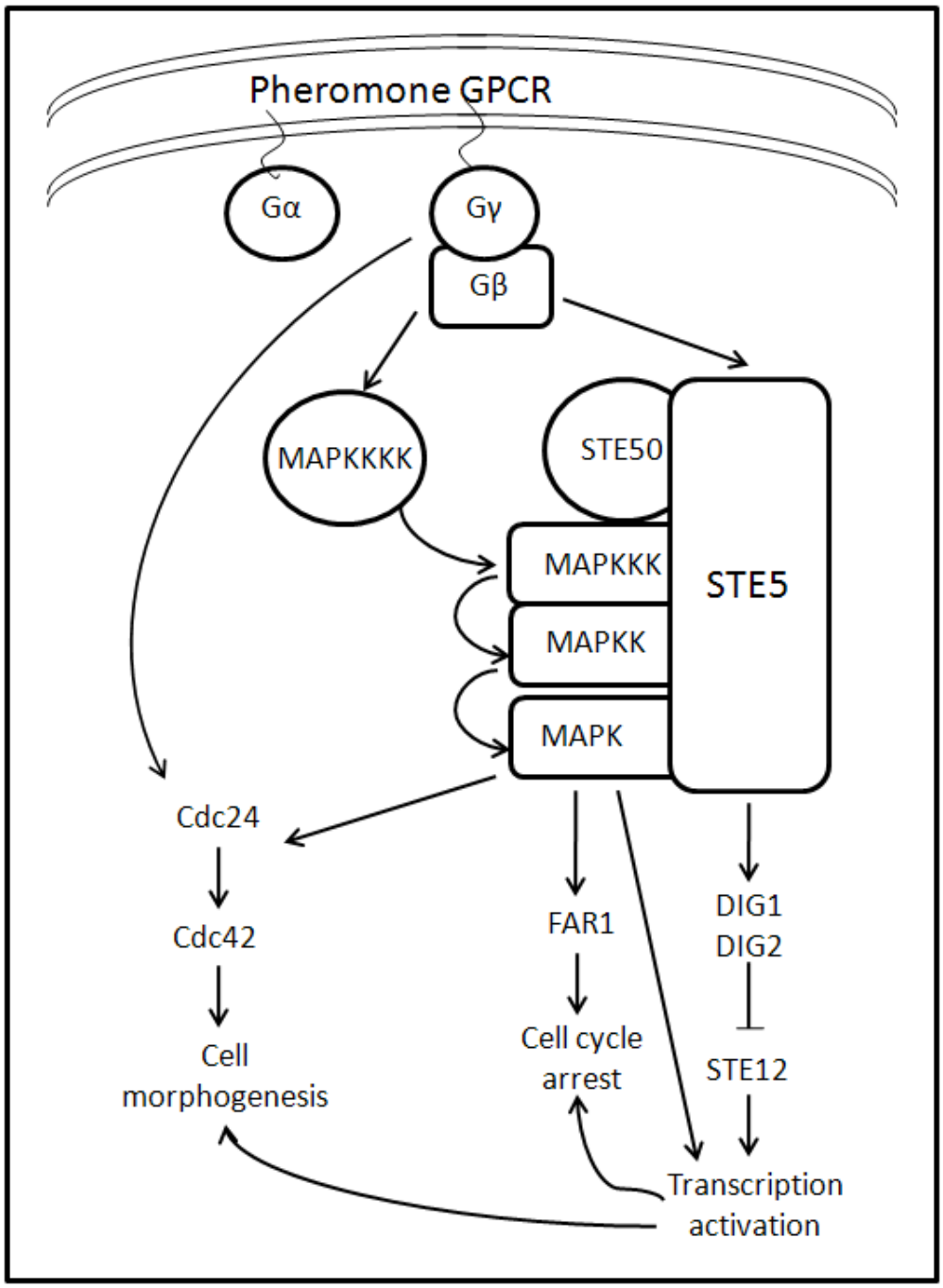


Figure 1.12: Overview of the MAP kinase cascade in the budding yeast species *S. cerevisiae*. [Adapted from Elion (2000), Gustin *et al*. (1998) and Leberer *et al*. (1997).]

The MAP kinases are activated sequentially via phosphorylation, STE50→ STE11→ STE7→ FUS3. STE20 is activated via Gβγ binding and activates STE11 via phosphorylation it also has roles independent of the MAPK cascade (Gustin *et al.* 1998; Pryciak and Huntress 1998). Gβγ recruits four cell morphogenesis proteins, FAR1, Cdc24, Cdc42 and Bem1. FAR1 may bind to Gβ and act as a scaffold for the other three proteins. STE20

associates with these morphogenesis proteins to elicit cell conformational changes i.e. promoting chemotropic growth of projection tips towards the pheromone source (Elion 2000; Gustin *et al.* 1998).

The MAPK, FUS3 activates STE12, DIG1 and DIG2 transcription factor complexes. STE12 [in *S. cerevisiae* and *P. marneffei* (STEA in *E. nidulans*, Cph1 in *C. albicans*)] binds the promoter regions of genes required for mating i.e. *MAT* genes, but can also act upstream to promote expression of pheromone precursor genes, possibly in a positive feedback loop. DIG1 and DIG2 also appear to negatively control STE12 activity (Banuett 1998; Borneman *et al.* 2001; Coria *et al.* 2006; Pryciak and Huntress 1998).

In *C. neoformans* the Gβ subunit acts to activate the MAPK cascade and Gα, encoded by *gpa1*, has no role in pheromone sensing. In *S. pombe gpb1* encodes Gβ, but this is involved in nutrient sensing. Instead Gα plays the active role in mating as well as a Ras1 homolog, which only has a role in cAMP cycling in *S. cerevisiae* (Lengeler *et al.* 2000a).

Detection of pheromone leads to transient arrest of the cell cycle, modulation of transcription (including genes involved in cell and nuclear fusion and conjugation genes) (Bobrowicz *et al.* 2002), and changes in cellular morphology, including the production and polarised growth of trichogynes towards a gradient of pheromone produced by their prospective mating partner as observed in *N. crassa* (Bistis 1956; Bistis 1996; Bistis 1998; Leberer *et al.* 1997; Shiu and Glass 2000).

1.4.7 Fruiting Body Development

Formation of a fruiting body is stimulated by upstream genes as described above. Fruiting body formation also involves many specific genes, some of which are as follows.

1.4.7.1 Ppo Proteins and Psi factors

Homologs of the *ppo* genes have been found in members of the Ascomycota and the Basidiomycota including asexual species, suggesting the feedback loop involving these genes is important for many fungal lineages. However, most studies have focussed on *E. nidulans* and it is to this species that this section mainly refers.

The *ppoA*, *ppoB* and *ppoC* genes encode putative fatty acid oxygenases that contribute to the formation of precocious sexual inducer (Psi) factors which induce premature

sexual sporulation. The Psi factors are hydroxylated oleic acid molecules (Psiβ) or hydroxylated lineolic acid molecules (Psiα). The position of the hydroxy groups denotes the class of factor, PsiB have 8' hydroxy groups, PsiA have 5'8' dihydroxy groups and PsiC have a lactone ring on the 5' of PsiA. The ratio of the Psi factors controls the ratio of asexual to sexual spore production. PsiBα and PsiCα promote sexual development, whereas PsiAα represses sexual development (Calvo *et al.* 2001; Tsitsigiannis *et al.* 2005; Tsitsigiannis *et al.* 2004b).

PpoA is involved in the production of PsiBα. PpoB is involved in the production of PsiBβ, but is not essential, PpoB also has a role in sexual spore production. PpoC is also involved in PsiBβ production and is repressed by PpoA and PpoB (Tsitsigiannis *et al.* 2005).

Expression of *ppoA* is regulated by VeA and the COP9 signalosome (see section 1.4.7.2). Deletion of *ppoB* repressed *ppoA* and upregulated *ppoC*, indicating that PpoB may partly regulate *ppoA* and *ppoC* expression. Strains with deletion of the *ppoB* gene exhibited changes in the ratio of asexual to sexual sporulation presumably due to altered PpoA and PpoC protein levels. This suggests the *ppo* genes are involved in an autoregulatory feedback loops. PpoC acts to repress *nsdD* expression and promotes asexual spore production (Calvo *et al.* 2001; Calvo *et al.* 1999; Champe and el-Zayat 1989; Tsitsigiannis *et al.* 2005; Tsitsigiannis *et al.* 2004a; Tsitsigiannis *et al.* 2004b).

1.4.7.2 COP9 Signalosome

The COP9 signalosome (CSN) is an eight-subunit multiprotein complex. This complex is involved in the maturation of cleistothecia and is essential for later stages of the sexual cycle. The complex is also involved in many other cellular processes including cell cycle progression. The complex has similarity to a photomorphogenesis protein in *Arabidopsis* species and also has metalloprotease activity and is capable of phosphorylating signalling regulators, possibly as part of the pheromone response pathway (Busch *et al.* 2003; Busch *et al.* 2007; Wei and Deng 2003).

1.4.8 Meiosis and Ascospore Production

Certain genes are known to be involved in the production and development of ascospores or meiosis. These include the following.

1.4.8.1 TubB

The *tubB* gene encodes an α-tubulin protein that is not required for early processes in the sexual cycle. A *tubB* deletion strain of *E. nidulans* develops normally until asci form. However, after that point the *ΔtubB* mutants form asci with only one nuclear mass. This means that either, one of the two nuclei was unable to undergo karyogamy earlier or, that karyogamy proceeded but the diploid nucleus was unable to undergo meiosis. Like *veA* and *nsdD*, *tubB* is essential for the completion of the sexual cycle in *E. nidulans* (Kirk and Morris 1991).

1.4.8.2 Cro

Cro1 is a cytosolic protein that has been found in *P. anserina* and *S. cerevisiae* (SHE4). Cro1 is not involved in fertilization. Instead, the *cro1-1* gene is mainly expressed in young crosiers (Figure 1.1) (when cells partition and contain two nuclei) and also when ascospores mature. In *cro1-1* deleted cells, asci are enlarged and multinucleate. This affects perithecial development, with fewer asci being produced. However, even the multinucleate crosiers are able to undergo mitosis synchronously (Berteaux-Lecellier *et al.* 1998; Simonet and Zickler 1978).

1.4.8.3 Meiotic Genes

Over 50 genes are estimated to be required for meiosis in ascomycetes (Galagan *et al.* 2005). These include various genes involved in DNA replication (Borde *et al.* 2000). Once replication has been completed, a DNA double-strand break (DSB) may be formed, this initiates homologous (meiotic) recombination in *S. cerevisiae* via a DNA topoisomerase-II-like enzyme (Spo11). This process involves 9 genes that form a complex, which activates Spo11 by recruiting it onto the DNA, Spo11 then forms a DSB (Sasanuma *et al.* 2008). Three genes (*mre11*, *rad50* and *xrs2*) control Spo11 association with, and translocation along, DNA. This MRX complex is also responsible for bridging the break site, thereby keeping the DNA molecules together so they can be ligated once homologous recombination between chromosomes has been completed (Borde 2007). Homologous recombination is essential during meiosis. It allows chromosomes to find their homologous partner and it also allows physical connections between homologues, which ensures their alignment on the spindle apparatus. Without homologous recombination there could be gene disruption and therefore functional loss which may be lethal to the organism. In *S. cerevisiae* homologous recombination is mediated by

either Rad51 alone or in concert with Dmc1, both these genes have been shown to be essential for meiosis. Rad51, Mre11, Rad50 and Xrs2 are also essential in mitotic recombination (Tsubouchi and Roeder 2006).

1.5 Asexuality in Fungi

By definition, the Ascomycota should only contain species capable of undergoing a sexual cycle and producing asci. However, it has repeatedly been shown that various asexual species have arisen from sexual ancestor species (Chang *et al.* 1991; Geiser *et al.* 1998a; Geiser *et al.* 1996; Guadet *et al.* 1989; LoBuglio *et al.* 1993; LoBuglio and Taylor 1993; Seifert *et al.* 2007; Taylor 1995; Turgeon 1998).

If asexual species arose from sexual lineages, it is therefore not surprising that we find evidence of previous or current sexual-related processes in the analysis of their genomes. One such example was first reported by Selker *et al*. (1987). They described a gene silencing process called repeat induced point (RIP) mutation in *N. crassa* (Selker *et al.* 1987). This is an efficient and irreversible process that occurs during recombination, prior to meiosis, and acts to mutate duplicated genes in a genome. "RIPing" involves the mutation of duplicated DNA sequences, via C:G to T:A transitions in both the original sequence and the inserted sequence, which increases the chances of introducing amber and ochre stop codons. This results in the loss of the original and duplicate sequence and therefore potentially results in non-functional gene products with premature stop codons or mutated or disabled regulator regions (Montiel *et al.* 2006).

While the full genetic pathway of RIPing is currently unknown, one gene has been shown to be essential. This gene encodes a putative cytosine DNA methyltransferase called RID (RIP defective) (Freitag *et al.* 2002). Since the discovery of RIPing in *N. crassa* similar genetic signatures have been found in other species. These include species with known sexual cycles, such as *E. nidulans*, *P. anserina*, *N. fumigata*, and also intriguingly asexual species, such as *A. oryzae*, *A. niger* and *P. chrysogenum*. All these species were also found to have potential RID homologs (Braumann *et al.* 2008b; Clutterbuck 2004; Galagan and Selker 2004; Hamann *et al.* 2000; Monroy and Sheppard 2005; Montiel *et al.* 2006; Neuveglise *et al.* 1996; Nielsen *et al.* 2001). The rate of mutation varies both within and between species i.e. linked sequences are mutated at a much faster rate than unlinked sequences (Selker *et al.* 1987). *N. crassa* has a much higher mutation rate compared to *N. fumigata* although this might reflect the relative rarity of sexual

reproduction in the latter species (Monroy and Sheppard 2005; O'Gorman *et al.* 2009). *A. niger* seems to have been subject to RIPing relatively recently as no RIPed sequences examined so far, in the isolate used for genome sequencing, show degeneration to the point of non-functionality. RIPing in *A. niger* also seems to be strong but localised i.e. a few sequences are affected strongly. This contrasts to the situation in *P. chrysogenum* where the effects of RIPing seems to be milder but more widespread in the genome (Braumann *et al.* 2008b). Evidence of RIPing is further evidence that these species possess, or have recently lost, a sexual cycle (Clutterbuck 2004).

Various possible reasons for the loss of a sexual cycle in a sexual species can be proposed, including the following.

1.5.1 Mutations in Key Genes

Logue *et al.* (2005) showed *Candida parapsilopsis* has a mutation leading to at least two premature stop codons being encoded in the *MAT**α**1* gene. This mutation leads to a non-functional protein and may thereby provide a functional reason for asexuality although other alternative explanations are also possible. However, genome projects have shown that mutation of *MAT* genes or other 'sex-related' genes is not observed in many other asexual species. Examples of genome-wide surveys of genes involved with sexual development include *A. oryzae* (Galagan *et al.* 2005; Machida *et al.* 2005), *A. niger* (Pel *et al.* 2007), *C. albicans* (Tzung *et al.* 2001), *C. glabrata* (Muller *et al.* 2008) and *P. marneffei* (Woo *et al.* 2006). In all these species, only one possible example of mutation of a key sexual developmental gene was found, namely a *pro1* mutation encoding a premature stop codon in *A. niger* (Pel *et al.* 2007). Meanwhile, studies specifically assessing *MAT* gene functionality have been made in *Fusarium avenaceum*, *F. culmorum*, *F. poae*, *F. semitectum* (Kerényi *et al.* 2004), *B. sacchari* (Sharon *et al.* 1996), *Alternaria brassicae*, *A. brassicicola* and *A. tenuissima* (Berbee *et al.* 2003). In all of these species apparently intact, non-mutated *MAT* genes were present and in most of these species expression of *MAT* genes was also shown. The presence of pheromone precursor genes and their expression has also been shown in some of the above species. However, there is estimated to be 200 to 400 genes involved in sexual reproduction (Dyer *et al.* 1992). A mutation in any such essential gene would result in asexuality. Thus, mutation in other, as yet, unidentified genes may be responsible for asexuality. Two hundred mutants have been found in *N. crassa* that disrupt, but not stop, the sexual

cycle. Some of these genes do not appear to be directly involved in the sexual cycle making identifying them in other species difficult. An example of this has been shown in *N. crassa* where mutants with female sterility also have vegetative morphological defects. This may reflect an involvement of common cytoskeletal or signalling proteins for sexual and asexual development, but are not necessary for either e.g. *ppo* genes (Raju 1992; Tsitsigiannis *et al.* 2004a; Tsitsigiannis *et al.* 2004b).

Decline in sexual reproductive ability may also occur via gene mutation and therefore lead eventually to lack of function. Certain isolates of *Candida parapsilopsis* and *Cryptococcus neoformans* have been shown to have defective or malfunctioning *MAT* or pheromone genes, and this phenomenon may be widespread in other species (Logue *et al.* 2005; Nielsen *et al.* 2003). Many asexual species seem to be phylogenetically distributed within teleomorphic genera. These include *Bipolaris* within *Cochliobolus* (Turgeon 1998), *Penicillium* within *Eupenicillium* and *Talaromyces* (Berbee *et al.* 1995; LoBuglio and Taylor 1993), *Cercospora* within *Mycosphaerella* (Greonewald *et al.* 2006) and *Phaeoacremonium* within *Togninia* (Mostert *et al.* 2006). It is therefore possible that multiple, independent, or possibly single gene losses have resulted in a polyphyletic asexual genus arising within a sexual genus.

For many fungal species, the early stages of sexual morphogenesis can occur independent of the *MAT* genes. Most species are able to produce both antheridia (male structures) as well as ascogonia (female structures) (Bistis 1996; Bobrowicz *et al.* 2002; Coppin *et al.* 2005). These species are called hermaphrodites. Some species, both homothallic and heterothallic, are only able to produce antheridia or ascogonia i.e. have maleness and femaleness superimposed onto mating type e.g. *Fusarium* species (Leslie and Summerell 2006). In these heterothallic species there are MAT1-1-female, MAT1-1-male, MAT1-2-female and MAT1-2-male, as well as MAT1-1 and MAT1-2 hermaphrodites. Males and females of opposite mating type must combine for viable sexual reproduction (Leslie and Summerell 2006; Nauta and Hoekstra 1992a). These species seem to be less common, possibly because the antheridia may be conidia, which also act as the asexual spores. Male sterility (loss of antheridia) would limit a species to sexual reproduction only when in close physical proximity to their partner. Inability to produce either sexual structure would render a species asexual in most circumstances.

1.5.2 Lack of Compatible Mating Partners

Another reason for the observed lack of sexual reproduction in some fungal species is that natural populations may not include isolates of both mating types, therefore precluding the possibility of sexual reproduction. Indeed this situation is seen in *Ascochyta rabiei*, where a MAT1-2 isolate is thought to have been recently introduced into Tunisia allowing *Didymella rabiei* to reproduce sexually where previously this was not possible due to lack of mating partners. A clonal population structure is observed for this species as a result, despite its sexuality (Barve *et al.* 2003; Rhaiem *et al.* 2008). A similar situation is seen for *Hypomyces solani* f. sp. *cucurbitae* (anamorph: *Fusarium solani* f. sp. *cucurbitae*) where isolates of opposite mating type have not been found in the same locations globally, but isolates are able to mate when brought together artificially (Snyder *et al.* 1975).

Whilst worldwide analysis of mating-type gene distribution in many fungi has often showed relatively equal numbers of compatible mating types (Greonewald *et al.* 2006; Linde *et al.* 2003; Paoletti *et al.* 2005; Woo *et al.* 2006), there are exceptions. In the basidiomycete *C. neoformans*, 1500 isolates from a worldwide sampling were examined for mating type. The vast majority of isolates were MAT-α, only two were MAT-a. One of the latter isolates could only mate with a subset of MAT-α isolates and the other was sterile due to a defective pheromone precursor gene, making sexual reproduction in the world very rare and thereby explaining the largely clonal population structure (Lengeler *et al.* 2000b; Nielsen *et al.* 2003). Intriguingly, there is evidence of 'same-sex' mating between isolates of *C. gattii* and *C. neoformans* possessing only α mating-types which is a possible reason why the absence of MATa isolates can be tolerated and sexual reproduction still proceed at low rates (Fraser *et al.* 2005; Lin *et al.* 2005).

Analysis of *MAT* gene distribution is further complicated by the sampling size. In the asexual species, *F. cumorum*, *MAT* genes have been shown to be present in a heterothallic-like arrangement and expressed at the mRNA level. However, when *MAT* gene distribution and population structure were examined, a disproportionately high number of MAT1-2 isolates were shown in Europe whereas a 1:1 distribution was seen in the USA. Population genetics showed a clonal structure in Hungary, but evidence of recent past or present recombination elsewhere, despite the bias in *MAT* gene

distribution for this species (Kerényi *et al.* 2004; Lengeler *et al.* 2000b; Nielsen *et al.* 2003; Tóth *et al.* 2004).

1.5.3 Slow Decline in Sexual Reproduction

It has been proposed that a gradual 'slow decline' in expression of genes required for sexual reproduction in populations of fungi may be responsible for a gradual shift towards asexuality as an expression threshold may have to be reached to stimulate and complete the sexual cycle (Dyer and Paoletti 2005). This phenomenon has been shown for *stuA* (Miller *et al.* 1992). Some preliminary data is available, investigating the expression of genes in sexual versus asexual species. However, use of quantitative Reverse Transcriptase (RT) PCR to compare RNA levels is problematic. This is highlighted by a study by Pyrzak *et al*. (2008) who compared *E. nidulans MAT1-1-1* and *MAT1-2-1* gene expression to that of (the then thought to be asexual) *N. fumigata*. The result of this quantitative RT-PCR indicated much lower expression levels in *N. fumigata* compared to *E. nidulans* after 6 days of incubation under conditions favouring *E. nidulans* sexual reproduction and possible mutation in the promoter region was thought to partially explain the supposed asexuality of *N. fumigata* (Pyrzak *et al.* 2008). However, it is now known that the sexual cycle in *N. fumigata* takes up to 6 months (O'Gorman *et al.* 2009), so *N. fumigata MAT* gene expression levels may not be at their peak after 6 days. *C. heterostrophus* does not reproduce sexually in liquid media and it was found that the *MAT* genes were not being transcribed in these conditions in this species, but RNA was detectable in minimal media, as were sexual structures (LeubnerMetzger *et al.* 1997). Unless conditions under which sexual reproduction is known to occur in a particular species are used, no conclusions can be drawn from using quantitative RT-PCR to determine intracellular RNA levels.

1.5.4 Sexual Species Not Yet Discovered

A further reason for asexuality may be that a 'cryptic' sexual stage is present, but has simply been overlooked and not yet discovered. There are some examples where a sexual state has been belatedly discovered. These include the discovery in the late 1980s of a sexual teleomorph state '*Tapesia yallundae*' in the previously assumed asexual anamorph species *Pseudocercospora herpotrichoides*, causal agent of eyespot of cereals.

Apothecia were found to develop only on straw stubble left over winter and had previously remained overlooked (Dyer *et al.* 2001a).

The barley pathogen, *Septoria passerinia* has also been shown to have a rare, cryptic sexual cycle belonging to the *Mycosphaerella* genus (Ware *et al.* 2007).

The teleomorphs of the plant pathogens, *Glomerella acutata* (anamorph: *Colletotrichum acutatum*) and *Glomerella tuncata* (anamorph: *Colletotrichum tuncatum*) were only discovered in 2001 and 2006, respectively (Armstrong-Cho and Banniza 2006; Gueber and Correll 2001).

A similar story of discovery of a sexual state in previously 'asexual' species has been seen in *Togninia minima* (anamorph: *Phaeoacremonium aleophilum*) the cause of Petri disease in grapevines, *Cordyceps bassiana* (anamorph: *Beauveria bassiana*) a species used as a biological control against insects and *Fusarium tucumaniae*, the cause of soybean sudden death syndrome (Covert *et al.* 2007; Huang *et al.* 2002; Mostert *et al.* 2006).

It may also be that the teleomorphic and anamorphic states of a species have already been found separately, but not linked together yet due to taxonomic oversight. This was the case for the important wheat pathogen *Mycosphaerella graminicola* which was first discovered in 1842, but not linked with its anamorph *Septoria trictici* until 1972 (Sanderson 1972). The important industrial cellulase producer *Trichoderma reesei* was not linked to its teleomorph, *Hypocrea jecorina* until the mid 1990s, despite *T. reesei* being used in industry since World War 2 (Druzhinina *et al.* 2006; Kuhls *et al.* 1996). This also occurred for the foodstuff, soil and plant contaminant *Byssochlamys spectabilis* (anamorph: *Paecilomyces variotii*) which was formerly known as *Talaromyces spectabilis*. It was not until the anamorphic and teleomorphic states were connected that the phylogenetic placement and name of *T. spectabilis* could be updated (Houbraken *et al.* 2008).

Another reason for an apparent lack of a sexual cycle could be that suitable conditions have not been found for a particular species. *C. albicans* has been shown to have seemingly intact *MAT* and pheromone genes, and a recombining population structure. Although no sexual cycle has been found in the wild, mating has been induced in the laboratory showing that lack of functional genes is not the reason for a lack of sexual reproduction in the wild (Hull *et al.* 2000; Magee and Magee 2000; Tzung *et al.* 2001).

However, in order to induce mating, isolates had to be genetically manipulated. Hull *et al.* (2000) deleted either the MTL**a** or MTLα regions, the isolates were then injected into mice and sexual structures formed. Magee and Magee (2000) made isolates homozygous by chromosome loss, so that only one mating-type locus was present in the diploid isolate. The isolates were then incubated at 30°C, which induces a white to opaque morphological change, this increased the mating frequency by up to 1000-fold (Hull *et al.* 2000; Magee and Magee 2000; Magee and Magee 2004). It has been suggested that certain host niches such as skin provide the environmental conditions necessary to inducing mating in *C. albicans* (Magee and Magee 2004).

1.5.5 Benefits of Asexual Reproduction Out-Weigh Costs

As discussed earlier (section 1.2), there may be benefits to a purely asexual reproductive lifestyle under certain environmental conditions because asexual reproduction requires less metabolic energy and maintains combinations of favourable genes due to clonality. Instead, asexual species might retain the ability to generate variation and undergo recombination through the parasexual cycle.

It has been suggested that in saprophytic species where the environment changes very quickly it might be of short-term benefit to effect recombination via the parasexual cycle (Jinks 1952b). The parasexual cycle has the benefit of providing recombination (albeit at a very low rate) without having to go through the temporally longer cycle of producing sexual structures, fertilization, meiosis etc. Whilst the fusion of nuclei is a rare event it has been shown that nuclei within the same cytoplasm can cooperate as a heterokaryon and allow the organism to survive in conditions where the parental homokaryons cannot. However, the parasexual cycle is only beneficial in the short-term as fusion is limited to isolates of the same vegetative compatibility group, and therefore leads to isolation from individuals differing in at least one HET locus (Burnett 2003; Jinks 1952b).

A variety of genes encoding proteins which contain HET domains have been found during genome sequencing of various *Aspergillus* species, *P. chrysogenum* as well as *N. crassa* and *P. anserina* genomes (Debeaupuis *et al.* 1997; Fedorova *et al.* 2005; Pontecorvo 1953; Xiang and Glass 2002). These genes may be HET loci themselves or other genes involved in vegetative incompatibility. Genome sequencing has identified ~50 proteins with HET domains from *N. crassa*, *N. fumigata, E. nidulans* and *P. anserina* all initiate vegetative incompatibility by interactions between allelic HET domain

proteins. In *P. anserina* and *N. crassa* two loci may interact to mediate non-allelic vegetative incompatibility. In *P. anserina*, *het-r* with *het-v* and *het-e* with *het-c* can do this, while in *N. crassa het-c* with *pin-c* and *un-24* with *het-6* are non-allelic *het* genes. In *N. crassa*, *MATA-1* and *MATa-1* can also act as allelic *het* genes. Homologs of these genes have been found in asexual and sexual Aspergilli. Pál *et al*. (2007) investigated the number of homologs of these, and other HET domain encoding genes, in *Aspergillus* species. BLAST searches focussed specifically on homologs of one incompatibility gene (*het-6*), one suppressor gene (*tol*) and an incompatibility related gene (*pin-c*) which is linked to *het-c*. It was found that homologs of a modifer gene *modA* which affects *het* gene interaction is not present in most Aspergilli, except *A. oryzae* where a sequence with low homology to *modA* was found. Significantly asexual *Aspergillus* species were found to have more homologs of the *het-6*, *tol* and *pin-c* genes than sexual species. This may reflect a need for these asexual species to increase genetic variation as this cannot be achieved via sexual reproduction. The parasexual cycle in *A. niger* and *P. anserina* which has large numbers of homologs for the aforementioned three genes have been shown to decrease the spread of mycoviruses as lineages become isolated. *E. nidulans* can remove such viruses via the production of sexual progeny, but *A. niger* lacks this ability and it may be too metabolically energy expensive for *P. anserina* to sexually reproduce in certain environments (Coenen *et al.* 1997; Fedorova *et al.* 2005; Galagan *et al.* 2003; Galagan *et al.* 2005; Glass and Dementhon 2006; Glass *et al.* 2000; Pál *et al.* 2007; Paoletti and Clave 2007; van Diepeningen *et al.* 1997).

1.5.6 Strain Improvement

As described, approximately 20% of all fungal species, and some 40% of species placed phylogenetically within the Ascomycota, are only known to reproduce by asexual means. However, it would be of great significance if it were possible to discover sexual cycles in some of these supposedly asexual species. This would provide insights into the population biology of these species, with the knowledge that in heterothallic species there would be the possibility to generate increased variation through recombination. This would allow the species to respond to changing conditions and evolve, which is highly significant in pathogenic fungi (Dyer and Paoletti 2005). Secondly, the sexual cycle would provide a valuable experimental tool to perform classical genetic crosses, thereby allowing experimenters to determine the genetic basis of traits of interests i.e. whether mono- or polygenic resistance. Finally, the sexual cycle could be used for strain

improvement of species of industrial or biotechnological importance. Thus, crosses could be performed and ascospore progeny selected showing an increase (or decrease) in the phenotype of interest. Thus, genetic manipulation and investigation of species via the sexual cycle, is much easier and faster than with asexual species. For example, the discovery of a sexual cycle in the cause of eyespot of cereals, *T. yallundae* has allowed the assessment of the risk of this species to develop fungicide resistance and advise disease management strategies. This was achieved by monitoring the development of the sexual cycle in the field (Dyer *et al.* 2001a) and by using the sexual cycle to determine the genetic basis of resistance to triazole fungicides (Dyer *et al.* 2000).

Another example is the previously mentioned wheat pathogen *M. graminicola*, in which the sexual cycle has been used to advance genetic identification of, and investigation into, resistance genes in wheat (Brading *et al.* 2002; Sanderson 1972).

It has been shown that *MAT* genes can be swapped between species and that *MAT* genes can successfully restore a functional sexual cycle (Große and Krappmann 2008; Pyrzak *et al.* 2008; Sharon *et al.* 1996). It may therefore be possible to make an asexual species either homothallic or heterothallic, a homothallic species heterothallic or vice versa. Indeed, *MAT* genes have been deleted from the homothallic species *G. zeae* and *E. nidulans* (Figure 1.7), resulting in the production of heterothallic isolates that could be successfully outcrossed (Lee *et al.* 2003; Paoletti *et al.* 2007). This offers the exciting possibility of allowing gene recombination and strain improvement to proceed in species that were limited in this ability due to lack of a sexual cycle, or a sexual cycle that was difficult to induce in the laboratory (Arnaise *et al.* 2001).

1.6 *Aspergillus* and *Penicillium* Species

The overall aim of this thesis is to investigate the evolution and genetic basis of asexuality in *Aspergillus* and *Penicillium* species. These species have been selected due to recent breakthroughs in the genomic sequencing and cloning of *MAT* genes from these species, together with their economic importance (Archer and Dyer 2004).

1.6.1 The Genus *Aspergillus*

The genus *Aspergillus* contains over 250 species (Geiser *et al.* 2007). This includes over 70 teleomorphic species, split into 11 genera with asexual species dispersed throughout (Figure 1.13) (Frisvad and Samson 2000; Gams 1993; Houbraken *et al.* 2008; Seifert and

Lévesque 2004; Udagawa and Uchiyama 2002). The vast majority of these teleomorphs are homothallic, with only 8 being heterothallic. Five of the heterothallic species belong to the genus *Neosartorya* [*N. fennelliae* (Kwon and Kim 1974), *N. spathulata* (Takada and Udagawa 1985) *N. udagawae* (Horie *et al.* 1995), *N. nishimurae* (Takada *et al.* 2001) and *N. fumigata* (O'Gorman *et al.* 2009)] the one species is in the genus *Emericella*, *E. heterothallica* (Kwon *et al.* 1964; Samson *et al.* 2007), and two species are in the genus *Petromyces* (Horn *et al.* 2009a; Horn *et al.* 2009b).

The genus *Aspergillus* is characterised by the production of unbranched, aseptate conidiophores with a swollen apex (vesicle), covered by a layer of sporulating cells (phialides or sterigmata) on which the conidia are borne. This structure is known as an aspergillum (Figure 4.1) (Raper and Fennell 1965).

Raper and Fennell (1965) divided this genus into 'groups', these were later formalised and split into subgenera and sections and linked with their related teleomorphic genera (Gams 1993). These divisions were primarily based on conidia colour and shape of vesicles, but have been largely confirmed with molecular data (Chang *et al.* 1991; Geiser *et al.* 1998a; Seifert and Lévesque 2004).

This genus contains many important species, including the opportunistic human pathogen *N. fumigata* (section *Fumigati*), and also the widely studied laboratory model species *E. nidulans* (Kontoyiannis and Bodey 2002; Latgé 1999; Pontecorvo 1953; Todd *et al.* 2007). *A. niger* (section *Nigri*) is a member of a the so-called 'black Aspergilli' and is widely used in biotechnology to produce food ingredients, pharmaceuticals and industrial enzymes e.g. citric acid (Pel *et al.* 2007). *A. flavus* (section *Flavi*) is an important plant and crop contaminant and aflatoxin producer (Accinelli *et al.* 2008). The closely related species *A. oryzae* (section *Flavi*) does not produce aflatoxins and has been used for over 4000 years to produce soy sauce and sake (Abe and Gomi 2008). *A. terreus* (section *Terrei*) is an emerging opportunistic pathogen causing invasive aspergillosis in immunocompromised patients, and is hard to treat due to its resistance to amphotericin B, the fungicide of choice (Hinrikson *et al.* 2005; Kontoyiannis and Lewis 2002; Sutton *et al.* 1999). *A. terreus* also produces lovastatin, a commercially used cholesterol-lowering drug, as well as other therapeutic agents (Alberts *et al.* 1980; Kennedy *et al.* 1999; Manzoni and Rollini 2002). *A. clavatus* (section *Clavati*) is an important crop contaminant and is responsible for an allergic reaction in malt workers and farmers (Blyth 1978; Grant *et al.* 1976; Latgé 1999). This species also produces

ribotoxins, which are being developed as immunotherapy tools (Carreras-Sangra *et al.* 2008; Martínez-Ruiz *et al.* 1999).

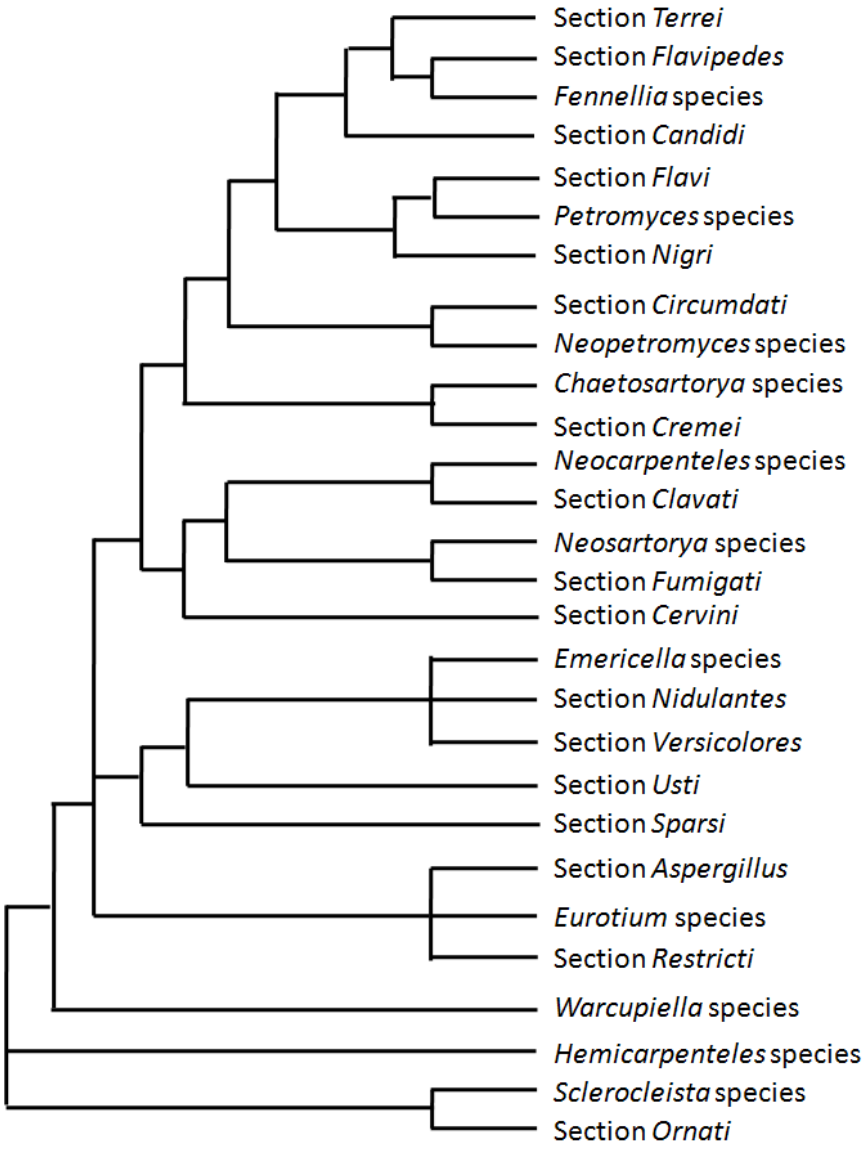


Figure 1.13: Phylogeny of the genus *Aspergillus*, with its 11 associated teleomorphic genera. [Adapted from Frisvad and Samson (2000), Peterson (2008), Samson *et al*. (2007), Tamura *et al*. (2000), Udagawa and Uchiyama (2002) and Varga *et al*. (2000a).]

All of the aforementioned *Aspergillus* species have had their genome sequenced, and all (except *E. nidulans* and *N. fischeri*) appear to have heterothallic-like *MAT* gene arrangements. This is surprising given that previously only six species are thought to be heterothallic in this genus, with *N. fumigata* being recently added to this list (O'Gorman *et al.* 2009). The sequencing of *A. flavus* has allowed the identification of *MAT* genes in

the closely related species *A. parasiticus* (section *Flavi*) and *P. alliaceus* and their *MAT* gene arrangements to be ascertained (Ramirez-Prado *et al.* 2008).

The genome sequencing of the Aspergilli has been undertaken by different institutions. The Institute of Genome Research (TIGR) sequenced *N. fumigata* (joint effort with The Sanger Centre and The Institut Pasteur), *N. fischeri*, *A. clavatus* and *A. flavus* (Nierman *et al.* 2005; Payne *et al.* 2008). The Broad Institute sequenced *E. nidulans* and *A. terreus* (Galagan *et al.* 2005). The Joint Genome Institute sequenced *A. niger* (Pel *et al.* 2007), while *A. oryzae* was a joint effort between the *A. oryzae* Consortium, the National Institute of Technology and Evaluation and the National Institute of Advanced Industrial Science and Technology (Machida *et al.* 2005). The publication of these genomes has allowed investigation into the evolutionary origins of these species, including the discovery that *A. oryzae, A. flavus* and *A. niger* have genomes 20% larger than the other Aspergilli sequenced. No evidence of genome or chromosome duplication was found and the origin(s) of these extra sequences remains elusive (Galagan *et al.* 2005; Khaldi and Wolfe 2008; Machida *et al.* 2005). Genome sequencing has allowed mating-type genes and other genes involved in the sexual cycle to be identified in these species. Genes involved in vegetative incompatibility suggested to have roles in mycovirus evasion, have also been found by *in silico* investigations (Galagan *et al.* 2005; Morita *et al.* 2007; Pál *et al.* 2007; Ramirez-Prado *et al.* 2008). Homologs of proteins involved in metabolic and cell developmental pathways were also identified in other species as a result of genome sequencing (Banuett *et al.* 2008; Braumann *et al.* 2008a; Maruyama and Kitamoto 2008; Zhang *et al.* 2008). Genome-wide expression profiling has also been performed to assess transcriptional changes under varying environmental conditions in *N. fumigata* (Sugui *et al.* 2008).

The sexual cycle may help to elucidate important differences between very closely related species. For example, phylogenetic analyses have shown *A. flavus* and *A. oryzae* to be almost identical and they also share very similar morphologies making their distinction difficult. Their classification as the same species or sister species is still controversial. As the former produces aflatoxins and is detrimental to human health, but the latter is used in various foodstuffs and does not produce aflatoxins. Investigation into a possible sexual cycle in *A. flavus* may help to control its aflatoxin production and have agricultural implications (Ramirez-Prado *et al.* 2008).

Aspergillus clavatus has been shown to be a sister species of *Neocarpenteles acanthosporum* (anamorphs: *A. acanthosporus*). This sexual species is phylogenetically distinct from the *Neosartorya* genus, which is also closely related to *A. clavatus*. *A. clavatus* and *N. acanthosporum*, also form the basal group to the *N. fischeri* and *N. fumigata* clade (Figure 1.13). Knowledge of the mode of sexual reproduction in *A. clavatus* may help evolutionary studies into the sexual history of *N. fumigata*, and possibly provide insights into why it is heterothallic when a sister species, *N. fischeri*, is homothallic (Peterson 2000b; Tamura *et al.* 2000; Udagawa and Uchiyama 2002).

The genus is also interesting as three homothallic species with *Aspergillus* anamorphs, *E. nidulans, N. fischeri* and *P. alliaceus*, have very different genome structures at their MAT loci (Figure 1.5) (Paoletti *et al.* 2007; Ramirez-Prado *et al.* 2008; Rydholm *et al.* 2007). The varying MAT locus arrangements seen in homothallic Aspergilli is intriguing given the comparative rarity of heterothallic Aspergilli but the apparent abundance of asexual species possessing MAT idiomorphs in a heterothallic-like arrangement. Further investigation into this genus may help elucidate the evolutionary ancestry of sexual strategy and also the origins of the *MAT* genes themselves.

All these factors have made the further study of sexual potential, sexual strategy and MAT idiomorph arrangements of *Aspergillus* species and their teleomorphs timely and very intriguing.

1.6.2 The Genus *Penicillium*

The genus *Penicillium* is a sister genus to *Aspergillus*. From a molecular genetic perspective, the genus *Penicillium* has been studied to a lesser extent than *Aspergillus*. Their close phylogenetic relationship now makes more detailed experimental and genomic studies feasible.

The genus *Penicillium* is paraphyletic and consists of over 220 species, although new species are being discovered regularly, many of which are important in the medical, biotechnology or food industry sectors (Peterson and Horn 2009; Pitt *et al.* 2000).

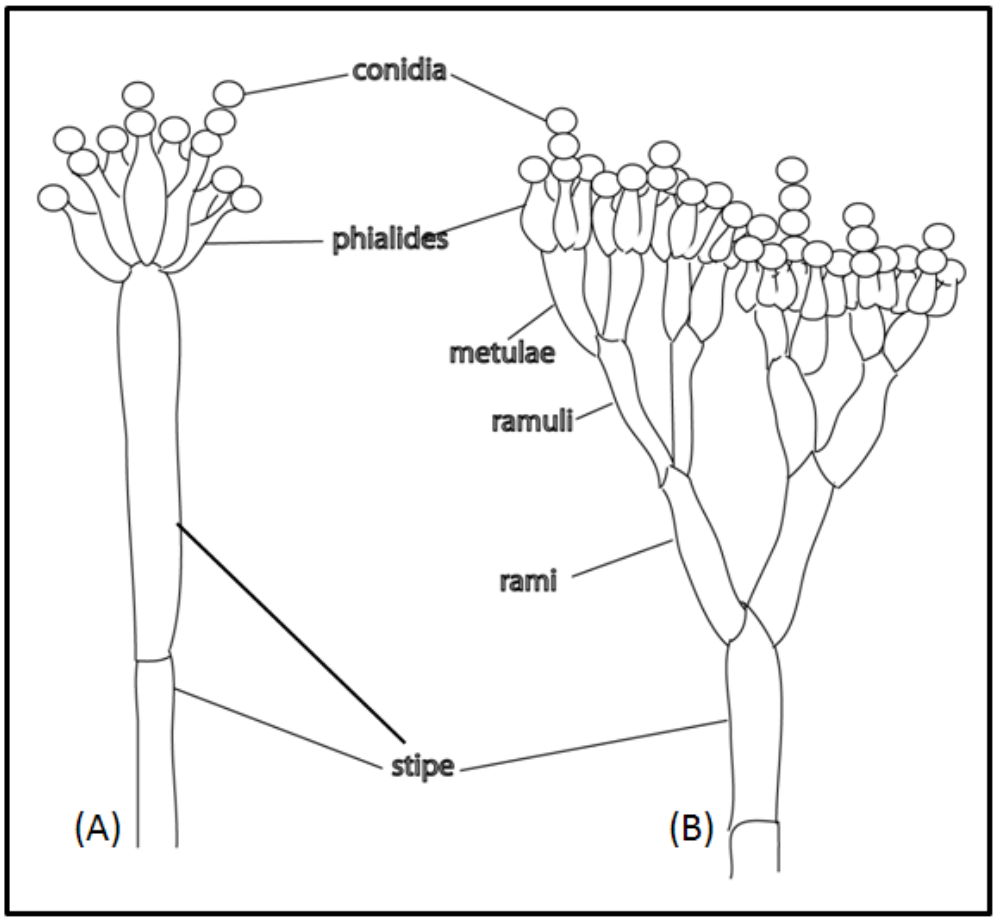


Figure 1.14: Structure of a penicillus. (A) is a monoverticillate penicillus, (B) is a quarterverticillate penicillus. [Adapted from Pitt (1979) and Raper and Thom (1949).]

This genus is characterised by the production of branched, septate conidiophores, with sporulating cells (phialides or sterigmata) clustered at the ends on which the conidia are located, this structure is known as a penicillus (Figure 1.14). Most *Penicillium* species produce green conidia so, unlike *Aspergillus* species, this morphological feature can not be used to divide the genus. Instead, the number of branch points within the penicillus was traditionally used, see below for a full explanation.

Penicillus morphology is based on the number of branch points between the stipe and the phialide e.g. Figure 1.14 (A) illustrates a monoverticillate species (one branch), (B) illustrates a quarterverticillate species (four branches). If the second branch is a metulae, the species is generally regarded as biverticillate i.e. did not include metulae to phialide branch, although Samson *et al*. (1976) referred to species with three branch points as biverticillate. The Raper and Thom (1949) and Pitt (1979) initial classification will be used throughout this thesis.

It has been suggested that evolution is towards a more complex penicillus structure, and ecologically away from a soil habitat towards free-living saproby. If this is correct then the subgenus *Furcatum* seems the simplest, least evolved anamorphic state of the genus *Penicillium*, which would agree with the phylogenetic data outlined below (Pitt 1993).

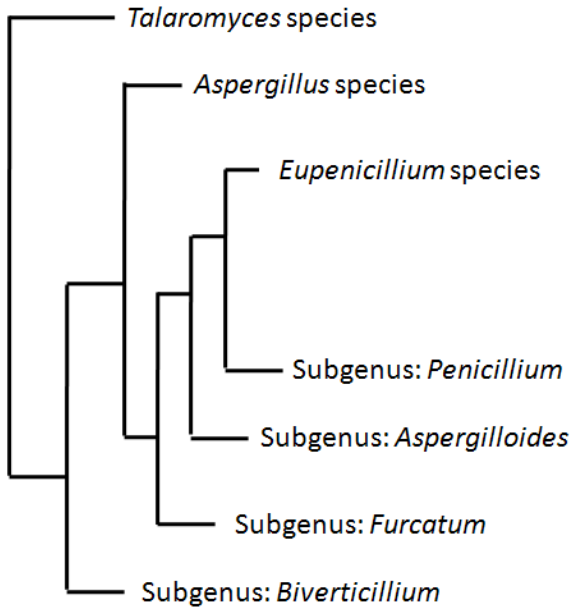


Figure 1.15: Phylogeny of the genera *Penicillium*, *Eupenicillium*, *Talaromyces* and *Aspergillus*. [Adapted from Peterson (1993).]

The genus *Penicillium* is divided into four subgenera: *Furcatum*, *Aspergilloides* and *Penicillium* forming a monophyletic clade, which group with the teleomorphic genus *Eupenicillium*. The fourth subgenus *Biverticillium* is paraphyletic to the three other subgenera and groups with the teleomorphic genus *Talaromyces* (Figure 1.15). Initial phylogeny of the four subgenera was based on penicillus morphology and is in general agreement with molecular data now available (Peterson 1993).

The subgenus *Aspergilloides* contains mostly monoverticillate species, however some species that produce both monoverticillate and biverticillate penicilli are included (Pitt 1979).

The subgenus *Furcatum* contains monoverticillate or biverticillate species, however these are generally irregular in shape when compared to subgenera *Biverticillium* or *Aspergilloides* (Pitt 1979).

The subgenus *Biverticillium* produces biverticillate penicilli, and contains the important opportunistic human pathogen *P. marneffei*, causing fatal infections particularly in SE Asia (Disalvo *et al.* 1973). The subgenus *Biverticillium* (and its related *Talaromyces* teleomorphs) appear to have split earliest from the *Aspergillus* (and related teleomorphs). ITS-5.8S rDNA sequencing has revealed that *Talaromyces* species seem to be more closely related to *Byssochlamys* species (with *Paecilomyces* anamorphs) than to *Aspergillus* teleomorphs or *Eupenicillium* species (Berbee *et al.* 1995; Pitt 1995). *Talaromyces* species do not produce cleistothecia, unlike *Eupenicillium* and most *Aspergillus* teleomorphs. Instead, *Talaromyces* species produce relatively simple 'gymnothecia' as their sexual structures, which consist of tightly woven hyphae surrounding the asci (Benjamin 1955). *Eupenicillium* species and the remaining three *Penicillium* subgenera form a monophyletic clade, which split from *Biverticillium* and *Talaromyces* species approximately 67 ± 21 million years ago (Berbee and Taylor 1993; Berbee *et al.* 1995; Peterson 2008) (based on the origin of the phylum Ascomycota being 400 ± 125 million years ago). From the remaining five groups, the genus *Aspergillus* (and related teleomorphs) is believed to be the earliest to diverge, followed by the subgenus *Furcatum* and then *Aspergilloides* (Figure 1.15) (Berbee and Taylor 1993; LoBuglio and Taylor 1993; Peterson 1993; Pitt 1993).

The subgenus *Penicillium* is also known as the terverticillate and (less commonly) quarterverticillate penicilli and this subgenus contains important medical and biotechnological species as well as species used in food manufacture. Examples include, *P. chrysogenum* (producer of the antibiotic penicillin), *P. griseofulvum* (producer of the antibiotic griseofulvin), *P. roqueforti* and *P. camemberti* (species used in the production of Roquefort and Camembert cheeses, respectively) (De Carli and Larizza 1988; Pitt 1979; Raper *et al.* 1944; Raper and Thom 1949).

Of the approximately 100 teleomorphic species that are closely related to *Penicillium* species, almost all of them exhibit homothallic, self-fertile, sexual breeding systems (Pitt *et al.* 2000). Currently only one heterothallic species is known, which is placed phylogenetically within the genus *Penicillium*. This species is *Talaromyces derxii* (anamorph: *Penicillium derxii* – subgenus *Biverticillium*) (Takada and Udagawa 1988). To date there are no known heterothallic *Eupenicillium* species with anamorphs in the subgenus *Penicillium*.

Mating-type genes, as well as pheromone receptor genes, and genes encoding MAPK cascade proteins, have been found in *P. marneffei*. No significant divergence or obvious mutation was discovered for the genes (or the proteins they encode). However, no pheromone precursor genes were found (Woo *et al.* 2006). A public sequencing project of *P. marneffei* by the National Center for Biotechnology Information (NCBI) is now underway, which may reveal the pheromone precursor genes in this species.

1.7 Aims of Thesis

The overall aim of this thesis is to gain insights into the evolution of reproductive strategy (sexuality and asexuality) within the *Aspergillus* and *Penicillium* taxa. This will be achieved by studying the following three main areas.

1. Investigation of evolution of sexual strategy in the Aspergilli. It is currently unclear whether the ancestral strategy of sexual reproduction in the Aspergilli is heterothallism or homothallism. This is in itself a fundamentally interesting question. But it is also anticipated that knowledge of this area will provide insights into the possible genetic reasons for asexuality in the Aspergilli. The evolution of reproductive strategy will be investigated with particular focus on *MAT* gene presence, organisation and function although other 'sex-related' genes will also be investigated in the Aspergilli.
2. Investigation of potential sexuality in the Penicillia. Representative 'asexual' *Penicillium* species will be assessed for the presence and distribution of *MAT* genes, as a guide to whether they might have the potential for sexual reproduction or whether some other genetic factor causing asexuality can be identified.
3. Investigation of whether sexuality can be induced under laboratory conditions for supposed 'asexual' species, given the identification of isolates of compatible mating type and the discoveries of sexual teleomorphs in other species once thought to reproduce solely by asexual means.

Chapter 2 Materials and Methods

Some materials and methods are specific to individual chapters and these will be described in the chapters. However, there are some general materials and experimental methods that were used throughout the study, these are as follows.

2.1 Materials

All growth media were sterilised by autoclaving at 117°C for 30 mins.

Malt Extract Agar (MEA) (for 1L)

20g Malt Extract powder (Sigma, UK), 1g Peptone (Oxoid, UK).
20g Agar (Oxoid, UK) made up to 1L with distilled water.

Malt Extract Media (MEM) (for 1L)

20g Malt Extract powder (Sigma, UK), 1g Peptone (Oxoid, UK) made up to 1L with distilled water.

Oatmeal Agar (OA) (for 1L)

40g Pinhead Porridge Oats simmered for 1 hour, strained through miracloth (Merck Chemicals Ltd., UK).
20g Agar (Oxoid) added after simmering, made up to 1L with distilled water.

Aspergillus Complete Media (ACM) (for 1L)

10g Glucose powder (Sigma, UK), 1g Yeast Extract powder (Oxoid, UK), 2g Peptone (Oxoid, UK), 1g Casamino acids (Sigma, UK), 0.075g Adenine (Sigma, UK), 10ml *Aspergillus* vitamin solution and 20ml *Aspergillus* salt solution.
20g Agar (Oxoid), adjusted to pH6.5 and made up to 1L using distilled water.

Aspergillus Vitamin Solution (for 1L)

400mg p-Aminobenzoic Acid, 50mg Thiamine HCl (Sigma, UK), 2mg d-Biotin (Sigma, UK), 100mg Nicotinic Acid (Sigma, UK), 250mg Pyridoxine Hydrochloride (Sigma, UK), 1.4g Choline Chloride (Sigma, UK) and 100mg Riboflavin (Sigma, UK) made up to 1L with distilled water.

Aspergillus Salt Solution (for 1L)

26g Potassium Chloride (VWR International, UK), 26g Magnesium Sulphate (Fisher, UK), 76g Potassium diHydrogen Phosphate (Fisher, UK) and 10ml *Aspergillus* Trace Elements Solution, made up to 1L with distilled water.

Aspergillus Trace Elements Soluton (for 1L)

40mg Sodium Tetraborate Decahydrate (VWR International, UK), 800mg Copper Sulphate Pentahydrate (Fisher, UK), 800mg Ferric Orthophosphate Monohydrate (Sigma, UK), 800mg Manganese Sulphate Tetrahydrate (Fisher, UK), 800mg Sodium Molybdate Dihydrate (Fisher, UK) and 8g Zinc Sulphate (Fisher, UK), made up to 1L with distilled water.

Luria Bertani Agar (LBA) (for 1L)

25g LB broth powder (Melford, UK).
15g Agar (Sigma, UK), adjusted to pH7 with NaOH and made up to 1L with distilled water.

Luria Bertani Broth (LBB) (for 1L)

25g LB broth powder (Melford, UK), adjusted to pH7 with NaOH and made up to 1L with distilled water.

Ampicillin Solution (Antibiotic) (for 1ml)

100mg ampicillin powder (Sigma, UK).
The 100mg/ml ampicillin stock was filter sterilised through a 0.2µm filter and stored at -20°C, until needed.

Loading Buffer (for 1ml)

25% w/v bromophenol blue, 25% w/v xylene cyanol, 40% w/v sucrose and made up to 1ml with distilled water.

Tris-Borate EDTA (TBE) (for 1L 10X buffer)

108g Tris (VWR International, UK), 55g Boric Acid (Fisher, UK), 9.3g EDTA and made up to 1L with distilled water. 10X buffer was stored at 28°C until needed, this was then diluted 1 in 10 to make a 1X working buffer solution.

2.2 Methods

2.2.1 Culture Growth on Solid Media

Cultures were grown on 9 cm Malt Extract Agar (MEA) Petri plates using either a swab to transfer mycelia from a pre-existing culture, silica stock or liquid nitrogen stock for inoculums as appropriate. MEA plates were grown in the light for one week at 28°C after which plates were sealed with parafilm, and incubated in the light at 28°C for a further week. MEA plates were then stored at 4°C.

2.2.2 Culture Growth in Liquid Media

Two mycelial plugs of approximately 0.5 cm in diameter were added to 250ml conical flasks containing 50ml of Malt Extract Media (MEM). Flasks were grown in the light at 28°C with shaking (200rpm) for 5 days, after which media was strained through miracloth and freeze-dried overnight, lyophilised samples were then stored at -80°C until DNA extraction was performed.

2.2.3 DNA Extraction

DNA was extracted using the DNeasy Plant Mini kit (Qiagen, UK) according to the manufacturer's instructions, as follows. Fungal mycelia were ground under liquid nitrogen, 20mg of ground material was resuspended in 404µl of buffer AP1 [including 4µl RNase A stock solution (100mg/ml)]. The resuspension was incubated at 65°C for 10 mins and was inverted every 2-3 mins during incubation. One hundred and thirty µl of buffer AP2 was added to the lysate, this was mixed by tube inversion and incubated on ice for 5 mins. Lysate was centrifuged at 13,000g for 5 mins. The supernatant was transferred to a QIAshredder mini spin column, placed in a 2ml collection tube, and centrifuged at 13,000g for 2 mins. The supernatant was transferred to a clean 1.5ml Eppendorf tube, 1.5 volumes of buffer AP3/E added and mixed by pipetting. Six hundred and fifty µl of solution was added to a DNeasy mini spin column and centrifuged at 8,000g for 1 min, flow-through was discarded. This step was repeated with any remaining mixture. The DNeasy column was transferred to a fresh 2ml collection tube, 150µl of buffer AW added and centrifuged at 8,000g for 1 min, flow-through was discarded. Five hundred µl of buffer AW was added to the column and centrifuged at 13,000g for 2 minutes. To elute, the DNeasy column was transferred to a new 1.5ml Eppendorf tube and 50µl of buffer AE added to the membrane. DNeasy column was

incubated at room temperature for 5 mins, then centrifuged at 8,000g for 1 min to yield a DNA suspension.

2.2.4 Amplification of *MAT, SLA2* and *APN2* Genomic Regions

Multiple alignments of previously identified MAT, SLA2 and APN2 proteins from the Aspergilli revealed the presence of conserved sequence within these proteins. This allowed degenerate or specific primers to be designed (Table 2.1) and a PCR strategy to be adopted to amplify particular regions of these genes.

Amplification of the *MAT1-1-1* alpha-domain, *MAT1-2-1* HMG-domain, and fragments of the *MAT1-2-4, SLA2* and *APN2* genes were performed using FastStart DNA Polymerase (Roche, UK) in PCRs with certain common reaction components, and involved the use of gene-specific degenerate primers (Table 2.1). Each 25µl reaction contained ~20ng genomic DNA, 2.5µl 10X PCR Buffer, 2µl (20mM) $MgCl_2$, 0.5µl (10mM each) dNTPs, 5µl (10µM or 20µM) degenerate/specific forward primer, 5µl (10µM or 20µM) degenerate/specific reverse primer, 0.2µl Taq Polymerase and 8.8µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: an initial denaturation step at 94°C for 5 min; 38 cycles consisting of 1 min at 94°C, 1 min at an appropriate annealing temperature and 30 seconds at 72°C; followed by a final extension step at 72°C for 5 min, all steps used a ramp rate of 60°C/min.

However, different conditions were used for specific PCRs. Thus, to amplify the putative *MAT1-1-1* alpha-domain region primer pair MAT5-6 (20µM) and MAT3-4 (20µM) was used (Table 2.1), at an annealing temperature of 50.8°C and predicted to amplify a 150bp region. To amplify the putative *MAT1-2-1* HMG-domain region, primer pair MAT5-7 (10µM) and MAT3-5 (10µM) was used (Table 2.1), at an annealing temperature of 53.5°C and predicted amplify a 270bp region. To amplify the putative *MAT1-2-4* gene, primer pair MAT124F (10µM) and MAT124R (10µM) was used (Table 2.1), at an annealing temperature of 50°C, this was predicted to amplify a 604bp region. To amplfy the putative *SLA2* gene fragment, primer pair aaSLA2 (10µM) and SLA2R (10µM) was used (Table 2.1), at an annealing temperature of 56°C and predicted to amplifly a 500bp region. To amplify the putative *APN2* gene fragment, primer pair aaAPN2 (10µM) and APN2R (10µM) was used (Table 2.1) at an annealing temperature of 56°C and predicted to amplify a 270bp region.

Products resulting from *MAT*, *SLA2* and *APN2* PCR amplifications were resolved via gel electrophoresis on 2% agarose gels containing 1% TBE Buffer. Eight µl of the resultant PCR product was added to 4µl loading buffer. Electrophoresis gels were run at 80V for 3 hours, at room temperature, before being stained for 20 mins in 1µl/ml ethidium bromide solution (10mg/ml), after which bands were visualised via UV light and photographed using a BioRad Chemidoc XRS (Bio-Rad Laboratories, Hemel Hempstead). Bands of interest were then excised from gels and purified as per section 2.2.8. Finally, the gel extraction products were ligated into plasmid pTOPO4, cloned into *E. coli* and sequenced using M13 Forward and M13 Reverse primers (sections 2.2.9 to 2.2.12)

Table 2.1: Primers used in the amplification of *MAT*, *SLA2* and *APN2* genomic regions

Primer name	Primer sequence 5' to 3'
MAT5-6	AARRTNCCNMGNCCNCCNAAYGC
MAT3-4	ARRAANCKNARNATNCCNSWYTT
MAT5-7	THSCNMGNCCNCCNAAYKSNTTYAT
MAT3-5	TTNCKNGGNKKRWANYKRTARTYNGG
MAT124F	AAGGCTACCCACATCATCGTTTCC
MAT124R	AGTTGTTCTGATGAGGAGCGTC
aaSLA2	AYMGNGARATGGCNGAYYTNGARG
SLA2R	CRTANSDNGGNSWNGCRTTYTG
aaAPN2	CARMGNAARGAYYTNMGNGAYGAYATG
APN2R	GGRTANCCNCCNAYYTGNYKNTC

All primers used in this study were obtained from Sigma, UK.

2.2.5 Amplification of MAT Regions (*SLA2-APN2* Positional PCR Strategy)

Previous analysis of MAT regions of filamentous ascomycete fungi has identified that mating-type genes are, in general, present at a conserved locus within the genome which is bordered by *SLA2* (cytoskeletal protein encoding gene) and *APN2* (a DNA lyase) genes (Figure 1.3) (Debuchy and Turgeon 2006). This allowed a novel strategy to be adopted in this study, using PCRs inwards from the *SLA2* and *APN2* genes to amplify the entire MAT region of the species of interest, and also determine the orientation of *MAT* gene within the MAT region, relative to the *SLA2* and *APN2* genes. Where it was not possible to amplify the entire MAT region between the *SLA2* and *APN2* genes, it was possible to use PCR 'inwards' from these flanking regions to known mating-type sequences within the MAT regions (identified from the degenerate PCR strategy outlined in section 2.2.4), which acted as 'bridges' to allow the whole MAT region to be identified.

The sequencing data obtained from the *MAT*, *SLA2* and *APN2* gene fragments (section 2.2.4) were used to design species-specific *MAT, SLA2* and *APN2* primers. These primers were used in combination to determine the relative *MAT* gene orientations within the idiomorphic region. The *SLA2* and *APN2* specific primers were also used to determine the full length of the idiomorph.

The PCR protocols used to amplify the idiomorph region were specific for each species and are therefore described in the relevant chapters.

Products resulting from amplification of MAT regions were resolved via gel electrophoresis on 0.8% agarose gels containing 1% TBE Buffer. Eight µl of the resultant PCR product was added to 4µl loading buffer. Electrophoresis gels were run at 120V for 2.5 hours, at room temperature, before being stained for 20 mins in 1µl/ml ethidium bromide solution (10mg/ml), after which bands were visualised via UV light and photographed using a BioRad Chemidoc XRS (Bio-Rad Laboratories, UK). If sequencing of the resultant PCR products was necessary, PCR purification was performed (section 2.2.7).

After amplification and sequencing of idiomorph sections, new primers were designed based on these resultant sequences. These new primers were either used to amplify new sections of the idiomorph, or the whole *SLA2-APN2* PCR product was used as a sequencing template. This chromosome walking strategy was used to sequence either the entire *SLA2-APN2* region or just the regions surrounding the *MAT* genes.

2.2.6 RAPD Analysis

RAPD-PCR DNA fingerprint analysis was performed on all *A. clavatus*, *A. terreus*, *P. chrysogenum* and *P. griseofulvum* isolates used in this study to determine if isolates were clonal.

Five RAPD primers were used to analyse isolates of *A. clavatus, A. terreus* and *P. chrysogenum* (OPW02 to OPAW13), whilst all 15 RAPD primers listed in Table 2.2 were used to analyse the clonality of *P. griseofulvum* [from kits OPW, OPAW, OPAJ and OPAX (Operon Biotechnologies, UK)].

RAPD PCRs were performed using DyNAzyme DNA Polymerase (Finnzymes, Finland) according to Murtagh *et al*. (1999). Each 25µl reaction contained ~1ng genomic DNA, 2.5µl 10X PCR Buffer, (containing 1.5mM $MgCl_2$), 0.5µl (10mM each) dNTPs, 1µl (40µM)

of RAPD primer, 0.25µl DNA Polymerase and 19.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: an initial denaturation step at 94°C for 5 min; 45 cycles consisting of 1 min at 94°C, 1 min at 35°C and 1 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 60°C/min.

Products resulting from RAPD PCRs were resolved via gel electrophoresis 2% agarose gels containing 1% TBE buffer. Eight µl of resultant PCR product was added to 4µl loading buffer. Electrophoresis gels were run at 80V for 3 hours, at room temperature, before being stained for 20 mins in 1µl/ml ethidium bromide solution (10mg/ml), after which bands were visualised via UV light and photographed using a BioRad Chemidoc XRS (Bio-Rad Laboratories, UK).

Table 2.2: Primers used in RAPD analysis

Operon Kit	Primer name	Primer Sequence 5' to 3'
OPW	OPW02	ACCCCGCCAA
OPW	OPW05	GGCGGATAAG
OPW	OPW08	GACTGCCTCT
OPW	OPW09	GTGACCGAGT
OPAW	OPAW13	CTACGATGCC
OPAJ	OPAJ02	TCGCACAGTC
OPAJ	OPAJ03	AGCACCTCGT
OPAJ	OPAJ05	CAGCGTTGCC
OPAX	OPAX11	TGATTGCGGG
OPAX	OPAX18	GTGTGCAGTG
OPAX	OPAX20	ACACTCGGCA
OPW	OPW03	GTCCGGAGTG
OPW	OPW04	CAGAAGCGGA
OPW	OPW06	AGGCCCGATG
OPW	OPW19	CAAGCGCTC

2.2.7 PCR Product Purification

Where appropriate, PCR products which required sequencing were purified using the QIAquick PCR Purification kit protocol (Qiagen, UK). An Eppendorf tube containing 100µl of PCR product was added to 500µl of buffer PB and mixed via inversion. Sample was then added to a QIAquick spin column, with a 2ml collection tube, this was centrifuged at 13,000g for 1 min and flow-through was discarded. Seven hundred and fifty µl of buffer PE was added to the column, this was centrifuged at 13,000g for 1 min. Flow-through was discarded and spin column was centrifuged at 13,000g for 1 min. To elute, the QIAquick column was transferred to a clean 1.5ml Eppendorf tube, 30µl of buffer EB

was added to the column and left to stand for 1 min. The QIAquick column was then centrifuged at 13,000g for 1 minute. The purified PCR fragments were then sequenced (section 2.2.12).

2.2.8 PCR Product Gel Extraction and Purification

Multiple PCR amplicons were produced in some amplifications, meaning that individual bands of interest had to be excised and purified. For gel extraction of gene fragments, PCR products were resolved on 2% agarose gels run at 20V for 12 hours, visualised and identified under UV light. It was hoped that the extensive running time of the agarose gels would allow identification of extra, unwanted PCR amplifications, and bands of interest were excised carefully from the gel using a clean razor blade.

DNA was extracted from these agarose gel segments using the QIAquick Gel Extraction kit protocol (Qiagen, UK). Duplicate Eppendorf tubes for each sample were prepared, both of which contained ~300mg of excised agarose gel and 3 volumes (~900µl) of buffer QG was then added. Samples were incubated at 50°C for 10 mins to dissolve the agarose gel. One volume of isopropanol was added to the sample, this was mixed via inversion. Sample was pippetted into a QIAquick spin column, with a 2ml collection tube, centrifuged at 13,000g for 1 min and flow-through was discarded. Five hundred µl of buffer QG was added, centrifuged at 13,000g for 1 min, and flow-through was discarded. Seven hundred and fifty µl of buffer PE was added to the column, centrifuged at 13,000g for 1 min and flow-through was discarded. To dry the QIAquick column, the column was centrifuged at 13,000g for 1 min. To elute, the QIAquick column was placed into a clean 1.5ml Eppendorf tube, with 30µl of buffer EB and incubated for 1 min, then centrifuged at 13,000g for 1 min.

2.2.9 Cloning of Gel Extract Products

After addition of 3'A-overhangs had been conducted on a Techne Genuis thermal cycler. The 5µl reaction contained 4µl of gel extracted product, 0.5µl 10X PCR Red Hot Buffer (including 15mM $MgCl_2$) (Abgene, UK), 0.2µl Red Hot Taq Polymerase and 4.3nM dATP. The reaction was heated to 72°C for 10 mins.

PCR products were then cloned using a TOPO TA Cloning® Kit for Sequencing (Invitrogen, UK) following the manufacturer's instruction. Thus, 0.5ml Eppendorf tubes containing 4µl of gel extracted product (after addition of 3' A-overhangs) was added to 1µl of salt

solution and 1µl of TOPO4 vector reaction mix, this was mixed gently and incubated at room temperature for 5 mins before being placed on ice. Two µl of this mixture was added to a vial of ONE SHOT® chemically competent *E. coli* and mixed gently, cells were then incubated on ice for 20 mins. Cells were then heat shocked at 42°C for 40 sec and immediately transferred to ice. Two hundred and fifty µl of room temperature SOC media was added and tubes were shaken horizontally at 37°C for 1 hour.

For transformations, 20, 50 or 100µl of cells were added to 50µl SOC media. These mixtures were then spread on selective LBA plates (containing 100µg/ml ampicillin). A control containing 10µl reaction with 50µl SOC media was spread on non-selective LBA plates. Plates were incubated overnight at 37°C. After an overnight incubation, colonies were streaked out onto LBA selective plates (containing 100µg/ml ampicillin) and incubated overnight at 37°C again.

2.2.10 Colony PCR

To test whether colonies grown on selective LBA plates contained the correct insert, a small scraping of colony was made using a wooden tooth pick and placed in 20µl of LBB (containing 10µg/ml ampicillin) in a 0.5ml Eppendorf tube and mixed by inversion.

PCRs were then performed to determine the size of any plasmid insert, utilising Red Hot Taq Polymerase (Abgene, UK). Each 25µl reaction contain 1µl of the SOC-colony derived mixture, 2.5µl 10X Red Hot PCR Buffer (containing 15mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) M13 Forward primer (5' GTAAAACGACGGCCAG 3'), 2.5µl (10µM) M13 Reverse primer (5' CAGGAAACAGCTATGAC 3'), 0.25µl Red Hot Taq Polymerase and ~15.25µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following parameters: an initial denaturation step at 94°C for 5 min; 30 cycles of 1 min at 94°C, 1 min at 56°C and 1 min 30 sec at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min. Eight µl of the resultant PCR product was added to 4µl loading buffer. Products resulting from colony PCRs were resolved via gel electrophoresis in 2% agarose gels containing 1% TBE Buffer. Electrophoresis gels were run at 80V for 2.5 hours, at room temperature, before being stained for 20 mins in 1µl/ml ethidium bromide solution (10mg/ml), after which bands were visualised via UV light and photographed using a BioRad Chemidoc XRS (Bio-Rad Laboratories, UK).

2.2.11 Plasmid Purification

Colonies containing an insert of the correct predicted size were added to 20ml universal tubes containg 5ml LBB (containing 100µg/ml ampicillin) and incubated overnight at 37°C.

Plasmid purification was conducted using the GenElute Plasmid Mini Prep kit (Sigma, UK), according to manufacturer's instructions. Two ml of the cells from overnight incubation were centrifuged at 8,000g for 2 mins in 2ml Eppendorf tubes. Supernatant was discarded and another 2ml of cells from overnight incubation was added and centrifuged at 8,000g for 2 mins. This was repeated until all cells grown in overnight incubations had been centrifuged.

Cells were resuspended in 200µl of resuspension solution and vortexed, 200µl of lysis buffer was then added and mixture was gently inverted and left to clear for 5 mins. Three hundred and fifty µl of neutralisation solution was added, inverted to mix and centrifuged at 13,000g for 10 mins. To prepare the binding column, 500ml of column preparation solution was added to the binding column and centrifuged at 13,000g for 1 min, flow-through was discarded. Cleared lysate was added to binding column and centrifuged at 13,000g for 1 min, flow-through was discarded. Seven hundred and fifty µl of wash solution was added to spin column, centrifuged at 13,000g for 1 min and flow-through was discarded. Spin column was centrifuged at 13,000g for an additional 5 mins to dry the column. To elute, the spin column was transferred to a new 2ml collection tube and 20µl of elution buffer was added and the spin column was left to incubate for 1 min at room temperature. The spin column was centrifuged for 13,000g for 1 min.

2.2.12 Sequencing of DNA Fragments

DNA samples were sequenced using a BigDye® Terminator v3.1 (Applied Biosystems) kit following manufacturers protocol on a capillary based sequencer – 3100 genetic analyser (Biochemistry Department, University of Nottingham).

2.2.13 RT-PCR Culture Conditions

A plug of mycelium approximately 0.5 cm in diameter was taken from pre-grown MEA plates and placed onto 5 cm diameter Aspergillus Complete Media (ACM) Petri plates, which had a nitrile disc pre-placed on top of the agar. The plugs were placed in the

configuration shown in Figure 2.1 (A) when a single isolate was incubated. The plugs were placed in the configuration shown in Figure 2.1 (B) when two isolates were incubated together. Cultures were grown in the dark at 28°C for 24 hours, after which the plates were sealed with two overlaying strips of parafilm. The cultures were grown for a further 4, 6, 9 or 13 days, unless otherwise stated (Chapter 3), after which the cultures were harvested and RNA extracted. RT-PCR results obtained from single isolates after 5 days of incubations are shown throughout this thesis. Longer and multiple isolate incubations were analysed when no expression was seen after 5 days in a single isolate incubation.

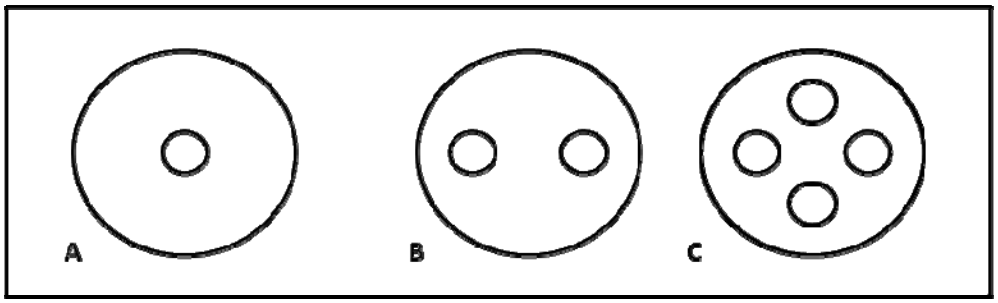


Figure 2.1: Plating configurations used to prepare cultures for RT-PCR analyses or induction of a sexual cycle. A. One plug, B. Two plugs and C. Four plugs

2.2.14 RNA Extraction for RT-PCR Analysis

RNA extraction was performed using an RNeasy Plant Mini kit (Qiagen, UK) according to the manufacturer's instructions. Thus, 50mg of fungal tissue was ground under liquid nitrogen and added to 450µl of buffer RLT (containing 10µl/ml β-Mercaptoethanol) and vortexed vigorously. The lysate was pippetted into a QIAshredder spin column, with a 2ml collection tube and centrifuged at 13,000g for 2 min. Supernatant was removed, without disturbing any pellet that had formed, and added to a new 2ml Eppendorf tube. To this, 0.5 volumes of 96-100% ethanol was added and mixed by pippetting. Of this, 650µl was added to a RNeasy mini column, with collection tube, this was centrifuged at 8,000g for 15 sec and flow-through was discarded. This step was repeated until all mixture had been centrifuged. To wash the spin column, 700µl of RW1 buffer was added to RNeasy column and centrifuged at 8,000g for 15 sec, flow-through was discarded. The RNeasy mini spin column was transferred to a new collection tube and 500µl of RPE buffer was added, this was then centrifuged at 8,000g for 15 sec and flow-through was discarded. To dry the RNeasy silica-gel membrane, another 500µl of RPE buffer was added, this was then centrifuged at 8,000g for 2 min and flow-through was discarded. RNeasy mini spin column was transferred to a new 2ml collection tube and centrifuged

at 13,000g for 1 min, to remove any of RPE buffer carryover. To elute, RNeasy mini spin column was transferred to a new 1.5ml Eppendorf tube and 30µl of RNase free water was added to the membrane, the column was then centrifuged at 8,000g for 1 min.

2.2.15 DNase Treatment

DNase treatment of the extracted RNA was performed using the RQ1 RNase-Free DNase kit (Promega, USA), according to the manufacturer's instructions. A DNA digestion was conducted using 8µl of extracted RNA, 1µl of RQ1 RNase-free DNase 10X reaction buffer and 1µl of RQ1 RNase-free DNase this was then incubated for 30 mins at 37°C. To terminate the DNA digestion, 1µl of DNase stop solution was added, and this was then incubated at 65°C for 10 mins. The total RNA was quantified on a Nanodrop™ UV-Vis Spectrophotometer (Thermo Scientific, USA) absorbance set at 260nm.

2.2.16 RT-PCR Analysis

Between 0.5pg and 1µg of total RNA was used to performed RT-PCR analysis using a OneStep kit (Qiagen, UK) according to the manufacturer's instructions. RNA was added to a 25µl reaction consisting of 5µl 5X Qiagen OneStep RT-PCR buffer, 1µl (10mM each) dNTP, 1.5µl (10µM) forward primer, 1.5µl (10µM) reverse primer, 1µl Qiagen RT-PCR enzyme mix (containing both Reverse Transcriptase and Standard Taq Polymerase), ~15µl of RNase free water.

The reverse transcription reaction was performed on a Techne Genius thermal cycler set at 50°C for 30 mins followed by 15 mins at 95°C. Reverse transcription was followed by PCR with the following cycling conditions: an initial denaturation step at 94°C for 1 min; 35 cycles consisting of 30s at 94°C, 30s at 50°C and 1 min at 72°C; followed by a final extension step of 72°C for 10 mins, all steps used a ramp rate of 70°C/min.

2.2.17 DNA Control for RT-PCR Analysis

PCRs were performed using FastStart Taq DNA Polymerase (Roche, UK). Each 25µl reaction contained ~10ng genomic DNA, 2.5µl 10X PCR Buffer, 1.5µl (20mM) $MgCl_2$, 0.5µl (10mM each) dNTPs, 2.5µl (10µM) forward primer, 2.5µl (10µM) reverse primer, 0.2µl Taq Polymerase and ~14.3µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: initial denaturation step at 94°C for 5 min; 40 cycles consisting of 1 min at 94°C, 1 min at 50°C

and 2 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min.

2.2.18 RT-PCR Gel Electrophoresis

Products resulting from RT-PCR amplification of RNA and PCR of control DNA were resolved via gel electrophoresis in 1.5% agarose gels containing 1% TBE Buffer. Eight µl of PCR product was added to 4µl loading buffer. Electrophoresis gels were run at 80V for 2.5 hours, at room temperature, before being stained for 20 minutes in 1µl/ml ethidium bromide solution (10mg/ml), after which bands were visualised via UV light and photographed using a BioRad Chemidoc XRS (Bio-Rad Laboratories, Hemel Hempstead).

2.2.19 Sexual Crosses Culture Conditions

Five cm Petri plates containing 10ml of ACM or Oatmeal Agar (OA) with plugs of 0.5 cm were plated in the configuration shown in Figure 2.1 (B) and (C), or mycelia from cultures were directly mixed on a plate (i.e. no plugs were used). Plates were incubated at 25°C or 28°C and in the light or dark. Plates were grown unsealed for 48 hours or 2 weeks and then sealed with two overlaying strips of parafilm, the plates were then incubated for 6 months to allow sexual structures to develop.

2.2.20 Phylogenetic Analyses

Nucleotide and amino acid sequences were aligned using ClustalW (Thompson *et al.* 1994). Phylogenetic and molecular evolution analyses were conducted using MEGA version 4 generating maximum parsimony phylogenetic trees (using default settings using bootstrapping of 500 replicates and excluding gaps) (Tamura *et al.* 2007).

Chapter 3 Mating-Type Genes in Sexual Aspergilli

3.1 Introduction

There are approximately 70 species with *Aspergillus* anamorphs that are known to produce sexual teleomorph stages. These are divided into 11 genera (Table 3.1). Only four of these genera contain more than three species namely, *Emericella*, *Eurotium, Petromyces* and *Neosartorya*. Some of the genera only contain the type species, e.g. *Neocarpenteles*, *Neopetromyces* and *Warcupiella* (Gams 1993; Pitt *et al.* 2000; Seifert and Lévesque 2004; Udagawa and Uchiyama 2002).

The majority of teleomorphic species within the *Aspergillus* clade are homothallic, with the model organism *Emericella nidulans* (anamorph: *A. nidulans*) being the most extensively studied (Todd *et al.* 2007). Six *Aspergillus* species have been shown to exhibit heterothallic breeding systems, these belong to two distinct genera, *Neosartorya* and *Emericella* (Kwon *et al.* 1964; Kwon and Kim 1974; O'Gorman *et al.* 2009; Takada and Udagawa 1985).

Classification of teleomorphic genera has traditionally been based on cleistothecia and ascospore morphology (Table 3.1). The traditional taxonomic relationships are largely supported by molecular data. As can be seen in Figure 3.1 mitosporic Aspergilli are interspersed within the meiosporic genera.

Currently only three *Aspergillus* species with known sexual stages have had their genomes sequenced, namely *Neosartorya fumigata* (anamorph: *A. fumigatus*), *Neosartorya fischeri* (anamorph: *A. fischeranius*) and *E. nidulans*.

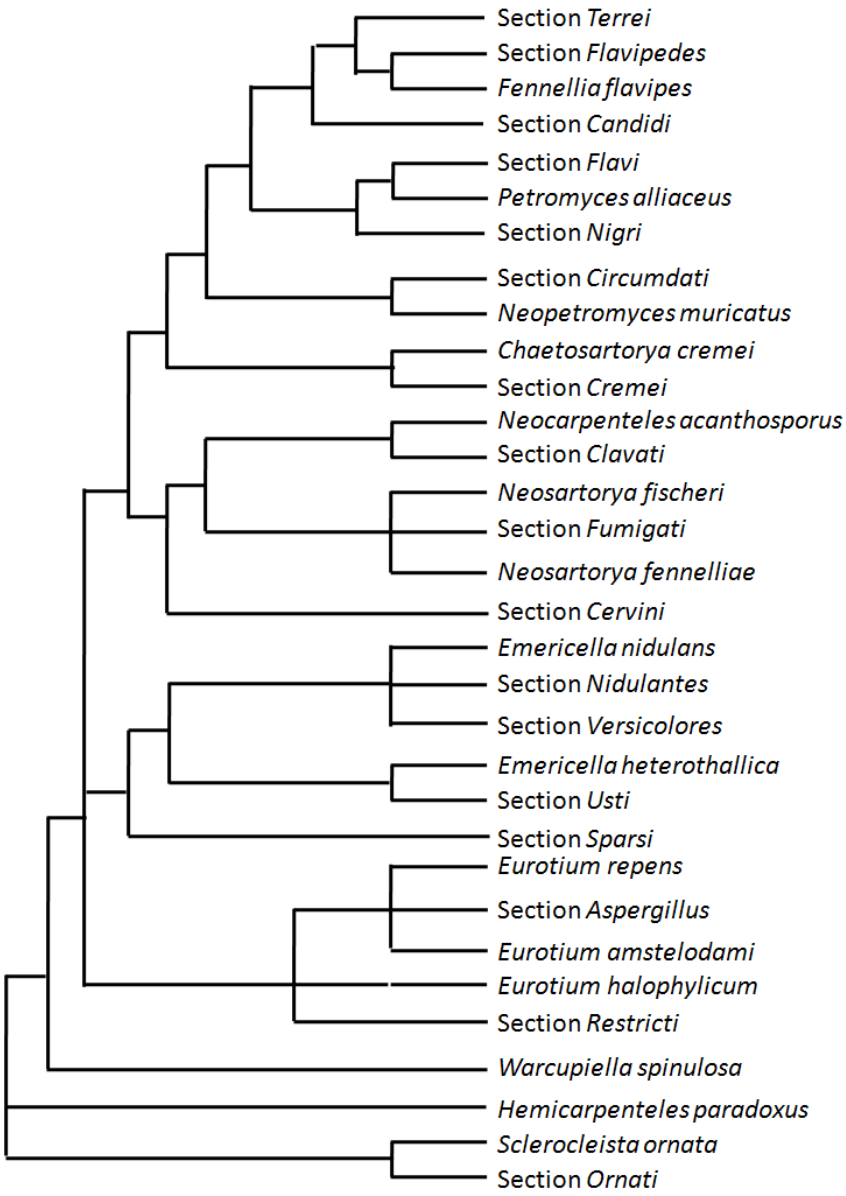


Figure 3.1: Phylogeny of the Genus *Aspergillus*, with its 11 associated teleomorphic genera. [Adapted from Frisvad and Samson (2000), Peterson (2008), Samson *et al*. (2007), Tamura *et al*. (2000), Udagawa and Uchiyama (2002) and Varga *et al*. (2000a).]

When considering the evolution of sexuality within the genus *Aspergillus*, there has been long standing debate over whether the ancestral sexual strategy is homothallic or heterothallic. The fact that the vast majority of sexual species are homothallic, combined with phylogenetic reconstruction analysis, lead Geiser *et al.* (1996) to propose that the group was derived from a homothallic ancestor. However, accumulating evidence from research on other ascomycete taxa suggests that evolution from a heterothallic ancestral strategy is more likely (Berbee *et al.* 2003; Yun *et al.* 1999). Genome analysis of *E. nidulans* and *N. fischeri* has revealed that these two homothallic

species, from two different genera, have different arrangements of MAT loci in their genomes (Figure 3.2) (Galagan *et al.* 2005; Paoletti *et al.* 2007; Rydholm *et al.* 2007). Meanwhile, the heterothallic *N. fumigata* has a MAT idiomorph arrangement characteristic of most other heterothallic euascomycetes (Figure 3.2). Given the apparent diversity of MAT locus organization amongst *Aspergillus* species, further investigation of this genus may give clues as to the origin of the *MAT* genes and the possible ancestral mating strategy of this clade, thereby helping to resolve this long standing evolutionary question.

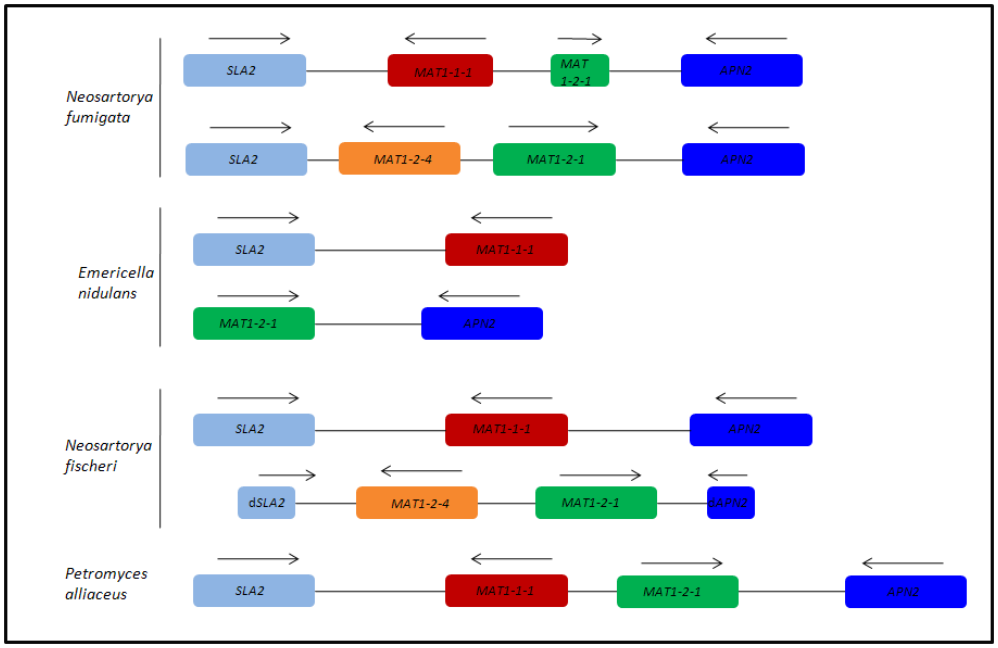


Figure 3.2: Arrangement of MAT idiomorphs in heterothallic species *N. fumigata* and homothallic species, *E. nidulans*, *N. fischeri* and *P. alliaceus*. *E. nidulans* and *N. fischeri* have unlinked MAT loci, with the *MAT* genes appearing on different chromosomes, whereas *P. alliaceus* has linked MAT loci. [Adapted from Paoletti *et al*. (2005 and 2007), Ramirez-Prado *et al*. (2008) and Rydholm *et al*. (2007).] Arrows indicate gene orientation.

One further question to be addressed concerns the status of the putative mating-type gene *MAT1-2-4*, which has been detected in the MAT-2 locus of *N. fumigata, N. fischeri*, *Penicillium marneffei*, *Talaromyces stipitatus*, *Coccidioides* species and *Ajellomyces capsulatus* (anamorph: *Histoplasma capsulatum*) but not in other *Aspergillus* or *Penicillium* species (Bubnick and Smulian 2007; Fraser *et al.* 2007b; Mandel *et al.* 2007; Paoletti *et al.* 2005; Rydholm *et al.* 2007; Woo *et al.* 2006). Although this ORF has been annotated in all of the former species, there has so far been only one experimental study investigating whether the *MAT1-2-4* gene was expressed in *Coccidiodes posadasii* (Mandel *et al.* 2007). There have not been any functional studies to determine whether

the gene is required for sexual development. Therefore, in the following chapter it was of interest to discover whether this gene was present in other members of the *Aspergillus* clade, and to assess whether the putative gene was transcribed to mRNA, which would indicate a functional role for this gene.

The focus of this chapter will be on determining *MAT* gene organisation of the MAT loci within three sexual genera. Two of the genera, *Emericella* and *Neosartorya*, were chosen as they contain both homothallic and heterothallic species, whilst a third genus, *Eurotium*, contains only homothallic species (Figure 3.3). These genera will now be described in more detail.

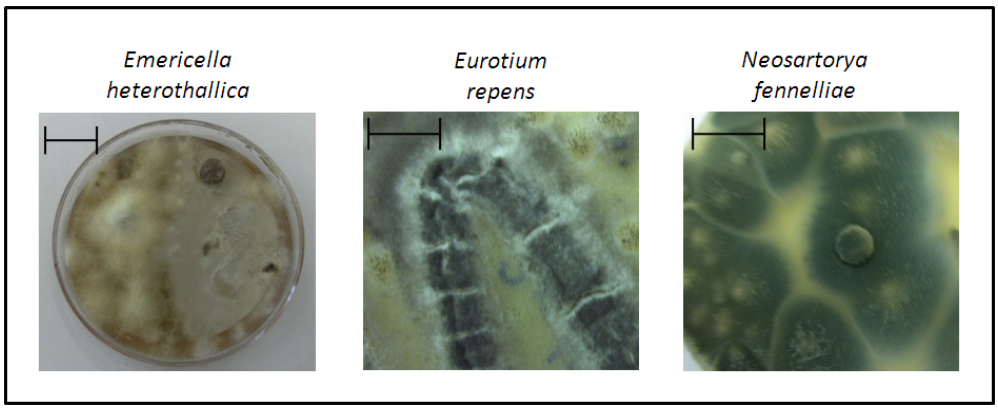


Figure 3.3: Colony morphology, showing conidial colours of representative species of *Aspergillus* with sexual stages. Cultures were grown for 7 days on ACM at 28°C in the light. Scale bar indicates approximately 1cm.

Table 3.1: Characteristics of the eleven sexual genera associated with *Aspergillus* anamorphs (Frisvad and Samson 2000; Klich 2001; Peterson 2008; Pitt *et al.* 2000; Samson 1979; Subramanian 1972; Udagawa and Uchiyama 2002; Wiley and Simmons 1973).

Teleomorphic genera	Example species	Cleistothecia - colour developmental features	Ascospores - morphological features	Associated asexual taxonomic group of Aspergilli
Chaetosartorya	*C. cremea* (anamorph: *A. cremeoflavus*)	Yellow or orange, loose network, ornamented, with short hyphae	Hyaline, lenticular with two broad equatorial ridges	Section *Cremi*
Emericella	*E. nidulans* (anamorph: *A. nidulans*)	Dark red or purple, globose, surrounded by Hülle cells	Red to purple, lenticular and smooth walled	Sections *Nidulantes* and *Usti*
Eurotium	*E. chevalieri* (anamorph: *A. chevalieri*)	Yellow, globose to subglobose	Lenticular, smooth walled	Sections *Aspergillus* and *Restricti*
Fennellia	*F. flavipes* (anamorph: *A. flavipes*)	Yellow, surrounded by Hülle cells	Globose, smooth, inconspicuous equatorial grooves, formed in clumps	Section *Flavipes*
Hemicarpenteles	*H. paradoxus* (anamorph: *A. paradoxus*)	Peridium surrounding cleistothecia, enveloped by	Colourless becoming brown, lenticular, crested	None

		sterile hyphae		
Neocarpenteles	*N. acanthosporum* (anamorph: *A. acanthosporus*)	Yellow-brown to grey-brown ascoma-bearing stromata	Hyaline, lenticular, two equatorial ridges	Section *Clavati*
Neopetromyces	*N. muricatus* (anamorph: *A. muricatus*)	Yellow, sclerotoid ascomata	Subglobose to broadly ellipsoidal, small equatorial ridges	Section *Circumdati*
Neosartorya	*N. fischeri* (anamorph: *A. fischerianus*)	White to cream, globose	Uncoloured, lenticular, almost sperhical, walls lightly to very rough, heat resistant	Section *Fumigati*
Petromyces	*P. alliaceus* (anamorph: *A. alliaceus*)	Globose	Uncoloured, elliptical, two equatorial ridges	Section *Flavi*
Sclerocleista	*S. ornara* (anamorph: *A. ornatus*)	White becoming purple, parenchymatous	Colourless becoming brown, lenticular	Section *Ornati*
Warcupiella	*W. spinulosa* (anamorph: *A. warcupii*)	White to olive, surrounded by loosely woven hyphae	Large, spiny, elliptical, without ridges	None

3.1.1 The Genus *Emericella*

The genus *Emericella* contains approximately 40 species. One of these, *E. heterothallica* (anamorph: *A. heterothallicus*) is heterothallic, whilst the remaining species are homothallic. Species within this genus usually have white cleistothecia, becoming dark purple at maturity, surrounded by masses of Hülle cells. Hülle cells may be red-brown or blue-violet and are the defining characteristic of this genus. However, Hülle cells are not present in all species in the genus. The conidial stage of species in this genus vary in colour being green, grey, purple or buff and having brown stipes. Conidia are generally dark green (Domsch *et al.* 1980; Malloch and Cain 1972b; Malloch and Cain 1972a; Pitt and Hocking 1997b; Raper and Fennell 1965).

Species within the *Emericella* genus are common in tropical and subtropical regions and are usually isolated from soil. Members of this genus have also been isolated from plant material and food products and are therefore known food spoilage organisms (Raper and Fennell 1965). Two species, *E. quadrinleata* and *E. nidulans*, have been reported to be opportunistic human pathogens (Verweij *et al.* 2008).

From molecular studies, *Emericella* species seem to be relatively closely related to the teleomorphic genus, *Eurotium* (Peterson 2008).

Emericella nidulans

Emericella nidulans is a homothallic species first reported in 1884 (Pontecorvo 1953; Schwarz 1928). Pontecorvo (1953) pioneered the use of this species as a model organism, demonstrating that *E. nidulans* was amenable to laboratory studies and genetic manipulation. Since then *E. nidulans* has proven to be an extremely valuable genetic model contributing to knowledge in numerous diverse areas of fungal genetics. An advantage of this species is its haploid genome, allowing fast gene deletion or insertion either via homologous or ectopic integration (Pontecorvo 1953; Todd *et al.* 2007). *E. nidulans* also has the ability to undergo the parasexual cycle. This provides an investigator with three reproductive pathways for genetic manipulation (Pontecorvo and Sermonti 1954). One potential disadvantage of *E. nidulans* is linked to its homothallic sexual cycle. To analyse the segregation of mutant phenotypes, the origin of ascospores has to be ascertained i.e. if they resulted from self-fertilisation or outcrossing. Auxotrophic markers have been developed for this species in order to identify hybrid cleistothecia (Todd *et al.* 2007).

Emericella heterothallica

The only known heterothallic species in the genus *Emericella* is *E. heterothallica*, which was first identified in forest soils in 1964 (Kwon *et al.* 1964). *E. heterothallica* was the first *Aspergillus* species with a heterothallic breeding system to be reported and will be a key focus of this chapter. *E. heterothallica* produces abundant yellow to red-orange Hülle cells and this was the primary reason it was classified within the genus *Emericella*. Cleistothecia are produced within the Hülle cell masses but these can take 4-8 weeks to develop. *E. heterothallica* produces lenticular, orange-brown ascospores with two pleated equatorial ridges. Colony colours vary between strains dividing the species into two distinct groups either yellow or orange-yellow becoming buff when mature, or reddish orange or orange-buff becoming orange when mature (Figure 3.3) (Kwon and Raper 1967b; Kwon and Raper 1967a; Raper and Fennell 1965).

Most *Emericella* species associate with sections *Nidulantes* and *Versicolores* (Figure 3.1). However, analysis of ITS-5.8S rDNA, calmodulin and β-tubulin DNA sequences revealed that *E. heterothallica* appeared to associate with species in the subgenus *Aspergillus*, section *Usti*, although actin gene sequencing did not support this classification (Peterson 2008).

3.1.2 The Genus *Eurotium*

The genus *Eurotium* contains approximately 20 species, all of which are homothallic xerophiles. Species within this genus include *E. rubrum*, *E. chevalieri*, *E. repens, E. herbarorium* and *E. amstelodami*. *Eurotium* anamorphic species are classified within the sections *Restricti* and *Aspergillus*. The latter was formerly known as the *Aspergillus glaucus* group (Peterson 2000a; Raper and Fennell 1965).

The genus *Eurotium* is characterised by the production of macroscopic, bright yellow, globose to subglobose cleistothecia. Cleistothecia are not universal throughout this genus for example, *E. aethicus* and *E. proliferans* do not produce cleistothecia, and instead produce abundant coiled ascogonia. *Eurotium* species produce lenticular, hyaline to yellow ascospores which may or may not have equatorial ridges (Domsch *et al.* 1980; Malloch and Cain 1972b; Malloch and Cain 1972a; Raper and Fennell 1965). The *Eurotium* genus is split into two groups based on ascospore morphology. Firstly, species that produce small ascospores (less than 6µm) (including *E. repens*, *E. ruber* and *E. amstelodami*) and secondly, species that produce large ascospores (more than 6µm)

(including *E. echinulatum* and *E. herbarorium*) (Raper and Fennell 1965). The ascospores produced by species in this genus have a low water potential, consequently they are able to survive at increased temperature and pressure. In fact, increased temperature has been shown to initiate ascospore germination (Eicher and Ludwig 2002; Yildiz and Çoksöyer 2002). *Eurotium* species are able to grow at low water activity and high salt and sugar concentrations (Butinar *et al.* 2005). These growth attributes, coupled with ascospores that are able to survive at high temperatures, helps to explain why many *Eurotium* species are food and feed contaminants (Butinar *et al.* 2005). Many species in this genus are also mycotoxin producers. Therefore their appearance in food, especially fruit juices, has health consequences (Eicher and Ludwig 2002; Yildiz and Çoksöyer 2002).

In this chapter, one species from this genus will be studied in depth to determine the arrangement of the *MAT* genes, namely *E. repens*.

Eurotium repens

Eurotium repens (anamorph: *A. repens* or *A. reptans*) is a homothallic species and its sexual strategy was confirmed in 1928 (Figure 3.3) (Schwarz 1928). This species produces small ascospores (less than 6µm) when compared to other species in this genus, which can be triggered to germinate by an increase in temperature (Eicher and Ludwig 2002; Raper and Fennell 1965). The ascospores of *E. repens* have convex surfaces, with equatorial ridges. The cleistothecia are usually very abundant and are usually produced on yellow to orange-red hyphae. On Czapek's agar the growth of this species is restricted and cleistothecia are usually aborted with only a few viable ascospores maturing (Raper and Fennell 1965).

3.1.3 The Genus *Neosartorya*

The genus *Neosartorya* contains approximately 20 thermotolerant species, of which five exhibit heterothallic breeding systems. This genus is mainly associated with species within the section *Fumigati* (Figure 3.1), which includes the opportunistic human pathogen *N. fumigata* (Kontoyiannis and Bodey 2002; Latgé 1999).

Members of the genus *Neosartorya* are generally abundant, ubiquitous, soil-borne species that are active in decomposing organic materials (Malloch and Cain 1972a). *Neosartorya* species are not restricted to the soil, some members of this genus have

been isolated from house dust, as a cause of human disease and, due to their heat resistant ascospores, are encountered as contaminants of fruit juices (Lonial *et al.* 1997; Scott and Bernard 1987; Udagawa *et al.* 1996).

All homothallic and heterothallic species within this genus produce globose or subglobose white cleistothecia that may become yellow at maturity. Ascospores are usually hyaline, although pale to yellowish brown are also seen. Most species produce ascospores that have two equatorial ridges which can be broad or narrow, more than two ridges are known also (Horie *et al.* 1995; Kwon and Kim 1974; Raper and Fennell 1965; Takada *et al.* 2001; Takada and Udagawa 1985). The heterothallic species within this genus are *N. fumigata* (O'Gorman *et al.* 2009), *N. fennelliae* (Kwon and Kim 1974), *N. spathulata* (Takada and Udagawa 1985) *N. udagawae* (Horie *et al.* 1995) and *N. nishimurae* (Takada *et al.* 2001). In this thesis the focus of studies will be on *N. fennelliae*.

<u>*Neosartorya fischeri*</u>

The type species of the genus *Neosartorya* is the homothallic *Neosartorya fischeri*, which has recently had its genome sequenced by The Institute of Genome Research. Mating-type genes have also been characterised from this species (Figure 3.2) (Rydholm *et al.* 2007). *N. fischeri* produces grey-green to pale blue conidia, and white or cream coloured cleistothecia. Cleistothecia and mature spherical ascospores are produced on MEA and other commonly used growth media within two weeks (Klich 2002; Raper and Fennell 1965). The ascospores produced by *N. fischeri* have been shown to be heat resistant, and their occurrence in fruit juices is common (Rajashekhara *et al.* 2000).

Neosartorya fischeri was first described in 1929 and is ubiquitous in soil. It is therefore often isolated from soil grown foods (Girardin *et al.* 1995). It is also a cause of keratinitis and pulmonary aspergillosis in transplant patients (Domsch *et al.* 1980; Lonial *et al.* 1997). *N. fischeri* is also an opportunistic pathogen of bone marrow transplant patients, although its occurrence is relatively rare (Lonial *et al.* 1997).

<u>*Neosartorya fumigata*</u>

Neosartorya fumigata is an opportunistic human pathogen (Kontoyiannis and Bodey 2002; Latgé 1999). This species is isolated from a range of environments including soils, food- and feed-stuffs (Domsch *et al.* 1980).

Neosartorya fumigata is a heterothallic species and has had its genome sequenced by a consortium consisting of The Institute of Genome Research, The Sanger Centre and The Institut Pasteur (Nierman *et al.* 2005). Genome sequencing lead to the identification of a series of 'sex-related' genes, and the concomitant discovery of MAT1-1 and MAT1-2 isolates in near equal proportions in worldwide collections led to much speculation concerning the potential for sexual reproduction in this species (Dyer and Paoletti 2005; Galagan *et al.* 2005; Große and Krappmann 2008; Kontoyiannis and Bodey 2002; Latgé 1999; Nierman *et al.* 2005; Paoletti *et al.* 2005; Pöggeler 2002; Pringle *et al.* 2005; Pyrzak *et al.* 2008; Rydholm *et al.* 2006; Varga 2003; Varga and Tóth 2003). A heterothallic sexual breeding system was confirmed in 2009, involving the production of greyish-yellow cleistothecia and yellowish white to greenish white, lenticular ascospores when cultured for six months on OA in the dark at 30°C (O'Gorman *et al.* 2009). The length of time required to produce cleistothecia is much longer than *N. fischeri* and *N. fennelliae* showing the variability of sexual fertility within this genus.

Neosartorya fennelliae

Neosartorya fennelliae (anamorph: *A. fennelliae*) was the second *Aspergillus* teleomorph discovered with a heterothallic breeding system. This species was discovered in the eyeballs of rabbits in 1972 and formally reported in 1974 (Kwon and Kim 1974). *N. fennelliae* was characterised as having white, globose cleistothecia which are either borne singly or in clusters. Lenitcular, hyaline ascospores are also produced (Kwon and Kim 1974). In the absence of mating, this species produces green condiophores and globose, subglobose to elliptical conidia (Kwon and Kim 1974). Due to the morphology of the cleistothecia and ascospores produced by this species, it was classified in the then wholly homothallic genus *Neosartorya*. Upon discovery, crosses of this species with homothallic *N. fischeri* and the then 'asexual' *N. fumigata* were performed. These crosses failed, although zones of clearing were also seen in some crosses (Kwon and Kim 1974). However, subsequently, a heterothallic mating strategy has been found in *N. fumigata* (O'Gorman *et al.* 2009; Paoletti *et al.* 2005), and abortive cleistothecia have been produced in crosses between *N. fumigata* and *N. fennelliae* (Varga 2003).

Given the differing mating-type gene arrangements seen in *N. fischeri* and *N. fumigata*, *N. fennelliae* will be investigated in this chapter. This may help elucidate the ancestral mating strategy of the genus *Neosartorya*.

3.1.4 Aims of this chapter

1) To determine the arrangement of *MAT* genes in homothallic and heterothallic species from a variety of genera with *Aspergillus* anamorphs.
2) To try and determine the ancestral sexual reproductive strategy within the genus *Aspergillus* and related teleomorphic genera.
3) To determine whether the putative *MAT1-2-4* gene is present within the teleomorphic genera under investigation and then to use RT-PCR to determine if this gene is expressed under conditions that induce sex in these species.

3.2 Materials and Methods

3.2.1 *Emericella heterothallica*

Isolates 50-3 and 50-5 of *E. heterothallica* were obtained from a culture collection held at the School of Biology, The University of Nottingham. These were derived from single-sporing of the reference isolates WB4982 (MAT-A) and WB5086 (MAT-a), from Kwon and Raper (1967a).

3.2.1.1 *MAT* Gene Isolation and Sequencing

Isolates were cultivated in MEM at 28°C, and DNA extracted as described in sections 2.2.2 and 2.2.3. *MAT* genes were amplified using the degenerate PCR strategy described in section 2.2.4. Resultant PCR products were resolved and visualised by gel electrophoresis, and bands of interest were excised and gel extracted as described in section 2.2.8. The gel extracted products were ligated into plasmid pTOPO4, then cloned into *E. coli* and sequenced from extracted plasmid DNA using M13 Forward and M13 Reverse primers as described in sections 2.2.9 to 2.2.12.

3.2.1.2 Cloning of Idiomorph Region and Idiomorph Orientation

The *SLA2-APN2* positional PCR strategy described in sections 2.2.4 and 2.2.5 was used to determine the orientation of the *MAT* genes relative to genes bordering the idiomorphs. Species-specific *SLA2* and *APN2* primers [EhSLA2-1, EhSLA2-2 and EhAPN2-1 (Table 3.2)] were designed from sequencing obtained using the protocol described in section 2.2.4 and sequenced using the protocols described in sections 2.2.8 to 2.2.12.

MAT-1 Idiomorph Orientation

PCRs to determine the *MAT1-1-1* gene orientation were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~20ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) of respective *MAT* gene primer, 2.5µl (10µM) of respective flanking gene primer, 0.25µl Phusion DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 98°C, 20 sec at 51.6°C and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min.

Primer pair EhM1F and EhSLA2-1 was used with inclusion of 4% DMSO, and primer pair EhM1R and EhAPN2-1 (Table 3.2) were also used to determine the relative *MAT1-1-1* gene orientation within the MAT-1 idiomorph. Resultant PCR products were resolved on 0.8% agarose gels and visualised by ethidium bromide staining (section 2.2.5).

MAT-2 Idiomorph Orientation

MAT1-2-1 Gene Orientation

PCRs to determine the *MAT1-2-1* gene orientation were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~20ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) of respective *MAT* gene primer, 2.5µl (10µM) of respective flanking gene primer, 0.25µl Phusion DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 98°C, 20 sec at an appropriate annealing temperature and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps were performed at a ramp rate of 70°C/min.

Primer pair EhM2F and EhAPN2-1 at an annealing temperature of 50°C, and primer pair EhM2R and EhSLA2-1 (Table 3.2) at an annealing temperature of 60.1°C were used to determine the relative *MAT1-2-1* gene orientation within the MAT-2 idiomorph. Resultant PCR products were resolved on 0.8% agarose gels and visualised by ethidium bromide staining (section 2.2.5).

MAT1-2-4 Gene Orientation

The previously identified *MAT1-2-4* gene sequences from *N. fumigata* and *N. fischeri* were used to design *MAT1-2-4* specific primers to amplify a fragment of this gene family (section 2.2.4), if present. After the whole *MAT1-2-4* gene was sequenced, *E. heterothallica* specific primers [EhM124R and EhM124R2 (Table 3.2)] were designed to determine gene orientation relative to the bordering *SLA2* and *APN2* genes.

PCRs to determine the *MAT1-2-4* gene orientation were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~20ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) of respective *MAT* gene primer, 2.5µl (10µM) of respective flanking gene primer, 0.25µl Phusion DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 98°C, 20 sec at 60.1°C and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps were performed at a ramp rate of 70°C/min.

Primer pairs M124F with EhSLA2-2 and EhM124R with EhAPN2-1 were used to determine the relative orientation of the *MAT1-2-4* gene within the MAT-2 idiomorph. Resultant PCR products were resolved on 0.8% agarose gels and visualised by ethidium bromide staining (section 2.2.5).

Table 3.2: List of primers used in amplification of *E. heterothallica* MAT regions and for RT-PCR studies.

Primer	Sequence 5' to 3'
EhM1F	CTATTGTCGATGCCACCCGA
EhM1R	CCACCCGATTACGTTGAGA
EhM2F	ATGTCGACCAGATCAAAACC
EhM2F2	ATTCATCCTTTATCGTCAGC
EhM2R	ATTCGGCCTTCCATTGTTTTCC
M124F	AAGGCTACCCACATCATCGTTTCC
EhM124R	TTCGTTGTCCTCGTTGGTC
EhM124R2	AGTTGTTTTGATGAGGAGCGTC
EhSLA2-1	ACTATTCGAAGCTCAGCTCG
EhSLA2-2	TACCGCAATCGCTAACAGCAGC
EhAPN2-1	AGTCTCCGAGAAAATCTACACC
Ate Actin Forward	ATTGTCGGTCGTCCCCG
Ate Actin Reverse	TCTGGGTCATCTTCTCACGG

3.2.1.3 RT-PCR Analysis

RT-PCR analysis was performed to determine whether gene expression and intron splicing occurred in any of the putative *MAT* genes found in *E. heterothallica*. Species-specific, gene-specific primers for the various *MAT* genes were designed using the sequencing data obtained during this study (Table 3.2).

Primer pairs were designed to span putative introns to confirm intron splicing. Samples were grown until cleistothecia were formed (approximately 4 week), after which time RNA was extracted and DNase treated according to the protocols described in sections 2.2.13 to 2.2.15. RT-PCR was performed and resultant PCR products were resolved on 1.5% agarose gels and visualised using ethidium bromide staining (sections 2.2.16 to 2.2.18).

For RT-PCR analysis of the putative *MAT1-1-1* gene primer pair EhM1F and EhM1R was used (Table 3.2). This was predicted to yield a 521bp fragment from unspliced genomic DNA and, after splicing of a 50bp intron, predicted to yield a 471bp fragment from processed mRNA. To analyse the putative *MAT1-2-1* gene primer pair EhM2F2 and EhM2R was used (Table 3.2). This was predicted to yield a 158bp fragment and a 107bp fragment from unspliced genomic DNA and processed mRNA, respectively (splicing of a 51bp intron). To analyse the putative *MAT1-2-4* gene primer pair M124F and EhM124R2 was used (Table 3.2). This was predicted to yield a 603bp fragment from unspliced genomic DNA, after splicing a 45bp intron, predicted to yield a 558bp fragment from processed mRNA (Tables 3.2 and 3.7).

RT-PCR analysis of the constitutively expressed actin gene was included as a control. Actin primers were designed using *Aspergillus terreus* actin sequencing data obtained from The Broad Institute genome sequencing project (http://www.broad.mit.edu/tools/data/seq.html) (gene locus ID: ATEG_06973.1). Primer pair Ate Actin Forward and Ate Actin Reverse was predicted to yield an approximate 330bp fragment from unspliced genomic DNA and an approximate 260bp fragment from processed mRNA, after splicing of a ~70bp intron (Tables 3.2 and 3.7).

3.2.2 *Eurotium repens*

Studies were conducted on two isolates of *E. repens*. Firstly, isolate 51-1 from a culture collection held at the School of Biology, The University of Nottingham. Secondly, isolate

CBS 529.65 (Nottingham Code: 51-2), which was originally isolated in 1965 from *Prunus domestica*, in France (Peterson 2008). This isolate was obtained directly from J. Houbraken and R. Samson (CBS, Netherlands) as part of an ongoing research collaboration. It was necessary to use data from two isolates due to contamination of stock cultures of isolate 51-1 during the progress of studies.

3.2.2.1 *MAT* Gene Isolation and Sequencing

Isolates were cultivated in MEM at 28°C and DNA extracted as described in sections 2.2.2 and 2.2.3. Conserved domains of the *MAT* genes were amplified using a degenerate PCR strategy as described in section 2.2.4. Resultant PCR products were resolved and visualised by gel electrophoresis as described in section 2.2.4, bands of interest were excised and gel extracted as described in section 2.2.8. The gel extracted products were ligated into plasmid pTOPO4, then cloned into *E. coli* and sequenced from extracted plasmid DNA using M13 Forward and M13 Reverse primers as described in sections 2.2.9 to 2.2.12.

3.2.2.2 Organisation of *E. repens* MAT Locus

Isolation of the MAT-2 Locus

The complete MAT-2 locus of the genome containing a high-mobility-group mating-type gene was obtained using the *SLA2-APN2* PCR strategy as described in section 2.2.5. Species-specific *SLA2* and *APN2* primers [ErSLA2 and ErAPN2 (Table 3.3)] were designed using the gene fragments amplified using the protocol described in section 2.2.4 and sequenced using the protocols described in sections 2.2.8 to 2.2.12.

PCRs were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~40ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) ErSLA2 primer, 2.5µl (10µM) ErAPN2 primer, 0.25µl DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 98°C, 20 sec at 58°C and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min.

MAT1-2-1 Gene Orientation

The orientation of the putative *MAT1-2-1* HMG-domain encoding gene relative to *SLA2* and *APN2* bordering genes was determined as follows.

All the *MAT1-2-1* gene orientation PCRs were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~20ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) of respective *MAT* gene primer, 2.5µl (10µM) of respective flanking gene primer, 0.25µl DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 98°C, 20 sec at an appropriate annealing temperature and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min.

Primer pair ErM2F and ErAPN2 at an annealing temperature of 50°C and primer pair ErM2R and ErSLA2 (Table 3.3) at an annealing temperature of 55.3°C were used to determine the relative orientation of the putative *MAT1-2-1* gene within the MAT-2 idiomorph. Resultant PCR products were resolved on 0.8% agarose gels and visualised using ethidium bromide staining (section 2.2.5).

MAT1-2-4 Gene Orientation

The previously identified *MAT1-2-4* gene sequences from *N. fumigata* and *N. fischeri* were used to design conserved *MAT1-2-4* gene-specific primers to amplify a fragment of this gene family (section 2.2.4), if present. After the whole *MAT1-2-4* gene was sequenced, *E. repens* specific primers [ErM124F and ErM124R (Table 3.3)] were designed to determine gene orientation relative to the bordering *SLA2* and *APN2* genes (section 2.2.5).

PCRs to determine the *MAT1-2-4* gene orientation were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~20ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) of respective *MAT* gene primer, 2.5µl (10µM) of respective flanking gene primer, 0.25µl DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 98°C, 20 sec

at 50°C and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min.

Primer pair ErM124F and ErSLA2 and primer pair ErM124R and ErAPN2 (Table 3.3) were used to determine the relative orientation of the putative *MAT1-2-4* gene within the MAT-2 idiomorph. Resultant PCR products were resolved on 0.8% agarose gels and visualised using ethidium bromide staining (section 2.2.5).

3.2.2.3 *MAT1-1-1* Gene Isolation and Seqeuncing

Thermal Asymmetric Interlaced (TAIL) PCR was used in combination with degenerate primers to sequence the entire *MAT1-1-1* gene. Successive rounds of TAIL-PCR were performed using degenerate primers together with specific nested primers designed from the 150bp fragment obtained from PCR with the *MAT1-1-1*-specific degenerate primers MAT5-6 and MAT3-4 (outlined in section 2.2.4) (Table 3.4) and fragments were sequenced using the protocols described in sections 2.2.8 to 2.2.12 (Liu and Whittier 1995). The PCRs were performed as follows.

Primary TAIL-PCR

Primary TAIL-PCRs were performed using FastStart Taq Polymerase (Roche, UK). Each 25µl reaction contained ~10ng genomic DNA, 2.5µl 10X PCR Buffer, 2µl (25mM) $MgCl_2$, 0.5µl (10mM each) dNTPs, 2µl (10µM) of respective specific primer, 12.5µl (10µM) of respective degenerate primer, 0.25µl Taq Polymerase and ~4.25µl H_2O.

The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: 1 cycle of 92°C for 2 min (60°C/min ramp rate); 1 cycle of 95°C for 1 min (60°C/min ramp rate); 5 cycles of 94°C for 15 sec, 62°C for 1 minute and 72°C for 2 min, with a 60°C/min ramp rate; 1 cycle of 94°C for 15 sec (60°C/min ramp rate), 44°C for 1 min (14°C/min ramp rate) and 72°C for 2 min (60°C/min ramp rate); 12 cycles of 94°C for 15 sec, 62°C for 1 min, 72°C for 2 min (+5 sec/cycle), 94°C for 15 sec, 62°C for 1 min, 72°C for 2 min (+5 sec/cycle), 94°C for 15 sec, 44°C for 1 min and 72°C for 2 min (+5 sec/cycle), with a 60°C/min ramp rate, followed by a final extension step of 72°C for 5 min at a 60°C/min ramp rate.

Secondary TAIL-PCR

Secondary TAIL-PCRs were performed using FastStart Taq Polymerase (Roche, UK). Each 25µl reaction contained 1µl of a 1/25 dilution of the primary TAIL-PCR product, 2.5µl 10X PCR Buffer, 1.5µl (25mM) $MgCl_2$, 0.0625µl (10mM each) dNTPs, 0.5µl (10µM) of respective specific primer, 10µl (10µM) of respective degnerate primer, 0.25 Taq Polymerase and ~9.2µl H_2O.

The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: 1 cycle of 92°C for 2 min (60°C/min ramp rate); 1 cycle of 95°C for 1 min (60°C/min ramp rate); 4 cycles of 94°C for 15 sec, 62°C for 1 min, 72°C 2 min, 94°C for 15 sec, 62°C for 1 min, 72°C for 2 min, 94°C for 15 sec, 44°C for 1 min and 72°C for 2 min, with a 60°C/min ramp rate; 8 cycles of 94°C for 15 sec, 62°C for 1 min, 72°C for 2 min (+ 5 sec/cycle), 94°C for 15 sec, 62°C for 1 min, 72°C for 2 min (+5 sec/cycle), 94°C for 15 sec, 44°C for 1 min and 72°C for 2 min (+ 5 sec/cycle), with a 60°C/min ramp rate, followed by a final extension step of 72°C for 5 min at a 60°C/min ramp rate.

Table 3.3: List of primers used in amplification of *E. repens* MAT loci and for RT-PCR analyses.

Primer	Sequence 5' to 3'
ErM1F	AGTAGCCCTACCTGATTCAGC
ErM1R	TACGCCTTGGCGAGGATGGC
ErM2F	AGTCCTTCGCACCTCGTGG
ErM2F2	TCCTCCGAATGCTTTCATCC
ErM2R	TACCATCGCTGGGGAAGAGACG
ErM2R2	TTCTCGTCTTTCCACTGCTTCCCA
ErM124F	ACAACCCTAGCACGAGGGG
ErM124R	ATCGCTTTCCGCTAAGAAGG
ErSLA2	ATGTCGACCATGCGCTCGACTCCC
ErAPN2	ATACCCGTAATGCAACCTGCGCTCC
Ate Actin Forward	ATTGTCGGTCGTCCCCG
Ate Actin Reverse	TCTGGGTCATCTTCTCACGG

Table 3.4: TAIL-PCR primers used for amplification of *E. repens* MAT loci.

Primer	Sequence 5' to 3'
N5	TGYCCNAAYMGNYT
TL1	TGWANGWGCNAAASGA
TL2	CTSNTTCGNTCTWGA
TL3	GTTNGWGCNAGWNAGA
TP1	NGTCGASWGANAWGAA
TP3	WGTGNAGWANCANAGA
ErTAIL1FA	ATCCGTTCAAAGCTAAATGG
ErTAIL1FB	TCAGAGGCGGAGTCGTCGAGG
ErTAIL1FC	TAGCCTGCAATTTTCTTCTAGG
ErTAIL1FD	TAGGTACAGTACGATCAGTC
ErTAIL1RA	TCTGGAGGAGCTTGGACG
ErTAIL1RB	AGACGAGAGACAAGTGTAGACG

3.2.2.4 RT-PCR Analysis

RT-PCR analysis was performed to determine whether gene expression and intron splicing occurred in any of the putative *MAT* genes found in *E. repens*. Species-specific, gene-specific primers for the various *MAT* genes were designed using the sequencing data obtained during this study (Table 3.3).

Primer pairs were designed to span putative introns to confirm intron splicing. Samples were grown until cleistothecia were formed (~2 weeks), after which time RNA was extracted and DNase treated according to the protocols described in sections 2.2.13 to 2.2.15. RT-PCR was performed and resultant PCR products were resolved on 1.5% agarose gels and visualised using ethidium bromide staining (sections 2.2.16 to 2.2.18).

For RT-PCR analysis of the putative *MAT1-1-1* gene, primer pair ErM1F and ErM1R was used (Table 3.3). This was predicted to yield a 260bp fragment from unspliced genomic DNA and, after splicing of a 54bp intron, predicted to yield a 206bp fragment from processed mRNA. To analyse the putative *MAT1-2-1* gene primer pair ErM2F2 and ErM2R was used (Table 3.3). This was predicted to yield a 174bp fragment and a 120bp fragment from unspliced genomic DNA and processed mRNA respectively (splicing of a 54bp intron). To analyse the putative *MAT1-2-4* gene primer pair ErM124F and ErM124R was used (Table 3.3). This was predicted to yield a 604bp fragment from unspliced genomic DNA, after splicing a 45bp intron, predicted to yield a 559bp fragment from the processed mRNA (Tables 3.3 and 3.8).

RT-PCR analysis of the constitutively expressed actin gene was included as a control. Actin controls were designed using *A. terreus* actin sequencing data from The Broad Institute genome sequencing project (gene locus ID: ATEG_06973.1). Primer pair Ate Actin Forward and Ate Actin Reverse was predicted to yield an approximate 330bp fragment from unspliced genomic DNA and an approximate 260bp fragment from processed mRNA, after splicing of a 70bp intron (Tables 3.3 and 3.8).

3.2.3 *Neosartorya fennelliae*

Isolates 54-1 (CBS 410.89, MATA) and 54-2 (CBS 411.89, MATa) of *N. fennelliae* were obtained from a culture collection at the School of Biology, The University of Nottingham. Both were originally isolated from Marine Sludge in Japan, 1981 (Takada and Udagawa 1985).

3.2.3.1 *MAT* Gene Sequencing

Conserved domains of the *MAT* genes were amplified using a degenerate PCR strategy as described in section 2.2.4. Resultant PCR products were resolved and visualised by gel electrophoresis as described in section 2.2.4, bands of interest were excised and gel extracted as described in section 2.2.8. The gel extracted products were ligated into plasmid pTOPO4, then cloned into *E. coli* and sequenced from extracted plasmid DNA using M13 Forward and M13 Reverse primers as described in sections 2.2.9 to 2.2.12.

3.2.3.2 MAT Region Amplification and Orientation of Genes Within Idiomorph

Whole Idiomorphic Region

The idiomorphs of *N. fennelliae* were amplified using the *SLA2-APN2* positional PCR strategy, as described in section 2.2.5. Species-specific *SLA2* and *APN2* primers [NfSLA2 and NfAPN2 (Table 3.5)] were designed from the gene fragments amplified using the protocol described in section 2.2.4 and sequenced using the protocols described in sections 2.2.8 to 2.2.12.

PCRs were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~40ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) NfSLA2 primer, 2.5µl (10µM) NfAPN2 primer, 0.25µl DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters:

initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 98°C, 20 sec at 58°C and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min. Resultant PCR products were resolved on 0.8% agarose gels and visualised using ethidium bromide staining (section 2.2.5).

MAT-1 Idiomorph Orientation

The orientation of the putative alpha-domain encoding *MAT1-1-1* gene relative to the *SLA2* and *APN2* bordering genes was determined as follows and section 2.2.5.

All PCRs to determine the *MAT1-1-1* gene orientation were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~20ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) of respective *MAT* gene primer, 2.5µl (10µM) of respective flanking gene primer, 0.25µl DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 98°C, 20 sec at 50°C and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min.

Primer pair NfM1F and NfSLA2 and primer pair NfM1R and NfAPN2 were used to determine the relative orientation of the putative *MAT1-1-1* gene within the MAT-1 idiomorph. Resultant PCR products were resolved on 0.8% agarose gels and visualised using ethidium bromide staining (section 2.2.5).

MAT-2 Idiomorph Orientation

The orientation of the putative HMG-domain encoding *MAT1-2-1* gene relative to the *SLA2* and *APN2* bordering genes was determined as follows and section 2.2.5.

All PCRs to determine the *MAT1-2-1* gene orientation were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~20ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) of respective *MAT* gene primer, 2.5µl (10µM) of respective flanking gene primer, 0.25µl DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 98°C, 20 sec

at an appropriate annealing temperature and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min.

Primer pair NfM2F and NfSLA2 at an annealing temperature of 50°C and primer pair NfM2R and NfAPN2 at an annealing tempertature of 53.5°C were used to determine the relative orientation of the putative *MAT1-2-1* gene within the MAT-2 idiomorph. Resultant PCR products were resolved on 0.8% agarose gels and visualised using ethidium bromide staining (section 2.2.5).

Table 3.5: List of primers used in amplification of *N. fennelliae MAT* regions and for RT-PCR studies.

Primer	Sequence 5' to 3'
NfM1F	TGGTTTCTCGTGCCAAACGCAC
NfM1R	TGATGGAGTACGCTTTCGCGAGG
NfM2F	AAGATCAAGGAAGCATATCCTG
NfM2F2	GCACCACCACCCCAAGATCAAGGAAGC
NfM2R	AGCCTTGACTTCTTCAGCTTCGGC
NfM2R2	GCTTCGGCTTTCCACTGCTTCCCA
NfSLA2	ACGTCGATCACGCATTGGACTCTC
NfAPN2	TGGCTGGGACTGCTACTTCAGTTTACC
Ate Actin Forward	ATTGTCGGTCGTCCCCG
Ate Actin Reverse	TCTGGGTCATCTTCTCACGG

3.2.3.3 RT-PCR Analysis

RT-PCR analysis was performed to determine whether gene expression and intron splicing occurred in any of the putative *MAT* genes found in *N. fennelliae*. Species-specific, gene-specific primers for the various *MAT* genes were designed using the sequencing data obtained during this study (Tables 3.6 and 3.10)

Primer pairs were designed to span putative introns to confirm intron splicing. Samples were grown until cleistothecia were formed (~3 weeks), after which time RNA was extracted and DNase treated according to the protocols described in sections 2.2.13 to 2.2.15. RT-PCR was performed and resultant PCR products were resolved on 1.5% agarose gels and visualised using ethidium bromide staining (sections 2.2.16 to 2.2.18).

For RT-PCR analysis of the putative *MAT1-1-1* gene primer pair NfM1F and NfM1R was used (Table 3.5). This was predicted to yield a 243bp fragment from unspliced genomic DNA and, after splicing of a 51bp intron, predicted to yield a 192bp fragment from processed mRNA. To analyse the putative *MAT1-2-1* gene primer pair NfM2F2 and NfM2R2 was used (Tables 3.5 and 3.8). This was predicted to yield a 141bp fragment and

a 90bp fragment from unspliced genomic DNA and processed mRNA, respectively (splicing of a 51bp intron).

RT-PCR analysis of the constitutively expressed actin gene was included as a control. Actin primers were desgined using *A. terreus* actin seqeucing data obtained from The Broad Institute genome sequencing project (gene locus ID: ATEG_06973.1). Primer pair Ate Actin Forward and Ate Actin Reverse was predicted to yield an approximate 330bp fragment from unspliced genomic DNA, and an approximate 260bp fragment from processed mRNA, after splicing of a 70bp intron (Tables 3.5 and 3.8).

3.2.4 Phylogenetic Analyses

Multiple alignments and related molecular evolution analyses and tree constructions were conducted using MEGA version 4 (Tamura *et al.* 2007). This involved maximum parsimony analyses under default values (with gaps excluded and bootstrapping of 500 replicates), section 2.2.20.

3.3 Results

3.3.1 *Emericella heterothallica*

3.3.1.1 *MAT1-1-1* Gene Isolation and Sequence Analysis

A 146bp putative *MAT1-1-1* gene fragment was successfully amplified from *E. heterothallica* isolate 50-5 using degenerate primers MAT5-6 and MAT3-4. This fragment was subsequently cloned and sequenced. Meanwhile, using the *SLA2-APN2* PCR cloning strategy (section 2.2.5), it was possible to amplify the entire MAT-1 idiomorph containing the putative *MAT1-1-1* gene. The idiomorph was then partially sequenced by chromosome walking outwards from the *MAT1-1-1* gene fragment to determine whether the complete putative *MAT1-1-1* gene was present (section 2.2.5), including the alpha-domain encoding sequence, necessary for function. In total 1532bp of the MAT1-1-1 region was sequenced from isolate 50-5, containing a 1139bp open reading frame, including one putative intron, which was predicted to encode a 362 amino acid MAT1-1-1 protein (Figure 3.4).

PSORT II (http://psort.nibb.ac.jp/) and TFSITESCAN (http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl) programs were used to interrogate resultant sequences for

nuclear-targeting and promoter-region motifs. The putative ATG translation start site of the MAT1-1-1 protein was consistent with the requirements of the Kozak rule (Kozak 1986). Analysis of the putative MAT1-1-1 protein revealed no clear nuclear targeting signals, but a transcriptional activator GATA box sequence was also found in the promoter region 108bp upstream of the start site.

```
1     agtacgatag ctagatcgat cagtcgacta gccgtcgact acgatcagca
51    tcgagcgata gctagctatc gactgatagc atcgatcagc tacgagctag
101   cattcagcga tcgagctagc agtctagcag ctagcatgcg actagcgatc
                                        |M||E||N ||E||L||S|
151   gagctagcga tgcagctagc gatcgagctg acatggaaaa cgagctctcc
      |P||L||Q|| R||A||F||N ||L||F||L| |L||S||M|| P||P||D||Q
201   cccttacagc gtgcttttaa cttatttcta ttgtcgatgc cacccgacca
      ||L||D||E| |L||V||K|| Y||I||Q||V ||G||K||A| |Q||E||I||
251   gcttgatgaa cttgtcaagt atatccaagt tggcaaagct caggagatct
      S||S||P||V ||H||D||W| |D||I||P|| A||A||R||L ||D||T||A|
301   cctcccctgt ccatgactgg gatattcccg cagcgcgact ggacactgcg
      |Q||D||N|| Q||H||T||V ||V||L||P| |D||S||A|| V||T||R||P
351   caggacaacc aacacaccgt ggtactgcca gattccgcgg tcactagacc
      ||S||S||S| |R||G||K|| R||S||H||D ||G||R||R| |P||L||N||
401   atcatcttcg cggggtaagc ggtcgcacga tggaaggcgg ccgttgaatt
      W||F||I||A ||F||R||
451   ggttcatcgc attcagaagt aagtatcact ttgaacgaaa agaaaaacac
                          S| |Y||Y||S|| V||I||F||P ||D||L||T|
501   aagctgattc ttttatagGT tactactctg ttatcttccc ggacctcact
      |Q||K||A|| K||S||G||I ||L||R||F| |L||W||Q|| A||D||P||F
551   caaaaagcca agtccggcat cctccgcttc ctgtggcaag cggacccatt
      ||K||A||K| |W||A||I|| L||A||K||A ||Y||S||I| |I||R||D||
601   caaggctaag tgggccattc tggccaaagc atactccata atacgtgata
      K||H||D||D ||E||V||S| |L||E||S|| F||L||T||L ||N||A||P|
651   agcatgatga tgaagtcagt ctcgagagct tcctgactct aaatgcccca
      |L||I||G|| I||L||D||P ||D||R||Y| |L||N||V|| I||G||W||Q
701   ctcatcggga tcctagaccc cgatcgatat ctcaacgtaa tcgggtggca
      ||L||A||P| |D||D||Q|| Q||Q||Y||T ||M||A||R| |V||K||T||
751   actcgctccg gacgatcagc agcaatatac gatggcacgg gtaaagaccc
      P||A||S||L ||E||A||E| |S||S||T|| N||Y||S||V ||D||D||L|
801   ccgcatctct agaagctgaa tcgtcaacca actactccgt tgacgacctg
      |V||K||H|| C||Y||A||T ||G||Y||V| |T||I||G|| K||G||K||S
851   gtcaagcatt gctatgccac cgggtacgtg actattggaa aggggaagtc
      ||K||A||I| |K||H||H|| N||A||P||T ||M||A||F| |A||V||Q||
901   caaggcgatc aagcaccaca atgcaccaac aatggcattt gctgtacaac
      P||A||L||V ||I||H||K| |D||D||S|| L||Q||V||S ||G||N||N|
1001  ctgctctggt catccacaaa gatgattcgc tccaggtatc gggcaacaac
      |T||I||V|| S||T||I||Y ||S||A||E| |V||D||M|| E||Y||P||S
1051  acgatcgtgt cgaccatata ttcagcggag gttgatatgg aatacccaag
      ||F||E||P| |T||E||D|| T||L||L||P ||N||P||S| |D||L||S||
1101  ttttgagccg acagaggaca cgctcctacc gaatccctca gacctgtcct
      S||N||V||A ||N||D||T| |T||L||G|| A||M||E||N ||I||F||V|
1151  ccaacgtagc aaacgatacc accctaggag ccatggagaa cattttcgtt
      |C||H||R|| Q||Q||P||S ||N||D||P| |G||A||G|| N||D||F||M
1201  tgccaccgtc agcagccgag caacgatcca ggagcaggga acgacttcat
```

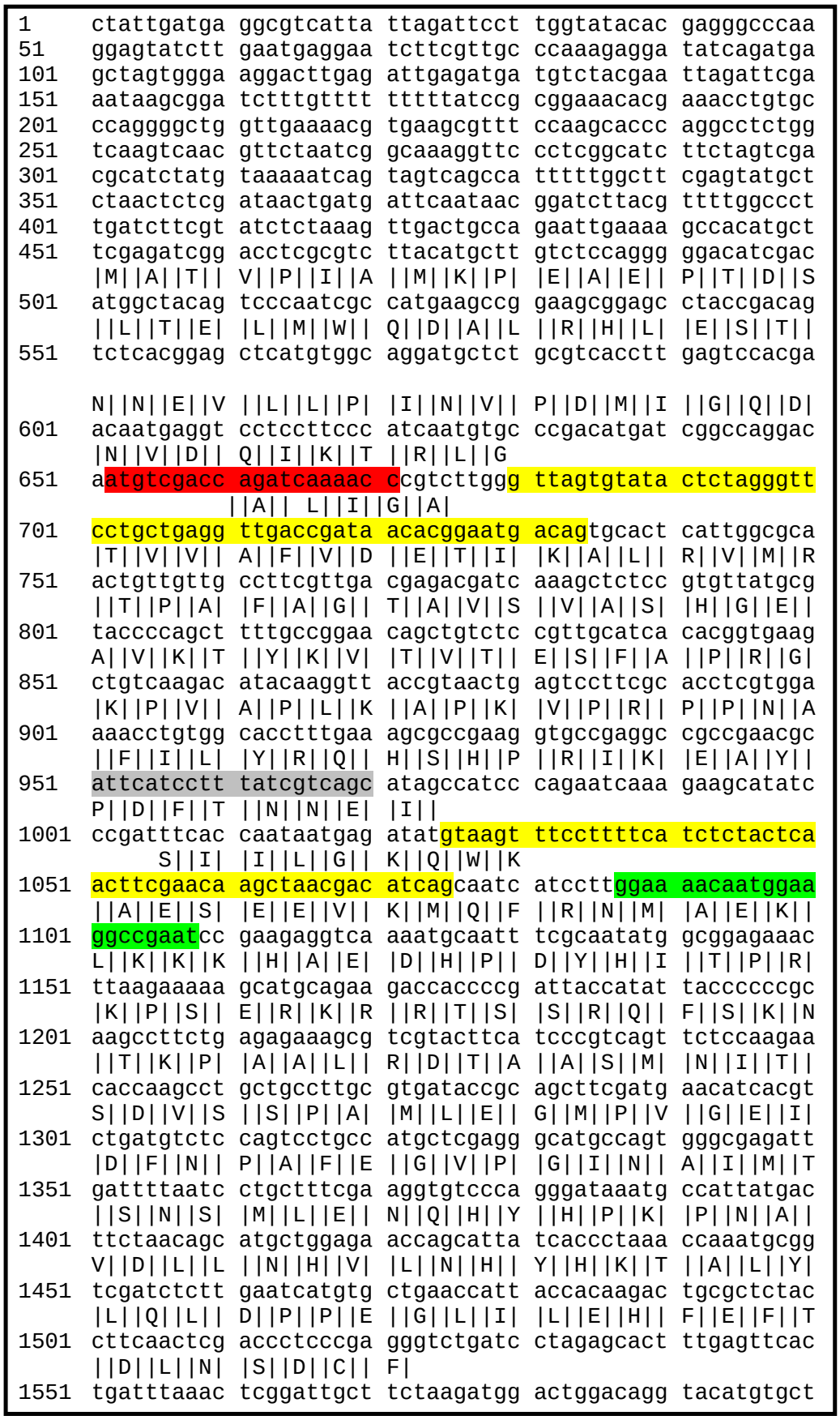
```
     ||P||D||N| |I||G||F|| P||G||I||I ||N||A||S| |P||Q||A||
1251 gccggacaat atcgggttcc ctggcattat taacgcctcc cctcaggctc
     l||M||P||Y ||D||P||L| |Y||C||D|| P||F||E||A ||F||D||I|
1301 taatgcccta cgatcctctg tattgcgatc cattcgaggc tttcgacata
     |K||Q||F|| L||D||V|
1351 aagcaatttc tagacgtata aagtgcatcg atgcatgatc gactacagcg
1401 atcagcgatc gagctagcag tcagctatcg atcgagctta cgatcagcta
1451 tcgagtctag cgatcgatct agctagcatc agcgtatcga tctagcgtat
1501 cgatcagcta gcagtcgagc tatgcgatct ag
```

Figure 3.4: DNA sequence of the MAT1-1-1 region of *E. heterothallica* isolate 50-5. The putative encoded amino acid sequence is indicated above the relevant base pair sequence. Primer EhM1F is highlighted red (forward), primer EhM1R is highlighted green (reverse) and putative intron is highlighted in yellow. Also included is 182bp upstream and 161bp downstream of the putative translation ATG start site and end site, respectively.

3.3.1.2 *MAT1-2-1* Gene Isolation and Sequence Analysis

A 269bp putative *MAT1-2-1* gene fragment was succesfully amplified from *E. heterothallica* isolate 50-3 using degenerate primers MAT5-7 and MAT3-5. This fragment was subsequently sequenced. Meanwhile, using the *SLA2-APN2* PCR strategy (section 2.2.5) to amplify the entire MAT-2 idiomorph containing the putative *MAT1-2-1* gene. 9208bp of the MAT-2 idiomorph was sequenced by a chromosome walking strategy outwards from the *MAT1-2-1* gene fragment to determine whether a complete putative *MAT1-2-1* gene was present (section 2.2.5), including the conserved HMG-domain encoding region, necessary for function. Analysis of sequence from isolate 50-3 revealed a 1075bp open reading frame including two introns, which was predicted to encode a 321 amino acid MAT1-2-1 protein (Figure 3.5).

PSORT II and TFSITESCAN programs were used to interrogate resultant sequences for nuclear-targeting and promoter-region motifs. The putative ATG translation start site of the MAT1-2-1 protein was consistent with the requirements of the Kozak rule (Kozak 1986). Analysis of the putative MAT1-2-1 protein revealed three nuclear targeting signals (KKKH at position 182, RKRR at position 202 and PSERKRR at position 199) and a transcriptional activator GATA box sequence 141bp upstream of the start site. Possession of nuclear targeting sites is consistent with a role of the *MAT1-2-1* gene as a transcriptional activator. The entire MAT-2 idiomorph was sequenced during this study, this sequence can be found in Appendix 1, Figure 1.


1 ctattgatga ggcgtcatta ttagattcct tggtatacac gagggcccaa
51 ggagtatctt gaatgaggaa tcttcgttgc ccaaagagga tatcagatga
101 gctagtggga aggacttgag attgagatga tgtctacgaa ttagattcga
151 aataagcgga tctttgtttt tttttatccg cggaaacacg aaacctgtgc
201 ccaggggctg gttgaaaacg tgaagcgttt ccaagcaccc aggcctctgg
251 tcaagtcaac gttctaatcg gcaaaggttc cctcggcatc ttctagtcga
301 cgcatctatg taaaaatcag tagtcagcca tttttggctt cgagtatgct
351 ctaactctcg ataactgatg attcaataac ggatcttacg ttttggccct
401 tgatcttcgt atctctaaag ttgactgcca gaattgaaaa gccacatgct
451 tcgagatcgg acctcgcgtc ttacatgctt gtctccaggg ggacatcgac
501 atggctacag tcccaatcgc catgaagccg gaagcggagc ctaccgacag
551 tctcacggag ctcatgtggc aggatgctct gcgtcacctt gagtccacga
601 acaatgaggt cctccttccc atcaatgtgc ccgacatgat cggccaggac
651 aatgtcgacc agatcaaaac ccgtcttggg ttagtgtata ctctagggtt
701 cctgctgagg ttgaccgata acacggaatg acagtgcact cattggcgca
751 actgttgttg ccttcgttga cgagacgatc aaagctctcc gtgttatgcg
801 taccccagct tttgccggaa cagctgtctc cgttgcatca cacggtgaag
851 ctgtcaagac atacaaggtt accgtaactg agtccttcgc acctcgtgga
901 aaacctgtgg caccttttgaa agcgccgaag gtgccgaggc cgccgaacgc
951 attcatcctt tatcgtcagc atagccatcc cagaatcaaa gaagcatatc
1001 ccgatttcac caataatgag atatgtaagt ttccttttca tctctactca
1051 acttcgaaca agctaacgac atcagcaatc atccttggaa aacaatggaa
1101 ggccgaatcc gaagaggtca aaatgcaatt tcgcaatatg gcggagaaac
1151 ttaagaaaaa gcatgcagaa gaccaccccg attaccatat tacccccgc
1201 aagccttctg agagaaagcg tcgtacttca tcccgtcagt tctccaagaa
1251 caccaagcct gctgccttgc gtgataccgc agcttcgatg aacatcacgt
1301 ctgatgtctc cagtcctgcc atgctcgagg gcatgccagt gggcgagatt
1351 gattttaatc ctgctttcga aggtgtccca gggataaatg ccattatgac
1401 ttctaacagc atgctggaga accagcatta tcaccctaaa ccaaatgcgg
1451 tcgatctctt gaatcatgtg ctgaaccatt accacaagac tgcgctctac
1501 cttcaactcg accctcccga gggtctgatc ctagagcact ttgagttcac
1551 tgatttaaac tcggattgct tctaagatgg actggacagg tacatgtgct

```
1601  tccccccccc ttttacgacg ctttcggagt atataaacaa tggatccctt
1651  gagcaagcaa aatccagtgt acaggaagtc atccgcaccc ttgagagagg
1701  ttcgaaattg tcctttaaat cgaaatgtac catattaaag tatggatgat
1751  ttgtcgatgg ggtgaagaac gatggtaacg tgcagatccc ctcgaattct
1801  ttggtcttct aagaccagaa gaaatctttt ccttaaaaaa tccgtgaaat
1851  taccgcgaga aatatacggt caattttgtg ctgcaaattt tgggatagga
1901  catcagccac ctacctgatc gaaaagctag ctatctatcg atatctccta
1951  gatcgatcta gatctatcta gatctatcga aatcttgttc gatctatcgt
2001  atcgtatcgt atcctagcta tcctacatct aaaaaatctc gatttcgatc
2051  tctaggatcg atcgtatcga tcgaa
```

Figure 3.5: DNA sequence of the MAT1-2-1 region of *E. heterothallica* isolate 50-3. The putative encoded amino acid sequence is indicated above the relevant base pair sequence. Primer EhM2F is highlighted red (forward), primer EhM2F2 is highlighted grey (forward), primer EhM2R is highlighted green (reverse) and putative introns are highlighted in yellow. Also incluced is 500bp upstream of the putative translation ATG start site and downstream of the end site, respectively.

3.3.1.3 *MAT1-2-4* Gene Isolation and Sequence Analysis

Analysis of the complete MAT-2 idiomorph from *E. heterothallica* isolate 50-3 revealed the presence of a putative *MAT1-2-4* gene between the *SLA2* and *MAT1-2-1* gene (Figure 3.9). Analysis of sequence from isolate 50-3 revealed a 771bp open reading frame including one putative intron, which was predicted to encode a 242 amino acid MAT1-2-4 protein (Figure 3.6).

PSORT II and TFSITESCAN programs were used to interrogate resultant sequences for nuclear-targeting and promoter-region motifs. The putative ATG translation start site of the MAT1-2-4 protein was consistent with the requirements of the Kozak rule (Kozak 1986). Analysis of the MAT1-2-4 protein revealed no clear nuclear targeting signals, mitochondrial targeting sequences or RNA binding sequences. There was a transcriptional activator TATA box sequence 35bp upstream of the start site.

```
1     cccctggaga caagcatgta agacgcgagg tccgatctcg aagcatgtgg
51    cttttcaatt ctggcagtca actttagaga tacgaagatc aagggccaaa
101   acgtaagatc cgttattgaa tcatcagtta tcgagagtta gagcatactc
151   gaagccaaaa atggctgact actgattttt acatagatgc gtcgactaga
201   agatgccgag ggaacctttg ccgattagaa cgttgacttg accagaggcc
251   tgggtgcttg gaaacgcttc acgttttcaa ccagcccctg ggcacaggtt
301   tcgtgtttcc gcggataaaa aaaaacaaag atccgcttat ttcgaatcta
351   attcgtagac atcatctcaa tctcaagtcc ttcccactag ctcatctgat
401   atcctctttg ggcaacgaag attcctcatt caagatactc cttgggccct
451   cgtgtatacc aaggaatcta ataatgacgc ctcatcaata ggtcttcagt
      |M||D||Y|| E||R||D||V ||L||L||W| |Y||L||G|| T||V||A||D
501   atggattatg aacgagatgt gcttctgtgg tatcttggta ctgtcgccga
      ||D||A||R| |Y||P||P|| D||L||R||N ||K||A||T| |H||I||I||
551   cgatgcccgt tacccgccag acttgagaaa caaggctacc cacatcatcg
      V||S||F||M ||R||H||R| |N||A||Y|| R||L||L||P ||Q||A||T|
601   tttccttcat gcgccacaga aacgcttaca gactcctgcc ccaggctact
```

```
     |E||L||A|| R||G||E||L ||V||M||Y| |P||F||Q|| Q||V||G||N
651  gaattggcca gaggagagct agtcatgtat ccctttcagc aggttggtaa
     ||I||P||G| |N||I||G|| L||P||V||R ||R||F||S| |Q||N||I||
701  cattccagga aacatcgggc tccctgtgcg gcgtttcagt caaaacattc
     H||A||I||T ||T||A||F| |G||I||I|| P||T||N||E ||D||N||E|
751  atgcaattac aactgctttt ggcattattc cgaccaacga ggacaacgaa
     |G||H||P|| I||E||L||I ||S||I||L| |D||P||A|| I||E||A||S
801  ggtcacccaa tcgagctcat cagcatcctt gatccagcta ttgaagcaag
     ||M||N||D| |N||Q||K|| F||E||F||H ||R||L||L| |L||V||K||
851  catgaatgac aaccagaaat ttgagttcca ccgcctactt cttgtcaagg
     E||R||Q||A ||N||A||D| |L||A||R|| S||V||Q||R ||Y||G||Y|
901  aacgacaggc aaatgccgat ctcgcgagat ccgttcagcg ttacggttac
     |H||Y||I|| F||R||A||G ||L||Q||Q| |Y||Y||M|| T
951  cactacatct ttagagctgg tcttcagcag tactacatga cgtatgtatt
                           ||K| |T||V||V||
1001 tctcactttc tatccgaatt ctgcttacag ttttagtaaa actgtcgtgg
     E||M||L||N ||F||W||T| |P||D||P|| R||G||N||A ||Y||R||V|
1051 agatgctcaa cttctggact ccggatcctc gtggtaatgc ctatcgtgtt
     |R||V||Q|| R||I||C||Y ||A||A||I| |E||T||R|| L||R||L||N
1101 cgtgtccagc gcatctgtta tgcggccatc gagacacgtc ttcgactcaa
     ||N||L||K| |K||T||L|| L||I||K||T ||T||R||S| |L||P||N||
1151 caacctcaag aagacgctcc tcatcaaaac aactcgctct ctgcctaatg
     D||A||L||R ||F||C||K| |Y||I||C|| A||L||L||T ||G||L||L|
1201 atgcgctacg cttctgtaag tacatctgtg ctctgctgac gggcttgcta
     |N||L||A|| R||G||L||D |
1251 aatcttgcca ggggcttgga ttgagtagtc gatggatcga tagcgatcga
1301 tcgagatcga tcgagactat ttagcgattt cgagagatct atcgagatcg
1351 atatcgagat ctagatcgat ctagatcgag atctagaatc gatctctcca
1401 gagatcttca gatacgatct atactagact ttcagagcat atagcagata
1451 gatcagagat cgagatctag gactgatcgg aatcagatcg agtacgatga
1501 tcggatcgat gatcgaggat cggatcgagg atagcgatcg aggatagatc
1551 ggatcgatcg agagatctag agctgatcga gatcagatca gatcagatta
1601 tatcgagaga tcaagctaga ctgagcatga cgatcgagct agcattgact
1651 agctatagac tgagatcgac tgagcatgac tgacgtagat cgacccagac
1701 tagcaagact gagagggtgt agcggatttt gatcgagtta gcgtgatatc
1751 gagatcgatc gagagatgga tagc
```

Figure 3.6: DNA sequence of the MAT1-2-4 region of *E. heterothallica* isolate 50-3. The putative encoded amino acid sequence is indicated above the relevant base pair sequence. Primer M124F is highlighted red (forward), primer EhM124R is highlighted green (reverse), primer EhM124R2 is highlighted purple (reverse) and putative intron is highlighted in yellow. Also included is 500bp upstream of the translation ATG start site and downstream of the end site, respectively.

3.3.1.4 Orientation of *MAT* Genes Within The Idiomorph

A variety of PCR amplifications were attempted in order to determine the orientations of the putative *MAT1-1-1* gene, *MAT1-2-1* gene and *MAT1-2-4* gene relative to the flanking *SLA2* and *APN2* genes (see sections 2.2.5 and 3.2.1.2 for protocols).

Amplicons of approximately 4kb and 5.5kb in size were produced by primer pair EhM1F and EhSLA2-2 and primer pair EhM1R and EhAPN2-1, respecitvely (Figure 3.7). This allowed the *MAT1-1-1* gene orientation to be deduced as shown in Figure 3.9. Amplicons of 2.5kb and 4kb in size were produced by primer pairs EhM2F with EhAPN2-

1 and EhM2R with EhSLA2-1, respectively (Figure 3.8). Amplicons of 4.5kb and 3kb in size were produced by primer pairs M124F with EhSLA2-2 and EhM124R with EhAPN2-1, respectively (Figure 3.8). This allowed the *MAT1-2-1* and *MAT1-2-4* gene orientations to be deduced as shown in Figure 3.9, confirming the data from sequencing of the MAT-2 idiomorph (Appendix 1, Figure 1).

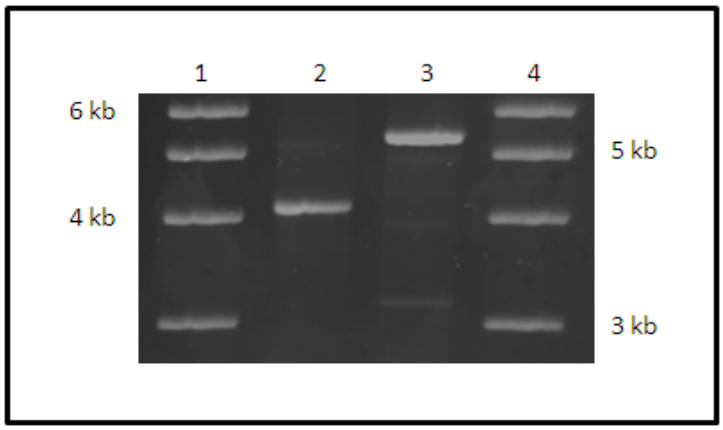


Figure 3.7: 0.8% agarose gel showing the results of PCR amplifications to determine *MAT1-1-1* gene orientation within the MAT-1 idiomorph of *E. heterothallica* isolate 50-5. Lanes 1 and 4: 1kb ladder. Lane 2: Amplicons produced by primers EhM1F and EhSLA2-2. Lane 3: Amplicons produced by primers EhM1R and EhAPN2-1.

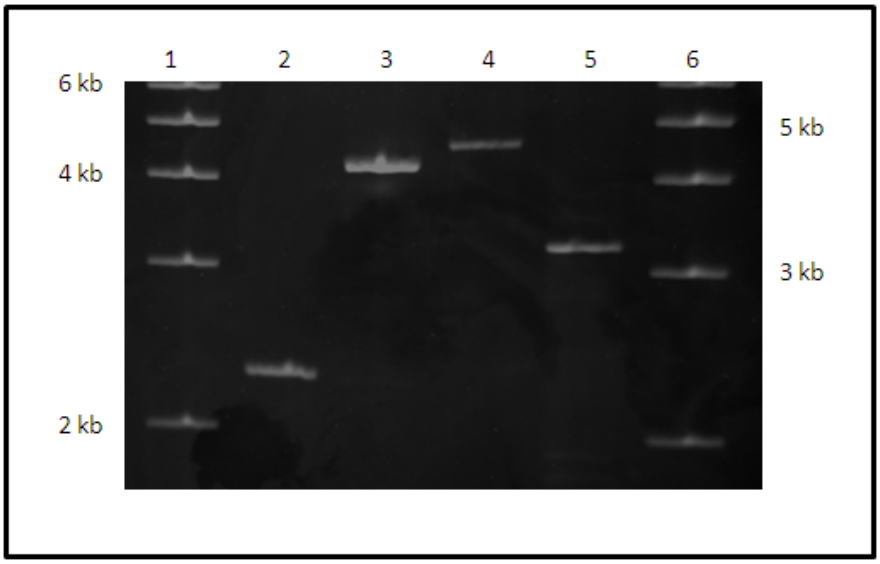


Figure 3.8: 0.8% agarose gel showing the results of PCR amplifications to determine *MAT1-2-1* and *MAT1-2-4* gene orientations within the MAT-2 idiomorph of *E. heterothallica* isolate 50-3. Lanes 1 and 6: 1kb ladder. Lane 2: Amplicons produced by primers EhM2F and EhAPN2-1. Lane 3: Amplicons produced by primers EhM2R and EhSLA2-1. Lane 4: Amplicons produced by primers M124F and EhSLA2-2. Lane 5: Amplicons produced by primers EhM124R and EhAPN2-1.

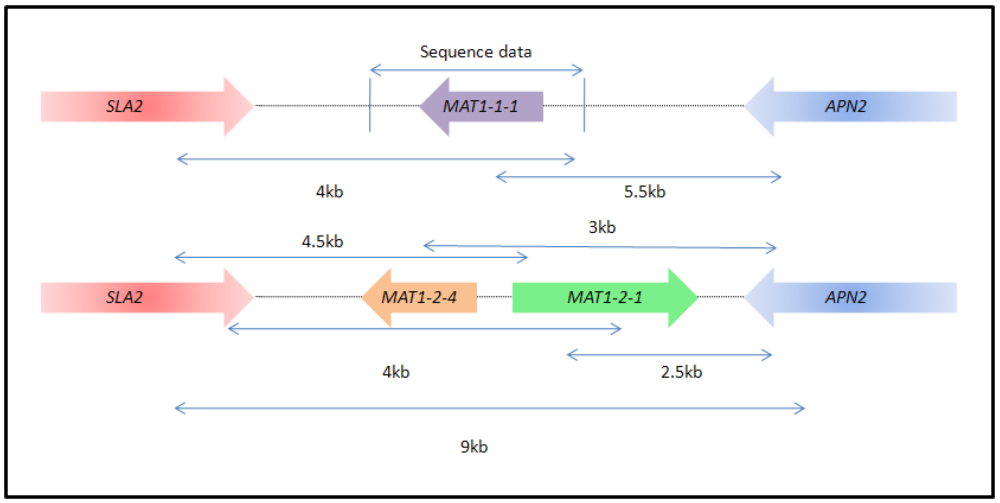


Figure 3.9: Schematic showing the organisation of the MAT-1 idiomorph (top) and the MAT-2 idiomorph (bottom) of *E. heterothallica* (distances in kb).

3.3.1.5 RT-PCR Analysis

The RT-PCR analysis provided clear evidence of the expression of the *MAT1-1-1* and *MAT1-2-1* genes of *E. heterothallica*, together with actin controls. Amplicons of smaller size, relative to genomic controls, were produced by RT-PCR corresponding to the predicted sizes of mRNA transcripts allowing for splicing of a 50bp intron from *MAT1-1-1* and a 51bp intron from *MAT1-2-1* genes (Figure 3.10 and Table 3.6). However, no expression could be detected for the putative *MAT1-2-4* gene as judged by lack of a RT-PCR products (Figure 3.10). This suggested that under the growth conditions assayed in this study, this gene was not expressed in isolate 50-3.

Table 3.6: Primers used in RT-PCR experiments and predicted fragment lengths of *E. heterothallica* amplification products.

Forward primer	Reverse primer	Genomic DNA fragment length (bp)	Processed mRNA fragment length (bp)
EhM1F	EhM1R	521	471
EhM2F2	EhM2R	158	107
M124F	EhM124R2	603	557
Ate Actin Forward	Ate Actin Reverse	~330	~260

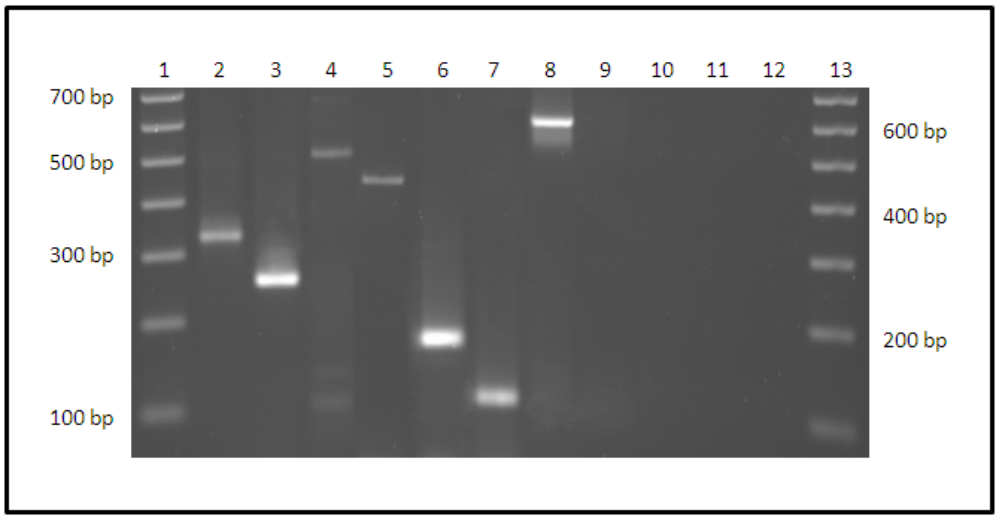


Figure 3.10: 1.5% agarose gel showing results of *MAT1-1-1, MAT1-2-1* and *MAT1-2-4* RT-PCR gene analysis of *E. heterothallica* isolates 50-5 and 50-3. Lanes 1 and 13: 100bp ladder. Lane 2: Genomic DNA Actin Control. Lane 3: RNA Actin Control. Lanes 4 and 5: Amplification with *MAT1-1-1* specific primers of genomic DNA and RNA extracts, respectively. Lanes 6 and 7: Amplification with *MAT1-2-1* specific primers of genomic DNA and RNA extracts, respectively. Lanes 8 and 9: Amplification with *MAT1-2-4* specific primers of genomic DNA and RNA extracts, respectively. Lane 10: *MAT1-1-1* RT-PCR water control with no RNA. Lane 11: *MAT1-2-1* RT-PCR water control with no RNA. Lane 12: *MAT1-2-4* RT-PCR water control with no RNA.

3.3.2 *Eurotium repens*

3.3.2.1 *MAT1-2-1* Gene Isolation and Sequence Analysis

PCR amplification with primers ErAPN2 and ErSLA2 produced a fragment of approximately 8-9kb in length (Figure3.14) from isolate 51-1. This fragment was sequenced by chromosome walking inwards from the *SLA2* and *APN2* genes (section 2.2.5), with new sequencing primers designed as necessary as sequencing continued inwards. This resulted in sequencing of more than 9000bp of the MAT locus of isolate 51-1. Meanwhile, a 274bp putative *MAT1-2-1* gene was successfully amplified from *E. repens* isolate 51-2 using degenerate primers MAT5-7 and MAT3-5. This fragment was sequenced and an outward PCR chromosome walking strategy based on the flanking *SLA2* and *APN2* genes (section 2.2.5) was used to gain further sequence of the putative *MAT1-2-1* gene. In total 3453bp of the MAT-2 locus was sequenced encompassing the 1078bp, *MAT1-2-1* gene, which contained two putative introns and was predicted to encode a 321 amino acid MAT1-2-1 protein (Figure 3.11). Sequence from isolate 51-2 ceased when siginificant nucleotide identity (99-100%) was seen compared to

sequencing obtained from isolate 51-1, when the sequences from the two isolates were pooled 9437bp of the MAT-2 locus had been sequenced (see Appendix 1, Figure 2).

PSORT II and TFSITESCAN programs were used to interrogate resultant sequences for nuclear-targeting and promoter-region motifs. The putative ATG translation start site of the MAT1-2-1 protein was not consistent with the requirements of the Kozak rule (Kozak 1986). Analysis of the putative MAT1-2-1 protein revealed three nuclear targeting signals (KKKH at position 183, KKRR at position 203 and PYEKKRR at position 200) and a transcriptional activator GATA box sequence 73bp upstream of the start site. Possession of nuclear targeting sites is consistent with a role of the *MAT1-2-1* gene as a transcriptional activator. A second possible start ATG codon was detected 7 amino acids inwards of the proposed MAT1-2-1 start site. This alternative start site did obey the Kozak rule, however this would have produced a truncated MAT1-2-1 protein when compared to other known MAT1-2-1 proteins.

```
1     gcatataatg atcctcgctg atgctagctc gatgctcgaa tgctagcgtg
51    atcgatcagg gcaaaaactt aagtaccttc tcgaaagaga aggcacttag
101   agatacagca tcctggaggg gggaaaaatg acaggctact gtttttata
151   tggcacatgg ggcgggtgtt cgaacttgag ggaattggcg ggtttcaatg
201   ttaacttgca ggaagtgaat gcttaagacg cgcttcacgt tttctcccag
251   cccctgacac ggttcgtctg atgctagcta gctagcatcg atcggatcgg
301   atcggctagc ttaggctagc taggctaggc gatgcagtct gcatgcgcta
351   gctcggctga gagtcgagct gagctcggat cgagctgcga tcgacgtctg
401   agctagcgat ctagcagcta gcgagaggat agatgcatgg ctagcagtca
451   tgcatgagct gatcgcttgc gtagctgcgt cgctgcgatg cgatcgctta
      |M||A||T|| V||P||I||A ||M||R||S| |D||A||Q|| S||P||E||S
501   atggcaacgg taccaatcgc tatgcgttca gatgcccagt cgcctgagag
||I||T||E| |L||L||W|| Q||D||A||L ||R||H||L| |F||S||T||
551   catcacggag ctcctctggc aggacgcgct acgacattta ttcagtacaa
      N||E||E||V ||L||P||P| |I||N||V|| T||D||M||I ||G||Q||D|
601   acgaagaagt cctgccaccg attaatgtga cggacatgat cgggcaagac
      |N||V||E|| R||L||K||S ||R||L||G
651   aacgtagagc gtctaaagtc gagactcggg tcgaatcgat cgatagctag
                                        ||A||L ||L||G||A|
701   ctagctagct agactagatc gatcgatgca gcagtgctct tcttggcgca
      |P||V||V|| S||F||V||D ||E||S||L| |N||A||L|| R||V||L||R
751   cctgttgtct ctttcgtaga tgagtcactg aatgcactaa gggtcttacg
      ||T||P||E| |F||S||G|| S||A||I||S ||V||A||S| |M||G||G||
801   aaccccggag ttttcgggat ccgcaattag cgtagcctca atgggaggag
      A||Q||T||S ||K||R||S| |T||V||T|| E||S||F||A ||P||R||G|
851   cccagacttc taagaggtct accgtaactg agtccttcgc acctcgtgga
      |K||P||V|| G||P||L||K ||A||P||K| |V||P||R|| P||P||N||A
901   aaacctgtgg gacctttgaa agcgccgaag gtcccgcgtc ctccgaatgc
      ||F||I||L ||Y||R||Q|| R||H||H||P ||K||I||K| |E||A||Y||
951   tttcatcctg tatcgtcagc gtcatcaccc caagatcaag gaagcatatc
      P||D||Y||S ||N||N||D| |I||
1001  ctgactattc gaacaacgat atttgtaagt tgcttgccta tatattttct
```

```
                                        S| |V||M||L|| G||K||Q||W
1051  ttacggttat ttttactaat atgcctagcc gtcatgcttg ggaagcagtg
      ||K||D||E| |N||E||E|| I||K||A||Q ||F||R||N| |L||A||E||
1101  gaaagacgag aatgaagaga tcaaggccca attccgaaac ctagcagaag
      E||L||K||K ||K||H||A| |E||D||H|| P||D||Y||H ||Y||T||P|
1151  agctcaagaa gaagcacgcc gaagatcatc ctgactatca ttacacccct
      |R||K||P|| Y||E||K||K ||R||R||N| |S||S||R|| C||C||S||K
1201  cgcaagcctt atgagaaaaa gcgacgcaat tcgtcgcggt gctgttcaaa
      ||A||P||Q ||R||F||F|| C||N||P||S ||Y||W||A| |M||T||Y||
1251  agcacctcag cgatttttt gcaatccgtc atactgggca atgacttaca
      I||P||S||D ||V||S||S| |P||A||M|| V||D||G||M ||P||V||G|
1301  tcccttccga cgtctcttcc ccagcgatgg tagacggcat gccagtggga
      |A||Q||F|| D||D||C||Y ||F||S||Q| |M||D||G|| E||N||A||I
1351  gctcaattcg atgattgtta tttcagtcaa atggacggtg aaaacgcgat
      ||M||P||Q| |R||T||L|| P||A||N||T ||C||A||N| |F||D||P||
1401  aatgccccaa cgaactttgc cggcgaatac atgcgccaat ttcgatcctt
      F||A||F||D ||L||F||Q| |Q||V||Q|| S||D||Y||H ||K||T||A|
1451  tcgcgttcga tctttttcag caggtacaaa gcgactatca taaaacggca
      |L||Y||R|| Q||L||A||P ||A||E||Q| |F||V||G|| E||S||F||E
1501  ctatatagac aattggctcc agcagaacag ttcgtcggag aatcatttga
      ||F||L||D| |L||I||S|| D||Y||Y|
1551  gtttctggac ctcatttcgg attattatta agatgcatga tcgatcgatc
1601  gatccgatcg atcgatacga tcagcattca gctacgatca gctatcgatc
1651  tagcatcgat caggcatcgg actagcgatc gatcgagcta gcgatcagct
1701  agcatcgagc tagctagtcg agctagctac gatctagcta tcgatcgact
1751  catcagtagc atacgatcga tcgatgcata gctagcatgc atcgatacag
1801  tcagcgatcg agcatcgatc tagcatgcat cagctagcgc atcgatcgac
1851  agtcgactag atcgagattt tcagactagt taaaaagact cgatcagcgc
1901  atcgactagc gcatcgatca gactagctac gctagcatcg agcatgatca
1951  tgactagcat cgagcatgca tgcatgcatg catctagtcg actagctagc
2001  agcatgcgta tcgagcatgc atctaagcta gctacgagct agctagctag
2051  cgatcagtac gattacgcta gcgactag
```

Figure 3.11: DNA sequence of MAT1-2-1 region of *E. repens* isolate 51-2. The putative encoded amino acid sequence is indicated above the relevant base pair sequence. Primer ErM2F is highlighted red (forward), primer ErM2F2 is highlighted purple (forward), primer ErM2R is highlighted green (reverse), primer ErM2R2 is highlighted in blue (reverse) and putative introns are highlighted in yellow. Also included is 500bp upstream and downstream of the putative translation ATG start site and end site.

3.3.2.2 *MAT1-2-4* Gene Isolation and Sequence Analysis

A 604bp fragment of a putative *MAT1-2-4* family gene was successfully amplified using primers MAT124F and MAT124R from isolate 51-2 (section 2.2.4). This fragment was sequenced and an outward PCR chromosome walking strategy based on the flanking *SLA2* and *APN2* genes (section 2.2.5) was used to gain further sequence from the putative MAT1-2-4 region. This resulted in identification of a *MAT1-2-4* gene, containing one putative intron and was predicted to encode a 242 amino acid MAT1-2-4 protein (Figure 3.12)

PSORT II and TFSITESCAN programs were used to interrogate resultant sequences for nuclear-targeting and promoter-region motifs. Analysis of the putative MAT1-2-4 protein revealed no clear nuclear localisation signals, no RNA binding signals and no mitochondrial targeting sequences. However, a transcriptional activator GATA box sequence 103bp and a TATA box sequence 46bp upstream of the putative ATG translation start site were identified. The entire MAT-2 locus was sequenced during this study, the sequence can be found in Appendix 1, Figure 2.

```
1     acgcaagcga tcagctcatg catgactgct agccatgcat ctatcctctc
51    gctagctgct agatcgctag ctcagacgtc gatcgcagct cgatccgagc
101   tcagctcgac tctcagccga gctagcgcat gcagactgca tcgcctagcc
151   tagctagcct aagctagccg atccgatccg atcgatgcta gctagctagc
201   atcagacgaa ccgtgtcagg ggctgggaga aaacgtgaag cgcgtcttaa
251   gcattcactt cctgcaagtt aacattgaaa cccgccaatt ccctcaagtt
301   cgaacacccg ccccatgtgc catataaaaa acagtagcct gtcatttttc
351   ccccctccag gatgctgtat ctctaagtgc cttctctttc gagaaggtac
401   ttaagttttt gccctgatcg atcacgctag cattcgagca tcgagctagc
451   atcagcgagg atcattatat gcatgcatcg atcgatcgac tagcgatcta
      |M||D||Y|| E||R||D||V ||L||L||W| |Y||L||G|| T||V||A||D
501   atggactacg aacgtgatgt actcctctgg tacctgggca cggtcgcaga
      ||A||A||K| |Y||N||P|| E||L||R||N ||R||A||T| |N||I||I||
551   tgccgcgaaa tacaaccctg aattacgtaa tagagccaca aatatcatcg
      A||S||F||M ||K||K||R| |N||A||Y|| R||K||K||A ||D||A||T|
601   catcgttcat gaagaaacgg aacgcatacc gtaagaaggc tgacgccaca
      |T||L||A|| R||G||E||L ||V||M||Y| |P||F||Q|| Q||V||A||N
651   accctagcac gaggggagct ggtcatgtat cctttccaac aagtagcgaa
      ||I||P||R| |N||K||G|| L||P||V||R ||R||F||P| |Q||N||I||
701   tattccgagg aataaaggac tccctgtacg acgctttccg cagaatatca
      N||A||I||T ||C||||F|| G||I||I||P ||C||N||D| |D||Y||E|A
751   atgctatcac gtgtgctttt ggtataatac catgcaacga cgactacgaa
      |G||H||P|| I||E||L||I ||S||I||L| |D||P||A|| I||E||N||S
801   ggacatccca ttgagttaat cagtatcctg gatccagcaa tagagaactc
      ||M||N||D| |N||Q||H|| N||E||F||H ||R||A||L| |L||V||N||
851   gatgaacgat aatcaacaca atgaattcca ccgagcactt cttgtcaacg
      E||K||Q||A ||N||A||D| |F||A||R|| S||V||Q||R ||Y||G||Y|
901   aaaaacaggc gaacgcggac tttgctagga gcgtacagcg ctatggctac
      |H||Y||I|| F||G|A||G| |L||Q||Q|| Y||Y||M||| T
951   cattacatct tcggtgcagg actgcagcag tattatatga cgtagtcgat
                                                ||K| |T||V||N||
1001  cgatcgatgc tagctagcta ggctagctag ctagagaaag acggttaacg
      E||M||K||N ||F||N||T| |P||D||P|| R||G||N||A ||Y||R||V|
1051  aaatgaaaaa ttttaatacc ccagatccgc gggggaatgc ataccgagtc
      |R||V||Q|| K||V||C||Y ||A||A||I| |E||R||R|| L||R||L||K
1101  cgcgtacaga aggtatgcta tgcggcgata gagcggcggc tacgtcttaa
      ||K||L||E| |K||T||L|| L||I||R||T ||T||R||S| |F||P||A||
1151  aaaactggag aagactctcc tcataaggac aacaagaagt ttccctgcag
      D||N||F||R ||F||T||P| |W||I||S|| N||L||L||S ||G||K||R|
1201  acaactttcg tttcaccccg tggatctcaa accttcttag cggaaagcga
      |C||L||N|| R||G||L||E |
1251  tgcctaaatc gaggcttgga ataagatgct agatcgatcg catgctagca
1301  tcgatcagct agcattcagc tagcatcgat caggctagca tcgactagtc
```

```
1351  agctagctac agcatcgttt cagctagcta gcacattgca tcaggcatcg
1401  atctagcgca tgcatcgatc gatcagctag tcgcatcgat ctagcatgca
1451  tcagcgcatg catgctagct agctagcatg catcgatcag gcatcgactt
1501  agcagcatgc tacgctgatg ctagctagcg catgcatcag agcatcgact
1551  actaggcatg actacgatag atctagcatg catcaggcat cgatcgactg
1601  catcgatcat cgatcgatca tgcatcgatc gatcgcatgc tacgatgcat
1651  cagtcatgca tgcatcagct agcatcgagc tagctacgat cgcatcgatc
1701  gacgcatcga tcgatcgcta gctagcttgc atcgatcagc tgctagcatg
1751  catcgctagc tactgcatgc g
```

Figure 3.12: DNA sequence of MAT1-2-4 region of *E. repens* isolate 51-2. The putative encoded amino acid sequence is indicated above the relevant base pair sequence. Primer ErM124F is highlighted red (forward), primer ErM124R is highlighted green (reverse) and putative intron is highlighted in yellow. Also included is 500bp upstream and downstream of the ATG start site and end site.

3.3.2.3 *MAT1-1-1* Gene Isolation and Sequence Analysis

Pooling of the sequence data from *E. repens* isolates 51-1 and 51-2 provided sequence of the entire 9437bp MAT region between the *APN2* and *SLA2* genes (Figures 3.11 and 3.12, Appendix 1, Figure 2). Bioinformatic analysis of this region failed to detect the presence of a *MAT1-1-1* family alpha-domain gene, so it was concluded that any *MAT1-1-1* gene was absent from the 'normal' predicted *SLA2-APN2* location of *MAT* genes.

Given the failure to identify a *MAT1-1-1* gene at the MAT locus, alternative methods were used to try and identify any alpha-domain encoding gene. Amplification using degenerate primers MAT5-6 with MAT3-4 produced a 151bp putative alpha-domain *MAT1-1-1* gene fragment. TAIL-PCR was then used to chromosome walk upstream and downstream of this fragment to determine whether a complete putative *MAT1-1-1* gene was present including the conserved alpha-domain encoding sequence, necessary for function. In total 1598bp was sequenced from the MAT1-1-1 region, including a putative 1125bp, putative *MAT1-1-1* gene, which contained one putative intron and was predicted to encode a 356 amino acid MAT1-1-1 protein (Figure 3.13).

PSORT II and TFSITESCAN programs were used to interrogate resultant sequences for nuclear-targeting and promoter-region motifs. The putative ATG translation start site of the *MAT1-1-1* gene was consistent with the requirements of the Kozak rule (Kozak 1986). Analysis of the putative MAT1-1-1 protein revealed three nuclear targeting signals (KKRR at position 83, KRRR at position 84 and RRRP at position 85) and a transcriptional activator GATA box sequence was located 283bp upstream of the putative start site. Possession of nuclear targeting sites is consistent with a role of the *MAT1-1-1* gene as a transcriptional activator.

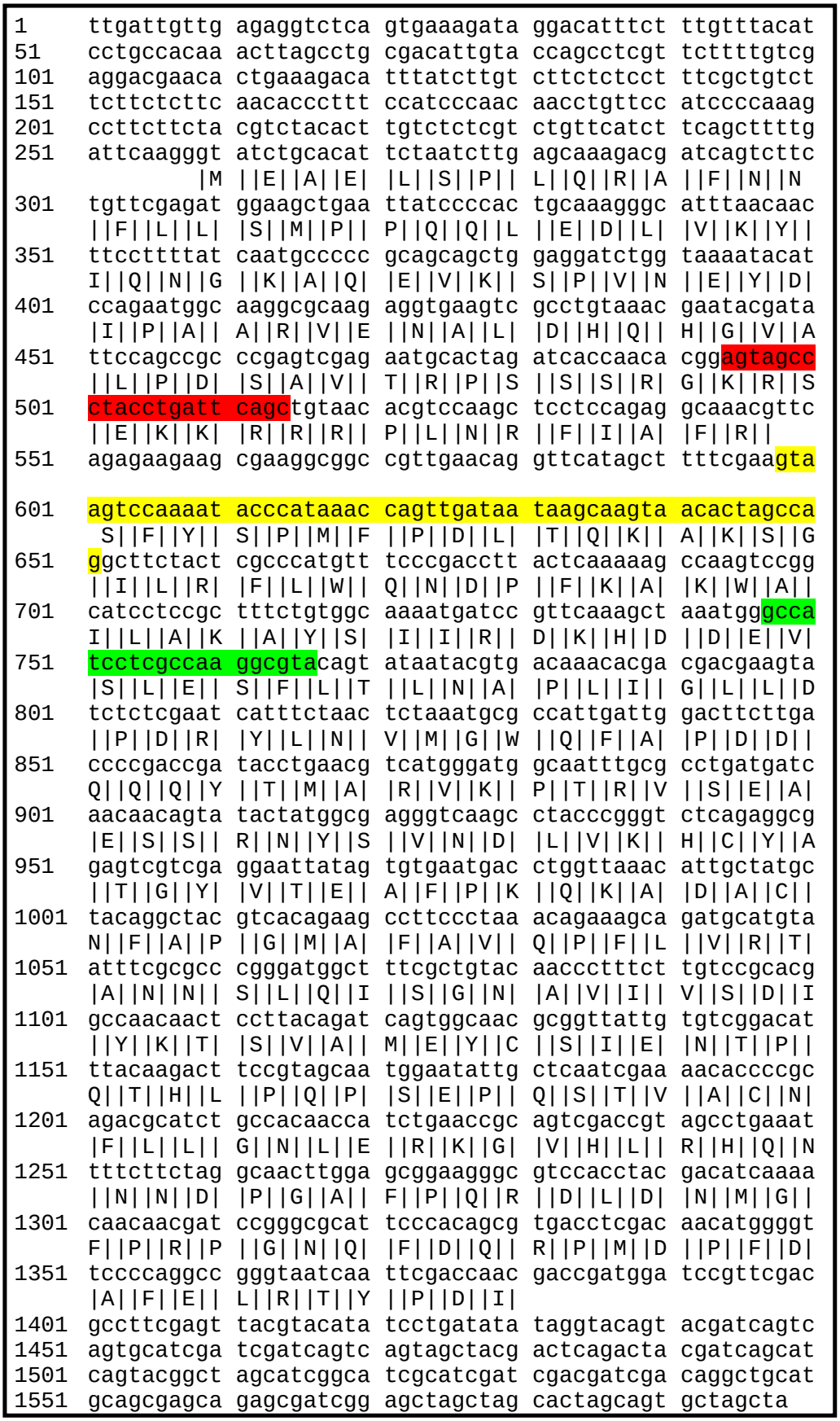

1 ttgattgttg agaggtctca gtgaaagata ggacatttct ttgtttacat
51 cctgccacaa acttagcctg cgacattgta ccagcctcgt tcttttgtcg
101 aggacgaaca ctgaaagaca tttatcttgt cttctctcct ttcgctgtct
151 tcttctcttc aacacccttt ccatcccaac aacctgttcc atccccaaag
201 ccttcttcta cgtctacact tgtctctcgt ctgttcatct tcagcttttg
251 attcaagggt atctgcacat tctaatcttg agcaaagacg atcagtcttc
|M ||E||A||E| |L||S||P|| L||Q||R||A ||F||N||N
301 tgttcgagat ggaagctgaa ttatccccac tgcaaagggc atttaacaac
||F||L||L| |S||M||P|| P||Q||Q||L ||E||D||L| |V||K||Y||
351 ttccttttat caatgccccc gcagcagctg gaggatctgg taaaatacat
I||Q||N||G ||K||A||Q| |E||V||K|| S||P||V||N ||E||Y||D|
401 ccagaatggc aaggcgcaag aggtgaagtc gcctgtaaac gaatacgata
|I||P||A|| A||R||V||E ||N||A||L| |D||H||Q|| H||G||V||A
451 ttccagccgc ccgagtcgag aatgcactag atcaccaaca cggagtagcc
||L||P||D| |S||A||V|| T||R||P||S ||S||S||R| G||K||R||S
501 ctacctgatt cagctgtaac acgtccaagc tcctccagag gcaaacgttc
||E||K||K| |R||R||R|| P||L||N||R ||F||I||A| |F||R||
551 agagaagaag cgaaggcggc cgttgaacag gttcatagct tttcgaagta
601 agtccaaaat acccataaac cagttgataa taagcaagta acactagcca
S||F||Y|| S||P||M||F ||P||D||L| |T||Q||K|| A||K||S||G
651 ggcttctact cgcccatgtt tcccgacctt actcaaaaag ccaagtccgg
||I||L||R| |F||L||W|| Q||N||D||P ||F||K||A| |K||W||A||
701 catcctccgc tttctgtggc aaaatgatcc gttcaaagct aaatgggcca
I||L||A||K ||A||Y||S| |I||I||R|| D||K||H||D ||D||E||V|
751 tcctcgccaa ggcgtacagt ataatacgtg acaaacacga cgacgaagta
|S||L||E|| S||F||L||T ||L||N||A| |P||L||I|| G||L||L||D
801 tctctcgaat catttctaac tctaaatgcg ccattgattg gacttcttga
||P||D||R| |Y||L||N|| V||M||G||W ||Q||F||A| |P||D||D||
851 ccccgaccga tacctgaacg tcatgggatg gcaatttgcg cctgatgatc
Q||Q||Q||Y ||T||M||A| |R||V||K|| P||T||R||V ||S||E||A|
901 aacaacagta tactatggcg agggtcaagc ctacccgggt ctcagaggcg
|E||S||S|| R||N||Y||S ||V||N||D| |L||V||K|| H||C||Y||A
951 gagtcgtcga ggaattatag tgtgaatgac ctggttaaac attgctatgc
||T||G||Y| |V||T||E|| A||F||P||K ||Q||K||A| |D||A||C||
1001 tacaggctac gtcacagaag ccttccctaa acagaaagca gatgcatgta
N||F||A||P ||G||M||A| |F||A||V|| Q||P||F||L ||V||R||T|
1051 atttcgcgcc cgggatggct ttcgctgtac aacccttctc tgtccgcacg
|A||N||N|| S||L||Q||I ||S||G||N| |A||V||I|| V||S||D||I
1101 gccaacaact ccttacagat cagtggcaac gcggttattg tgtcggacat
||Y||K||T| |S||V||A|| M||E||Y||C ||S||I||E| |N||T||P||
1151 ttacaagact tccgtagcaa tggaatattg ctcaatcgaa aacaccccgc
Q||T||H||L ||P||Q||P| |S||E||P|| Q||S||T||V ||A||C||N|
1201 agacgcatct gccacaacca tctgaaccgc agtcgaccgt agcctgaaat
|F||L||L|| G||N||L||E ||R||K||G| |V||H||L|| R||H||Q||N
1251 tttcttctag gcaacttgga gcggaagggc gtccacctac gacatcaaaa
||N||N||D| |P||G||A|| F||P||Q||R ||D||L||D| |N||M||G||
1301 caacaacgat ccgggcgcat tcccacagcg tgacctcgac aacatggggt
F||P||R||P ||G||N||Q| |F||D||Q|| R||P||M||D ||P||F||D|
1351 tccccaggcc gggtaatcaa ttcgaccaac gaccgatgga tccgttcgac
|A||F||E|| L||R||T||Y ||P||D||I|
1401 gccttcgagt tacgtacata tcctgatata taggtacagt acgatcagtc
1451 agtgcatcga tcgatcagtc agtagctacg actcagacta cgatcagcat
1501 cagtacggct agcatcggca tcgcatcgat cgacgatcga caggctgcat
1551 gcagcgagca gagcgatcgg agctagctag cactagcagt gctagcta

Figure 3.13: DNA sequence of the MAT1-1-1 region of *E. repens* isolate 51-2. The putative encoded amino acid sequence is indicated above the relevant base pair sequence. Primer ErM1F is highlighted red (forward), primer ErM1R is highlighted green (reverse) and putative intron is highlighted in yellow. Also included is 308bp upstream of the translation ATG start site and 165bp downstream of the end site.

3.3.2.4 Orientation of *MAT* Genes Within The Idiomorph

A variety of PCR amplifications were attempted in order to determine the orientation of the *MAT1-2-1* and *MAT1-2-4* gene relative to the flanking *SLA2* and *APN2* genes (see sections 2.2.5 and 3.2.2.2 for protocols).

Amplicons of approximately 9kb were produced by primers ErSLA2 with ErAPN2 (Figure 3.14). Amplicons of approximately 3.2kb and 6kb in size were produced by primers ErM2F with ErAPN2 and ErM2R with ErSLA2, respectively (Figure 3.14). Amplicons of approximately 4.7kb and 4.9kb were produced by primers ErM124F with ErSLA2 and ErM124R with ErAPN2, respectively (Figure 3.14). The results allowed the positions and orientations of the *MAT1-2-1* and *MAT1-2-4* genes to be deduced (Figure 3.15). The results confirmed the gene orientations predicted from sequencing the entire *SLA2-APN2* region of isolate 51-1 and 51-2 (Appendix 1, Figure 2).

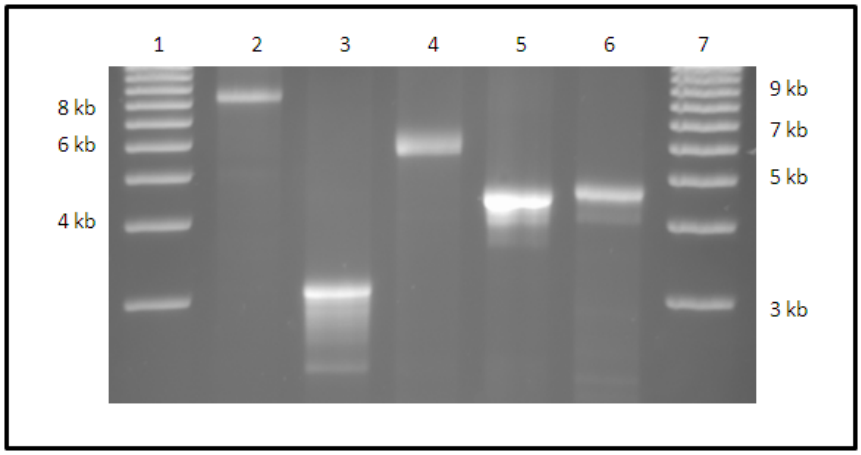


Figure 3.14: 0.8% agarose gel showing the results of PCR amplification to determine *MAT1-2-1* and *MAT1-2-4* gene orientations within the MAT-2 idiomorph of *E. repens* isolate 51-2. Lanes 1 and 7: 1kb ladder. Lane 2: Amplicons produced by primers ErAPN2 and ErSLA2. Lane 3: Amplicons produced by primers ErM2F and ErAPN2. Lane 4: Amplicons produced by primers ErM2R and ErSLA2. Lane 5: Amplicons produced by primers ErM124F and ErSLA2. Lane 6: ErM124R and ErAPN2.

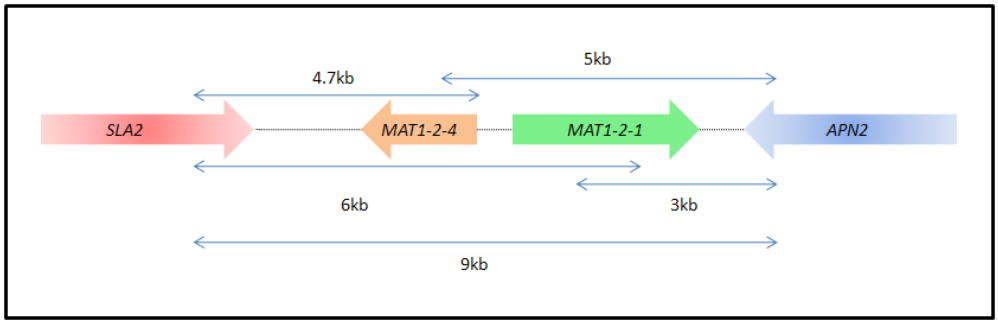


Figure 3.15: Schematic showing the organisation of the MAT-2 locus of *E. repens* (distances in kb).

3.3.2.5 RT-PCR Analysis

The RT-PCR analysis provided clear evidence of the expression of the *MAT1-1-1* and *MAT1-2-1* genes of *E. repens*, together with the actin control. Amplicons of a smaller size, relative to genomic DNA controls, were produced by RT-PCR corresponding to the predicted sizes of mRNA transcripts allowing for splicing of 54bp introns from the *MAT1-1-1* gene and *MAT1-2-1* genes (Figure 3.16 and Table 3.7). However, no expression could be detected from the putative *MAT1-2-4* gene as judged by lack of a RT-PCR product (Figure 3.16). This suggests that under the conditions tested in this study this gene is not expressed in isolate 51-2.

Table 3.7: Primers used in RT-PCR experiments and predicted fragments lengths of *E. repens* amplification products.

Forward primer	Reverse primer	Genomic DNA fragment length (bp)	Processed mRNA fragment length (bp)
ErM1F	ErM1R	260	208
ErM2F2	ErM2R2	174	120
ErM124F	ErM124R	604	559
Ate Actin Forward	Ate Actin Reverse	~330	~260

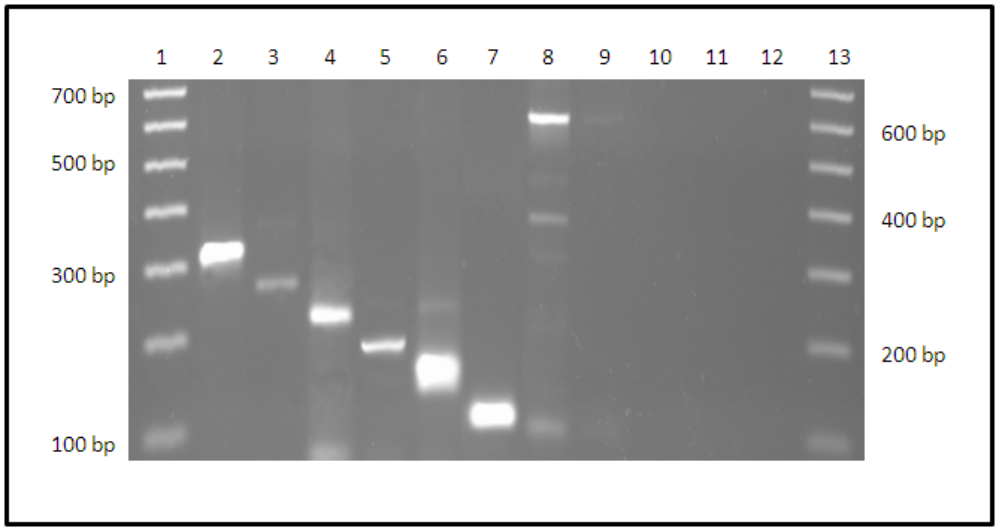


Figure 3.16: 0.8% aragose gel showing results of *MAT1-1-1, MAT1-2-1* and *MAT1-2-4* RT-PCR gene analysis of *E. repens* isolate 51-2. Lanes 1 and 13: 100bp ladder. Lane 2: Genomic DNA Actin Control. Lane 3: RNA Actin Control. Lanes 4 and 5: Amplification with *MAT1-1-1* specific primers of genomic DNA and RNA extracts, respectively. Lanes 6 and 7: Amplification with *MAT1-2-1* specific primers of genomic DNA and RNA extracts, respectively. Lanes 8 and 9: Amplification with *MAT1-2-4* specific primers of genomic DNA and RNA extracts, respectively. Lane 10: *MAT1-1-1* RT-PCR water control with no RNA. Lane 11: *MAT1-2-1* RT-PCR water control with no RNA. Lane 12: *MAT1-2-4* RT-PCR water control with no RNA.

3.3.3 *Neosartorya fennelliae*

3.3.3.1 *MAT1-1-1* Gene Isolation and Sequencing

A 148bp putative *MAT1-1-1* gene fragment was succesfully amplified from *N. fennelliae* isolate 54-1 using degenerate primers MAT5-6 and MAT3-4. This fragment was subsequently sequenced. The NfSLA2 and NfAPN2 primer pair produced a PCR product approximately 8-9kb in size. The idiomorph was then partially sequenced by PCR chromosome walking outwards from the *MAT1-1-1* gene fragment to determine whether a complete putative *MAT1-1-1* gene was present (section 2.2.4), including the conserved alpha-domain encoding sequence, necessary for function. In total a 2027bp region was sequenced, encompassing the complete 1160bp open reading frame, which contained one putative intron, and was predicted to encode a 369 amino acid MAT1-1-1 protein (Figure 3.17).

PSORT II and TFSITESCAN programs were used to interrogate resultant sequences for nuclear-targeting and promoter-region motifs. The ATG translation start site of this protein was consistent with the requirements of the Kozak rule (Kozak 1986). Analysis of the putative MAT1-1-1 protein revealed one nuclear localisation sequence (KKKP at

postion 82). A transcriptional activator GATA box sequence was detected 293bp upstream of the ATG start site. Possession of a nuclear targeting site is consistent with a role of the *MAT1-1-1* gene as a transcriptional activator.

```
1     catggtttag atgcggattt ccggtactcc aaccactgag ggatttctga
51    agattagctg gtgatctcga gcacagtgat atgtggatga cacactgact
101   tcatccaaca gcagcacatc gcatacgttg tatgtctcac acacacaaga
151   tcatgtaatt attatagatt atactatgtg cagaattaca agatttataa
201   aatcaagtag cgtaagaaat aataagggga agtctactga gtggagaggg
251   ccggggaggg aggcggtgaa ttgattgtcg agaggtctca gtgaaggata
301   ggacatttct ttgtttacat cctgccacaa actcagcctg ggacatcgta
351   cccagccttc gtttttttatc gaggacaaac actgaaagag atttgtcctt
401   tttcttctcc tttatcttct cctttcgtct tcttcttctc ttcaaccctt
451   tccatcccaa caactcgtcg gatcttcaaa gtctccttct gcgtctacac
501   ttgcctttcg tctgttcatc ttcagcttct cattcaagag tttctgcaca
                                                | M||D||A||A
551   gtctaatctc gagaaaagac ggtcagtctt ctgttcgaaa tggacgccgc
      ||I||S||P| |L||E||R|| A||F||N||T ||F||L||T| |T||M||P||
601   aatctctccc ctcgagcgtg ctttcaacac cttcttgacg accatgcctg
      A||E||Q||L ||E||E||L| |L||Q||Y|| L||Q||D||T ||K||A||Q|
651   cagagcagct ggaggagctt ctgcagtacc tccaagacac caaagcgcag
      |E||N||N|| G||M||Q||L ||P||D||A| |T||P||D|| N||A||A||N
701   gaaaacaatg gtatgcagct cccagatgca actcctgaca atgctgcaaa
      ||Y||A||L| |D||N||G|| N||G||A||A ||V||P||V| |A||A||T||
751   ctacgctttg gacaacggta atggtgctgc cgttcctgtt gccgcgactc
      P||R||T||L ||V||S||R| |A||K||R|| T||Q||E||G ||K||K||R|
801   ctcgtactct ggtttctcgt gccaaacgca cccaggaagg aaagaaaaga
      |P||L||N|| S||F||I||A ||F||R||
851   cctcttaaca gcttcatcgc attcagaagt gagtccaact ttaccaccaa
                R ||L||Y||S| |V||I||F||
901   gtatagatat acaggctaat aaagaatagg gttgtactct gtcatctttc
      P||D||I||T ||Q||K||A| |K||S||G|| I||L||R||F ||L||W||Q|
951   cggacatcac ccaaaaggcc aagtcgggca ttcttcgctt cttgtggcag
      |N||D||P|| F||K||A||K ||W||A||I| |L||A||K|| A||Y||S||I
1001  aatgaccctt tcaaggccaa atgggcaatc ctcgcgaaag cgtactccat
      ||I||R||D| |D||H||G|| G||E||V||S ||L||D||Q| |F||L||E||
1051  catccgcgat gaccatggtg gtgaggtctc tttggatcag ttcctggaga
      I||T||A||K ||F||I||G| |L||F||E|| P||T||R||Y ||L||D||A|
1101  ttactgccaa gttcatcggc ctctttgaac ccactcgcta cctcgacgcg
      |M||G||W|| Q||L||N||F ||D||A||E| |Q||Q||Y|| T||M||A||K
1151  atgggttggc agttgaactt cgatgccgag cagcaataca caatggctaa
      ||V||K||I| |T||T||I|| P||E||A||D ||I||S||T| |N||Y||S||
1201  ggtcaaaatc acaactatcc ctgaagccga tatctcaacc aactactcgg
      V||D||D||V ||V||K||H| |C||Y||D|| T||G||Y||V ||S||E||K|
1251  ttgatgatgt cgtgaaacac tgctatgata ctggctatgt ctccgagaaa
      |P||G||K|| H||S||A||N ||N||G||N| |N||A||A|| T||M||A||F
1301  ccaggcaagc acagcgcaaa taacggcaac aatgctgcca ccatggcctt
      ||A||A||Q| |P||T||F|| V||V||K||A ||E||N||G| |I||Q||I||
1351  cgctgctcag ccgactttcg ttgtcaaggc ggagaacggc attcagatca
      T||G||D||D ||A||T||I| |V||T||D|| D||E||F||A ||T||P||E|
1401  ctggcgacga tgctactatt gtgactgacg atgagttcgc aactcctgaa
      |V||D||F|| P||T||P||E ||E||T||N| |D||T||P|| T||P||D||P
1451  gttgacttcc caactcccga agagacaaac gacactccaa cccctgatcc
```

```
     ||V||E||A| |E||P||V|| V||D||N||N ||P||L||P| |Y||F||D||
1501 cgtcgaggca gaaccagtgg tcgacaacaa ccccttgcct tattttgatg
     A||P||G||V ||P||D||G| |Q||Q||L|| Q||L||E||L ||F||Q||G|
1551 cacctggtgt gcctgacggt cagcagcttc aacttgaact cttccagggc
     |L||D||F|| N||L||D||N ||M||Q||L| |P||F||I|| E||D||L||A
1601 cttgacttca atctcgacaa catgcaactc ccatttatcg aagacttggc
     ||L||Y||D| |P||A||A|| A||F||P||L ||V||M||D| |Y||D||P||
1651 cctttacgat ccggcagccg catttcctct cgtgatggac tatgacccac
     L||E||E||P ||P||F||G| |V||F||D|| I||D||Q||Y ||I||N||V|
1701 tcgaagagcc tccttttggc gtctttgaca ttgatcaata catcaatgtc
1751 tgaccgacca tgcactgagg tccggccacg actccttggg tgtctcgagt
1801 tcactcctgc agatcatctt cgcgcagaag gaatactcaa cgatgtctca
1851 agctctttca catgtacact atctgatctt gtttccgaat gtactctatt
1901 catttcccat cagcatttcg agcaatcgcg ttacgaatcg agcgtgattg
1951 gttttcgtga cgacatcagc tttcgaacgc atacgaactc ctatgaattg
2001 ggggccgttc aatgtaattc tctaccc
```

Figure 3.17: DNA sequence of the MAT1-1-1 region of *N. fennelliae* isolate 54-1. The putative encoded amino acid sequence is indicated above the relevant base pair sequence. Primer NfM1F is highlighted red (forward), primer NfM1R is highlighted green (reverse) and putative intron is highlighted in yellow. Also included is 589bp upstream of the translation ATG start site, and 274bp downstream of the end site.

3.3.3.2 *MAT1-2-1* Gene Isolation and Sequence Analysis

A 271bp putative *MAT1-2-1* gene fragment was succesfully amplified from *N. fennelliae* isolate 54-2 using degenerate primers MAT5-7 and MAT3-5. The NfSLA2 and NfAPN2 primer pair produced a PCR amplicon approximately 9kb in size. The idiomorph was then partially sequenced by chromosome walking outwards from the *MAT1-2-1* gene fragment to determine whether a complete putative *MAT1-2-1* gene was present, including the conserved HMG-domain encoding sequence, necessary for function. In total a 1865bp region was sequenced, encompassing the complete 1072bp open reading frame, which contained two putative introns and was predicted to encode a 322 amino acid MAT1-2-1 protein (Figure 3.18).

PSORT II and TFSITESCAN programs were used to interrogate resultant sequences for nuclear-targeting and promoter-region motifs. The ATG translation start site of this protein was consistent with the requirements of the Kozak rule (Kozak 1986). Analysis of the putative MAT1-2-1 protein revealed three nuclear localisation signals (KKKH at position 183, RKRR at position 203 and PSERKRR at position 200). A transcriptional activator GATA box sequence was found 131bp upstream of the start site. Possession of nuclear targeting signals is consistent with a role of the *MAT1-2-1* gene as a transcriptional activator.

However, no *MAT1-2-4* gene was found in the sequenced region adjoining the *MAT1-2-1* gene. Also, it was not possible to obtain any PCR product from amplifications with primers MAT124F and MAT124R (section 2.2.4). Therefore, it appears that there is no *MAT1-2-4* gene in the MAT-2 idiomorph of *N. fennelliae* isolate 54-2.

```
1     atattttgcg ctgtccgaaa gataccttcc ttcttaccct tcttctttcg
51    attttcgact ctctgcaaag tcgacagttg ggctgcggag ttccatactt
101   caagatctca acattattag cagtaatctt gtctgcagac cgagatcaaa
       |M||A||T| |V||P||I|| A||M||K||S ||A||A||E| |S||T||D||
151   aatggctaca gttccaatcg ccatgaagtc ggcagcggaa tctacagaca
      T||L||T||E ||L||L||W| |Q||D||A|| L||R||H||L ||E||S||T|
201   ctctcacaga gctcttgtgg caggatgctt tgcgtcacct ggagtccacg
      |N||N||E|| V||L||L||P ||I||N||V| |T||D||M|| I||G||Q||D
251   aacaatgagg tcctcctccc catcaatgtg accgacatga tcggccagga
      ||N||V||D| |K||I||K|| N||R||L||G
301   caatgtcgac aagatcaaaa atcgtcttgg gtaagtgttt actctaaagc
           ||A|| L||I||G||A
351   aactggcaag cttgattaat aacacggaat gccagtgcac tcattggcgc
      ||P||V||V| |A||F||V|| D||E||S||V ||K||A||L| |R||V||M||
401   tcctgttgtc gcctttgtcg acgagtcggt caaagctctc cgtgttatgc
      R||T||P||G ||F||S||G| |T||A||I|| W||V||A||S ||H||G||A|
451   gcaccccagg gttttccgga accgccatct gggttgcatc tcacggtgca
      |A||L||N|| A||G||K||V ||E||A||S| |E||S||F|| K||P||R||G
501   gctctcaatg cgggcaaggt tgaagcaagt gagtcgttca aacctcgcgg
      ||K||P||A| |G||P||M|| K||A||P||K ||V||P||R| |P||P||N||
551   aaaacctgca ggtcctatga aggcaccgaa ggtcccgcgt cctccgaatg
      A||F||I||L ||Y||R||Q| |H||H||H|| P||K||I||K ||E||A||Y|
601   cattcattct gtaccgtcag caccaccacc ccaagatcaa ggaagcatat
      |P||D||F|| S||N||N||D ||I||
651   cctgactttt cgaacaacga tatctgtaag ttgcttgtct atatctttgt
            S||I ||M||L||G| |K||Q||W||
701   atgcttacat ttactaacat gcatagccat catgcttggg aagcagtgga
      K||A||E||A ||E||E||V| |K||A||Q|| F||R||N||L ||A||E||E|
751   aagccgaagc tgaagaagtc aaggctcaat tcagaaacct agcagaagaa
      |L||K||K|| K||H||A||E ||D||H||P| |D||Y||H|| Y||P||P||R
801   ctcaagaaga agcatgccga agaccatcca gactatcatt acccccccg
      ||K||P||S| |E||R||K|| R||R||A||S ||S||R||L| |F||S||K||
851   caagccttct gagagaaagc gtcgtgcttc gtcccgtctg ttctccaaga
      N||T||K||P ||A||A||L| |L||D||T|| P||A||S||T ||N||V||A|
901   acaccaagcc tgctgccttg ctcgatactc cggcttcgac gaacgtcgcg
      |S||D||V|| S||T||P||A ||M||L||Q| |G||M||P|| V||G||E||I
951   tctgacgtct ccactcctgc catgctccag ggcatgccag tgggcgagat
      ||D||F||H| |A||A||F|| E||G||V||P ||D||M||D| |A||I||M||
1001  tgacttccat gcggctttcg aaggtgttcc agatatggat gccatcatga
      T||P||N||G ||M||P||E| |D||Q||Q|| Y||R||F||E ||P||N||A|
1051  ctcctaacgg catgcccgag gaccagcagt atcgttttga accaaatgcg
      |F||D||L|| M||H||Q||M ||Q||N||D| |Y||N||K|| A||G||L||Y
1101  ttcgatctca tgcatcagat gcagaacgat tacaacaagg ctggcctcta
      ||R||Q||L| |S||L||P|| E||G||Q||I ||A||E||N| |F||E||F||
1151  ccggcaactc agccttcccg agggtcagat tgcagagaac ttcgagttca
      T||D||F||I ||T||D||C| |F|
1201  ctgacttcat caccgattgc ttctaagctc ggcaggtaca tgtgcttctt
1251  ctctgcttgt cttcttgccc cttctgttgt tgttacttga gcaagcaccg
```

```
1301  tgtgtacaat ttctacatca gcacctctag ccgagggctt caacacatcg
1351  tggcctttga tactgtacca tactggcgcg gatgatttgt tttcttcatt
1401  ttgatcttat cctgggcgat ggaatgatct ctgacgactc tggagacttg
1451  ttttcagaag acatcaggcg gatcagtgtc attacaccga aaatatcaga
1501  cttctagata gtcacttaga taggacatac agacagagaa atacaaacaa
1551  cggagttaat cagtatgtgt tccctcgagt gtccacactc tttttcctca
1601  ttctcgacat catacagtaa gttcaacgtg cctgcggcac taggactgcc
1651  ccccccttgct gctcaacgtt acccgacagc ttctgccagg cgtgtctcag
1701  tacattcgag attcccatga catcctagca tggctggtcc tcgaaggcct
1751  ggcaggagcc cttcgtgaat ctgtaccagc ctagctagct gtcaatacca
1801  ggctacggaa catggtagct ttaccgggga atctctcgtt acaatgttaa
1851  ttcgtgtcca ggggg
```

Figure 3.18: DNA sequence of the MAT1-2-1 region of *N. fennelliae* isolate 54-2. The putative encoded amino acid sequence is indicated above the relevant base pair sequence. Primer NfM2F is highlighted red (forward), primer NfM2F2 is highlighted purple (forward), primer NfM2R is highlighted green (reverse), primer NfM2R2 is highlighted blue (reverse) and putative Introns are highlighted in yellow. Also included is 151bp upstream of the putative ATG translation start site and 639bp downstream of the end site.

3.3.3.3 Orientation of *MAT* Genes Within The Idiomorph

A variety of PCR amplifications were attempted in order to determine the orientation of the putative *MAT1-1-1* gene and *MAT1-2-1* gene relative to the flanking *SLA2* and *APN2* genes (see sections 2.2.5 and 3.2.3.2 for protocols). Amplicons of approximately 4.5kb in size were produced by primers NfM1F with NfSLA2 and NfM1R with NfAPN2 (Figure 3.19). This allowed the orientation of the *MAT1-1-1* gene to be deduced as shown in Figure 3.21. Amplicons of approximately 3kb and 6.2kb were produced by primers NfM2F with NfSLA2 and NfM2R with NfAPN2, respectively (Figure 3.20). This allowed the orientations of the *MAT1-2-1* gene to be deduced as shown in Figure 3.21.

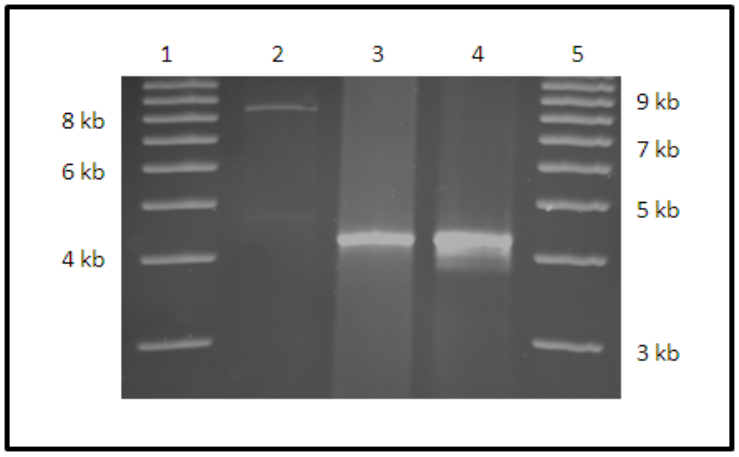


Figure 3.19: 0.8% agarose gel showing results of PCR amplifications to determine the *MAT1-1-1* gene orientation within the MAT-1 idiomorph of *N. fennelliae* isolate 54-1. Lanes 1 and 5: 1kb ladders. Lane 2: Amplicons produced by primers NfSLA2 and NfAPN2. Lane 3: Amplicons produced by primers NfM1F and NfSLA2. Lane 4: Amplicons produced by primers NfM1R and NfAPN2.

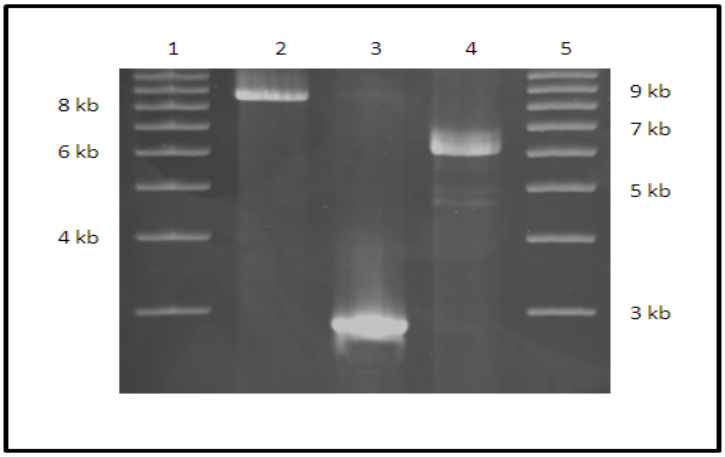


Figure 3.20: 0.8% agarose gel showing results of PCR amplifications to determine the *MAT1-2-1* gene orientation within the MAT-2 idiomorph of *N. fennelliae* isolate 54-2. Lanes 1 and 5: 1kb ladder. Lane 2: Amplicons produced by primers NfSLA2 and NfAPN2. Lane 3: Amplicons produced by primers NfM2F and NfSLA2. Lane 4: Amplicons produced by primers NfM2R and NfAPN2.

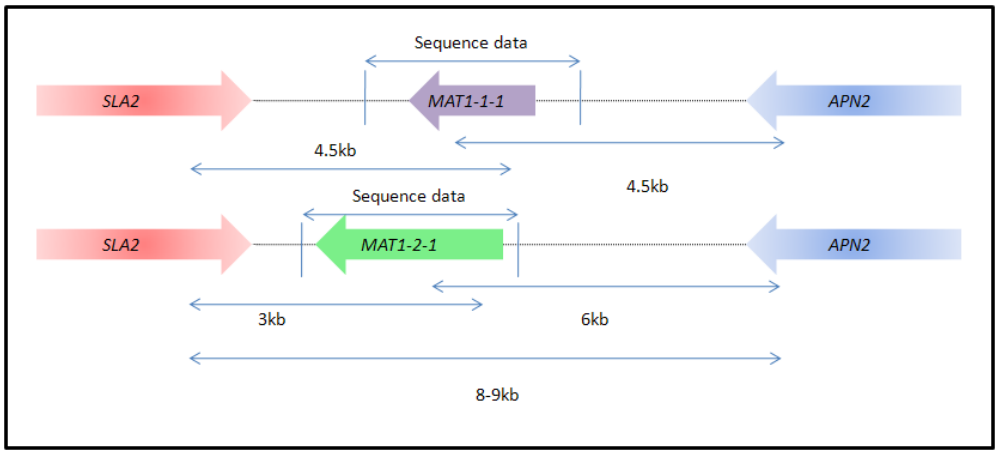


Figure 3.21: Schematic showing the organisation of the MAT-1 and MAT-2 idiomorph orientations of *N. fennelliae*, lengths are shown in kb.

3.3.3.4 RT-PCR Analysis

The RT-PCR analysis provided clear evidence of the expression of both the *MAT1-1-1* and *MAT1-2-1* genes of *N. fennelliae*, together with the actin control. Amplicons of a smaller size, relative to genomic controls were produced by RT-PCR corresponding to the predicted sizes of mRNA transcripts allowing for splicing of a 51bp intron from *MAT1-1-1* and *MAT1-2-1* genes (Figure 3.22 and Table 3.8). Splicing of these introns indicated that both *MAT1-1-1* and *MAT1-2-1* genes are expressed in *N. fennelliae* under the conditions assayed.

Table 3.8: Primers used in RT-PCR experiments and predicted fragment lengths of *N. fennelliae* amplification products.

Forward primer	Reverse primer	Genomic DNA fragment length (bp)	Processed mRNA fragment length (bp)
NfM1F	NfM1R	243	192
NfM2F2	NfM2R2	141	90
Ate Actin Forward	Ate Actin Reverse	~330	~260

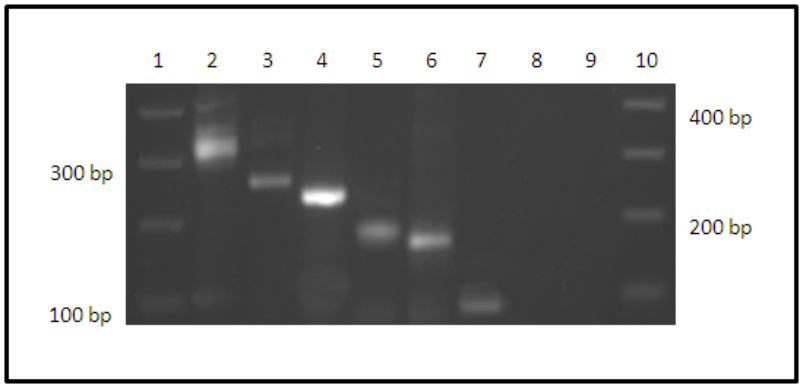


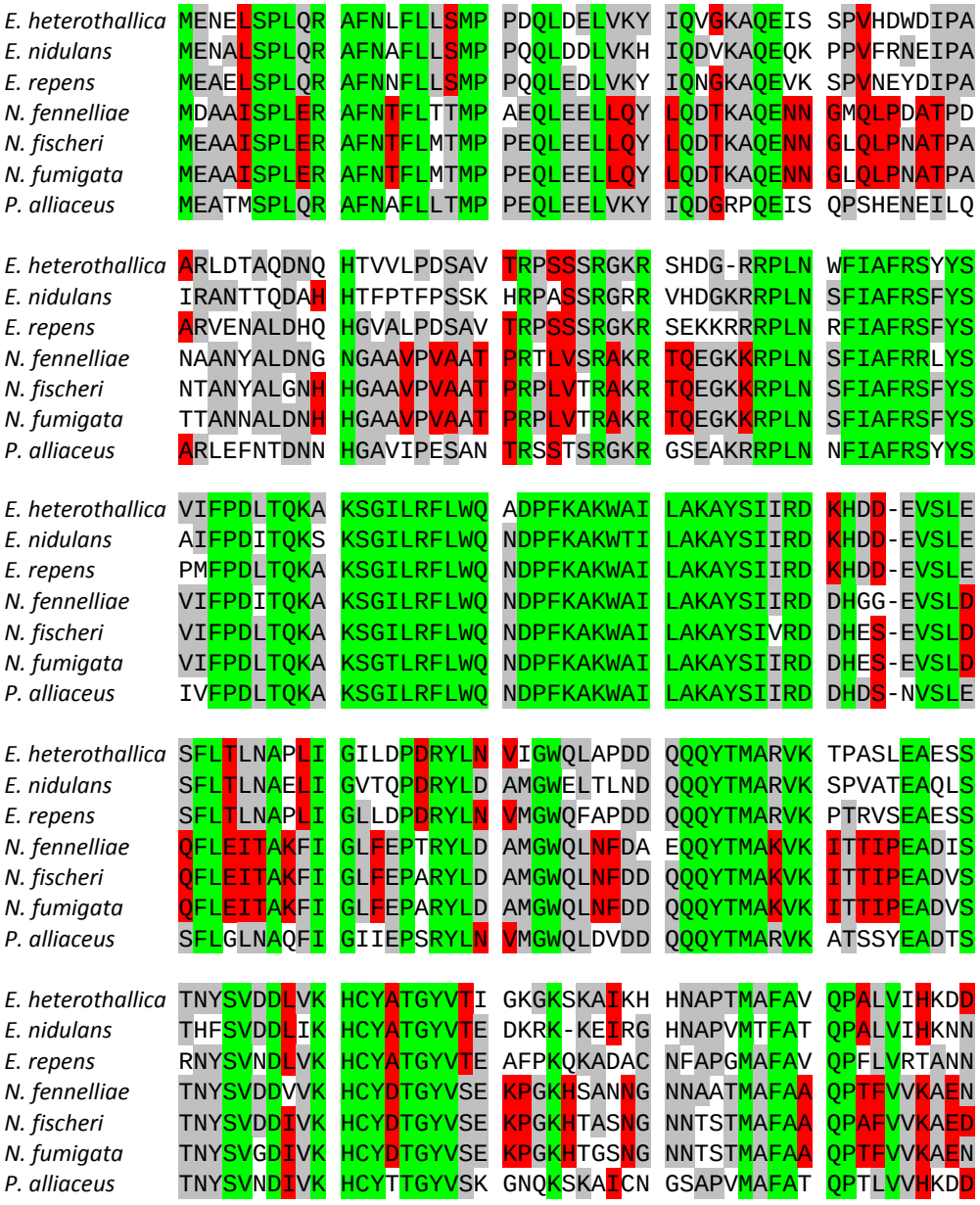
Figure 3.22: 1.5% agarose gel showing results of *MAT1-1-1* and *MAT1-2-1* genes RT-PCR analysis of *N. fennelliae* isolates 54-1 and 54-2. Lanes 1 and 10: 100bp ladder. Lane 2: Genomic DNA Actin control. Lane 3: RNA Actin control. Lanes 4 and 5: Amplification with *MAT1-1-1* specific primers of genomic DNA and RNA extracts, respectively. Lanes 6 and 7: Amplification with *MAT1-2-1* specific primers of genomic DNA and RNA extracts, respectively. Lane 8: *MAT1-1-1* RT-PCR water control with no RNA. Lane 9: *MAT1-2-1* RT-PCR water control with no RNA.

3.3.4 *MAT1-1-1* Family Alpha-Domain Gene Phylogeny

A multiple alignment was made of the amino acid sequences of the alpha-domain MAT1-1-1 proteins identified from all the species of *Aspergillus* under investigation, together with sequences from four other sexual *Aspergillus* species for which data is already published (Figure 2.23). This revealed 52-96% nucleotide identity and 45-96% amino acid identity between the species (Tables 3.9 and 3.10). *N. fischeri* and *N. fumigata* shared the greatest amino acid identity, although *N. fennelliae* was highly similar to both species. *E. nidulans* and *N. fennelliae* shared the least amino acid homology (45%). Similar relationships were found for nucleotide sequences (Table 3.9) (see Appendix 1, Figure 3 for nucleotide multiple alignments).

Phylogenetic analysis showed *E. repens* to be the most divergent species, whilst *E. heterothallica* and *E. nidulans* formed a distinct group (Figure 3.24), which is consistent with traditional taxonomic analysis of cleistothecial and ascospore morphology. Meanwhile, *N. fumigata, N. fischeri* and *N. fennelliae* form a clade with strong bootstrap

support, this is also not surprising given previous phylogenetic analyses of these species (Figures 3.1 and 3.24). Phylogenetic analysis was also conducted using the nucleotide sequences of the *MAT1-1-1* genes. In this analysis *E. repens* and *E. heterothallica* were sister species, forming a clade basal to the other sexual Aspergilli analysed (data not shown).



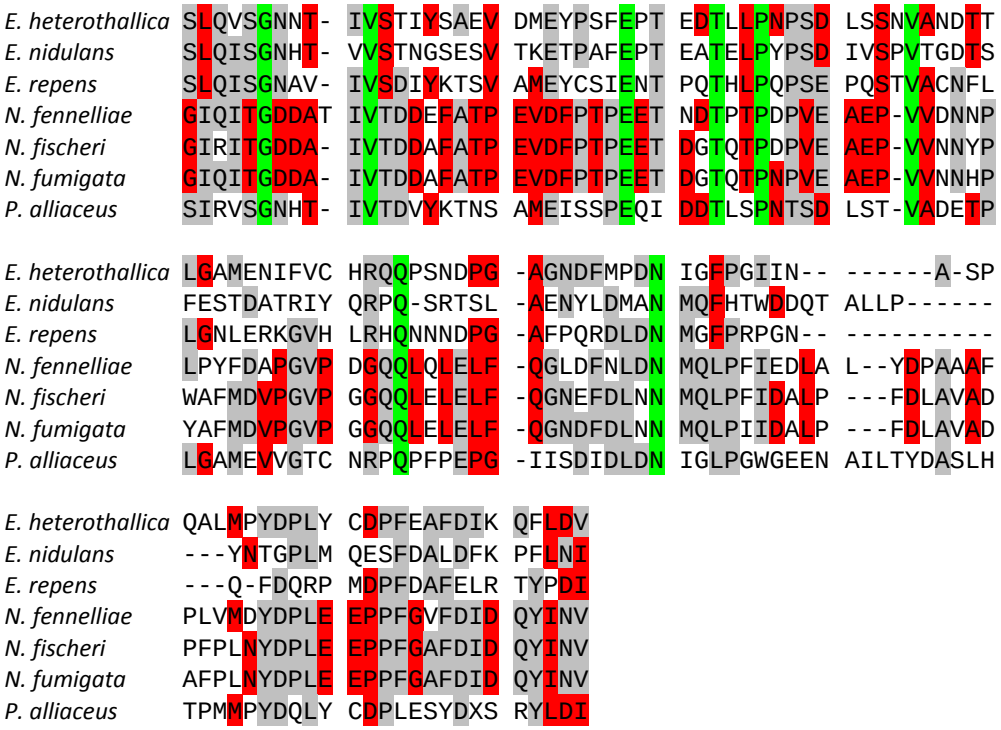


Figure 3.23: Amino acid alignment of the putative MAT1-1-1 protein of *E. heterothallica, E. nidulans, E. repens, N. fennelliae, N. fischeri, N. fumigata* and *P. alliaceus*. Green highlighting indicates where nucleotides are conserved between all species. Grey highlighting indicates where nucleotides are conserved between four or more species. Red highlighting indicates where nucleotides are conserved between three species. For culture identification codes and GenBank accession numbers used for alignments, see Appendix 4.

Table 3.9: Percentage nucleotide identity between the putative *MAT1-1-1* genes of *E. heterothallica, E. nidulans, E. repens, N. fennelliae, N. fischeri, N. fumigata* and *P. alliaceus*.

	E. nidulans	*E. repens*	*N. fennelliae*	*N. fischeri*	*N. fumigata*	*P. alliaceus*
E. heterothallica	58	54	52	56	57	62
E. nidulans		54	57	58	58	57
E. repens			63	57	58	56
N. fennelliae				90	88	59
N. fischeri					96	61
N. fumigata						61

Table 3.10: Percentage amino acid identity table between putative MAT1-1-1 proteins of *E. heterothallica, E. nidulans, E. repens, N. fennelliae, N. fischeri, N. fumigata* and *P. alliaceus*.

	E. nidulans	*E. repens*	*N. fennelliae*	*N. fischeri*	*N. fumigata*	*P. alliaceus*
E. heterothallica	61	69	49	51	51	64
E. nidulans		56	45	47	48	50
E. repens			48	50	50	59
N. fennelliae				84	83	49
N. fischeri					96	50
N. fumigata						51

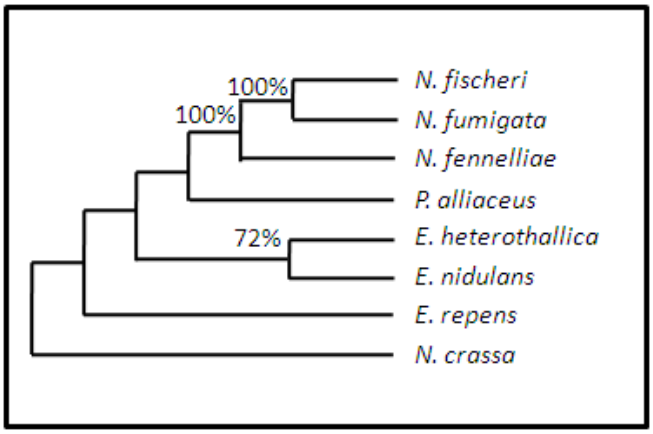


Figure 3.24: Phylogenetic analysis of the amino acid sequences of the putative MAT1-1-1 proteins of *E. heterothallica, E. nidulans, E. repens, N. fennelliae, N. fischeri, N. fumigata* and *P. alliaceus*. *N. crassa* MATA is included as an outgroup. Bootstrap values greater than 70% are indicated. Maximum parsimony analysis was performed using MEGA version 4 program, with bootstrapping of 500 replicates and excluding gaps (section 2.2.20) (Tamura *et al.* 2007). Culture identification codes and GenBank accession numbers used to construct this phylogeny, see Appendix 4.

3.3.5 *MAT1-2-1* Family HMG-Domain Gene Phylogeny

A multiple alignment was made using the amino acid sequences of the HMG-domain MAT1-2-1 proteins identified from all *Aspergillus* species under investigation, together with sequences from four other sexual Aspergilli for which data was already published (Figure 3.25). This revealed 57-88% nucleotide identity and 48-85% amino acid identity between the species (Figure 3.25 and Tables 3.11 and 3.12). *N. fennelliae* shared 88% amino acid identity with both *N. fumigata* and *N. fischeri. E. nidulans* and *E. heterothallica* shared the least amino acid identity (48%). Similar relationships were found for nucleotide sequences (Table 3.11) (see Appendix 1, Figure 4 for nucleotide alignments).

Phylogenetic analysis showed *E. nidulans* to be the most divergent species. *N. fumigata* and *E. heterothallica* form a group as do *N. fischeri* and *N. fennelliae* (Figure 3.26). The phylogenetic analysis was also conducted using the nucleotide sequences of the *MAT1-2-1* gene, in this analysis *P. alliaceus* formed the basal species to the other Aspergilli, but other groupings remained the same (results not shown).

```
E. heterothallica MATVPIAMKP EAEPTDSLTE LMWQDALRHL ESTNNEVLLP INVPDMIGQD
E. nidulans       MAAVSIAMKS PTQSPDSITE LLWKDALRHL GSTNDEVLLP TNVVDIIGQD
E. repens         MATVPIAMRS DAQSPESITE LLWQDALRHL FSTNEEVLPP INVTDMIGQD
N. fennelliae     MATVPIAMKS AAESTDTLTE LLWQDALRHL ESTNNEVLLP INVTDMIGQD
N. fischeri       MATIPITMKS AVDSTDTFTE LLWQDALRHL ESMNNEVLLP INVTDMIGQD
N. fumigata       MATVPIAMKP AAESTDTLTE LLWQDALRHL ESTNNEVLLP INVTDMIGQD
P. alliaceus      MATVPIAMRS AAESTDTLTE LLWQDALRHL ESTNNEVLLP INVTNMIGQR

E. heterothallica NVDQIKTRLG ALIGATVVAF VDETIKALRV MRTPAFAGTA VSVASHGEAV
E. nidulans       NVEKIKSRLS ALLGAPVVSF VDESINALRV LRTPTFSGSS ISVASPSRAL
E. repens         NVERLKSRLG ALLGAPVVSF VDESLNALRV LRTPEFSGSA ISVASMGGAQ
N. fennelliae     NVDKIKNRLG ALIGAPVVAF VDESVKALRV MRTPGFSGTA IWVASHGAAL
N. fischeri       NVDKIKTRLS ALIGAPVVAF VDKSIEALRV MRTPAFSGRA ISVASHGAAL
N. fumigata       NVDKIKTRLG ALIGAPVVAF VDETIKALRV MRTPAFSGTA VSVASHGEAV
P. alliaceus      NVDKIKTRLG ALIGAPVVTF IDETINALRV MRTPAFSGSV VSIASHDGIS

E. heterothallica KTYKVTVTES FAPRGKPVAP LKAPKVPRPP NAFILYRQHS HPRIKEAYPD
E. nidulans       DSWPSEPPNK PRP-----AS MKPAKIPRPP NAFILYRQHH YPKVKEARPD
E. repens         TSKRSTVTES FAPRGKPVGP LKAPKVPRPP NAFILYRQRH HPKIKEAYPD
N. fennelliae     NAGKVEASES FKPRGKPAGP MKAPKVPRPP NAFILYRQHH HPKIKEAYPD
N. fischeri       NADKVAATES FKPRGKPAGP MKAPKVPRPP NAFILYRQRH HPKIKEEYPD
N. fumigata       KTNKVTVTES FAPRGKPVGP LKAPKVPRPP NAFILYRQHH HPKIKEAYPD
P. alliaceus      NLKREVTESS ARMHGRPALS AKSVKVPRPP NAFILYRQHH HPRIKEAYPD

E. heterothallica FTNNEISIIL GKQWKAESEE VKMQFRNMAE KLKKKHAEDH PDYHITPRKP
E. nidulans       LSNNEISVII GKKWRAEPEE GKLHFKNLAE EFKKKHAEEY PDYQYTPRKP
E. repens         YSNNDISVML GKQWKDENEE IKAQFRNLAE ELKKKHAEDH PDYHYTPRKP
N. fennelliae     FSNNDISIML GKQWKAEAEE VKAQFRNLAE ELKKKHAEDH PDYHYPPRKP
N. fischeri       FSNNDISIML GKQWKDEPEE VKAQFRNLAE ELKKKHAEDH PNYYYTPRKP
N. fumigata       YSNNDISVML GKQWKDENEE IKTQFRNLAE ELKKKHAEDH PDYHYTPRKP
P. alliaceus      FTNNEISIML GKQWKAESEE AKVQFRSMAE ELKRKHAEDH PDYHYTPRKP

E. heterothallica SERKRRTSSR QFSKN-TKPA ALRDTAASMN ITSDVSSPAM LEGMPVGEID
E. nidulans       SEKKRRAASR ISPKNSKRTV ALENPGSMTA PSSNVFTPQM YPGIQNGQLA
E. repens         YEKKRRNSSR CCSKAPQRFF CNPSYWAMTY IPSDVSSPAM VDGMPVGAQF
N. fennelliae     SERKRRASSR LFSKN-TKPA ALLDTPASTN VASDVSTPAM LQGMPVGEID
N. fischeri       SERKRRASSR QFSKN-TKSA AVLDIPASMN VASDVSTPAM HQGMPVGEID
N. fumigata       SERKRRTSSR QFSKN-TKPA ALRDTPASMN ISSDVSTPAM LEGMPVGEID
P. alliaceus      SEKKRRASSR QCSK----PN KRQKSPALTN DTSDTSTPSM YSGIQLDNTP
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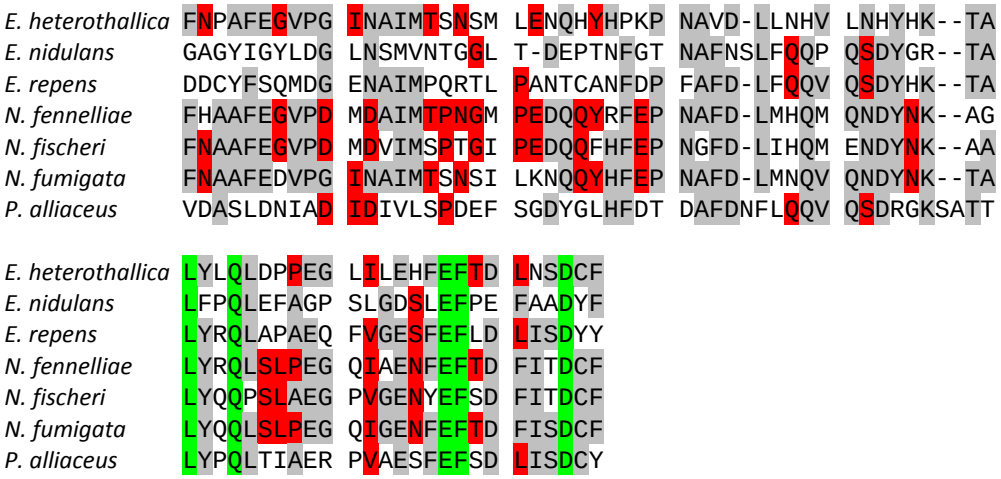

Figure 3.25: Amino acid alignment of the putative MAT1-2-1 proteins of *E. heterothallica, E. nidulans, E. repens, N. fennelliae, N. fischeri, N. fumigata* and *P. alliaceus*. Green highlighting indicates where nucleotides are conserved between all species. Grey highlighting indicates where nucleotides are conserved between four or more species. Red highlighting indicates where nucleotides are conserved between three species. For culture identification codes and GenBank accession numbers used for alignments, see Appendix 4.

Table 3.11: Percentage nucleotide identity between the putative *MAT1-2-1* genes of *E. heterothallica, E. nidulans, E. repens, N. fennelliae, N. fischeri, N. fumigata* and *P. alliaceus*.

	E. nidulans	*E. repens*	*N. fennelliae*	*N. fischeri*	*N. fumigata*	*P. alliaceus*
E. heterothallica	58	63	80	77	87	65
E. nidulans		59	61	60	62	63
E. repens			67	65	72	57
N. fennelliae				88	88	64
N. fischeri					84	64
N. fumigata						65

Table 3.12: Percentage amino acid identity between the putative MAT1-2-1 proteins of *E. heterothallica, E. nidulans, E. repens, N. fennelliae, N. fischeri, N. fumigata* and *P. alliaceus*.

	E. nidulans	*E. repens*	*N. fennelliae*	*N. fischeri*	*N. fumigata*	*P. alliaceus*
E. heterothallica	48	63	75	70	85	60
E. nidulans		57	53	52	53	51
E. repens			65	63	70	60
N. fennelliae				85	84	64
N. fischeri					79	61
N. fumigata						64

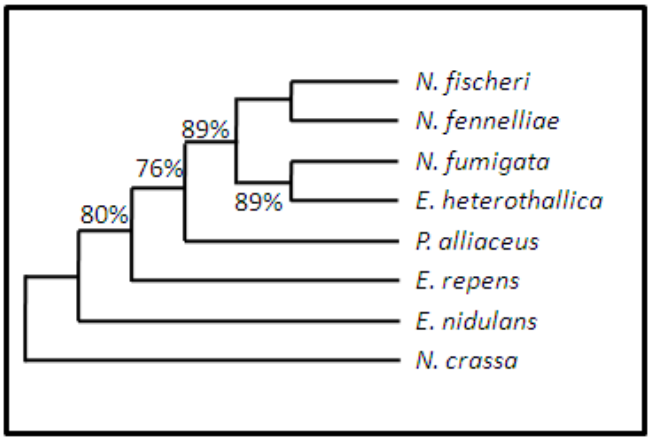

Figure 3.26: Phylogenetic analysis of the amino acid sequences of the putative MAT1-2-1 proteins of *E. heterothallica, E. nidulans, E. repens, N. fennelliae, N. fischeri, N. fumigata* and *P. alliaceus*. *N. crassa* MATa is included as an outgroup. Bootstrap values greater than 70% are indicated. Maximum parsimony and evolutionary analysis was performed using MEGA version 4 program, with bootstrapping of 500 replicates and excluding gaps (section 2.2.20) (Tamura *et al.* 2007). For culture identification codes and GenBank Accession numbers used to construct this phylogeny, see Appendix 4.

3.3.6 *MAT1-2-4* Family Gene Phylogeny

A multiple alignment was made using the amino acid sequences of the MAT1-2-4 protein newly identified from *E. repens* and *E. heterothallica,* together with sequences from *N. fumigata* and *N. fischeri* in which MAT1-2-4 has previously been identified (Figure 3.27). This revealed 63-98% nucleotide identity and 77-96% amino acid identity (Tables 3.13 and 3.14). *E. heterothallica* and *N. fumigata* shared 98% nucleotide identity and 96% amino acid identity, see Appendix 1, Figure 5 for nucleotide alignment.

Phylogenetic analysis showed that *N. fumigata* and *E. heterothallica* form a strongly supported group. The same phylogeny was found when nucleotide sequences were analysed (results not shown).

```
E. heterothallica MDYERDVLLW YLGTVADDAR YPPDLRNKAT HIIVSFMRHR NAYRLLPQAT
E. repens         MDYERDVLLW YLGTVADAAK YNPELRNRAT NIIASFMKKR NAYRKKADAT
N. fischeri       MDYERDVLLW YLGTVADNAH YRPDLRNKAT YIIISFMRHG NAYRLMAEAT
N. fumigata       MDYERDVLLW YLGTVADDAR YPPGLRNKAT HIIVSFMRHR NAYRLLAQAT

E. heterothallica ELARGELVMY PFQQVGNIPG NIGLPVRRFS QNIHAITTAF GIIPTNEDNE
E. repens         TLARGELVMY PFQQVANIPR NKGLPVRRFP QNINAITCAF GIIPCNDDYE
N. fischeri       ALARGELVMY PFQQVNNIPR NLGLPVRRFG QNIQAITAAF GIIPTNADYE
N. fumigata       ELARGELVMY PFQQVGNIPR NIGLPVRRFS QNIRAITTAF GIIPTNEDNE

E. heterothallica GHPIELISIL DPAIEASMND NQKFEFHRLL LVKERQANAD LARSVQRYGY
E. repens         GHPIELISIL DPAIENSMND NQHNEFHRAL LVNEKQANAD FARSVQRYGY
N. fischeri       GHPIELISVL DPAIEESMND NLRLEFHRAL LVKEQRANAD LARSVQRYGY
N. fumigata       GHPIELISIL DPAIEASMND NQKFEFHRAL LVKERQANAD LARSVQRYGY
```

Figure 3.27: Amino acid alignment of the putative MAT1-2-4 proteins of *E. heterothallica, E.repens, N. fischeri* and *N. fumigata*. Green highlighting indicates where nucleotides are conserved between all species. Red highlighting indicates where nucleotides are conserved between three species. (Sequencing was obtained from The Broad Institute genome sequencing project. (http://www.broad.mit.edu), *N. fumigata MAT1-2-4*, gene locus ID: Afu3g06160 , *N. fischeri MAT1-2-4*, gene locus ID: NFIA_024400).

Table 3.13: Percentage nucleotide identity between the putative *MAT1-2-4* genes of *E. heterothallica, E. repens, N. fischeri* and *N. fumigata*.

	E. repens	*N. fischeri*	*N. fumigata*
E. heterothallica	65	86	98
E. repens		63	64
N. fischeri			86

Table 3.14: Percentage amino acid identity between the putative MAT1-2-4 proteins of *E. heterothallica, E. repens, N. fischeri* and *N. fumigata*.

	E. repens	*N. fischeri*	*N. fumigata*
E. heterothallica	77	83	96
E. repens		80	79
N. fischeri			84

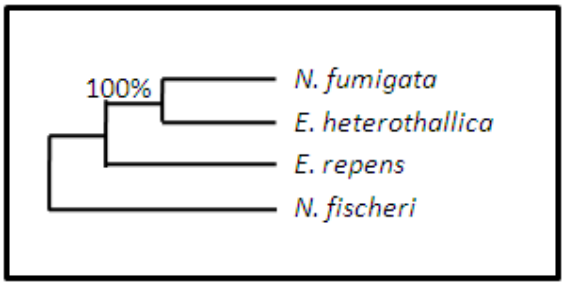

Figure 3.28: Phylogenetic analysis of the amino acid sequences of the putative MAT1-2-4 proteins comparing *E. heterothallica, E. repens, N. fischeri* and *N. fumigata*. Bootstrap values greater than 70% are indicated. Maximum parsimony analyses was performed using MEGA version 4 program, with bootstrapping of 500 replicates and excluding gaps (section 2.2.20) (Tamura *et al.* 2007). (Sequencing was obtained from The Broad Institute genome sequencing project. *N. fumigata MAT1-2-4*, gene locus ID: Afu3g06160 , *N. fischeri MAT1-2-4*, gene locus ID: NFIA_024400).

3.4 Discussion

The aim of this chapter was to determine the genetic structure of MAT loci or MAT idiomorphs of *Aspergillus* species known to reproduce by sexual means. This was performed in an attempt to determine the ancestral sexual strategy of the Aspergilli.

The other aim of this chapter was to assess the possession and expression of the putative *MAT1-2-4* gene. The results gained in this chapter have provided insights into both of these evolutionary questions.

3.4.1 Status of the *MAT1-2-4* Gene

It has been noted that the MAT regions seem to have particularly fast evolutionary rates compared to the rest of the genome, with the acquisition of extra genes within the MAT idiomorphs being common (Butler 2007; Cozijnsen and Howlett 2003; Debuchy *et al.* 1993; Fraser *et al.* 2007b; Galagan *et al.* 2003; Glass *et al.* 1990a; Kanematsu *et al.* 2007; Mandel *et al.* 2007; Yun *et al.* 2000; Yun *et al.* 1999).

The putative *MAT1-2-4* gene has previously been identified within the MAT-2 idiomorph of *P. marneffei*, *N. fumigata, N. fischeri*, *Ajellomyces capsulatus* and *Coccidiodes* species. However, in *Penicillium* and *Aspergillus* species, no expression studies have yet been made (Bubnick and Smulian 2007; Fraser *et al.* 2007b; Mandel *et al.* 2007; Paoletti *et al.* 2005; Woo *et al.* 2006). One aim of the present study was to assess the presence of this putative gene within other *Aspergillus* species, and also to investigate whether the *MAT1-2-4* ORF encodes a functional protein rather than being a pseudogene.

Results showed firstly that the *MAT1-2-4* ORF is present a in conserved positions in the MAT-2 idiomorphs of *E. repens, E. heterothallica, P. marneffei, N. fumigata* and *N. fischeri*. Secondly, the *MAT1-2-4* ORF DNA sequences are very well conserved, to a much greater extent compared to the *MAT* genes. This conservation of DNA and amino acid sequences is consistent with the *MAT1-2-4* ORF being a gene. Furthermore, the position of an intron is conserved between *E. repens, E. heterothallica, N. fumigata* and *N. fischeri* this is also consistent with *MAT1-2-4* being a functional gene.

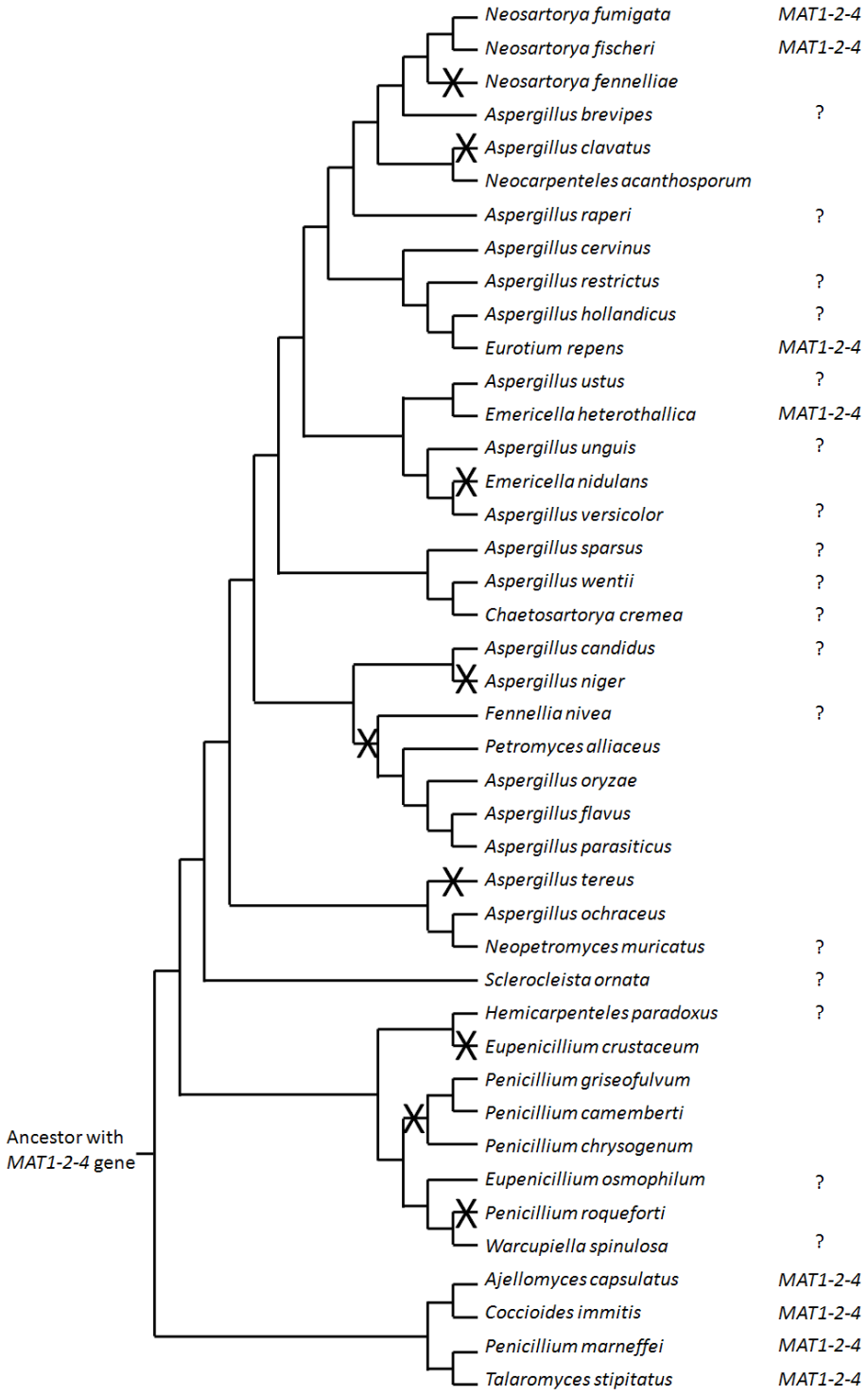

Figure 3.29: Phylogeny of sexual and asexual *Aspergillus* and *Penicillium* species based on β-tubulin sequence data. '?' denotes where sequence data are not available and presence of *MAT1-2-4* remains to be determined, '*MAT1-2-4*' denotes where the *MAT1-2-4* gene is present, 'X' denotes where *MAT1-2-4* gene has been lost. For Culture Identification Codes and GenBank Accession numbers used to contruct this phylogeny see Appendix 4. The tree was constructed using maximum parsimony analyses (excluding gaps)

However, RT-PCR analyses failed to demonstrate expression of the putative *MAT1-2-4* gene in *E. repens* or *E. heterothallica*. Expression of the putative *MAT1-2-4* gene has been demonstrated in *Coccidiodes posadasii* although at a level 24 times lower than that seen for the actin control. It is possible that expression of this gene is at a level lower than the threshold needed in the RT-PCR analysis used in this study (Mandel *et al.* 2007).

There may be other reasons for not detecting expression. Firstly, it may be that the *MAT1-2-4* gene is expressed to a greater level under different conditions not assayed in this study. Secondly, the precise boundaries of the ORF of this gene are still unclear. It is possible that the start site is in a different position to the one used in this study. An alternative possible start site could be 58 amino acids into the MAT1-2-4 protein detailed in this study. If this alternative translation start site is correct, the *MAT1-2-4* forward primer used in the RT-PCR analyses is located outside of the transcribed and translated sequence. Further experimental work using new primers will need to be performed to either confirm no expression of the *MAT1-2-4* gene or if expression is at a very low level. Longer culturing times may need to be used as expression did increase over time in the *C. posadasii* study (Mandel *et al.* 2007).

The conservation of DNA and amino acid sequences of the four *Aspergillus* species suggests that the *MAT1-2-4* ORF may be a genuine gene. However, PSORT II analysis gives no clues as to the function of this gene as no clear nuclear-localization, RNA-binding or mitochondrial targeting signals could be found. The fact that the gene is limited to the MAT1-2 genotype strongly suggests some role specifically linked to mating identity and sexual reproduction.

From Figure 3.29 I suggest that the *MAT1-2-4* gene is ancestral to the *Aspergillus*/*Penicillium* clade. Chapters 4 and 5 will show that this gene is not present in any of the other species that were analysed. Where data is available the known presence or absence of the *MAT1-2-4* gene for each species is shown. Predictions can be made regarding *MAT1-2-4* gene absence or presence in, as yet, unsequenced species but will require experimental validation.

The eight species that were found to contain the putative *MAT1-2-4* gene (*E. heterothallica, E. repens, N. fischeri, N. fumigata, P. marneffei, Talaromyces stipitatus, Ajellomyces capsulatus, Coccioides immits*) do not group together in the phylogenetic analysis performed in Figure 3.29. The *MAT1-2-4* gene was not detected in *N. fennelliae*

and *A. clavatus* both of which form a monophyletic clade with *N. fumigata* and *N. fischeri*. In contrast, the *MAT1-2-4* gene is present in *E.heterothallica*, which clusters with different species not possessing this gene. Figure 3.29 shows 9 losses of the *MAT1-2-4* gene. However, more losses of his gene may be reported as more species are analysed.

3.4.2 Evolution of *MAT* Gene Regions Within the Aspergilli

An interesting observation is that phylogenetic analyses of the *MAT* genes produced non-congruent phylogenies for some species i.e. the *MAT1-1-1* and *MAT1-2-1* genes produced slightly different phylogenies. Analyses of sequence for both MAT1-1-1 and MAT1-2-1 proteins indicated that *P. alliaceus* is basal to the *Neosartorya* species clade, which is consistent with previous phylogenetic studies (Peterson 2008). The MAT1-1-1 amino acid sequence analysis also groups *E. heterothallica* and *E. nidulans* together which has been suggested from morphological and other genetic phylogenetic analyses (Kwon *et al.* 1964; Peterson 2008). Neither of the phylogenies derived from MAT1-1-1 or MAT1-2-1 amino acid sequences were significantly divergent from other phylogenies produced involving these species (Peterson 2008; Samson *et al.* 2007; Tamura *et al.* 2000; Varga *et al.* 2000b).

Chapter 4 Mating-Type and Other 'Sex-Related' Genes in Asexual Aspergilli

4.1 Introduction

The taxonomic placement of the genus *Aspergillus* is as follows (Hibbett *et al.* 2007; James *et al.* 2006a).

Kingdom: Fungi
Subkingdom: Dikarya
Phylum: Ascomycota
Subphylum: Pezizomycotina
Class: Eurotiomycetes
Subclass: Eurotiomycetidae
Order: Eurotiales
Family: mitosporic Trichocoma
Genus: *Aspergillus*

The genus *Aspergillus* contains over 250 species, and includes over 70 teleomorphic species that are interspersed among the anamorphic species (Figure 4.2). The anamorphic state of species in this genus was first described in 1729 and is characterised by the production of an unbranched, usually aseptate condiophore (Figure 4.1). At the apex, the conidiophore becomes (depending on species) globose, hemispherical, elliptical or clavate (club-shaped), forming a structure termed the vesicle. Projecting from the vesicle are fertile structures known as sterigmata, these produce the asexual spores known as conidia. The whole asexual structure is known as an aspergillum (Figure 4.1) (Raper and Fennell 1965).

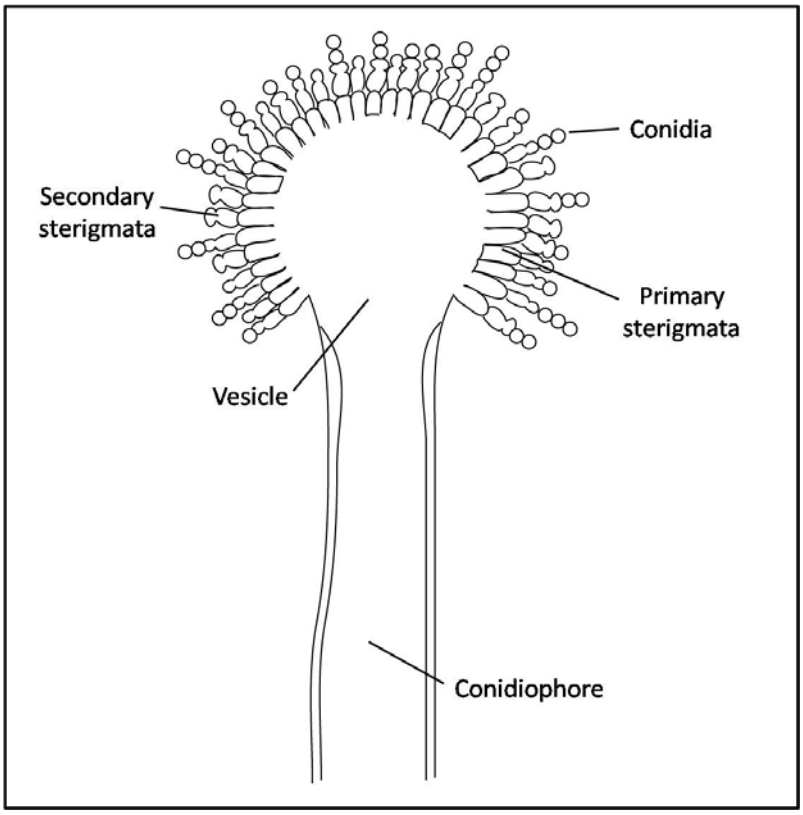

Figure 4.1: Diagrammatic representation of an aspergillum. [Adapted from Raper and Fennell (1965).]

Conidia colour has often been used to classify *Aspergillus* species into their subgenera and sections, although vesicle shape and presence or absence of secondary sterigmata have been used also (Table 4.1). Species in the genus *Aspergillus* are haploid, sabrobic and can be opportunistic human, animal and plant pathogens (Klich 2001; Klich 2002; Raper and Fennell 1965).

There are six subgenera, split into 17 sections (Figure 4.2 and Table 4.1), within the genus *Aspergillus.* Although Peterson (2008) suggested that sections *Clavati*, *Cervini* and *Fumigati* could be merged into section *Fumigati* (Peterson 2008). In this thesis the sections will be kept separate, because sections *Clavati* and *Fumigati* are each associated with different teleomorphic genera (Figure 4.2) (Peterson 2008; Samson *et al.* 2007; Udagawa and Uchiyama 2002). The subgenera are indicated in Table 4.1, but are not considered further as the subgenus *Nidulantes* and subgenus *Circumdati* are paraphyletic (Figure 4.2) and therefore only sections will be used. Section divisions have been largely supported by multilocus phylogenetic analyses (Houbraken *et al.* 2007; Peterson 2008; Samson *et al.* 2007; Varga *et al.* 2007a; Varga *et al.* 2007b).

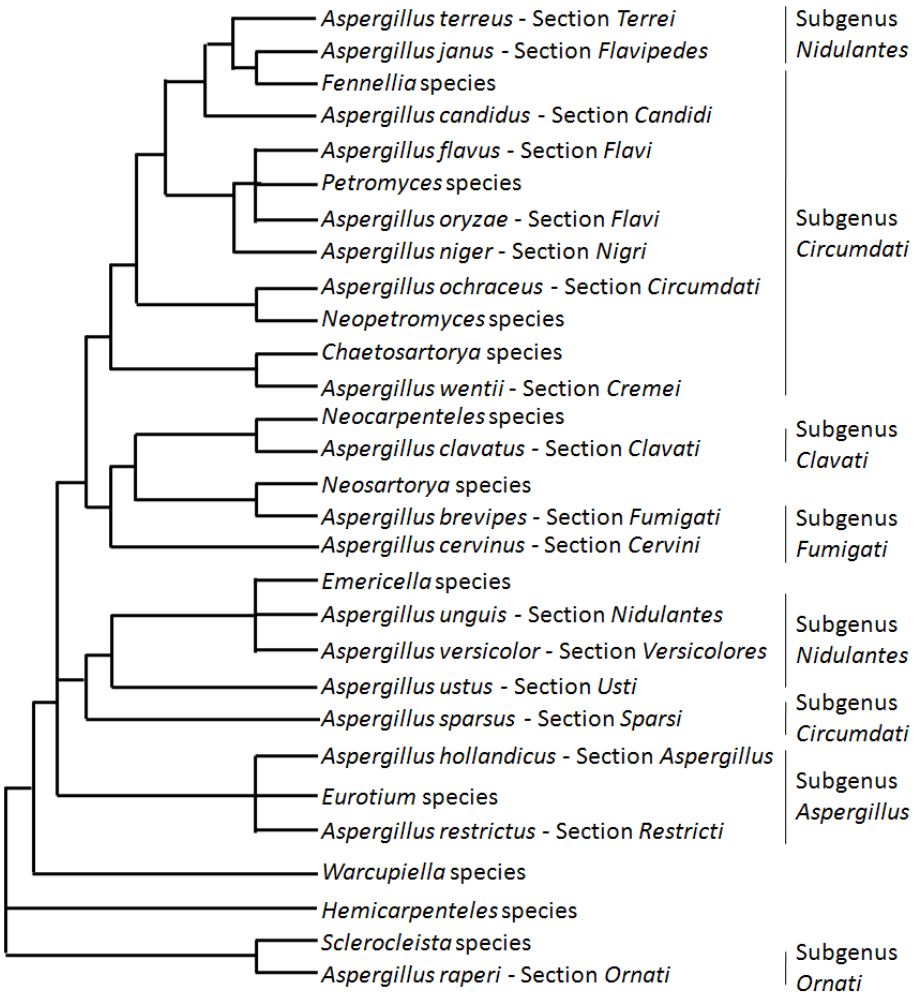

Figure 4.2: Phylogeny of the genus *Aspergillus*, with its 11 associated teleomorphic genera. [Adapted from Frisvad and Samson (2000), Peterson (2008), Samson *et al*. (2007), Tamura *et al*. (2000), Udagawa and Uchiyama (2002) and Varga *et al*. (2000a).]

Table 4.1: Morphological characteristics of the *Aspergillus* subgenera.

Subgenus	Sections	Distinguishing morphological features
Aspergillus	*Aspergillus* *Restrictus*	Xerophilic, grey-green conidia
Fumigati	*Fumigati* *Cervini*	Vesicles predominantly pyriform, grey-green, blue-green or orange conidia
Ornati	*Ornati*	Grey-green, yellow-green or olive brown conidia
Clavati	*Clavati*	Vesicles predominantly clavate, grey-green conidia
Nidulantes	*Nidulantes* *Versicolores* *Usti* *Terrei* *Flavipedes*	Green, grey-green, blue-green, buff or orange-brown conidia
Circumdati	*Flavi* *Nigri* *Circumdati* *Candidi* *Cremei* *Sparsi*	Vesicles spherical to pyriform, buff, yellow, olive, green or near black conidia.

The genus *Aspergillus* contains numerous species that are often abundant and usually ubiquitous, although some species are relatively rare (Klich 2002; Raper and Fennell 1965). *Aspergillus* species are able to produce a variety of secondary metabolites. These include mycotoxins, which are capable of causing human and animal disease, as well as compounds with antibacterial, antitumoric and antifungal activities. The ability of *Aspergillus* species to produce beneficial and detrimental compounds means that their worldwide distribution is arguably a double-edged sword. It also means that many *Aspergillus* species are important medical and biotechnology organisms. Consequently, some species in this genus have been extensively studied, and eight Aspergilli, including asexual and sexual species have had their genomes sequenced (Table 4.2). The species relevant to further studies in this chapter will now be described in more detail.

Table 4.2: Genome sizes of eight *Aspergillus* species that have been genome sequenced.

Species	Isolate sequence[a]	Genome size[a] (Mb)	Sequencing undertaken by
A. clavatus	NRRL 1	27.86	TIGR[b]
A. flavus	NRRL 3357	36.79	TIGR[b]
A.niger	ATCC 1015	37.3	JGI[c]
A. oryzae	ATCC 42149	37.12	*A. oryzae* Consortium, NITE[d], NIAIST[e]
A. terreus	NIH 2624	29.33	The Broad Institute
E. nidulans	FGSC A4	30.07	The Broad Institute
N. fischeri	NRRL 181	27.86	TIGR[b]
N. fumigata	Af293	29.38	TIGR[b], Sanger, Institut Pasteur

[a]Information from The Broad Institute, additional isolates for certain species have been sequenced (www.broad.mit.edu/node/304). [b]The Institute of Genome Research (http://www.tigr.org/db.shtml). [c]DOE Joint Genome Institute (http://www.jgi.doe.gov/). [d]National Institute of Technology and Evaluation (http://www.nite.go.jp/index-e.htm). [e]National Institute of Advanced Industrial Science and Technology (http://www.aist.go.jp/index_en.html).

4.1.1 *Aspergillus clavatus*

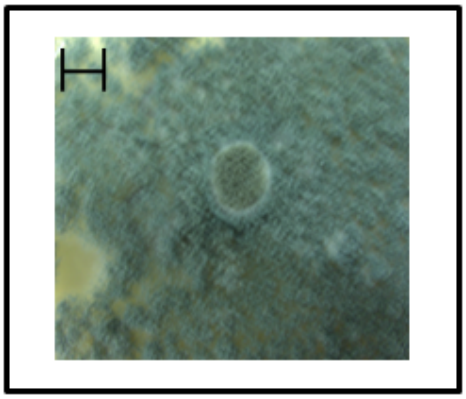

Figure 4.3: Culture of *Aspergillus clavatus* isolate NRRL 3072 grown for 1 week on MEA in the dark. Scale bar indicates approximately 1cm.

The section *Clavati* is characterised by the production of clavate vesicles and grey-green conidia. The species in this section produce large blue-green conidia heads (Figure 4.3) and species are determined by the size of the conidiophores they produce (Raper and Fennell 1965). The type species of this section is *A. clavatus*, which is also the most economically important species in this section (see below).

The ability of *A. clavatus* to grow in highly alkaline conditions contributes to its ubiquitous occurrence in dung and certain soils worldwide. This species is also able to grow on rotten fruits especially apples, as well as stored products with high moisture content e.g. corn, millet and rice (Domsch *et al.* 1980; Flannigan and Pearce 1994; Raper and Fennell 1965). *A. clavatus* as well as other species in the section *Clavati* are able to produce a variety of mycotoxins. These include the fruit oxidation preventative, kojic acid, cytochalasins (actin inhibitor), tremorigenic compounds and the genotoxin and tumorigenic compound, patulin. Patulin has been found in fruit juices produced worldwide as a consequence of fungal contamination (Beretta *et al.* 2000; Harrison 1989; Lopez-Diaz and Flannigan 1997; Smith and Moss 1985; Varga *et al.* 2003; Waksman *et al.* 1942).

Aspergillus clavatus seems to be particularly well adapted to survive the malting process and some of the mycotoxins retain functionality even after the kilning process used in malting (Flannigan and Pearce 1994; Lopez-Diaz and Flannigan 1997). This survival ability can lead to extrinsic allergic alveolitis causing a medical conidition known as farmers' or malters' lung (Blyth 1978; Channell *et al.* 1969; Grant *et al.* 1976; Riddle *et al.* 1968). Automation in the farming and malting industries has lead to a decrease in allergic reactions caused by this species. *A. clavatus* and *A. giganteus* are able to produce ribotoxins, these compounds are currently being investigated as possible immunotherapy tools (Carreras-Sangra *et al.* 2008; Martínez-Ruiz *et al.* 1999).

The section *Clavati* is a sister group to the teleomorphic genus *Neocarpenteles* (Figure 4.2) (Peterson 2000a; Peterson 2008; Varga *et al.* 2007a). *Neocarpenteles acanthosporum* (anamorph: *A. acanthosporus*) is the only species currently classified in this genus and produces hyaline ascospores, which have two equatorial ridges. Cleistothecia take 3-4 weeks to mature and appear yellow-brown (Udagawa and Uchiyama 2002).

The section *Clavati* also forms a basal clade to species in the section *Fumigati* and the genus *Neosartorya* (Figure 4.2). The genus *Neosartorya* contains both homothallic and heterothallic species (Samson *et al.* 2007), including *N. fennelliae* which has already been investigated in Chapter 3 of this thesis. The only known species in the genus *Neocarpenteles* is homothallic however, no molecular investigation into its *MAT* gene arrangement has yet been undertaken (Udagawa and Uchiyama 2002; Varga *et al.* 2007a). An understanding of the sexual reproductive strategy, if any, of *A. clavatus* may

help in elucidating the evolution of sexual strategy and *MAT* genes in the Aspergilli, with particular relevance to the genus *Neosartorya*, which contains the opportunistic human pathogen *N. fumigata* (Kontoyiannis and Bodey 2002; Latgé 1999).

4.1.2 *Aspergillus terreus*

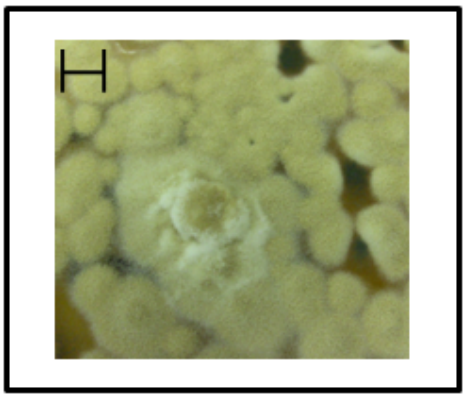

Figure 4.4: Culture of *Aspergillus terreus* isolate NRRL 6450 grown for 1 week on MEA in the dark. Scale bar indicates approximately 1cm.

Aspergillus terreus (section *Terrei*) produces buff, cinnamon to orange-brown conidia, with smooth colourless conidiophores (Figure 4.4). *A. terreus* is believed to be the only species in section *Terrei* (Peterson 2000a; Raper and Fennell 1965). It is an ubiquitous soil-borne, saprobic species, and is an important apple rot fungus, which is often isolated from stored grains and other products exposed to moisture (Del Prado and Christensen 1952; Raper and Fennell 1965; Tandon and Bhatnagar 1958; Varga *et al.* 2005).

Aspergillus terreus is also a medically important species, causing invasive aspergillosis in immunocompromised patients, particularly those with haematological malignancies. This is of particular significance as, unlike *N. fumigata*, this species is intrinsically resistant to amphotericin B, the antifungal drug of choice (Iwen *et al.* 1993; Iwen *et al.* 1998; Kontoyiannis and Lewis 2002; Sutton *et al.* 1999; Torres *et al.* 2003).

Aspergillus terreus produces a variety of mycotoxins, for example citrinin (carcinogenic), ochratoxin (neprotoxic) and patulin. Due to its presence on apples, *A. terreus* also contributes to the worldwide contamination of fruit juices by patulin (Peraica *et al.* 2008; Sankawa *et al.* 1983; Turner and Aldridge 1983). As well as mycotoxins, this species can also produce an array of medically and industrially important secondary metabolites. These include lovastatin, which was the first cholesterol lowering drug to

be discovered (Alberts *et al.* 1980; Kennedy *et al.* 1999; Manzoni and Rollini 2002). *A. terreus* also produces territrem B and terrulactone which are acetylcholinesterase inhibitors (Chen and Ling 1996). *A. terreus* is also able to produce the enzyme α-L-rhamnosidase (Rase). This enzyme has a range of activities including removing the bitter component of grapefruit (naringin), eliminating kesperidin crystals from orange juices, enhancing the aroma of wines, and enzymatically catalyzing the hydrolysis of natural glycosides [see Elinbaum *et al.* (2002) for references]. Making Rase, and thereby *A. terreus* an industrially important species.

Section *Terrei* is not a sister group to any known teleomorphic genera, although it does form the basal lineage to the section *Flavipedes* which includes the teleomorphic genus *Fennellia* (Figure 4.2) (Peterson 2000a; Peterson 2008). The lack of closely related teleomorphic genera makes *A. terreus* intriguing to study. It raises questions such as if this species has retained the ability to reproduce sexually, does it have a homothallic or heterothallic mating strategy? Also, if the ability to sexually reproduce has been lost, it would be interesting to know why it has been lost, i.e. lack of a mating partner or mutation in genes or their promoters.

4.1.3 *Aspergillus flavus*

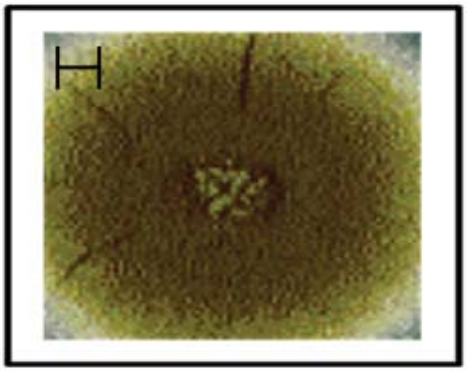

Figure 4.5: Culture of *A. flavus* (picture sourced from Hedayati *et al*. (2007)). Scale bar indicates approximately 1cm.

Aspergillus flavus (section *Flavi*) is an ubiquitous saprobe with a worldwide distribution (Domsch *et al.* 1980; Hedayati *et al.* 2007; Payne 2008; Payne *et al.* 2008; Raper and Fennell 1965). *A. flavus* produces colourless conidiophores and vesicles that are clavate when young and become globose when mature. Conidia are pale yellow or yellow green (Figure 4.5) (Klich 2002; Raper and Fennell 1965).

Section *Flavi* is closely related phylogenetically to the sexual genus *Petromyces* (Figure 4.2). The type species of the genus *Petromyces* is *P. alliaceus* (anamorph: *A. alliaceus*), which produces ellipsoidal, colourless ascospores that may take a few months to mature (Klich 2002).

Aspergillus flavus is commonly found as a contaminant on economically important crops e.g. maize and peanuts, and is one of the most widely reported food-borne fungi (Domsch *et al.* 1980; Hedayati *et al.* 2007; Payne 2008; Payne *et al.* 2008). *A. flavus* is the second [after *N. fumigata* (anamorph: *A. fumigatus*)] leading cause of invasive aspergillosis accounting for approximately 30% of cases, and is the most common species of *Aspergillus* to cause superficial fungal infections (Hedayati *et al.* 2007). The contamination of feed-stuffs and opportunistic human and animal infection by *A. flavus* is especially significant because this species produces aflatoxins and other toxic secondary metabolites including sterigmatocysin, cyclopiazonic acid, kojic acid, aspertoxin and aspergillic acid (Yu *et al.* 2004). Aflatoxin B1 is the most toxic aflatoxin and is a potent hepatocarcinogenic compound (Bennett and Klich 2003). There is isolate variation of aflatoxin production within *A. flavus* (Hedayati *et al.* 2007). *A. flavus* isolates are resistant to the antifungal amphotericin B *in vitro*, and they appear less susceptible compared to other fungal species *in vivo*. *A. flavus* also appears to be resistant *in vitro* and less susceptible *in vivo* to itraconazole, voriconazole and the echinocandin group of antifungal agents (Hedayati *et al.* 2007).

Aspergillus flavus isolates can be divided into two taxonomically distinct groups (Geiser *et al.* 2000; Geiser *et al.* 1998b). It is unclear whether these two groups vary in their clinical significance, drug resistance or virulence. *A. flavus* is only known to reproduce by asexual means, and a parasexual cycle can be induced in the lab (Geiser *et al.* 1998b). Geiser *et al.* (1998b) suggested that *A. flavus* has a recombining population structure, but the amount of recombination could not be determined (Geiser *et al.* 1998b). Most incidences of *A. flavus* outbreaks (both clinical and crop contaminants) result from growth of only a single or a few isolates. Some reports suggest that there is less genetic diversity in *A. flavus* isolates compared to *N. fumigata* isolates (Hedayati *et al.* 2007).

4.1.4 Previous Molecular Investigations in Asexual Aspergilli

Table 4.2 shows the genome sizes of the eight Aspergilli sequenced so far. As can be seen *A. oryzae*, *A. flavus* and *A. niger* have genomes 20-30% larger than the other five

species. The genomes of the closely related species, *A. oryzae* and *A. flavus* have been compared. Both genomes were found to be enriched with genes for secondary metabolism, but do not differ greatly as might be expected given their phylogenetic relatedness. The aflatoxin biosynthesis cluster in *A. oryzae* is thought to be non-functional due to deletions and frameshift mutations (Tominaga *et al.* 2006). Forty three and 129 species-specific genes were detected for *A. flavus* and *A*. *oryzae*, respectively, and over 160 genes are differentially expressed between these two species (Payne 2008; Payne *et al.* 2006).

The origins of the additional genome content of *A. oryzae, A. flavus* and *A. niger* has been investigated, but the source (or sources) of these sequences is still unclear. The extra sequences do not appear to be the result of chromosomal rearrangement or duplication and a single evolutionary ancestor of this extra genome sequence seems unlikely (Khaldi and Wolfe 2008).

Comparison of two genomes from *A. niger* isolates (CBS 513.88 and ATCC 1015) has revealed differences in the retrotransposon ANiTa1 copy number and sequence. CBS 513.88 is an industrial isolate and has undergone selection pressure to increase enzyme production etc., ATCC 1015 is an environmental isolate and the isolate-specific sequence differences may be a result of the evolutionary backgrounds of both isolates (Braumann *et al.* 2008a).

4.1.5 Mating-Type and Other 'Sex-Related' Genes in Asexual Aspergilli

There are ongoing investigations to determine whether a sexual cycle might be present in supposedly asexual *Aspergillus* species. A MAT locus has been found via genome sequencing in all five of the asexual Aspergilli sequenced so far, suggesting a possible latent potential for sexuality (Dyer 2007; Galagan *et al.* 2005; Machida *et al.* 2005; Payne *et al.* 2006; Payne *et al.* 2008; Pel *et al.* 2007; Ramirez-Prado *et al.* 2008). There is precedent for the discovery of a sexual cycle based on studies of *N. fumigata* (O'Gorman *et al.* 2009). The detection of isolates with compatible mating-type genes in the same geographical locations, together with evidence of recombination and the discovery of a plethora of auxiliary sexual genes (via genome sequence data mining) in the opportunistic human pathogen *N. fumigata* led to years of speculation as to the sexual capacity of this species (Dyer and Paoletti 2005; Galagan *et al.* 2005; Große and Krappmann 2008; Kontoyiannis and Bodey 2002; Latgé 1999; Nierman *et al.* 2005;

Paoletti *et al.* 2005; Pöggeler 2002; Pringle *et al.* 2005; Pyrzak *et al.* 2008; Rydholm *et al.* 2006; Varga 2003; Varga and Tóth 2003). The situation was confused by suggestions that both the *MAT1-2-1* and *nsdD* genes may not be functional in *N. fumigata* as a result of defective promoters (Große and Krappmann 2008; Pyrzak *et al.* 2008). It was not until 2009 that a sexual cycle was found and a heterothallic sexual reproductive mode was confirmed (O'Gorman *et al.* 2009).

BLAST genome searching of the genome sequences of five asexual *Aspergillus* species sequenced so far has revealed the presence of *MAT1-1-1*-like genes in all of these species (Dyer 2007) (Table 4.3). The corresponding *MAT1-2-1* encoding genes have since been found by experimental means in *A. flavus* and *A. oryzae*, and expression of *A. flavus MAT1-1-1* and *MAT1-2-1* genes detected by RT-PCR (Ramirez-Prado *et al.* 2008). *MAT1-1-1* and *MAT1-2-1* genes have also been detected in *A. parasiticus* (section *Flavi*) (Ramirez-Prado *et al.* 2008). However, no *MAT1-2-1*-like genes have yet been found in *A. clavatus* or *A. terreus*.

Putative genes encoding a α-factor pheromone precursor, and both **a** and α-factor-like pheromone receptors have also been annotated in all of the asexual *Aspergillus* genomes sequenced so far (Tables 4.4 to 4.6). Two genes encoding transcriptions factors (VeA and NsdD), that are essential to the sexual cycle and act upstream of the *MAT* genes have also been annotated (Tables 4.7 and 4.8). However, it is not yet known whether pheromone precursor and receptor genes or other sex-related transcription factors are expressed in any of the 'asexual' Aspergilli.

Table 4.3: Details of putative *MAT1-1-1* genes from *Aspergillus* species for which genome sequence data is available.

Species	Length of ORF (bp)	Position of Introns in Protein	Protein length (amino acids)	Protein size (kDa)	Gene locus[a]	Accession number[b]
A. clavatus	1166	98	372	41.3	ACLA_034110	XM_001270633
A. flavus	1165	97	371	41.7	AFL2G_11189.2	EU357934
A. niger	1155	97	368	41.1	gw1_4.505	XM_001394939
A. oryzae	1163	97	371	41.7	AO090020000089	XM_001824409
A. terreus	1123	79	357	39.8	ATEG_08812.1	XM_001217397
E. nidulans	1139	98	361	40.9	AN2755.3	XM_655267
N. fischeri	1155	97	368	41.0	NFIA_07110	XM_001263835
N. fumigata	1158	97	368	40.8		AY898661

[a]Gene locus information from The Broad Institute (http://www.broad.mit.edu/tools/data/seq.html).

[b]Accession numbers from GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez).

Table 4.4: Details of putative *ppgA* α-factor pheromone precursor genes from *Aspergillus* species for which genome sequence data is available.

Species	Length of ORF (bp)	Position of Introns in Protein	Protein length (amino acids)	Protein size (kDa)	Gene locus[a]	Accession number[b]
A. clavatus	315		105	10.9	ACLA_088140	
A. flavus	309		103	10.7	AFL2G_02931.2	
A. niger	321		107	11.3	e_gw1_10.940	
A. oryzae	494	22	103	11.0	AO090003000007	
A. terreus	506	110 119	136	14.8	ATEG_07407.1	
E. nidulans	384		128		AN5791.3	BK001308
N. fischeri	306		102	10.8	NFIA_051990	
N. fumigata	306		102	10.8	Afu6g06360	XM_745435

[a]Gene locus information from The Broad Institute. [b]Accession numbers from GenBank.

Table 4.5: Details of putative *ste3* (*preA*) **a**-factor pheromone receptor genes from *Aspergillus* species for which genome sequence data is available.

Species	Length of ORF (bp)	Position of Introns in Protein	Protein length (amino acids)	Protein size (kDa)	Gene locus[a]	Accession number[b]
A. clavatus	1482	188 323	433	49.0	ACLA_012620	
A. flavus	1809	188 351	465	52.7	AFL2G_06286.2	
A. niger	1489	188 349	457	51.5	fge1_pg_C_14000056	
A. oryzae	1503	188 351	465	52.7	AO090701000699	
A. terreus	1433	184 342	442	50.4	ATEG_08338.1	
E. nidulans	1544	179 292	426	47.3	AN7743.3	BK001309
N. fischeri	1329	188 349	405	45.2	NFIA_079400	
N. fumigata	1485	188 349	460	51.3	Afu5g07880	XM_748755

[a]Gene locus information from The Broad Institute. [b]Accession numbers from GenBank.

Table 4.6: Details of putative *ste2* (*preB*) α-factor pheromone receptor genes from *Aspergillus* species for which genome sequence data is available

Species	Length of ORF (bp)	Position of Introns in Protein	Protein length (amino acids)	Protein size (kDa)	Gene locus[a]	Accession number[b]
A. clavatus	1173	249	370	41.2	ACLA_041790	
A. flavus	1162	249	369	40.9	AFL2G_06216.2	
A. niger	1194	248	378	40.9	fge_1_pg_C_ 11000212	
A. oryzae	1161	249	369	40.9	AO090701000605	
A. terreus	1175	249	373	41.5	ATEG_03500.1	
E. nidulans	1202	249	430	47.7	AN2520.3	BK001310
N. fischeri	1173	249	369	40.5	NFIA_062970	
N. fumigata	1173	249	369	40.3	Afu3g14330	XM_749100

[a]Gene locus information from The Broad Institute. [b]Accession numbers from GenBank.

Table 4.7: Details of putative *nsdD* GATA transcription factor genes from *ASerpgillus* species for which genome sequence data is available.

Species	Length of ORF (bp)	Position of Introns in Protein	Protein length (amino acids)	Protein size (kDa)	Gene locus[a]	Accession number[b]
A. clavatus	1833	11 318 428	498	53.2	ACLA_041500	XM_001271357
A. flavus	1575	274 384	453	48.5	AFL2G_03635.2	
A. niger	1851	9 322 433	503	53.7	fge1_pg_C_ 2000697	XM_001400053
A. oryzae	1578	274 384	453	48.6	AO090012000768	
A. terreus	1521	251 361	430	46.8	ATEG_04043.1	XM_001213221
E. nidulans	1560	11 282 392	461	49.3	AN3152.3	XM_655664
N. fischeri	1803	11 313 332	493	52.9	NFIA_063400	
N. fumigata	1804	11 313 423	493	53.0	Afu3g13870	XM_749144

[a]Gene locus information from The Broad Institute. [b]Accession numbers from GenBank.

Table 4.8: Details of putative *veA* transcription factor genes from *Aspergillus* species for which genome sequence data is available

Species	Length of ORF (bp)	Position of Introns in Protein	Protein length (amino acids)	Protein size (kDa)	Gene locus[a]	Accession number[b]
A. clavatus	1797	50	578	63.7	ACLA_022920	XM_001269003
A. flavus	1787	34 178	549	60.5	AFL2G_07468.2	DG519574
A. niger	1734	50	555	61.3	Est_GW_Plus_C_31478	XM_001392590
A. oryzae	1784	50	574	63.0	AO090001000237	
A. terreus	1767	50	590	62.5	ATEG_00439.1	XM_001210525
E. nidulans	1664	16	537	59.3	AN1052.3	U95045
N. fischeri	1779	50	570	63.1	NFIA_013050	
N. fumigata	1778	50	570	63.0	Afu1g12490	XM_747526

[a]Gene locus information from The Broad Institute. [b]Accession numbers from GenBank.

4.1.6 Evolution of MAT Regions

As described in Chapter 3, there has been much discussion and dispute over the evolution of mating strategies and mating-type regions within the genus *Aspergillus* and the Eurotiomycetes in general. When *Cochliobolus*, *Ajellomyces*, *Aspergillus* and *Penicillium* species are treated together, heterothallism has been suggested as the ancestral strategy (Fraser *et al.* 2007b). However, when just *Aspergillus* (or *Aspergillus* and *Penicillium*) species are considered, homothallism (which is the sexual strategy of most sexual Aspergilli, see Chapter 3) has been suggested as the ancestral sexual strategy (Galagan *et al.* 2005; Geiser *et al.* 1998a; Varga *et al.* 2003). If this is the case, then heterothallic Aspergilli (of which there appear to be a growing number) would have to have re-evolved from a homothallic ancestor (Figure 4.8). Further investigation into MAT loci organisation and potential sexual strategies of *Aspergillus* species needs to be undertaken to try and elucidate the ancestral sexual strategy.

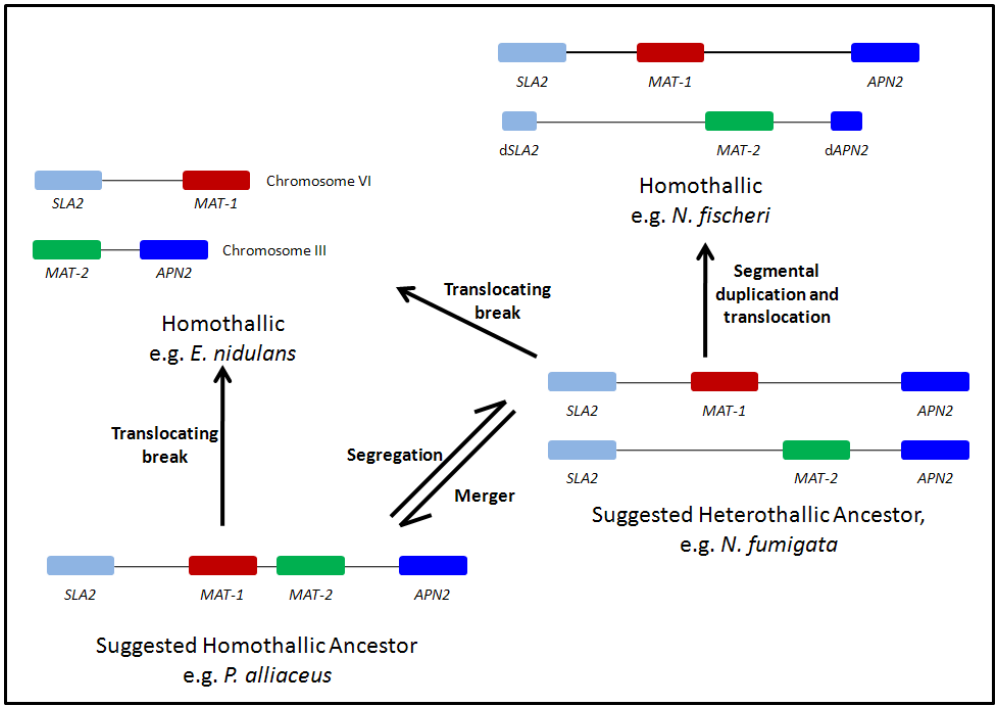

Figure 4.6: Possible routes of *MAT* evolution using *Aspergillus* species as examples [Adapted from Galagan *et al*. (2005), Paoletti *et al*. (2005 and 2007), Ramirez-Prado *et al*. (2008) and Rydholm *et al*. (2007).]

4.1.7 Aims of this Chapter

This chapter has two main aims. Firstly, to determine the reasons for asexuality in selected 'asexual' Aspergilli or conversely to determine whether some supposedly asexual species have a latent potential for sexuality. Secondly, to determine MAT loci organisation of certain asexual Aspergilli in order to gain insights into the evolution of breeding systems within the Aspergilli. This will be achieved by the following experimental objectives.

1. Determination of whether MAT1-2 genotypes can be detected in *A. clavatus* and *A. terreus*.
2. Determination of the distribution of MAT1-1 and MAT1-2 genotypes of *A. clavatus* and *A. terreus* in a worldwide sampling of isolates.
3. Use of RT-PCR to determine if mating-type, pheromone precursor and pheromone receptor genes of the 'asexual' species *A. clavatus, A. terreus* and *A. flavus* are expressed, and if so under what conditions.

4. Use of RT-PCR to determine if the genes that encode two additional transcription factors required for sex in *E. nidulans*, the *veA* and *nsdD* genes, are expressed in the asexual species *A. flavus, A. clavatus* and *A. terreus*.
5. Undertaking of bioinformatic analyses to determine whether genes required for sex can be detected in the genomes of the 'asexual' *A. flavus*, *A. terreus* and *A. clavatus*.
6. Incubation of compatible mating type isolates of *A. clavatus* and *A. terreus* together to determine if sexual structures are produced under the conditions tested.

4.2 Materials and Methods

4.2.1 *Aspergillus clavatus*

4.2.1.1 *MAT* Gene Screening

Initially, 20 *A. clavatus* isolates (Table 4.9) were screened for the presence of a *MAT1-2-1* HMG-domain encoding gene using *MAT* gene-specific degenerate primers (MAT5-7 and MAT3-5) as described in section 2.2.4. Eight microlitre aliquots of the resultant PCRs were resolved on 2% agarose gels and gels were visualised as described in section 2.2.4. Bands of interest were then excised from gels and purified as per section 2.2.8. The gel extracted products were ligated into plasmid pTOPO4, then cloned into *E. coli* and sequenced from extracted plasmid DNA using M13 Forward and M13 Reverse primers as described in sections 2.2.9 to 2.2.12.

Detection of *MAT1-2-1* amplicons allowed the sequencing of the entire putative *MAT1-2-1* gene (see results section 4.3.1.2). The gene sequence was then used to design the specific primer pair AclM2F and AclM2R (Table 4.10), which were used in a diagnostic PCR to test for the presence of MAT1-2 genotypes. Meanwhile, the putative *MAT1-1-1* gene sequence data was obtained from The Broad Institute *A. clavatus* genome screening project (http://www.broad.mit.edu/tools/data/seq.html) (gene locus ID: ACLA_034110) and specific *MAT1-1-1* primers (AclM1F and AclM1R, Table 4.10) were designed to detect the presence of isolates of MAT1-1 genotype. These primers were then used in a PCR diagnostic to screen 20 worldwide isolates (Table 4.9) to determine

whether isolates were of MAT1-1 or MAT1-2 identity based on the presence of an alpha-domain or HMG-domain encoding gene, respectively.

All PCRs were performed using 25µl reaction volumes containing ~10ng genomic DNA, 2.5µl 10X PCR Buffer (containing 20mM $MgCl_2$), 0.5µl (10mM each) dNTPs, 2.5µl (10µM) of respective *MAT* forward primer, 2.5µl (10µM) of respective *MAT* reverse primer, 0.2µl FastStart Taq Polymerase (Roche, UK) and ~15.8µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: an initial denaturation step at 94°C for 5 min; 35 cycles consisting of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C; followed by a final extension step at 72°C for 5 min, all steps were performed at a ramp rate of 70°C/min. Resultant PCR products were resoved on 2% agarose gels and visualized by ethidium bromide staining (section 2.2.4).

Table 4.9: Isolates of *A. clavatus* used in *MAT* gene screening. Isolates were kindly provided by Dr A. Rokas, Vanderbilt University, USA.

Culture ID Code	Other Culture ID Codes	Source of Isolate	Geographical Source of Isolate
NRRL 4	ATCC 10058 ATCC 16926 CBS 514.65 IFO 33020 IMI 232883 IMI 321306 JCM 10251 MUCL 15642 NRRL 2254 NRRL A-1432 QM 6884 Thom 4754.3 WB 2254	Dung	British Guiana
NRRL 5811	ATCC 18327 CBS 344.67 IMI 129967 QM 8964 VKM F-1136	Soil	Moldavia, Romania
NRRL 1823			Atypical strain
NRRL A-251		Soil	Venezuela
NRRL A-404		Corn	Ontario, Canada
NRRL A-803		Soil	Nicaragua
NRRL A-2905	QM 872	Japanese tarpaulin	New Guinea
NRRL A-3072		Soil	Liberia
NRRL A-3880		Soil	Formosa
NRRL A-7813		Soil	Jamaica
NRRL A-8053		Insect dung from dead yellow pine limb	Indiana, USA
NRRL A-14401		Raw sewage	Ithaca, New York,

			USA
NRRL A-14611		Stored corn	J. Ullands Farm, Austin, Minnesota, USA
NRRL A-22162		Corn	Iowa, USA
NRRL A-22359		European corn borer	In a light trap in Missouri, USA
NRRL A-22620		Soil	Lucknow, India
NRRL A-24204		Corn	North Carolina, USA
NRRL A-25278		Air	Budapest, Hungary
NRRL A-26725		Wheat	Nebraska, USA
NRRL A-28596		Wheat	Oklahoma, USA

4.2.1.2 Cloning of Idiomorph Region and Idiomorph Orientation

Species-specific *SLA2* and *APN2* primers [AclSLA2 and AclAPN2 (Table 4.10)] were designed using sequence data obtained from The Broad Institute *A. clavatus* genome sequencing project. The putative *SLA2* gene was found at gene locus ID: ACLA_034100, and the putative *APN2* gene was found at gene locus ID: ACLA_034130.

Whole Idiomorphic Region

The idiomorph region of *A. clavatus* was amplified by PCR following the *SLA2* and *APN2* flanking gene strategy as described in section 2.2.5. Primers AclSLA2 and AclAPN2 (Table 4.10) were used to amplify the whole idiomorphic region from both MAT1-1 and MAT1-2 isolates in PCRs utilising Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~40ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) AclSLA2 primer, 2.5µl (10µM) AclAPN2 primer, 0.25µl DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: an initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 98°C, 20 sec at 58°C and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps were performed at a ramp rate of 70°C/min. Resultant PCR products were resolved on 0.8% agarose gels and visualised by ethidium bromide staining (section 2.2.5).

MAT1-2-1 Gene Orientation

To determine the orientation of the *MAT1-2-1* gene relative to genes bordering the MAT-2 idiomorph, the *SLA2-APN2* positional PCR strategy, described in section 2.2.5, was used.

All PCRs to determine the *MAT1-2-1* gene orientation were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~20ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) of respective *MAT1-2-1* gene primer, 2.5µl (10µM) of respective flanking gene primer, 0.25µl DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: an initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 98°C, 20 sec at 58°C and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps were performed at a ramp rate of 70°C/min. Resultant PCR products were resolved on 0.8% agarose gels and visualised by ethidium bromide staining (section 2.2.5).

Table 4.10: Primers used for *A. clavatus MAT* gene screening, determination of idiomorph orientation and RT-PCR analysis.

Primer	Sequence 5' to 3'
AclM1F	CAGTTGTTCGTAGCAGACGGGG
AclM1R	CGGTGGAGTATGCTTTGGCGAGG
AclM2F	ATCAAGGCTCTTCGTGTCATGC
AclM2R	ATGCTTCTTCTTCATATCTTCTGCC
AclSLA2	TGTTGACGACGCTCTTTACGAATTGG
AclAPN2	TGGGATTGCTACTTCAGTCTGCCTA
Acl Actin Forward	TTCCATTGTCGGTCGTCC
Acl Actin Forward 2	ATTGTCGGTCGTCCCCG
Acl Actin Reverse	ATCTTCATCAGGTAATCCGTCAG
Acl Actin Reverse 2	TCTGGGTCATCTTCTCACGG
Acl PpgAF	TCTCCGTCGTCCTCGCTACTC
Acl PpgAR	TTCACTTCCTCAGCGGCTTCAG
Acl STE2F	TCTTCTTCACCTTGGTCCTGG
Acl STE2R	AGTAGCATGTGGTGTTTGGCG
Acl STE3F	AAATCAAGGTTCCTGCGACTG
Acl STE3R	TTCCACGGCTTTGGCTCG
Acl VeAF	ATGCAGCAGCCGGAGCG
Acl VeAR	TGATGTCGGTCTTGTGGAGATC
Acl NsdDF	ATGGCAATGTACCAAGAAGA
Acl NsdDR	TGTGGCAACGACCAGGAGG

4.2.1.3 RT-PCR Analysis

RT-PCR analysis was performed to determine whether gene expression and intron splicing of the putative *MAT*, pheromone precursor, pheromone receptor and transcription factor encoding genes (Tables 4.3 to 4.8) occurred in *A. clavatus*.

Species-specific, gene-specific primers were designed using the sequencing data obtained during this study and from The Broad Institute *A. clavatus* genome sequencing project.

Where possible primer pairs were designed to span putative introns to confirm intron splicing. Samples were grown, RNA extracted and DNase treated using the protocols described in sections 2.2.13 to 2.2.15. RT-PCR was performed using the protocols in sections 2.2.16 and 2.2.17, and resulting PCR products were resolved on 1.5% agarose gels and visualised by ethidium bromide staining (section 2.2.18).

For analysis of the putative *MAT1-1-1* gene primer pair AclM1F and AclM1R was used (Table 4.10). This was predicted to yield a 244bp fragment from unspliced genomic DNA and a 192bp fragment from processed mRNA, after splicing of a 52bp intron. For analysis of the putative *MAT1-2-1* gene primer pair AclM2F and AclM2R was used (Table 4.10). This was predicted to yield a 388bp fragment and a 336bp fragment from unspliced genomic DNA and processed mRNA respectively (splicing of a 52bp intron). For analysis of the putative pheromone precursor gene, *ppgA*, (gene locus ID: ACLA_088140) primer pair AclPpgAF and AclPpgAR was used (Table 4.10). This was predicted to yield a 253bp fragment from unspliced genomic DNA and processed mRNA as no intron was predicted from the sequencing data. For analysis of the putative pheromone receptor gene *ste3* (gene locus ID: ACLA_012620) primer pair AclSTE3F and AclSTE3R (Table 4.10) was used. This was predicted to yield a 715bp fragment and a 574bp fragment from unspliced genomic DNA and processed mRNA respectively (splicing of 76bp and 65bp introns). To analyse the other putative pheromone receptor gene, *ste2* (gene locus ID: ACLA_041790) primer pair AclSTE2F and AclSTE2R (Table 4.10) was used. From unspliced genomic DNA this was predicted to yield a 693bp fragment, whereas from processed mRNA a 630bp fragment was predicted (splicing of a 63bp intron). For analysis of the putative GATA transcription factor encoding gene *nsdD* (gene locus ID: ACLA_041500) primer pair AclNsdF and AclNsdR was used (Table 4.10). These primers were predicted to yield a 269bp fragment from unspliced DNA and, after splicing a 169bp intron, to yield

a 100bp fragment from processed mRNA. A further transcription factor encoding gene, *veA* (gene locus ID: ACLA_022920) was also subjected to RT-PCR analysis. Primer pair AclVeAF and AclVeAR (Table 4.10) was predicted to yield a 207bp fragment from unspliced genomic DNA. After splicing of a 65bp intron, a 142bp fragment was predicted from processed mRNA (Tables 4.10 and 4.13).

RT-PCR analysis of the constiutively expressed actin gene was included as a control. Actin primers were designed using *A. clavatus* actin sequencing data obtained from The Broad Institute genome sequencing project (gene locus ID: ACLA_095800). Primer pair Acl Actin Forward and Acl Actin Reverse was predicted to yield a 555bp fragment from unspliced genomic DNA, and a 480bp fragment from processed mRNA, after splicing of a 75bp intron. Another pair of actin primers was also used, Acl Actin Forward 2 and Acl Actin Reverse 2, which were predicted to yield a 340bp fragment from unspliced genomic DNA and a 265bp fragment from processed mRNA (Tables 4.10 and 4.14).

4.2.2 *Aspergillus terreus*

4.2.2.1 *MAT* Gene Screening

Initially 25 *A. terreus* isolates (Table 4.11) were screened for the presence of a *MAT1-2-1* HMG-domain encoding sequence using *MAT* gene-specific degenerate primers (MAT5-7 and MAT3-5), as described in section 2.2.4. Eight microlitre aliquots of the resultant PCRs were resolved on 2% agarose gels and gels visualised as described in section 2.2.4. Bands of interest were then excised from gels and purified as per section 2.2.8. The gel extracted products were ligated into plasmid pTOPO4, then cloned into *E. coli* and sequenced from extracted plasmid DNA using M13 Forward and M13 Reverse primers as described in sections 2.2.9 to 2.2.12.

Detection of *MAT1-2-1* gene fragments allowed sequencing of the entire putative *MAT1-2-1* gene (see results section 4.3.2.2). This gene sequence was then used to design the specific primer pair AteM2F and AteM2R (Table 4.12) to test for the presence of isolates of MAT1-2 genotype. Meanwhile, putative *MAT1-1-1* gene sequencing data was obtained from The Broad Institute *A. terreus* genome sequencing project (gene locus ID: ATEG_08812.1) and specific *MAT1-1-1* primers AteM1F and AteM1R (Table 4.12) were designed using this sequence data to detect the presence of isolates of MAT1-1 genotype. These primers were then used in a PCR diagnostic to screen 25 worldwide

isolates (Table 4.12) to determine whether isolates were of MAT1-1 or MAT1-2 identity based on the presence of an alpha-domain or HMG-domain encoding gene respectively.

MAT1-1-1 Gene Screening Protocol

PCRs to determine whether an isolate possessed an alpha-domain encoding sequence were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~10ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl of 100% DMSO, 0.5µl (10mM each) dNTPs, 5µl (10µM) AteM1F primer, 5µl (10µM) AteM1R primer, 0.25µl DNA Polymerase and ~7.25µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: an initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 98°C, 20 sec at 53.5°C and 30 sec at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min.

MAT1-2-1 Gene Screening Protocol

PCRs to determine whether an isolate possessed a HMG-domain encoding sequence were performed using FastStart Taq DNA Polymerase (Roche, UK). Each 25µl reaction contained ~10ng genomic DNA, 2.5µl 10X PCR Buffer (containing 20mM $MgCl_2$), 0.5µl (10mM each) dNTPs, 2.5µl (10µM) AteM2F primer, 2.5µl (10µM) AteM2R primer, 0.2µl Taq Polymerase and ~15.8µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: an initial denaturation step at 94°C for 5 min; 35 cycles consisting of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C; followed by a final extension step at 72°C for 5 min, all steps used a ramp rate of 70°C/min.

Resultant PCR products were resolved on a 2% agarose gels and visualised by ethidium bromide staining (section 2.2.4).

Table 4.11: Isolates of *A. terreus* used in *MAT* gene screening. Isolates were kindly provided by Dr A. Balajee, Centers for Disease Control and Prevention, USA.

Culture ID Code	Environmental or Clinical isolate	Source of Isolate
CBS 106.25	Environmental	Unknown
CBS 469.81	Clinical	Thailand
CBS 503.65	Environmental	Texas
CBS 118.27	Environmental	Unknown
CBS 134.60	Environmental	Unknown
UAB 1	Clinical	Alabama
UAB 17	Clinical	Alabama

UAB 20	Clinical	Alabama
UAB 23	Clinical	Alabama
UAB 26	Clinical	Alabama
IBT 6450	Environmental	Unknown
IBT 12713	Environmental	New Mexico
IBT 13121	Environmental	Japan
IBT 14590	Environmental	Unknown
IBT 15722	Environmental	Unknown
IBT 16744	Environmental	Galapagos Islands
IBT 16745	Environmental	Unknown
IBT 21125	Environmental	Unknown
IBT 22556	Environmental	Unknown
IBT 23544	Environmental	Unknown
IBT 26384	Environmental	Unknown
IBT 26915	Environmental	Panama
MD 6	Clinical	Unknown
MD 11	Clinical	Texas
MD 29	Clinical	Texas

4.2.2.2 Idiomorph Orientation

Species-specific *SLA2* and *APN2* primers [AteSLA2 and AteAPN2 (Table 4.12)] were designed using sequence data obtained from The Broad Institute *A. terreus* genome sequencing project. The putative *SLA2* gene was found at gene locus ID: ATEG_08813.1 and the putative *APN2* gene was found at gene locus ID: ATEG_08811.1.

MAT1-2-1 Gene Orientation

To determine the orientation of the *MAT1-2-1* gene relative to the genes bordering the MAT-2 idiomorph, the *SLA2-APN2* positional PCR strategy described in section 2.2.5 was used.

All PCRs to determine *MAT1-2-1* gene orientation were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~20ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) of respective *MAT1-2-1* gene primer, 2.5µl (10µM) of respective flanking gene primer, 0.25µl DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: an initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 98°C, 20 sec at an appropriate annealing temperature and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min.

Resultant PCR products were resolved on 0.8% agarose gels and visualised by ethidium bromide staining (section 2.2.5).

Different conditions were used for specific PCRs. Primer pair AteM2F and AteAPN2 was used at annealing temperature of 56°C, whereas the primer pair AteM2R and AteSLA2 was used at an annealing temperature of 53°C.

Table 4.12: Primers used for *A. terreus MAT* gene screening, determination of idiomorph orientation and RT-PCR analysis.

Primer	Sequence 5' to 3'
AteM1F	GCGAGGCAGACACATTCAGGAT
AteM1R	CGAGGATGCCAATAAAACCAGC
AteM2F	TCTATCGCCAGCACCATCATCC
AteM2R	CTTGTTGTGGTGGTGGTCGTTCT
AteSLA2	TGAGAAGGACGAGGAGATTGAAGTG
AteAPN2	AAGAAGCGAACCTATTCCTCCCGTC
Ate Actin Forward	ATTGTCGGTCGTCCCCG
Ate Actin Reverse	TCTGGGTCATCTTCTCACGG
Ate PpgAF	TCATTACCCTGGTTCTTGCC
Ate PpgAR	TTGACCTTGAGACAGCCCTGG
Ate STE2F	ATCTGCCAGCTCATCTACTTCACG
Ate STE2R	TGACGAGGGAGAAGATGTTGG
Ate STE3F	TTTCCTCTCCTTTGTTATGCTCC
Ate STE3R	AGATCAGGTTCAATGGAGTCGAC
Ate VeAF	AACACTCCGTCAGTCGCATCAC
Ate VeAR	TCTTGTGAACGTCGTCGTTGG
Ate NsdDF	ATGGCGATGTACCAAGAAGA
Ate NsdDR	TGTGGCAACGGCCAGGAGG

4.2.2.3 RT-PCR Analysis

RT-PCR analysis was performed to determine whether gene expression of the putative *MAT*, pheromone precursor, pheromone receptor and transcription factor encoding genes (Tables 4.3 to 4.8) occurred in *A. terreus*. Species-specific and gene-specific primers were designed using the sequence data obtained during this study or from The Broad Institute *A. terreus* genome sequencing project

Where possible primer pairs were designed to span putative introns to confirm intron splicing. Samples were grown, RNA extracted and DNase treated using the protocols described in sections 2.2.13 and 2.2.15. RT-PCR was performed using the protocols described in sections 2.2.16 and 2.2.17, and bands were resolved on 1.5% agarose gels and visualised by ethidium bromide staining (section 2.2.18).

To analyse the putative *MAT1-1-1* gene primer pair AteM1F and AteM1R was used (Table 4.12). These were predicted to yield a 314bp fragment from unspliced genomic DNA and, after splicing of a 54bp intron, predicted to yield a 260bp fragment from processed mRNA. To analyse the putative *MAT1-2-1* gene primer pair AteM2F and AteM2R was used (Table 4.12). From unspliced genomic DNA this was predicted to yield a 513bp fragment. After splicing a 52bp intron, processed mRNA was predicted to produce a 461bp fragment. To analyse the putative pheromone precursor *ppgA* (gene locus ID: ATEG_07407.1), primer pair AtePpgAF and AtePpgAR was used (Table 4.12). A 232bp fragment was predicted to be amplified from both genomic DNA and mRNA as no intron was predicted within the putative gene. To analyse the putative pheromone receptor encoding gene *ste3* (gene locus ID: ATEG_08338.1) primer pair AteSTE3F and AteSTE3R was used (Table 4.12). This was predicted to yield a 605bp fragment in unspliced genomic DNA and a 557bp fragment in processed mRNA (splicing of a 48bp intron). To analyse the other putative pheromone receptor *ste2* (gene locus ID: ATEG_03500.1) primer pair AteSTE2F and AteSTE2R was used (Table 4.12). This was predicted to yield a 617bp fragment from unspliced genomic DNA and a 559bp fragment from processed mRNA (splicing of a 58bp intron). To analyse the putative GATA transcription factor encoding gene, *nsdD* (gene locus ID: ATEG_04043.1) primer pair AteNsdF and AteNsdR was used (Table 4.12). This was predicted to yield a 243bp fragment from unspliced genomic DNA and, after splicing a 143bp intron, predicted to yield a 100bp fragment from processed mRNA. A further transcription factor encoding gene, *veA* (gene locus ID: ATEG_00439.1) was also subjected to RT-PCR analysis. Primer pair AteVeAF and ATEVeAR was used (Table 4.12) and predicted to yield a 249bp fragment in unspliced genomic DNA. After splicing a 57bp intron, a 192bp fragment was predicted from processed mRNA (Tables 4.12 and 4.14).

RT-PCR analysis of constitutively expressed actin gene was included as a control. Actin primers were designed using *A. terreus* actin sequencing data obtained from The Broad Institute genome sequencing project (gene locus ID: ATEG_06973.1). Primer pair Ate Actin Forward and Ate Actin Reverse was used and predicted to yield a 331bp fragment from unspliced genomic DNA and a 265bp fragment from processed mRNA (splicing of a 66bp intron).

4.2.3 *Aspergillus flavus*

Aspergillus flavus isolates NRRL and ATCC were obtained from a culture collection at the School of Biology, The University of Nottingham. These isolates were assayed for the presence of *MAT1-1-1* and *MAT1-2-1* genes using the degenerate primers MAT5-6 with MAT3-4 and MAT5-7 with MAT3-5, respectively (section 2.2.4). Analysis of isolates resulted in a *MAT1-1-1* gene fragment being amplified from isolate NRRL and a *MAT1-2-1* gene fragment amplified from isolate ATCC.

Table 4.13: Primers used for *A. flavus* RT-PCR analysis.

Primer	Sequence 5' to 3'
Afl Actin Forward	ATTGTCGGTCGTCCCCG
Afl Actin Reverse	TCTGGGTCATCTTCTCACGG
Afl PpgAF	TCATTTCTGTCGTCGTTGCCG
Afl PpgAR	AACGTCTGGCTTCTTCAACAGC
Afl STE2F	ATCTGCCAGCTCATCTACTTCACG
Afl STE2R	TTCCACAAGGCTCCACGGGACG
Afl STE3F	TTGTCCCGAGCAAGCAGCAACG
Afl STE3R	ATGAGCCATACATCCTAGAAGCGTC
Afl VeAF	ATGCAGCAGCCGGAGCG
Afl VeAR	GTATGTCGGTCTTGTGGAGATC
Afl NsdDF	ATGGCAATGTACCAAGAAGA
Afl NsdDR	TGTGGCAACGACCAGGAGG

4.2.3.1 RT-PCR

RT-PCR analysis was performed to determine whether gene expression and intron splicing of the putative *MAT*, pheromone precursor, pheromone receptor and transcription factor encoding genes (Tables 4.3 to 4.8) occurred in *A. flavus*. Species-specific, gene-specific primers were designed using the sequencing data obtained from The Broad Institute *A. flavus* genome sequencing project.

Where possible primer pairs were designed to span putative introns to confirm intron splicing. Samples were grown, RNA extracted and DNase treated using the protocols described in sections 2.2.13 to 2.2.15. RT-PCR was performed using the protocols in sections 2.2.16 and 2.2.17 and bands were resolved on 1.5% agarose gels and visualised by ethidium bromide staining (section 2.2.18).

For analysis of the putative pheromone precursor gene, *ppgA*, (gene locus ID: AFL2G_02931.2) primer pair AflPpgAF and AflPpgAR was used (Table 4.13). This was

predicted to yield a 258bp fragment from genomic unspliced DNA and mRNA as no intron was predicted from the sequencing data. For analysis of the putative pheromone receptor gene *ste3* (gene locus ID: AFL2G_06286.2) primer pair AflSTE3F and AflSTE3R was used (Table 4.13). This was predicted to yield a 631bp fragment and a 576bp fragment from unspliced genomic DNA and processed mRNA, respectively (splicing of a 55bp intron). For analysis of the other putative pheromone receptor gene, *ste2* (gene locus ID: AFL2G_06217.2) primer pair AflSTE2F and AflSTE2R was used (Table 4.13). From unspliced genomic DNA these primers were predicted to yield a 709bp fragment, whereas for processed mRNA a 652bp fragment was predicted (splicing of a 56bp intron). For analysis of the putative GATA transcription factor encoding gene *nsdD* (gene locus ID: AFL2G_03635.2) primer pair AflNsdF and AflNsdR was used (Table 4.13). These primers were predicted to yield a 238bp fragment from unspliced genomic DNA and, after splicing a 138bp intron, predicted to yield a 100bp fragment from processed mRNA. A further transcription factor encoding gene, *veA* (gene locus ID: AFL2G_07468.2) was also subjected to RT-PCR analysis. Primer pair AfVeAF and AfVeAR was used and predicted to yield a 206bp fragment from unspliced genomic DNA. After splicing a 66bp intron, a 140bp fragment was predicted from processed mRNA (Tables 4.13 and 4.17).

RT-PCR analysis of the constitutively expressed actin gene was included as a control. Actin primers were designed using *A. flavus* actin sequencing data obtained from The Broad Institute genome sequencing project (gene locus ID: AFL2G_05717.2). Primer pair Afl Actin Forward and Afl Actin Reverse was predicted yield a 328bp fragment from unspliced genomic DNA, and a 265bp fragment from processed mRNA (Tables 4.13 and 4.17).

4.2.4 Data-Mining of Genome Sequences for Sex-Related Genes

Previous studies by Galagan *et al.* (2005) and Pel *et al.* (2007) have identified over 60 genes known to be involved with sexual reproduction in ascomycete fungi. These genes, via BLAST analysis, were used to interrogate the genome sequences of *A. terreus, A. clavatus* and *A. flavus* available from The Broad Institute (www.broad.mit.edu/node/304). E-values were scored for the homologous genes (where available) from *E. nidulans* and *N. fumigata*. E-value cut off was 0.001.

4.2.5 Attempted Induction of Sexual Cycle

Attempts were made to induce the sexual cycle of *A. clavatus* isolates and *A. terreus* isolates (Table 4.14) under conditions known to induce sex in *N. fumigata* (O'Gorman *et al.* 2009) and described in section 2.2.19. All crosses were set up by mixing and plug plating configurations, as described in section 2.2.13, Figure 2.1 (B).

Table 4.14: Isolates of *A. clavatus* and *A. terreus* used in sexual crosses.

Species	MAT1-1 Genotype Isolates	MAT1-2 Genotype Isolates
A. clavatus	NRRL A-25278	NRRL A-22620
	NRRL A-25278	NRRL A-803
	NRRL A-25278	NRRL 4
	NRRL A-25278	NRRL A-24204
	NRRL A-251	NRRL A-22620
	NRRL A-251	NRRL A-803
	NRRL A-251	NRRL 4
	NRRL A-251	NRRL A-24204
	NRRL A-3880	NRRL A-22620
	NRRL A-3880	NRRL A-803
	NRRL A-3880	NRRL 4
	NRRL A-3880	NRRL A-24204
	NRRL A-3072	NRRL A-22620
	NRRL A-3072	NRRL A-803
	NRRL A-3072	NRRL 4
	NRRL A-3072	NRRL A-24204
A. terreus	MD 29	IBT 6450
	MD 29	MD 11
	IBT 16744	IBT 6450
	IBT 16744	MD 11

4.3 Results

4.3.1 *Aspergillus clavatus*

4.3.1.1 *MAT* Gene Screening

A 270bp putative *MAT1-2-1* gene fragment was successfully amplified from certain isolates of *A.* clavatus using degenerate primers MAT5-7 and MAT3-5. This allowed the isolation of the entire *MAT1-2-1* gene sequence (see section 4.3.1.2) and design of a PCR diagnostic to determine the mating type of *A. clavatus* (section 4.2.1.1).

The *MAT* PCR diagnostic using primer pairs AclM1F with AclM1R, or AclM2F with AclM2R successfully amplified putative *MAT* gene fragments from all 20 worldwide isolates of *A. clavatus*. Primers were designed in such a way that MAT1-1 genotypes were predicted to produce a 244bp fragment, whereas MAT1-2 genotypes were predicted to produce a 388bp product (Figure 4.7).

Of the 20 worldwide isolates screened, 10 isolates produced a *MAT1-1-1* fragment only, and 10 isolates produced a *MAT1-2-1* fragment only (Table 4.15), giving a 1:1 ratio of worldwide *MAT* gene distribution. To ensure that all of these were independent isolates, (i.e. not clonal) RAPD analysis was performed using the primers listed in Table 2.2 and the protocol described in 2.2.6. Results showed all isolates to produce distinct RAPD-PCR fingerprints, and therefore none of the isolates were clonal (data not shown).

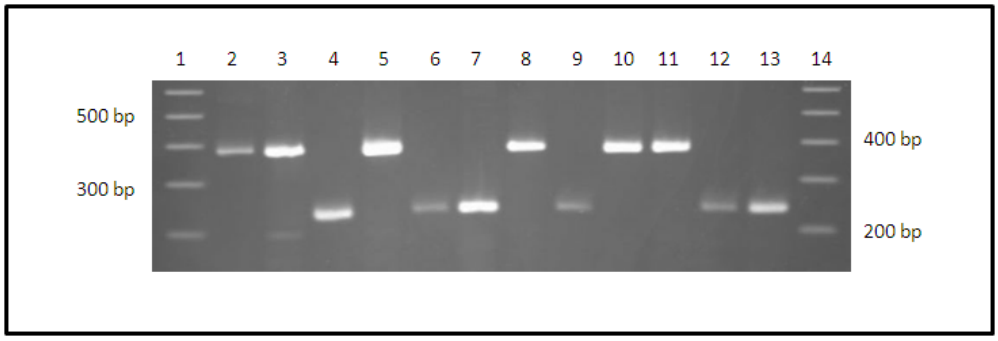

Figure 4.7: 2% agarose gel showing representative results of amplification of *MAT* gene fragments from various *A. clavatus* isolates. Lanes 1 and 14: 100bp ladder. Lane 2: NRRL 4. Lane 3: NRRL A-24204. Lane 4: NRRL A-22359. Lane 5: NRRL A-22620. Lane 6: NRRL A-2905. Lane 7: NRRL 5811. Lane 8: NRRL A-803. Lane 9: NRRL A-251. Lane 10: NRRL A-14611. Lane 11: NRRL 1823. Lane 12:NRRL A-3880. Lane 13: NRRL A-3072.

Table 4.15: MAT genotype of isolates of *A. clavatus* as determined by *MAT* PCR diagnostic test.

Culture ID Code	MAT Genotype[a]
NRRL 4	MAT1-2
NRRL 5811	MAT1-1
NRRL 1823	MAT1-2
NRRL A-251	MAT1-1
NRRL A-404	MAT1-2
NRRL A-803	MAT1-2
NRRL A-2905	MAT1-1
NRRL A-3072	MAT1-1
NRRL A-3880	MAT1-1
NRRL A-7813	MAT1-2
NRRL A-8053	MAT1-2
NRRL A-14401	MAT1-2
NRRL A-14611	MAT1-2
NRRL A-22162	MAT1-1
NRRL A-22359	MAT1-1

NRRL A-22620	MAT1-2
NRRL A-24204	MAT1-2
NRRL A-25278	MAT1-1
NRRL A-26725	MAT1-1
NRRL A-28596	MAT1-1

[a]MAT genotype determined by the presence of a *MAT1-1-1* alpha-domain (MAT1-1), or a *MAT1-2-1* HMG-domain (MAT1-2) encoding genes.

4.3.1.2 *MAT1-2-1* Gene Isolation and Sequence Analysis

Using the *SLA2-APN2* positional PCR strategy (section 2.2.5), it was possible to amplify the entire MAT-2 idiomorph region from *A. clavatus* isolate NRRL A24204. The AclSLA2 and AclAPN2 primer pair produced a PCR fragment of approximately 8kb in length. The idiomorph was then partially sequenced by chromosome walking outwards from the *MAT1-2-1* gene fragment to determine whether a complete putative *MAT1-2-1* gene was present, including a conserved HMG-domain encoding sequence, necessary for functionality. In total a 3580bp MAT1-2-1 region was sequenced from isolate NRRL A-24204 containing a putative a 1037bp open reading frame, which included two putative introns, and was predicted to encode a 322 amino acid MAT1-2-1 protein (Figure 4.8).

PSORT II (http://psort.nibb.ac.jp/) and TFSITESCAN (http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl) programs were used to interrogate resultant sequences for nuclear-targeting and promoter-region motifs. The putative ATG translation start site of this protein was consistent with the requirements of the Kozak rule (Kozak 1986). Analysis of the putative MAT1-2-1 protein revealed three nuclear targeting signals (KKKH at position 183, RKRR at position 203 and PSERKRR at position 200), and a transcriptional activator TATA box sequence was also found in the promoter region 91bp upstream of the start site. Meanwhile, analysis of the putative MAT1-1-1 protein (identified from the Broad Institute *A. clavatus* genome sequencing project) revealed one nuclear targeting signal (RKRP at position 86) and a transcriptional activator TATA box sequence 4bp upstream of the start site. Possession of nuclear targeting sites is consistent with a role of the *MAT* genes as transcriptional activators.

However, no *MAT1-2-4* gene was found in the sequenced region adjoining the *MAT1-2-1* gene. Also, it was not possible to obtain any PCR product from amplifications with primers MAT124F and MAT124R (section 2.2.4). Therefore, it appears that there is no *MAT1-2-4* gene in the MAT-2 idiomorph of *A. clavatus* isolate NRRL A-24204.

```
1     caatatgggt aaccaataga gatgctgaca gtacgcaagt ccagctcaat
51    ggcggtatat ggcacgcagt tgtatcaatc caataaccta tcgaatcgtc
101   cagaaatgct cgacagtgat catgctatac tcacagaatc tcagtgcatc
151   tgagtgggag ccaatctgat gtataagaca agctttcgag atcatgagtc
201   taacgcgcct ctcaatggct gcgtaacata tcttggcatg aacacgatat
251   gcatcgccac gcggatcagt tctccagaag ttgcacatgt tgacgacaga
301   tttactagct atgtaagcaa cattgaaatg cagatatgaa gagatactta
351   cgtcatgtaa tactgctgaa gacctgctct gaagatgtag tggtagccat
401   aacgttgagt gcagattgca agatcttcat ttgccctctt atccattgca
451   agaagtgtct ggtgaaaccg aagtcttgtg ttgtcctcca taagtccttc
501   gatattgggg tcgaggatac tgatgagttc aataggatgc ccttcaaaat
551   ccgcagtagt cgggatgata ccaaaagtag ccgcgaccac atggacgttt
601   cgaccgaaac gacgaacagg aggtgctggg tatctgggcc cattcctaat
651   tcgccgaaaa gcagggacta tgagttcgtt tctggtcaaa gcagtgaact
701   gagcaataag tctatggacg tttccatgtc ttctgaagga agtagtaatg
751   tagatagctt gctgcctcaa gccaagggga gaattgggat tgcgagcgac
801   tgcaccaaga taccacagaa gagcatcttg ataagcttcc atttcgaacg
851   cctgctcttt gcgagctacg cgcagtacga ttgagaagtt ttggatgcgg
901   attatgcttg aacgacgagg atcaacagtg gatgaaatgc tcaagacagg
951   agatttgagg agatgtcgaa gagagagatt cgagaaaaac agatgcgaga
1001  tttctgtgtt tatttacttc acgagaggca agactcaggt gaccagtggg
1051  tggaggaaaa agtgaagaag ttgtctccag acattcacct gctcgcccta
1101  gtcaacaaag aaacctaccg acccctgtgg ttgacacagc ttattggcca
1151  tgtcttacca aatccaatac cctttctctc atccaggatg ctgtcttcaa
1201  tcactgtcaa tgagatacct cctctttcga tttgctcctt attcttccta
1251  tatctgaagt tgacggggtg atttggaaag cctcatcctt cgagatccta
                                              | M||A||T||L
1301  tcagatctca aacctccttg tctacaattc gaaattgaca tggctacact
      ||P||I||A| |M||K||S|| A||A||E||S ||T||D||T| |I||T||E||
1351  acccatcgca atgaagtcgg cagcggaatc cacagacact atcactgagc
      L||L||W||Q ||D||A||L| |R||H||L|| K||S||T||N ||N||E||V|
1401  ttctgtggca ggatgctttg cgtcacctca agtctaccaa caacgaggtc
      |L||L||P|| I||N||V||T ||D||M||I| |G||Q||G|| N||V||D||K
1451  ctcttgccca ttaatgtcac agacatgatt gggcagggca atgtcgacaa
      ||I||K||T| |R||L||S
1501  gataaaaact cgccttgggt aagtgtccga gtcgaagttc ttgatgagct
                              ||A||L| |I||G||A|| P||V||V||A
1551  cgactgctaa cacaggatgc tagtgcgctt attggtgccc ctgttgtggc
      ||F||V||D| |E||S||I|| K||A||L||R ||V||M||R| |T||P||A||
1601  atttgttgat gagtcgatca aggctcttcg tgtcatgcgc actccagcat
      F||S||G||T ||A||I||S| |V||A||S|| H||G||A||T ||F||E||E|
1651  tttcaggaac agccatatcc gtcgcatccc acggggcaac ttttgaggag
      |D||V||V|| T||E||L||K ||S||V||K| |S||R||S|| K||R||T||G
1701  gatgtggtta ctgaattgaa atccgtcaaa tcgcgctcaa agcgcacagg
      ||P||V||K| |P||P||K|| V||P||R||P ||P||N||A| |F||I||L||
1751  acccgtgaaa cctccaaagg ttccccgtcc tccaaatgca ttcatcctat
      Y||R||Q||Q ||H||H||P| |K||I||K|| E||T||Y||P ||N||F||S|
1801  accgacaaca acatcatccc aagatcaagg agacgtatcc gaacttttct
      |N||N||D|| I||
1851  aataatgata tctgtaagtt gcgtttagtc atcataaata tgctctgctt
                        C||V| |M||L||G|| K||Q||W||K ||S||E||P|
1901  agctgacacg attagctgtc atgcttggaa agcagtggaa atcagaacct
      |E||E||I|| K||A||Q||F ||R||S||L| |A||E||D|| M||K||K||K
1951  gaagagatca aagctcaatt ccgcagtctg gcagaagata tgaagaagaa
```

```
     ||H||A||E| |E||H||P|| D||Y||H||Y ||T||P||R| |K||P||S||
2001 gcatgctgaa gaacacccag actaccatta tacccccga aaaccttctg
     E||R||K||R ||R||A||P| |S||R||Q|| F||S||R||S ||T||K||S|
2051 aaagaaaacg tcgcgctcca tcgcgtcaat tttccagaag caccaagtct
     |V||G||V|| L||N||A||S ||T||P||T| |N||D||I|| P||G||S||F
2101 gttggcgtgc tgaatgcgtc gactccaacg aacgacattc ctggttcctt
     ||T||S||A| |M||G||S|| G||M||T||M ||G||D||T| |E||Y||N||
2151 cacatcagcc atggggtcag gcatgacaat gggcgacacc gaatacaatg
     G||G||H||D ||D||N||T| |D||M||N|| I||I||M||T ||S||H||G|
2201 gaggtcatga cgacaataca gatatgaata ttatcatgac ctctcatggc
     |V||P||E|| T||Q||Q||F ||Q||F||E| |P||N||T|| F||D||F||I
2251 gtaccagaaa cacagcagtt ccagttcgaa ccaaacacat tcgacttcat
     ||Q||Q||V| |Q||N||D|| Y||N||R||A ||A||L||F| |Q||Q||L||
2301 tcaacaagtg caaaacgatt acaatagagc cgccctcttc cagcagctga
     N||I||A||E ||G||P||F| |G||E||S|| F||E||F||S ||D||F||I|
2351 acattgctga gggccctttt ggggagtcct ttgagttctc cgactttatt
     |T||D||C|| C|
2401 actgactgct tcgatggccg gttcatccca gcaataccca agctttttt
2451 ttttttttt cctgctttta tttactgttc ttttgttgtt gttatttgag
2501 caagcatccc gtgtacaatt cgacatctca accctccttt aaagggctca
2551 ccacggcttc tctcttcttg ctgtaccata gcagcgtgcc caatttcttt
2601 acttgcttca acttagggat cacaaaggct ttggcacccg tgaagcatgt
2651 gtccccagga catcgacttt gattcctggc attgcctcga aagacccaaa
2701 ttctaaatgc tttacaagct tccacatctc atttaaacaa atacaaataa
2751 tgaaagtaaa tatggggcgt cgtgctaaag tcttgcttcg cacatactcg
2801 gtagtccagc aaacctcaag gtcgagctaa agttgagcgc gtttgcattc
2851 ggacctcctg cctctgcaag gttactcgac ggattctgca ttcccgttag
2901 ttccaatatc aacgcctctt atgtgtcgtt tgacgtggat tgaggggccc
2951 ttgcatgtgg cacctgctca ggaaccgttc ggctgtcata ccaatgctcc
3001 tatccgcgac atgcatcctt caggggcctt ggtatggctg agatgtgtta
3051 ctccagtgga tgctggtact gtagatccat tgattgaaga caaggttgcg
3101 aatggttcct atctctgtga gaaatattta tggaatttgg tctgctgacg
3151 gacgcttcca tctgatagct accttgtgtt gtgtgaatct atcgcggaaa
3201 aggctcaacg cctcgaacat tcgtccccaa taagatcaaa ccacattcct
3251 tactaaggga ttctaggaac aggaagagaa agggtatcgt gcacaattga
3301 aaaagaaaaa gaaaaaaacc gaagtctcaa caaacacata tctcacgaag
3351 cagcaacgat tccagtcgct tgcccagatg aatgtagaca tcgccactgc
3401 gtccccgttt gcttgtttac actaggacac tagagacgac gacagatcat
3451 aaagctcttc cgcagttgat accagctttc ttgatgtcag ctgaatccag
3501 ttcatgatgt ccctcgcacc cttggactga cgttcgtaaa agctttgaca
3551 gtcatctaat ggacaatgga tccatgttcc
```

Figure 4.8: DNA sequence of MAT1-2-1 region of *A. clavatus* isolate NRRL A-24204. The putative encoded amino acid sequence is indicated above the relevant base pair sequence. Primer AclM2F is highlighted red (forward), primer AclM2R is highlighted green (reverse) and putative introns are highlighted in yellow. Also included is 1339p upstream of the putative ATG start site and 1168bp downstream of the finish site.

4.3.1.3 Idiomorph Orientation

A variety of PCR amplifications were attempted in order to determine the orientation of the putative *MAT1-2-1* gene relative to the flanking *SLA2* and *APN2* genes (see section 2.2.5 for protocols).

Amplicons of approximately 3.2kb in size were produced by primers AclM2F and AclAPN2 and 5.5kb by primers AclM2R and AclSLA2 (Figure 4.9). This allowed the *MAT1-2-1* gene orientation to be deduced as shown in Figure 4.10. The *MAT1-1-1* gene orientation was already known from genome sequencing data from The Broad Insitute *A. clavatus* genome sequencing project. The species has a heterothallic-like MAT idiomorph arrangement, with the *MAT1-1-1* gene orientation in the same direction as, and downstream of, the *APN2* gene whereas the *MAT1-2-1* gene is orientated in the same direction as, and downstream of, the *SLA2* gene (Figure 4.10).

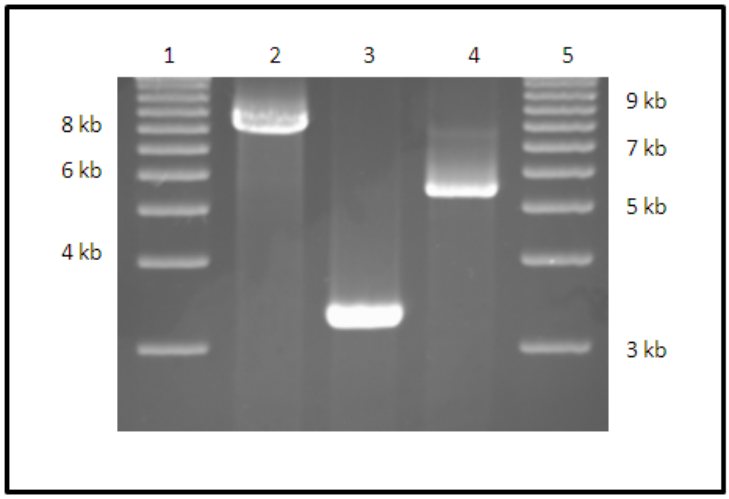

Figure 4.9: 0.8% agarose gel showing results of PCR amplification to determine *MAT1-2-1* gene orientation within the MAT-2 idiomorph of *A. clavatus* isolate NRRL A-24204. Lanes 1 and 5: 1kb ladder. Lane 2: Amplicon produced by primers AclSLA2 and AclAPN2. Lane 3: Amplicon produced by primers AclM2F and AclAPN2. Lane 4: Amplicon produced by primers AclM2R and AclSLA2.

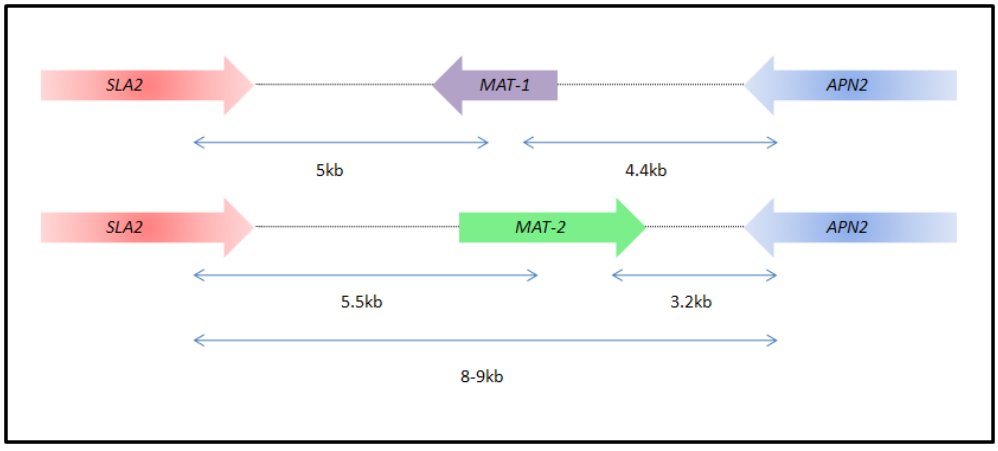

Figure 4.10: Schematic diagram showing the MAT-1 idiomorph (top) and the MAT-2 idiomorph (bottom) of *A. clavatus* (distances in kb).

4.3.1.4 RT-PCR Analysis

RT-PCR analysis provided clear evidence of the expression of the *MAT1-1-1*, *MAT1-2-1*, *ppgA*, *ste2*, *veA* and *nsdD* encoding genes of *A. clavatus*, together with the actin control gene. Amplicons of a smaller size, relative to genomic DNA controls, were produced by RT-PCR corresponding to the predicted sizes of mRNA transcripts allowing for splicing of a 52bp intron from *MAT1-1-1*, 52bp intron from *MAT1-2-1*, 63bp intron from *ste2*, 65bp intron from *veA*, and 169bp intron from *nsdD* (Figures 4.12 to 4.16). The only exception was the *ppgA* gene, for which no intron was predicted (Table 4.16)

Intriguingly, expression of the *ste2, ste3* and *ppgA* genes was detected in both isolates of NRRL A-24204 (MAT1-2 genotype) (Figure 4.16) and NRRL A22359 (MAT1-1 genotype) (data not shown) i.e. there was no apparent mating-type specific expression of the pheromone precursor and receptor genes.

One other unexpected result was that the pheromone receptor gene *ste3* did not produce the mRNA fragment size that was predicted from genome sequencing (gene locus ID: ACLA_012620). The genome annotation predicted three introns to be present. However, sequencing of the RT-PCR products showed that only two introns are spliced, producing a fragment 651bp in length (Figure 4.11). According to the genome annotation, if this third intron is not spliced the protein has premature stop codons. However, from sequencing of the RT-PCR products there are 17 base changes that convert these supposed stop codons into coding amino acids. If this region of the genome sequence is replaced with data from the RT-PCR sequencing a 469 amino acid protein is produced. There seems to be an apparent sequencing or annotation error in the Broad Institute genome sequencing data.

```
       |I||K||V ||P||A||T| |V||L||A|| F||V||M||L ||L||T||I|
1      aaatcaaggt tcctgcgact gttcttgcat tcgtcatgct cttgaccatt
       |L||P||I|| Q||A||F||V ||V||Y||Q| |N||I||L|| L||S||L||P
51     ctcccaatcc aggcgttcgt cgtctatcag aacattctat tgtcattgcc
       ||P||W||H| |S||T||S|| W||S||S||L ||H||G||P| |E||W||N||
101    gtggcactct tattcatgga gttccctcca tggaccagag tggaatacta
       T||I||I||K ||V||P||S| |N||G||Q|| A||F||F||D ||R||W||I|
151    tcatcaaagt tccgtcgaat ggccaggcat tctttgaccg ctggattcca
       |P||I||A|| A||G||Y||V ||L||F||I| |F||F||G|| C||C||R||D
201    atcgctgcgg gctatgtgct ctttatcttc tttggatgtt gcagggatgc
       ||A||F||R| |L||Y||G|| S||I||L||C ||F||L||G| |L||G||G||
251    tttccggctg tacggatcta ttctgtgctt tcttggccta ggcggacgtt
       R||F||A||A ||L||R||S| |S||P||H|| A||T||G||S ||S||G||G|
301    ttgctgctct tcgctcctcg ccgcacgcaa cgggatctag tggtggagga
       |G||S||T|| T||S||R||R ||L||L||F| |S||K||Q|| W||P||S||S
```

```
351   tcgacaacga gccgtgcaag gct**c**ctcttc tccaaa**ca**t ggccatcgtc
      ||V||R||T| |F||D||D|| S||A||P||S ||R||S||D| |S||Q||N||
401   a**gtgag**aaca ttcgacgact ctgcgcctag tagatcag**a**c ag**t**cagaact
      Y||A||D||I ||E||K||G| |I||I||F|| P||R||N||N ||Q||G||S|
451   acgcagatat cgaaaagggt a**tt**atcttcc cacgaaacaa ccaaggctca
      |K||R||S|| F||W||L||S ||L||R||W| |T||F||L|| G||R||R||F
501   aaaaggtcat tctggctctc acttcgatgg actttcttgg gccgcagatt
      ||H||S||A| |R||E||R|| A||P||S||P ||P||K||L| |S||V||P||
551   ccattccgct cgcgagag**a**g caccctcgcc tcccaag**t**tg tct**g**tcccag
      A||T||T||V ||S||T||S| |A||W||A|| G||T||S||Q ||S||R||G
601   cgacaacggt atctacgagc gcctgggccg gcacgagcca aagccgtgga
651   a
```

Figure 4.11: DNA seqeunce of RT-PCR fragment of the pheromone receptor gene *ste3* from *A. clavatus* NRRL A-22359. The putative encoded amino acid sequence is indicated above the relevant base pair sequence. Primer AclSTE3F is highlighted red (forward), primer AclSTE3R is highlighted in grey (reverse) and the intron annotation in The Broad Institute genome sequence but not spliced in the RT-PCR product is highlighted in yellow. The nucleotide differences between NRRL A-22359 and A-24204 compared with the genome sequence obtained from isolate NRRL 1 are highlighted in emboldened font.

Table 4.16: Primers used for *A. clavatus* RT-PCR analyses and predicted fragment lengths from genomic DNA and processed mRNA.

Forward primer	Reverse primer	Genomic DNA fragment length (bp)	Processed mRNA fragment length (bp)
AclM1F	AclM1R	244	192
AclM2F	AclM2R	388	336
Acl Actin Forward	Acl Actin Reverse	555	480
Acl Actin Forward 2	Acl Actin Reverse 2	340	265
Acl PpgAF	Acl PpgAR	253	253
Acl STE2F	Acl STE2R	693	630
Acl STE3F	Acl STE3R	715	574
Acl VeAF	Acl VeAR	207	142
Acl NsdDF	Acl NsdDR	269	100

MAT1-1-1

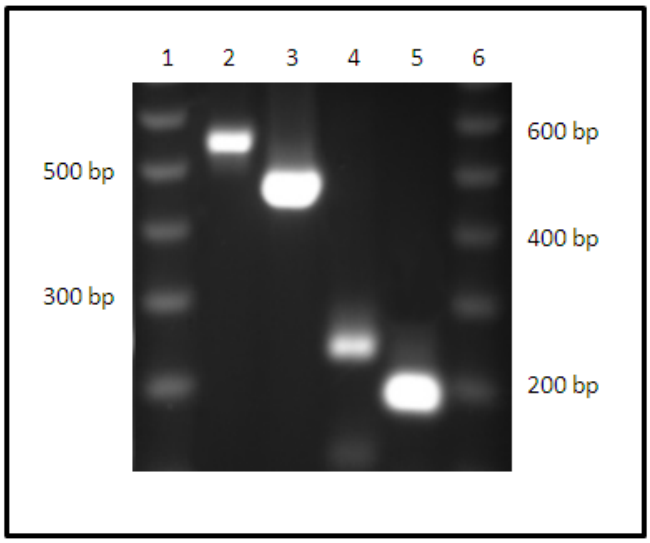

Figure 4.12: 1.5% agarose gel showing results of *MAT1-1-1* RT-PCR gene analysis of *A. clavatus* isolate NRRL A-22359. Lane 1 and 6: 100bp ladder. Lane 2: Genomic DNA Actin control. Lane 3: RNA Actin control. Lanes 4 and 5: Amplifications with *MAT1-1-1* specific primers of genomic DNA and RNA extracts, respectively.

MAT1-2-1

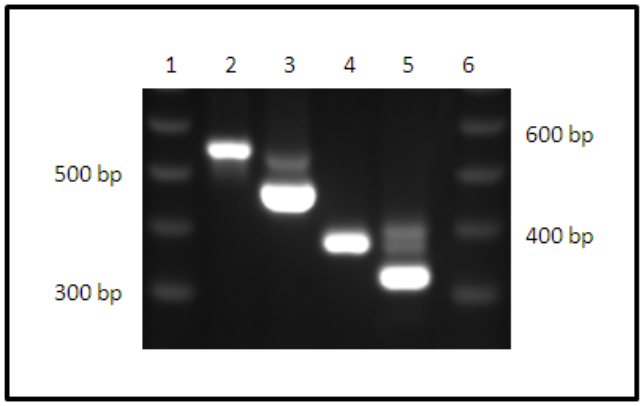

Figure 4.13: 1.5% agarose gel showing results of *MAT1-2-1* RT-PCR gene analysis of *A. clavatus* isolate NRRL A-24204. Lanes 1 and 6: 100bp ladder. Lane 2: Genomic DNA Actin Control. Lane 3: RNA Actin control. Lanes 4 and 5: Amplifications with *MAT1-2-1* specific primers of genomic DNA and RNA extracts, respectively.

Pheromone Precursor and Receptors (*ppgA, ste3* and *ste2*)

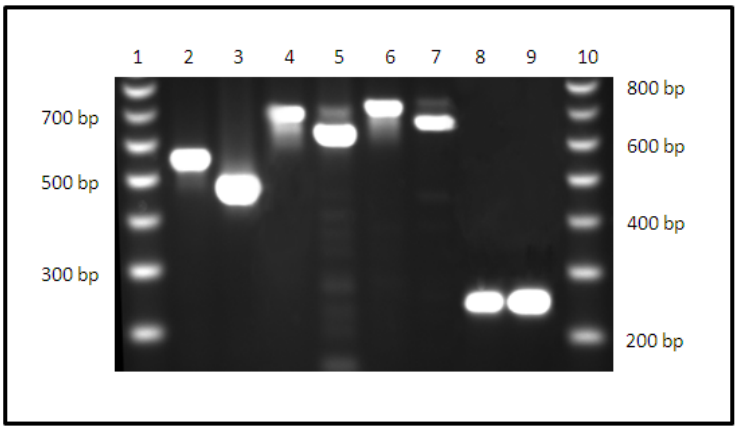

Figure 4.14: 1.5% agarose gel showing results of pheromone gene RT-PCR analysis of *A. clavatus* isolate NRRL A-24204. Lanes 1 and 10: 100bp ladder. Lane 2: Genomic DNA Actin control. Lane 3: RNA Actin control. Lanes 4 and 5: Amplifications with *ste2* specific primers of genomic DNA and RNA extracts, respectively. Lanes 6 and 7: Amplifications with *ste3* specific primers of genomic DNA and RNA extracts, respectively. Lanes 8 and 9: Amplifications with *ppgA* specific primers of genomic DNA and RNA extracts, respectively.

Transcription Factors

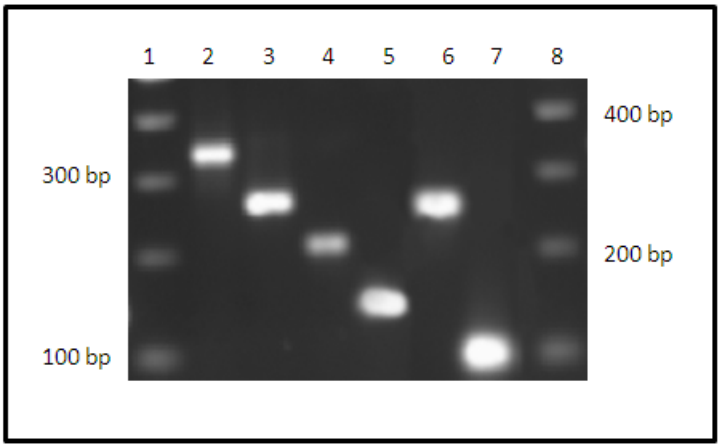

Figure 4.15: 1.5% agarose gel showing the results of transcription factor RT-PCR gene analysis of *A. clavatus* isolate A-24204. Lane 1 and 8: 100bp ladders. Lane 2: Genomic DNA Actin control. Lane 3: RNA Actin control. Lanes 4 and 5: Amplification with *veA* specific primers of genomic DNA and RNA extracts, respectively. Lanes 6 and 7: Amplification with *nsdD* specific primers of genomic DNA and RNA extracts, respectively.

4.3.1.5 Induction of Sexual Reproduction in *A. clavatus*

Aspergillus clavatus MAT1-1 isolates NRRL A-25278, NRRL A-3880, NRRL A-251 and NRRL A-3072 were incubated with the MAT1-2 isolates NRRL A-22620, NRRL 4, NRRL A-803 and NRRL A-24204. However after 6 months of culture at 25°C and 28°C in all

combinations of light and dark on OA and ACM, no sexual structures were found, although plates were not examined for micromorphological development e.g. ascogonial coils.

4.3.2 *Aspergillus terreus*

4.3.2.1 *MAT1-2-1* Gene Screening

A 270bp putative *MAT1-2-1* gene fragment was successfully amplified from certain isolates of *A. terreus* using degenerate primers MAT5-7 and MAT3-5. This allowed isolation of the entire *MAT1-2-1* gene sequence (see section 4.2.2.2) and design of a PCR diagnostic to determine the mating type of isolates of *A. terreus* (section 4.2.2.1).

The *MAT* PCR diagnostic using primer pairs AteM1F with AteM1R or AteM2F with AteM2R successfully amplified putative *MAT* gene fragments from all 25 worldwide isolates of *A. terreus*. Primers were designed in such a way that MAT1-1 genotypes were predicted to produce a 314bp fragment, whereas MAT1-2 genotypes should produce a 513bp product (Figure 4.16).

Of the 25 isolates screened, 15 isolates produced a *MAT1-1-1* fragment only, and 10 isolates produced a *MAT1-2-1* fragment only (Table 4.17). Using a Chi-squared test, it was confirmed that this *MAT* gene distribution is not significantly different from a 1:1 ratio (x^2 value = 1.0, p=0.3173, 1d.f). Therefore, on the basis of this, admittedly small sampling, *A. terreus* appears to have a 1:1 worldwide *MAT* gene distribution.

To ensure that all of these isolates were independent isolates, i.e. not clonal, RAPD analysis was performed (data not shown) using the primers listed in Table 2.2 and the protocol described in section 2.2.6. Results showed all isolates to produce distinct RAPD-PCR fingerprints and therefore none of the isolates were clonal (data not shown).

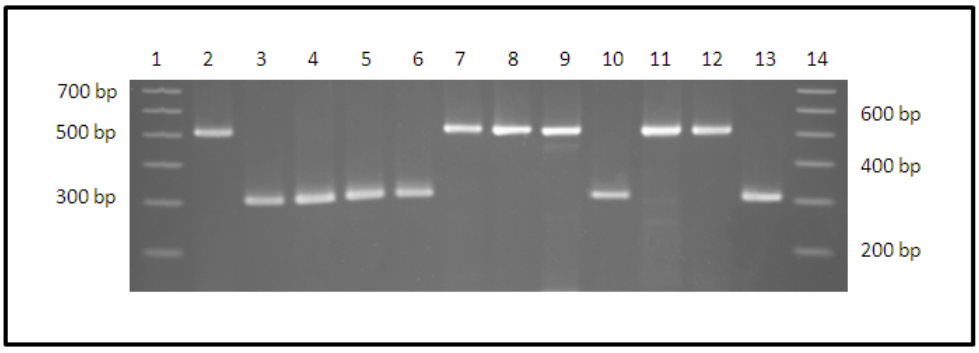

Figure 4.16: 2% agarose gel showing representative amplification of *MAT* gene fragments from

various *A. terreus* isolates. Lanes 1 and 14: 100bp ladders. Lane 2: IBT 6450. Lane 3: IBT 16744. Lane 4: IBT 21125. Lane 5: UAB 17. Lane 6: MD 29. Lane 7: IBT 13121. Lane 8: MD 11. Lane 9: IBT 26915. Lane 10: UAB 23. Lane 11: IBT 26384. Lane 12: UAB 1. Lane 13: IBT 22556.

Table 4.17: MAT genotype of isolates of *A. terreus* as determined by *MAT* PCR diagnostic test.

Culture ID Code	MAT Genotype[a]
CBS 106.25	MAT1-1
CBS 469.81	MAT1-1
CBS 403.65	MAT1-2
CBS 118.27	MAT1-2
CBS 134.60	MAT1-2
UAB 1	MAT1-2
UAB 17	MAT1-1
UAB 20	MAT1-2
UAB 23	MAT1-1
UAB 26	MAT1-2
IBT 6450	MAT1-2
IBT 12713	MAT1-1
IBT 13121	MAT1-2
IBT 14590	MAT1-1
IBT 15722	MAT1-1
IBT 16744	MAT1-1
IBT 16745	MAT1-1
IBT 21125	MAT1-1
IBT 22556	MAT1-1
IBT 23544	MAT1-1
IBT 26384	MAT1-1
IBT 26915	MAT1-2
MD 6	MAT1-1
MD 11	MAT1-2
MD 29	MAT1-1

[a]MAT genotype determined by the presence of a *MAT1-1-1* alpha-domain (MAT1-1), or a *MAT1-2-1* HMG-domain (MAT1-2) encoding genes.

4.3.2.2 *MAT1-2-1* Gene Isolation and Sequence Analysis

Using the *SLA2-APN2* positional PCR strategy (section 2.2.5) it was possible to amplify the entire MAT-2 idiomorph from *A. terreus* isolate IBT 6450, with amplicons of approximately 2.2kb in size being produced by primers AteM2F and AteAPN2, and approximately 4.2kb in size being produced by primers AteM2R and AteSLA2. The idiomorph was then partially sequenced by chromosome walking outwards from the *MAT1-2-1* gene fragment to determine whether a complete putative *MAT1-2-1* gene was present, including a conserved HMG-domain encoding sequence, necessary for functionality. In total 1710bp of the MAT1-2-1 region was sequenced from isolate IBT 6450 containing a putative 1091bp open reading frame, which includes two putative introns, and was predicted to encode a 310 amino acid MAT1-2-1 protein (Figure 4.17).

PSORT II and TFSITESCAN programs were used to interrogate resultant sequences for nuclear-targeting and promoter-region motifs. The putative ATG translation start site of this protein was consistent with the requirements of the Kozak rule (Kozak 1986). Analysis of the putative MAT1-2-1 protein revealed three nuclear targeting signals (KKKH at position 176, KKRR at position 196 and PSEKKRR at position 193). No promoter sequence analysis could be performed as only 10bp upstream of the start site was available for analysis. Meanwhile, analysis of the putative MAT1-1-1 protein identified from The Broad Institute *A. terreus* genome sequencing project revealed one nuclear targeting signal (KKRP at position 69) and a GATA transcriptional activator 336bp upstream of the start site. Possession of nuclear targeting sites is consistent with a role of these genes as transcriptional activators.

However, no *MAT1-2-4* gene was found in the sequenced region adjoining the *MAT1-2-1* gene. Also, it was not possible to obtain any PCR product from amplifications with primers MAT124F and MAT124R (section 2.2.4). Therefore, it appears that there is no *MAT1-2-4* gene in the MAT-2 idiomorph of *A. terreus* isolate IBT 6450.

```
                |M||P||A|| S||Q||I||S ||K||W||P| |G||V||P||
1     atgtctcatt atgcctgcat ctcaaatatc gaaatggccc ggtgtacctt
      S||T||D||K ||L||T||E| |L||L||W|| Q||D||A||L ||R||H||L|
51    ctacggacaa gctcacagag ttgctttggc aagatgcctt acgccacctc
      |E||S||T|| N||N||E||V ||L||L||P| |V||N||I|| T||E||I||I
101   gaatcaacca acaacgaagt tcttctcccg gtcaacataa ccgaaatcat
      ||G||Q||E| |N||V||N|| K||I||K||A ||R||L||G
151   cggccaggaa aacgtcaata agataaaggc tcgtcttggg tgagttgtcg
201   atatttgctt acccggaata acgaaacctt ttctcaactt gtgcttcatg
                                                        ||
251   aaacaacgtt gggagacctt ccatcgattt tgggctaacg ttggacagtg
      A||L||I||G ||A||P||V| |V||A||F|| I||D||E||T ||I||S||A|
301   ccctcatagg tgccccggtt gtggctttca ttgacgagac catcagcgcc
      |L||R||V|| M||R||T||P ||E||F||S| |G||T||V|| I||S||L||A
351   ctccgagtta tgcgcacccc agagttctca ggaaccgtaa tttctcttgc
      ||T||H||D| |R||M||S|| L||G||R||H ||S||P||D| |G||K||N||
401   cacacacgac agaatgtcac tcggaagaca ttcgcctgat gggaagaacg
      A||V||P||A ||K||P||V| |K||V||P|| R||P||P||N ||A||F||I|
451   cggtgccagc gaagcccgta aaagttcccc ggccacccaa tgccttcatt
      |L||Y||R|| Q||H||H||H ||P||M||V| |K||E||A|| H||P||H||L
501   ctctatcgcc agcaccatca tcctatggtc aaggaagcac atccacatct
      ||S||N||N| |E||I||
551   ttcaaacaat gagatctgta agtaaagctc agttctgctc tggcttcttc
                       S ||I||I||L| |G||K||Q|| W||K||S||E
601   ttcaagctga cggctacagc gatcattctg ggcaaacaat ggaaatccga
      ||G||D||E| |T||K||L|| H||F||R||N ||L||A||E| |E||L||K||
651   gggtgacgaa accaagctgc acttccgcaa tttggctgaa gagctcaaga
      K||K||H||A ||E||D||Y| |P||D||Y|| H||Y||S||P ||R||K||P|
701   agaagcacgc cgaagactat cccgattacc attattcgcc ccgaaagccg
```

```
     |S||E||K|| K||R||R||A ||S||S||R| |Q||S||K|| L||S||R||N
751  tccgagaaga agcgccgggc ttcctcccgt cagtcgaaac tcagcagaaa
     ||E||S||S| |P||S||T|| N||G||P||S ||N||V||S| |T||P||I||
801  cgaatcgtcc ccttcgacca atggcccatc gaacgtatcc actccaatca
     M||Y||P||D ||M||P||M| |G||D||S|| S||I||T||G ||A||T||F|
851  tgtacccgga catgccaatg ggagattcta gcattacagg ggctacattt
     |G||G||V|| F||T||A||G ||N||I||D| |V||H||A|| R||N||L||E
901  ggaggagttt ttactgctgg taatattgat gttcatgcta gaaatctcga
     ||F||D||P| |E||A||F|| D||T||L||L ||Q||Q||V| |Q||N||D||
951  atttgacccc gaagctttcg acaccctgct ccagcaggtg cagaacgacc
     H||H||H||N ||K||N||A| |T||M||Y|| Q||Q||F||S ||Y||P||D|
1001 accaccacaa caagaacgcg acaatgtacc aacagttcag ttacccggat
     |I||P||V|| G||D||S||F ||E||F||S| |E||F||V|| S||D||C||F
1051 atccctgttg gtgactcgtt tgagttctct gaattcgttt ccgactgctt
     |
1101 catgctgagc agctgatgcc cccctctatg ccttgtcatt gctcttcctc
1151 tggtgcaggt gtccctgaca gcgactccca cgagcaaaat gccggccctg
1201 taccctgata tccattcgca cggatcagag aggtgcgtac ccaaggtctc
1251 gggtcgatca aatctaacgt gattgtatag atattgtgtc gatccccatg
1301 agaacgacgt gcagatcctc gaattcttct gtccctttct ccgtcttcct
1351 tatctgtttt catacgtgag ataccattcc attcgtgact taaatactac
1401 attttgtttc acaagctctg ttattctaaa tggctggtct acccaacagg
1451 cgttatgttt gcggcaattg ctctcttcct tccgctgtac atctctatcc
1501 agtgatctta ctttagatta gtgaagcccg tggatttgat gatacttctt
1551 ctagggattg acaataagtt agaagataac ttggcgtctg gatcagtcga
1601 ttcagcgata agaggcctga tcagcgatcg ttcagtgcat gtacgagaca
1651 cagcccgcac gggggtccct ccgcgccaaa taaacaccat ggactcgagt
1701 tgtaccagag tattttgtgc
```

Figure 4.17: DNA sequence of MAT1-2-1 region of *A. terreus* isolate IBT 6450. The putative encoded amino acid sequence is indicated above the relevant base pair sequence. Primer AteM2F is highlighted red (forward), primer AteM2R is highlighted green (reverse) and putative introns are highlighted in yellow. Also included is 10bp upstream of the putative ATG start site and 619bp downstream of the putative finish site.

4.3.2.3 Idiomorph Orientation

For reasons unknown, it was not possible to amplify the entire MAT-2 idiomorph of *A. terreus* as a single product. Instead, the overall size of the idiomorph was derived from the sizes of the amplification products obtained with primers orientated outwards from the *MAT1-2-1* gene towards the *APN2* and *SLA2* genes. Successful PCR with primer pairs AteM2F with AteAPN2, and AteM2R with AteSLA2 also allowed the orientation of the *MAT1-2-1* gene within the idiomorph to be determined (Figure 4.19). The orientation of the *MAT1-1-1* gene within the MAT-1 idiomorph was obtained from genome sequence data from The Broad Institute *A. terreus* genome sequencing project.

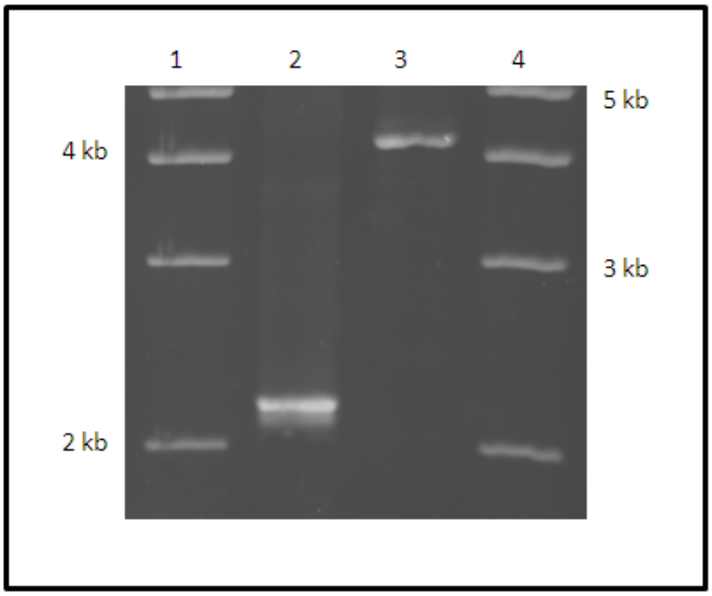

Figure 4.18: 1.5% agarose gel showing the results of PCR amplification to determine *MAT1-2-1* gene orientation within the MAT-2 idiomorph of *A. terreus* isolate IBT 6450. Lanes 1 and 4: 1kb ladder. Lane 2: Amplicon produced by AteM2F and AteAPN2. Lane 3: Amplicon produced by AteM2R and AteSLA2.

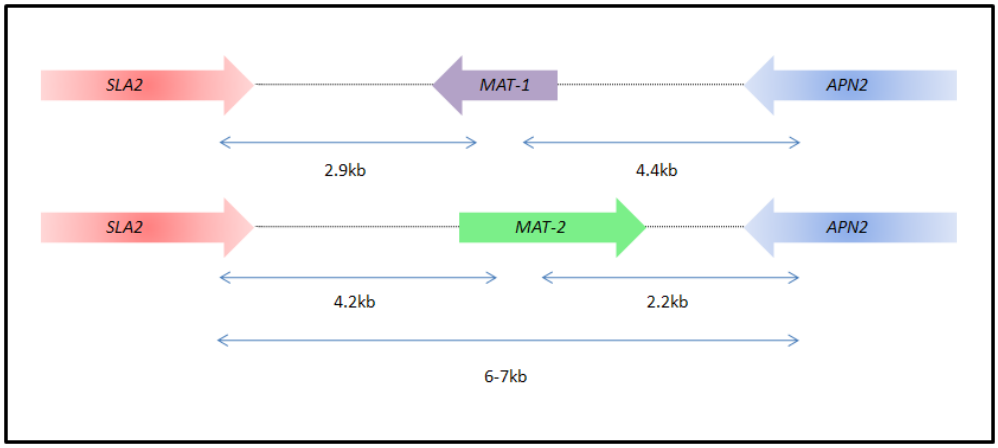

Figure 4.19: Schematic diagram showing the *MAT-1* idiomorph (top) and the *MAT-2* idiomorph (bottom) of *A. terreus* (distances in kb).

4.3.2.4 RT-PCR Analysis

RT-PCR analysis provided clear evidence of the expression of the *ste3*, *ste2*, *veA* and *nsdD* genes of MAT1-2 *A. terreus* isolate IBT 6450, together with the actin control gene. For these genes, amplicons of a smaller size were produced from RT-PCR amplification relative to amplifications from genomic DNA controls (Figures 4.22 and 4.23), corresponding to the predicted sizes of processed mRNA transcripts allowing for splicing of a 58bp intron from *ste2*, 48bp intron from *ste3*, 57bp intron from *veA* and 143bp intron from *nsdD* (Table 4.18).

However, no gene expression could be detected for the *MAT1-2-1* or *ppgA* genes as judged by a lack of RT-PCR amplification products (Figures 4.21 and 4.22). This suggests that, under the conditions assayed in this study, these genes are not expressed in isolate IBT 6450.

Similar results concerning the expression of *ste3, ste2, veA, ppgA* and *nsdD* genes were obtained for MAT1-1 isolate IBT 16744 (data not shown) i.e. there was no apparent mating type-specific expression of the pheromone receptor genes. However, expression and intron splicing (54bp) of the *MAT1-1-1* gene was detected in this isolate (Figure 4.20).

Table 4.18: Primers used for *A. terreus* RT-PCR analysis and predicted fragment lengths from genomic DNA and mRNA.

Forward primer	Reverse primer	Genomic DNA fragment length (bp)	Processed mRNA fragment length (bp)
AteM1F	AteM1R	314	260
AteM2F	AteM2R	513	461
Ate Actin Forward	Ate Actin Forward	331	265
Ate PpgAF	Ate PpgAR	232	232
Ate STE2F	Ate STE2R	617	559
Ate STE3F	Ate STE3R	605	557
Ate VeAF	Ate VeAR	249	192
Ate NsdDF	Ate NsdDR	243	100

MAT1-1-1

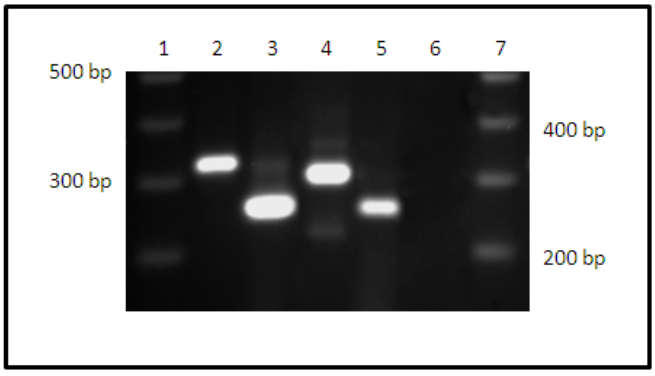

Figure 4.20: 1.5% agarose gel showing result of *MAT1-1-1* RT-PCR gene analysis of *A. terreus* isolate IBT 16744 . Lanes 1 and 7: 100bp ladder. Lane 2: Genomic DNA Actin control. Lane 3: RNA Actin Control. Lanes 4 and 5: Amplification with *MAT1-1-1* specific primers of genomic DNA and RNA extracts, respectively. Lane 6: *MAT1-1-1* RT-PCR water control with no RNA.

MAT1-2-1

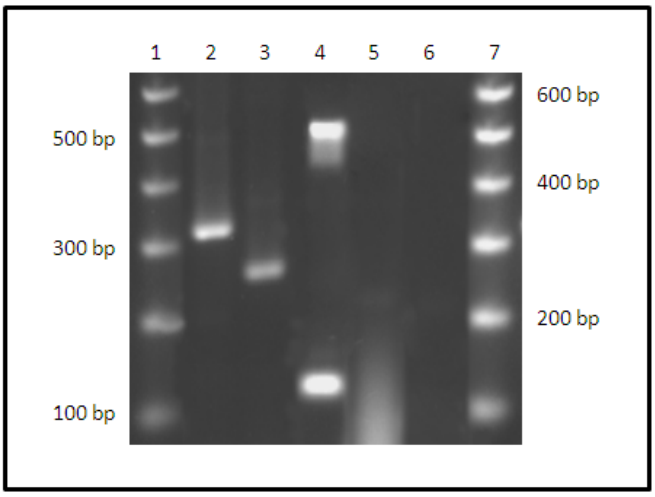

Figure 4.21: 1.5% agarose gel of *MAT1-2-1* RT-PCR gene analysis of *A. terreus* isolate IBT 6450 . Lanes 1 and 7: 100bp ladder. Lane 2: Genomic DNA Actin control. Lane 3: RNA Actin Control. Lanes 4 and 5: Amplification with *MAT1-2-1* specific primers of genomic DNA and RNA extracts, respectively. Lane 6: *MAT1-2-1* RT-PCR water control with no RNA.

Pheromone Precursor and Receptors (*ppgA, ste3* and *ste2*)

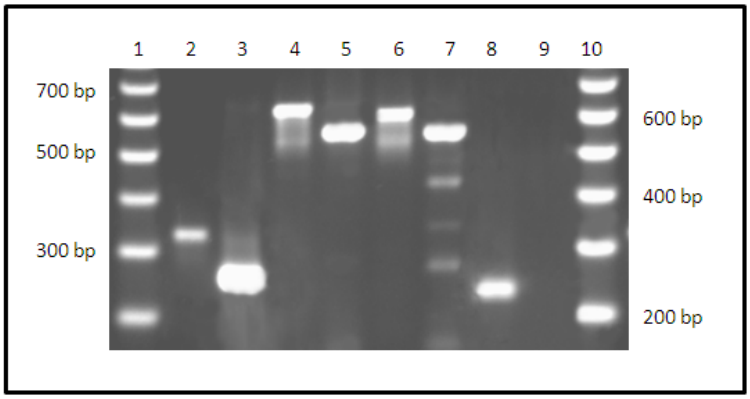

Figure 4.22: 1.5% agarose gel showing results of pheromone gene RT-PCR analysis of *A. terreus* isolate IBT 6450 . Lanes 1 and 10: 100bp ladder. Lane 2: Genomic DNA Actin control. Lane 3: RNA Actin control. Lanes 4 and 5: Amplification with *ste2* specific primers of genomic DNA and RNA extracts, respectively. Lane 6 and 7: Amplification with *ste3* specific primers of genomic DNA and RNA extracts, respectively. Lanes 8 and 9: Amplification with *ppgA* specific primers of genomic DNA and RNA extracts, respectively.

Transcription Factors

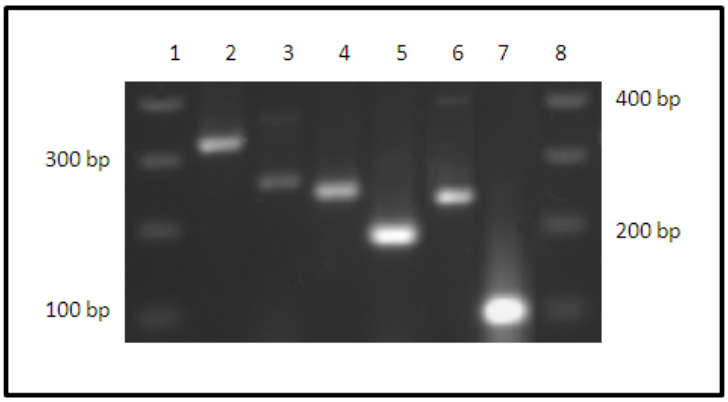

Figure 4.23: 1.5% agarose gel showing results of transcription factor RT-PCR gene analysis of *A. terreus* isolate IBT 6450. Lanes 1 and 8: 100bp ladder. Lane 2: Genomic DNA Actin control. Lane 3: RNA Actin Control. Lanes 4 and 5: Amplification with *veA* specific primers of genomic DNA and RNA extracts, respectively. Lanes 6 and 7: Amplification with *nsdD* specific primers of genomic DNA and RNA extracts, respectively.

4.3.2.5 Induction of Sexual Reproduction in *A. terreus*

Aspergillus terreus MAT1-1 isolates MD 29 and IBT 16744 were incubated with the MAT1-2 isolates IBT 6450 and MD 11. However after 6 months of culture at 25°C and 28°C in light and dark, on OA and ACM, no sexual structures were found, although plates were not examined for micromorphological development e.g. ascogonial coils.

4.3.3 *Aspergillus flavus*

4.3.3.1 RT-PCR Analysis

RT-PCR analysis of *A. flavus* provided no evidence of gene expression or splicing of either pheromone receptor genes, *ste2* and *ste3* or the pheromone precursor gene, *ppgA* under the conditions assayed in this study. However, amplicons predicted for processed mRNA were obtained for both of the transcription factors, *veA* and *nsdD*. *MAT* gene expression was not assessed as gene expression and intron splicing has already been reported in this species (Ramirez-Prado *et al.* 2008). Whilst results are shown only for the MAT1-1 isolate, NRRL, RT-PCR analyses was also performed for a MAT1-2 isolate, ATCC, and similar results were obtained (data not shown).

Table 4.19: Primers used in *A. flavus* RT-PCR and expected fragment sizes from genomic DNA and mRNA.

Forward primer	Reverse primer	Genomic DNA fragment length (bp)	Processed mRNA fragment length (bp)
Afl Actin Forward	Afl Actin Reverse	328	265
Afl STE2F	AflSTE2R	709	652
Afl STE3F	Afl STE3R	631	576
Afl PpgAF	Afl PpgAR	258	258
Afl VeAF	Afl VeAR	206	140
Afl NsdDF	Afl NsdDR	238	138

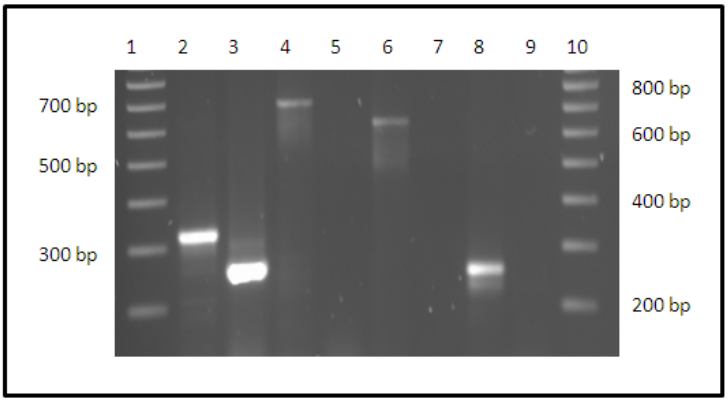

Figure 4.24: 1.5% agarose gel showing the results of pheromone gene RT-PCR analysis of *A. flavus* isolate NRRL. Lanes 1 and 10: 100bp ladder. Lane 2: Genomic DNA Actin Control. Lane 3: RNA Actin Control. Lanes 4 and 5: Amplification with *ste2* specific primers of genomic DNA and RNA extracts, respectively. Lanes 6 and 7: Amplification with *ste3* specific primers of genomic DNA and RNA extracts, respectively. Lanes 8 and 9: Amplification with *ppgA* specific primers of genomic DNA and RNA extracts, respectively.

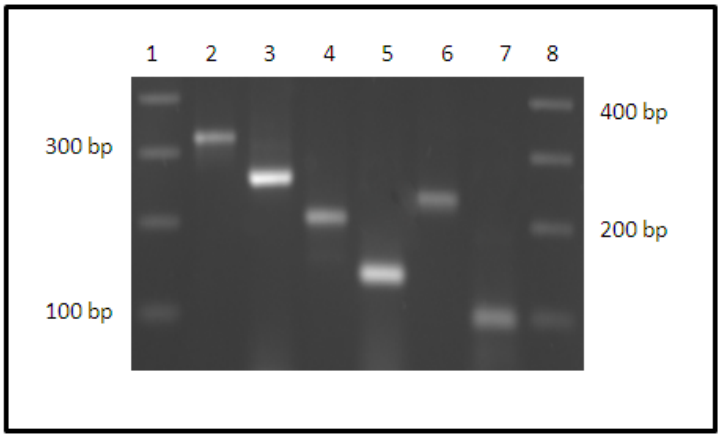

Figure 4.25: 1.5% agarose gel showing the results of transcription factor encoding gene RT-PCR analysis of *A. flavus* isolate NRRL. Lane 1 and 8: 100bp ladders. Lane 2: Genomic DNA Actin control. Lanes 3 and 4: Amplification with *veA* specific primers of genomic DNA and RNA extracts, respectively. Lanes 5 and 6: Amplification with *nsdD* specific primers of genomic DNA and RNA extracts, respectively.

4.3.4 *MAT1-2-1* Family HMG-Domain Gene Analyis

A multiple alignment was made using the amino acid sequences of the HMG-domain MAT1-2-1 proteins identified from *A. clavatus* and *A. terreus* together with sequence from three previously sequenced Aspergilli, one asexual and two sexual (Figure 4.26). This revealed 59-72% nucleotide identity and 51-69% amino acid identity (Tables 4.20 to 4.21). *A. clavatus* and *N. fumigata* shared the greatest nucleotide and amino acid identity (72% and 69%, respectively). *A. terreus* and *E. nidulans* shared the least nucleotide identity (59%) and *A. flavus* and *E. nidulans* share the least amino acid identity (51%). Nucleotide alignments can be found in Appendix 2, Figure 1).

```
A. clavatus     MATLPIAMKS AAESTDTITE LLWQDALRHL KSTNNEVLLP INVTDMIGQG
A. terreus      MPASQISKWP GVPSTDKLTE LLWQDALRHL ESTNNEVLLP VNITEIIGQE
N. fumigata     MATVPIAMKP AAESTDTLTE LLWQDALRHL ESTNNEVLLP INVTDMIGQD
E. nidulans     MAAVSIAMKS PTQSPDSITE LLWKDALRHL GSTNDEVLLP TNVVDIIGQD
A. flavus       MTTIPIAMKT TAESTDKLTE LLWQDALRHL ESTNNEVLLP INVTDMIGQS

A. clavatus     NVDKIKTRLS ALIGAPVVAF VDESIKALRV MRTPAFSGTA ISVASHGATF
A. terreus      NVNKIKARLG ALIGAPVVAF IDETISALRV MRTPEFSGTV ISLATHDRMS
N. fumigata     NVDKIKTRLG ALIGAPVVAF VDETIKALRV MRTPAFSGTA VSVASHGEAV
E. nidulans     NVEKIKSRLS ALLGAPVVSF VDESINALRV LRTPTFSGSS ISVASPSRAL
A. flavus       NVDKIRTRLG ALIGAPVVAF VDETINALRV MRTPAFSGSV VSVASHDRIS

A. clavatus     EEDVVTELKS VKSRSKRTGP VKPPKVPRPP NAFILYRQQH HPKIKETYPN
A. terreus      -------LGR HSPDGKNAVP AKPVKVPRPP NAFILYRQHH HPMVKEAHPH
N. fumigata     KTNKVTVTES FAPRGKPVGP LKAPKVPRPP NAFILYRQHH HPKIKEAYPD
E. nidulans     DSWP-----S EPPNKPRPAS MKPAKIPRPP NAFILYRQHH YPKVKEARPD
A. flavus       NLEKEITEAS GRTHGKSALP TK-SKVPRPP NAFILYRQHH HPRIKEAYPD

A. clavatus     FSNNDICVML GKQWKSEPEE IKAQFRSLAE DMKKKHAEEH PDYHYTPRKP
A. terreus      LSNNEISIIL GKQWKSEGDE TKLHFRNLAE ELKKKHAEDY PDYHYSPRKP
N. fumigata     YSNNDISVML GKQWKDENEE IKTQFRNLAE ELKKKHAEDH PDYHYTPRKP
E. nidulans     LSNNEISVII GKKWRAEPEE GKLHFKNLAE EFKKKHAEEY PDYQYTPRKP
A. flavus       FTNNEISIIL GKQWKAESEE VKMQFRNMAE KLKKKHAEDH PDYHYTPRKP

A. clavatus     SERKRRAPSR QFSRSTKSVG VLNASTPTND IP-GSFTSAM GSGMTMGDTE
A. terreus      SEKKRRASSR -QSKLS---- RNESSPSTNG PS-NVSTPIM YPDMPMG---
N. fumigata     SERKRRTSSR QFSKNTKPAA LRDTPASMNI SS-DVSTPAM LEGMPVGEID
E. nidulans     SEKKRRAASR ISPKNSKRTV ALENPGSMTA PSSNVFTPQM YPGIQNGQ-L
A. flavus       SEKKRRASSR QYSKPTK--- RQKSPALTND TS-DSSTPSM YSGMQLDNIP

A. clavatus     YNGGHDDNTD MNIIMTSHGV PETQQFQFEP NTFD-FIQQV QNDYN---RA
A. terreus      -DSSITGATF GGVFTAGNID VHARNLEFDP EAFDTLLQQV QNDHHHNKNA
N. fumigata     FNAAFEDVPG INAIMTSNSI LKNQQYHFEP NAFD-LMNQV QNDYN---KT
E. nidulans     AGAGYIGYLD GLNSMVNTGG LTDEPTNFGT NAFNSLFQQP QSDYG---RT
A. flavus       VDASLDNLAD IDIVLSPDEL PRDCGLQFDS VAFDNFLQQV QGDCG-KTAA
```

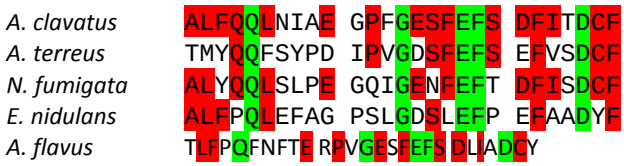

Figure 4.26: Amino acid alignment of the MAT1-2-1 protein of *A. clavatus*, *A. terreus*, *N. fumigata*, *E. nidulans* and *A. flavus.* Green highlighting indicates where amino acids are conserved between all species. Red highlighting indicates where amino acids are conserved between three or four species. For culture identification codes and GenBank accession numbers of sequences used to generate alignments, see Appendix 4.

Table 4.20: Percentage nucleotide identity between the putative *MAT1-2-1* genes of *A. clavatus, A. terreus, A. flavus, E. nidulans* and *N. fumigata*.

	A. terreus	*A. flavus*	*E. nidulans*	*N. fumigata*
A. clavatus	62	64	62	72
A. terreus		69	59	62
A. flavus			62	62
E. nidulans				63

Table 4.21: Percentage amino acid identity between the putative MAT1-2-1 proteins of *A. clavatus, A. terreus, A. flavus, E. nidulans* and *N. fumigata*.

	A. terreus	*A. flavus*	*E. nidulans*	*N. fumigata*
A. clavatus	55	62	52	69
A. terreus		61	52	60
A. flavus			51	64
E. nidulans				52

4.3.5 Data Mining and Comparison of *A. clavatus*, *A. flavus* and *A. terreus* Genome Sequences for Presence of Auxiliary Genes in the Sexual Cycle

A bioinformatic search was made against The Broad Institute genome sequence database of *A. clavatus, A. flavus* and *A. terreus* using 61 genes known to be involved in the sexual cycle of ascomycete fungi (Table 4.22).

Genes underwent BLAST searches using homologous genes from *E. nidulans* and *N. fumigata*, separately, as search query terms. Resulting E values were scored (E-value cut off was 0.001), as were nucleotide and amino acid identity for each gene and each test species when compared to the *E. nidulans* and *N. fumigata* search terms. The results of these BLAST and comparative analyses are shown in Tables 4.22 to 4.24. Each matching gene was also checked for the presence of any apparent mutation which might affect functionality of the genes e.g. point or frameshift mutations.

Table 4.22: Gene locus identifications of genes thought to be involved in the sexual cycle in *N. fumigata*, *E. nidulans*, *A. niger*, *A. oryzae* and *N. fischeri*, which were used as search terms against the *A. clavatus, A. terreus* and *A. flavus* genomes.

Gene/ Protein	Gene/Protein Description	*N. fumigata* gene locus identification[a]	*E. nidulans* gene locus identification[a]	*A. niger* gene locus idenitfication[a]	*A. oryzae* gene locus identification[a]	*N. fischeri* gene locus idenitification[a]	Homology between *N. fumigata* and *E. nidulans*[b] (%)	
							Nucleotide	Amino acid
Asd-1	Rhamnogalacturonase B, necessary for normal sexual development (Nelson *et al.* 1997b)	Afu4g03780	AN7135	est_GWPlus_C_ 80250	AO090011000 349	NFIA_029620	68	84
Asd-4 /areB	GATA, Zn-finger transcription factor (Feng *et al.* 2000)	Afu2g13380	AN6221	fge1_pg_C_200 0168	AO090026000 315	NFIA_088370	71	79
Bem1	Cell morphogenesis protein (Elion 2000)	Afu4g04120	AN7030	est_fge1_pg_C_ 80062	AO090206000 084	NFIA_029260	68	78
Cdc24	Cell morphogenesis protein (Elion 2000)	Afu4g11450	AN5592	gw1_17.225	AO090003001 078	NFIA_104750	72	80
Cdc42	Cell morphogenesis protein (Elion 2000)	Afu2g05740	AN7487	est_fge1_pg_C_ 21003	AO090001000 693	NFIA_070960	69	98
Cro1	Cytosolic protein, crozier cell wall partitioning (Berteaux-Lecellier *et al.* 1998)	Afu5g11600	AN0135	est_fge1_pg_C_ 100217	AO090026000 667	NFIA_075670	66	70
CsnD	COP9 signalosome subunit 4 (Busch *et al.* 2003)	Afu8g05500	AN1539	est_fge1_pg_C_ 180148	AO090005000 595	NFIA_098230	74	83
CsnE	COP9 signalosome subunit 5 (Busch *et al.* 2003)	Afu2g16250	AN2129	est_fge1_pg_C_ 60508	AO090102000 238	NFIA_091510	73	81
Dmc1	Homologous recombination mediator (Tsubouchi and Roeder 2006)	Afu7g02200	AN9092	fge1_pg_C_120 00033	AO090038000 596	NFIA_114920	71	90

DopA	Spatiotemporal organisation of multicellular structures (Pascon and Miller 2000)	Afu2g05020	AN2094	gw1_4.789	AO090003000304	NFIA_081740	65	64
EsdC	Necessary for sexual structure formation (Han *et al.* 2008)	Afu7g01930	AN9121	est_gw1_C_120139	AO090038000569	NFIA_114660	71	85
Far11	Transmembrane protein involved in mating cell fusion (Xiang and Glass 2002)	Afu6g04250	AN6611	e_gw1_6.643	AO09070100144	NFIA_050770	66	68
FphA	Red light phytochrome (Blumenstein *et al.* 2005)	Afu4g02900	AN9008	fge1_pg_C_8000259	AO090001000178	NFIA_030620	62	59
FluG	Extracellular signalling factor (Lee and Adams 1994)	Afu3g07140	AN4819	est-fge1_pm_C_80151	AO090020000217	NFIA_070130	67	70
Fus3 / Kss1/ MpkB	MAP kinase (Banuett 1998; Elion 2000)	Afu6g12820	AN3719	est_GWPlus_C_30521	AO090003000402	NFIA_058770	76	98
GanB/ GpaB	Alpha subunit of G-protein Negative regulator of asexual sporulation (Chang *et al.* 2004)	Afu1g12930	AN1016	est_fge1_pm_C_30278	AO090012000600	NFIA_012610	72	94
GpaA	Alpha subunit of G-protein (Elion 2000)	Afu1g13140	AN0651	est_GWPlus_C_31324	AO090012000577	NFIA_012380	82	98
GpgA	Gamma subunit of G-protein (Elion 2000)	Afu1g05210	AN2742	fge1_pm_C_1000451	NF	NFIA_019530	73	70
GprD	Negatively controls sexual development (Han *et al.* 2004)	Afu2g12640	AN3387	fge1_pm_C_2000010	AO090026000360	NFIA_087770	65	65

Kex1	Carboxypeptidase for alpha-factor processing (Latchiniansadek and Thomas 1994)	Afu1g08940	AN10184	est_GWPlus_C_32371	AO090005001632	NFIA_01670	66	71
Kex2	Endoprotease for alpha-factor processing (Jalving *et al.* 2000)	Afu4g12970	AN3583	est_fge1_pm_C_10265	AO090009000291	NFIA_103380	71	74
LsdA	Negatively regulates sexual cycle in high salt (Lee *et al.* 2001)	Afu5g10400	AN2330	NF	AO090010000503	NFIA_076830	55	48
MAT1-1-1	Mating-type alpha-box domain transcriptional activator (Coppin *et al.* 1997; Turgeon and Yoder 2000)	AY898661	AN2755	gw1_4.505	AO090020000089	NFIA_07110	57	47
MAT1-2-1	Mating-type HMG-box transcriptional activator (Coppin *et al.* 1997; Turgeon and Yoder 2000)	Afu3g06170	AN4734	NF	in house source[c]	NFIA_024390	63	54
MedA	Developmental regulator involved in sexual morphogenesis (Busby *et al.* 1996)	Afu2g13260	AN6230	est_GW1_C_20389	AO090026000285	NFIA_088430	66	65
Mre11	Part of MRX complex during meiosis (Borde 2007)	Afu6g11410	AN0556	e_gw1_3.1309	AO090023000471	NFIA_057100	70	76
MutA	Mutanase	Afu8g06030	AN7349	est_fge1_pg_C_30309	AO090005000538	NFIA_098820	40	33
NsdD	DNA binding, GAT-type transcription factor (Han *et*	Afu3g13870	AN3152	fge1_pg_C_2000697	AO090012000768	NFIA063400	63	67

	al. 2001)							
PhoA	Cyclin dependent kinase (Bussink and Osmani 1998)	Afu5g04130	AN8261	est_GWPlus_C_110084	AO090102000614	NFIA_037990	73	88
PpoA	Fatty acid oxygenase for Psi factor (Calvo *et al.* 2001)	Afu4g10770	AN1967	fge1_pm_C_17000075	AO090003001138	NFIA_105320	69	78
PpoB	Fatty acid oxygenase for Psi factor (Calvo *et al.* 2001)	NF	AN6320	NF	AO090010000591	NFIA_045480	/	/
PpoC	Fatty acid oxygenase for Psi factor (Calvo *et al.* 2001)	Afu3g12120	AN5028	e_gw1_2.1137	AO090003000772	NFIA_065200	65	65
PpgA	Alpha-facto like pheromone precursor (Casselton 2002)	Afu6g06360	AN5791	e_gw1_10.940	AO090003000007	NFIA_051990	57	44
Pro1/NosA	DNA-binding C_6 Zn-transcriptional activator (Masloff *et al.* 2002)	Afu4g09710	AN1848	est_fge1_pg_C_170014	AO090003001259	NFIA_106320	68	72
Pro11	Similar to WD40, calmodulin binding site (Pöggeler and Kück 2004)	Afu5g01780	AN8071	e_gw1_7.960	AO090003001346	NFIA_040320	70	78
Pro41	ER membrane protein involved in Ca^{2+}-dependent signalling (Nowrousian *et al.* 2007)	Afu6g13340	AN5479	est_GWPlus_C_30647	AO090003000458	NFIA_059260	62	58
Rad50	Part of MRX complex during meiosis (Borde 2007)	Afu4g12680	AN3619	NF	AO090009000296	NFIA_10640	68	79
Rad51	Homologous recombination mediator (Tsubouchi and Roeder 2006)	Afu1g10410	AN1237	NF	AO090038000386	NFIA_015260	75	98
Ram2	CAAX farnesyltransferase alpha-subunit, CAAX-prenyl	Afu4g07800	AN3867	e_gw1_13.237	AO090023000791	NFIA_108370	68	74

	cysteine carboxymethyltransferase, alpha-factor modulator (He *et al.* 1991; Song and White 2003)							
Rce1	CAAX prenylprotease, a-factor C-terminal processing (Manandhar *et al.* 2007)	Afu6g04890	AN6528	est_GWPlus_C_80659	AO090701000050	NFIA_051440	62	68
RosA	$Zn(II)_2Cys_6$ transcriptional activator, negatively regulates sexual development (Vienken *et al.* 2005)	Afu6g07010	AN5170	gw1_5.851	AO090012000945	NFIA_052650	56	48
SakA	Map kinase represses sexual development (Kawasaki *et al.* 2002)	Afu1g12940	AN1017	est_fge1_pg_C_30586	AO090020000466	NFIA_012610	66	86
SfaD	Beta-subunit G-protein (Elion 2000)	Afu5g12210	AN0081	est_fge1_pg_C_100120	AO090120000339	NFIA_02052	77	97
Spo11	DNA-topoisomerase-II-like enzyme (Sasanuma *et al.* 2008)	Afu5g04070	AN8259	e_gw1_11.802	AO090102000610	NFIA_038040	56	50
STE2	Alpha-factor binding pheromone receptor (Pöggeler and Kück 2001)	Afu3g14330	AN2520	fge1_og_C_1100212	AO090701000605	NFIA_062970	52	42
STE3	**a**-factor binding pheromone receptor (Pöggeler and Kück 2001)	Afu5g07880	AN7743	fge1_pg_C_140000056	AO090701000699	NFIA_079400	56	47
STE6	ATP binding cassette protein,	Afu5g06070	AN2300	est_GWPlus_C_	AO090009000	NFIA_03730	72	80

	transporter of **a**-factor (Berkower and Michaelis 1991; Quinby *et al.* 1999)			160549	651			
STE7	Map kinase kinase (Banuett 1998; Elion 2000; Lengeler *et al.* 2000a)	Afu3g05900	AN3422	est_GWPlus_C_41825	AO090020000060	NFIA_071330	68	81
STE11	Map kinase kinase kinase (Banuett 1998; Elion 2000; Lengeler *et al.* 2000a)	Afu5g06420	AN2269	est_GWPlus_C_160459	AO090009000610	NFIA_036710	70	80
STE12/ STEA	Binds promoter regions of genes involved in mating (Banuett 1998)	Afu5g06190	AN2290	est_fge_pm_C_160118	AO090009000638	NFIA_036920	70	77
STE13	Dipeptidylamino peptidase for alpha-facto processing (Anna-Arriola and Herskowitz 1994)	Afu3g07850	AN2946	est_gw1_C_22053	AO090005001482	NFIA_069310	68	68
STE14	ER membrane protein, carboxymethylates **a**-factor (Anderson *et al.* 2005; Romano and Michaelis 2001)	Afu2g08420	AN6162	e_gw1_9.788	AO090011000860	NFIA_084050	64	63
STE16/ Ram1	CAAX-farnesylation transferase, beta-subunit, **a**-factor modification(He *et al.* 1991)	Afu4g10330	AN2002	gw1_17.297	AO090003001188	NFIA_105740	66	69
STE20	Map kinase kinase kinase kinase (Banuett 1998; Elion 2000; Lengeler *et al.* 2000a)	Afu2g04680	AN2067	est_GWPlus_C_40790	AO090003000267	NFIA_081350	71	78
STE23	Dipeptidylamino peptidase	Afu5g02010	AN8044	fge1_pg_C_700	AO090003001	NFIA_040110	70	78

	for **a**-factor processing			0416	317			
STE24	CAAX prenylprotease, **a**-factor C- and N-terminal processing (Tam *et al.* 2001)	Afu4g07590	AN11231	fge1_pm_C_1300031	AO090023000816	NFIA_108610	75	89
STE50	Necessary for optimal Ste11 functioning (Banuett 1998; Elion 2000)	Afu2g17130	AN7252	fge1_pm_C_20000009	AO090102000104	NFIA_092440	67	76
StuA	Transcription factor affecting sexual morphogenesis (Dutton *et al.* 1997)	Afu2g07900	AN5836	fge1_pm_C_9000260	AO090011000905	NFIA_083590	70	73
TubB	Alpha-tubulin, asci development (Kirk and Morris 1991)	Afu2g_14990	AN7570	est_fge1_pm_C_60163	AO090012000417	NFIA_090230	72	92
VeA	Velvet activator, environmental sensing (Bayram *et al.* 2008b)	Afu1g12490	AN1052	est_gw_Plus_C_31478	AO090001000237	NFIA_01350	59	58
VelB	Interacts with VeA, links with secondary metabolism pathways (Bayram *et al.* 2008a)	Afu1g01970	AN0363	e_gw1_1.1811	AO090005000898	NFIA_022680	66	72

[a]From The Broad Insitute website, or NCBI database
[b]Nucleotide and amino acid identity is shown
[c]Provided from sources within The University of Nottingham
NF indicates gene/protein not found

Table 4.23: Gene locus number and E-values of genes identified form the genome sequence of *A. clavatus* with possible roles in sexual development.

Gene	*A. clavatus* Gene size		Gene locus number[a]	E. value compared to *N. fumigata*[a]		E. value compared to *E. nidulans*[a]		Homology compared to *N. fumigata*[b]		Homology compared to *E. nidulans*[b]	
	Nucleotide	Amino acid		Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid
Asd-1	2295	529	ACLA_054660	5.2581E-36	0	1.02633E-15	0	70	82	65	82
Asd-4	1038	314	ACLA_072190	0	0	1.18663E-13	0	81	88	71	78
Bem1	2457	608	ACLA_054270	0	0	4.96415E-30	0	78	86	66	79
Cdc24	3116	919	ACLA_050540	0	0	1.40459E-15	0	80	92	71	80
Cdc42	985	193	ACLA_068600	0	0	0	0	76	98	68	97
Cro1	2639	839	ACLA_014670	0	0	1.25503E-15	0	77	84	65	68
CsnD	1281	427	ACLA_058920	0	0	6.54641E-25	0	84	92	74	84
CsnE	1084	335	ACLA_075210	0	0	1.53872E-31	0	82	91	74	84
Dmc1	1538	338	ACLA_065330	0	0	1.817E-42	0	77	94	70	91
DopA	5290	1851	ACLA_089640	0	0	1.79209E-16	0	74	76	65	70
EsdC	842	262	ACLA_065620	0	0	4.22876E-19	0	86	95	72	85
Far11	3414	1077	ACLA_096570	0	0	0	0	74	80	65	67
FphA	4206	1383	ACLA_056010	0	0	3.2798E-23	0	72	74	62	59
FluG	1347	448	ACLA_074350	NF	0	NF	0	51	40	50	41

Fus3	1295	354	ACLA_086060	0	0	0	0	80	99	78	98
GanB	1416	357	ACLA_022530	0	0	4.87166E-29	0	79	96	71	94
GpaA	1251	354	ACLA_022330	0	0	0	0	84	99	81	97
GpgA	513	91	ACLA_029430	1.4E-45	1.0467E-23	8.75175E-22	6.7E-44	80	71	72	92
GprD	1390	446	ACLA_006460	NF	0	NF	0	54	44	54	44
Kex1	2007	614	ACLA_026530	0	0	2.45767E-16	0	80	87	67	71
Kex2	2608	845	ACLA_051950	0	0	1.88376E-14	0	78	85	70	73
LsdA	1099	348	ACLA_013340	1.27382E-10	0	NF	0	71	72	58	49
MAT1-1-1	1171	373	ACLA_034110	1.44941E-19	0	7.5902E-6	0	67	58	58	42
MAT1-2-1	1076	323	this study	NF	NF	NF	NF	72	70	62	53
MedA	3235	717	ACLA_072060	0	0	1.03833E-18	0	77	78	66	65
Mre11	2451	817	ACLA_085070	0	0	9.88737E-32	0	81	88	71	76
MutA	1502	480	ACLA_059460	0	0	1.50061E-4	0	71	63	41	33
NsdD	1838	499	ACLA_041500	0	0	2.04239E-22	0	76	84	64	67
PhoA	1157	332	ACLA_001050	0	0	0	0	82	99	75	96
PpoA	3552	1081	ACLA_049940	0	0	1.65415E-12	0	78	89	70	78
PpoB	/	/	NF	/	/	/	/	/	/	/	/
PpoC	4070	1118	ACLA_039980	0	0	3.5658E-29	0	74	80	64	68
PpgA	318	106	ACLA_088140	9.03355E-18	2.783E-10	NF	1.58825E-11	77	74	60	45

Pro1	2233	690	ACLA_048970	0	0	1.15863E-24	0	79	88	68	72
Pro11	2762	850	ACLA_003540	0	0	1.52916E-30	0	79	88	70	77
Pro41	1098	366	ACLA_086590	0	0	0	0	75	75	63	53
Rad50	4263	1383	ACLA_051660	0	0	0	0	80	90	72	79
Rad51	1257	348	ACLA_025130	0	0	0	0	80	99	75	98
Ram2	1187	358	ACLA_047170	6.81012E-31	0	5.18602E-7	0	77	86	67	74
Rce1	1328	352	ACLA_095650	1.4E-45	0	3.57106E-8	0	74	77	64	70
RosA	2147	673	ACLA_088790	1.33951E-36	0	NF	0	100	100	56	48
SakA	1672	366	ACLA_022520	0	0	2.27116E-25	0	76	97	68	86
SfaD	1617	431	ACLA_015240	0	0	0	0	83	92	77	96
Spo11	342	114	ACLA_001100	3.7664E-28	2.7669E-41	NF	2.50215E-33	76	69	61	59
STE2	1176	371	ACLA_041790	9.42987E-21	0	NF	0	70	69	52	44
STE3	1494	433	ACLA_012620	6.61074e-10	0	NF	0	68	64	55	46
STE3 (from this study)	/	470	This study	/	/	/	/	71	68	58	48
STE6	4232	1356	ACLA_009390	0	0	1.02777E-35	0	79	88	71	80
STE7	1831	536	ACLA_033870	0	0	9.52156E-25	0	79	91	69	80
STE11	2883	901	ACLA_009750	0	0	0	0	78	89	71	80
STE12	2435	692	ACLA_089520	0	0	1.4E-45	0	77	86	71	79
STE13	2895	915	ACLA_035780	0	0	8.1975E-11	0	78	79	67	70
STE14	904	283	ACLA_080250	0	0	1.12169E-13	0	79	82	65	65

STE16	1950	516	ACLA_049530	4.70344E-16	0	2.42672E-19	0	77	83	65	68
STE20	2655	828	ACLA_089970	0	0	3.67024E-25	0	79	87	69	79
STE23	3678	1157	ACLA_003260	0	0	1.19906E-28	0	79	87	71	78
STE24	1703	457	ACLA_108610	0	0	6.47881E-38	0	81	/3	74	90
STE50	1620	493	ACLA_076100	0	0	NF	0	78	91	67	75
StuA	2110	644	ACLA_079840	2.01379E-35	0	2.25989E-10	0	76	85	70	73
TubB	1842	452	ACLA_072490	0	0	0	0	77	95	72	91
VeA	1802	579	ACLA_022920	0	0	4.57473E-8	0	78	84	60	58
VelB	1263	359	ACLA_032540	0	0	8.7779E-9	0	80	96	67	73

[a]From The Broad Institute website.

[b]Nucleotide and amino acid identity is shown

NF indicates gene/protein not found by BLAST searching the Broad Institute database.

Table 4.24: Gene locus numbers and E-values of genes identified from the genome sequence of *A. flavus* with possible roles in sexual development.

Gene	Gene size in *A. flavus*		Gene locus Number[a]	E. value compared to *N. fumigata*[a]		E. value compared to *E. nidulans*[a]		Homology compared to *N. fumigata*[b]		Homology compared to *E. nidulans*[b]	
	Nucleotide	Amino acid		Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid
Asd-1	2233	529	AFL2G_05136	4.2002e-18	0	4.05542e-15	0	66	81	69	82
Asd-4	1047	314	AFL2G_06945	4.5e-44	0	1.18663e-13	0	72	80	70	76
Bem1	2326	602	AFL2G_12395	0	0	2.19468E-38	0	72	82	68	79
Cdc24	3217	917	AFL2G_01957	2.98027E-38	0	3.83108E-22	0	74	86	72	81
Cdc42	1106	193	AFL2G_09325	5.66321E-34	0	1.49587E-37	0	70	97	69	96
Cro1	2660	799	AFL2G_06639	7.05632E-5	0	1.88752E-11	0	68	73	67	70
CsnD	1361	416	AFL2G_00592	0	0	4.51884e-32	0	76	86	75	86
CsnE	/	/	NF	/	/	/	/	/	/	/	/
Dmc1	1277	319	AFL2G_09724	4.2E-45	0	7.0E-45	0	72	92	72	91
DopA	5926	1855	AFL2G_02676	2.35994E-28	0	2.69823E-12	0	68	69	67	72
EsdC	1393	266	AFL2G_07898	0	0	6.6025e-18	0	79	88	72	86
Far11	3417	1069	AFL2G_05794	9.106E-42	0	8.64418E-39	0	70	75	66	70
FphA	3839	1261	AFL2G_07412	7.38732E-9	0	NF	0	63	64	63	61
FluG	2657	864	AFL2G_11076	2.6563E-26	0	1.39904E-	0	70	71	68	70

						21					
Fus3	1529	355	AFL2G_02589	0	0	0	0	76	97	76	98
GanB	1868	324	AFL2G_03487	4.77247E-26	0	2.33E-43	0	75	90	74	90
GpaA	1242	329	AFL2G_03465	0	0	0	0	72	99	79	97
GpgA	915	91	AFL2G_02202	2.05968E-26	1.46056 E-25	1.71061E-38	1.4E-44	68	7Y	77	94
GprD	1314	416	AFL2G_06900	3.70969E-8	0	1.49213E-7	0	67	68	66	69
Kex1	2207	626	AFL2G_01517	6.97301E-26	0	2.11584E-4	0	69	76	66	71
Kex2	2731	837	AFL2G_10381	1.39924E-24	0	4.76737E-15	0	69	76	69	76
LsdA	886	357	AFL2G_11665	NF	0	NF	0	59	56	57	50
MAT1-1-1	1165	371	AFL2G_11189	6.17249E-25	0	8.49244E-15	0	63	52	61	47
MAT1-2-1	1066	321	EU357936	NF	NF	NF	NF	64	64	62	52
MedA	3318	719	AFL2G_06967	1.91136E-29	0	3.05231E-31	0	68	72	66	69
Mre11	2412	786	AFL2G_04312	0	0	0	0	72	81	70	77
MutA	1471	473	AFL2G_00539	6.77429e-13	0	NF	0	54	68	40	35
NsdD	1580	453	AFL2G_03635	1.57234E-26	0	4.7959E-17	0	69	74	68	68
PhoA	1311	381	AFL2G_09988	0	0	0	0	76	88	71	88
PpoA	3610	1080	AFL2G_01904	2.06903E-30	0	2.68122E-14	0	70	80	68	77
PpoB	3820	1140	AFL2G_11739	/	/	NF	0	/	/	49	40
PpoC	4043	1115	AFL2G_02242	6.26212E-18	0	7.76908E-18	0	64	67	66	69
PpgA	312	103	AFL2G_02931	NF	8.95323 E-9	NF	1.43651 E-12	66	61	58	46
Pro1	2205	690	AFL2G_01801	1.09214E-18	0	1.07504E-	0	69	75	69	72

						18					
Pro11	2674	828	AFL2G_01722	6.31349e-33	0	1.52916e-30	0	72	80	70	78
Pro41	2383	540	AFL2G_02532	2.20591e-38	0	1.01104e-26	0	61	62	58	58
Rad50	6438	1932	AFL2G_10377	0	0	0	0	73	83	72	80
Rad51	3871	511	AFL2G_07727	0	0	0	0	73	82	75	82
Ram2	1176	350	AFL2G_04602	7.88083E-6	0	1.31246E-7	0	68	74	67	72
Rce1	1307	258	AFL2G_05704	3.20481E-5	0	NF	0	65	74	63	72
RosA	2385	795	AFL2G_03812	NF	0	NF	0	55	39	50	36
SakA	1582	319	AFL2G_03486	9.04653E-28	0	8.02079E-16	1.24337E-34	70	90	68	76
SfaD	1347	354	AFL2G_08346	0	0	0	0	77	98	79	97
Spo11	363	121	AFL2G_09984	1.18882E-9	2.58976E-36	NF	1.51449E-38	70	69	64	63
STE2	1167	374	AFL2G_06217	NF	0	NF	0	58	41	55	41
STE3	1815	466	AFL2G_06286	NF	0	NF	0	59	52	56	45
STE6	4168	1344	AFL2G_10080	0	0	5.66708E-25	0	74	80	72	80
STE7	1750	453	AFL2G_11213	2.0E-44	0	1.4E-45	0	73	86	70	80
STE11	2866	596	AFL2G_10116	0	0	0	0	73	89	71	86
STE12	2417	700	AFL2G_10091	0	0	0	0	72	81	74	82
STE13	2871	917	AFL2G_01384	5.11061E-9	0	5.45061E-15	0	68	69	66	68
STE14	688	214	AFL2G_05571	2.50362E-8	1.0248E-40	6.42092E-6	5.17928E-30	66	49	64	50
STE16	1771	454	AFL2G_01858	8.49823E-10	0	1.38913E-11	0	69	74	67	76

STE20	2711	849	AFL2G_02712	0	0	0	0	73	80	71	80
STE23	3408	1109	AFL2G_01751	0	0	3.5248E-41	0	72	81	73	78
STE24	1505	412	AFL2G_04628	0	0	2.37533E-31	0	76	83	73	82
STE50	1598	486	AFL2G_09556	2.25814E-28	0	1.14544E-11	0	70	78	67	75
StuA	2108	634	AFL2G_05613	1.7337E-23	0	NF	0	71	78	70	74
TubB	1891	452	AFL2G_03324	0	0	0	0	74	93	77	91
VeA	1792	549	AFL2G_07468	1.38488E-17	0	7.14268E-7	0	69	66	59	56
VelB	1577	362	AFL2G_00875	1.51505e-16	0	5.21645e-4	0	71	84	66	71

[a]From The Broad Institute website or NCBI database.

[b]Nucleotide and amino acid identity is shown

NF indicates gene/protein not found by BLAST searching of the Broad Institute database.

Table 4.25: Gene locus numbers and E-values of genes identified from the genome sequence of *A. terreus* with possible roles ins exual development.

Gene	Gene size in *A. terreus*		Gene locus Number[a]	E. value compared to *N. fumigata*[a]		E. value compared to *E. nidulans*[a]		Homology compared to *N. fumigata*[b]		Homology compared to *E. nidulans*[b]	
	Nucleotide	Amino acid		Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid
Asd-1	2094	548	ATEG_02193	2.79276E-22	0	2.09616E-25	0	71	85	69	86
Asd-4	1525	263	ATEG_01312	1.26867E-16	0	NF	0	69	71	67	65
Bem1	2216	608	ATEG_10121	0	0	1.4E-45	0	72	82	69	80
Cdc24	3457	922	ATEG_03936	0	0	3.42406E-13	0	76	87	72	79
Cdc42	901	193	ATEG_06763	0	0	0	0	72	99	70	97
Cro1	2636	835	ATEG_02111	5.87355E-27	0	2.83875E-7	0	68	75	66	70
CsnD	1289	414	ATEG_07740	0	0	0	0	77	84	75	83
CsnE	1063	336	ATEG_02321	0	0	0	0	76	84	74	88
Dmc1	2240	608	ATEG_01853	2.21569E-31	0	2.9E-44	0	73	89	66	76
DopA	5697	1779	ATEG_06002	1.20739E-11	0	2.00511E-25	0	68	70	66	69
EsdC	841	263	ATEG_01821	0	0	3.39059E-38	0	79	92	75	86
Far11	3363	1043	ATEG_07050	3.09741E-29	0	1.95522	0	70	72	68	70
FphA	3771	1240	ATEG_07903	7.11585E-6	0	1.03523E-4	0	62	60	64	58
FluG	2686	878	ATEG_01762	3.30113E-16	0	1.9523E-11	0	65	61	62	61

Fus3	1265	355	ATEG_03316	0	0	0	0	78	98	80	99
GanB	1265	364	ATEG_00488	1.2078E-26	0	2.33E-43	0	72	83	72	84
GpaA	1243	354	ATEG_00509	0	0	0	0	80	99	81	97
GpgA	408	91	ATEG_02589	7.00661E-14	3.59747E-24	1.41857E-23	2.2E-44	73	71	73	92
GprD	1311	417	ATEG_01243	NF	0	6.35439E-13	0	65	65	70	66
Kex1	2004	626	ATEG_08515	0	0	1.35515E-5	0	70	75	66	72
Kex2	2599	842	ATEG_03179	6.18615E-33	0	7.72742E-17	0	69	74	69	75
LsdA	1121	332	ATEG_07594	7.56992E-6	0	NF	0	61	54	56	46
MAT1-1-1	1128	358	ATEG_08812	2.02259E-9	0	3.11359E-8	0	59	47	59	44
MAT1-2-1	1094	311	this study	NF	NF	NF	NF	64	60	58	54
MedA	1905	611	ATEG_01302	1.31937E-36	0	1.62119E-17	0	67	73	66	68
Mre11	2401	783	ATEG_05022	0	0	1.15E-43	0	75	84	70	78
MutA	1571	460	ATEG_05977	1.71441E-13	1.55254E-40	NF	0	30	14	55	38
NsdD	1524	431	ATEG_04043	1.63232E-29	0	1.35801E-26	0	69	74	67	67
PhoA	1330	386	ATEG_08001	0	0	0	0	73	91	76	88
PpoA	3553	1072	ATEG_03992	4.18274E-13	0	1.65415E-12	0	69	76	67	76
PpoB	3722	1125	ATEG_03171	/	/	NF	0	/	/	49	40
PpoC	3986	1114	ATEG_04755	4.48751E-28	0	2.95705E-14	0	66	69	68	71
PpgA	511	136	ATEG_07407	NF	4.44344E-8	NF	3.65303E-16	66	63	59	41

Pro1	2187	702	ATEG_00770	1.77/24E-20	0	6.15388E-11	0	70	73	68	70
Pro11	2639	819	ATEG_09797	0	0	2.57317E-35	0	71	80	71	78
Pro41	840	280	ATEG_03369	0	NF	2.8E-45	NF	44	49	46	16
Rad50	3685	1211	ATEG_03151	0	0	0	0	73	81	71	79
Rad51	1291	349	ATEG_00230	0	0	0	0	74	97	76	97
Ram2	1188	351	ATEG_05421	4.20144E-29	0	2.20852E-12	0	71	78	69	76
Rce1	823	258	ATEG_06959	2.21221E-12	0	NF	0	69	75	68	72
RosA	2236	663	ATEG_03734	NF	0	NF	0	62	47	54	43
SakA	1582	319	ATEG_00489	0	0	2.44776E-31	0	70	86	71	77
SfaD	1077	321	ATEG_02052	0	0	0	0	80	86	78	88
Spo11	/	/	NF	/	/	/	/	/	/	/	/
STE2	1180	374	ATEG_03500	NF	0	NF	0	60	53	56	48
STE3	1438	443	ATEG_08338	NF	0	NF	0	62	53	54	44
STE6	4149	1341	ATEG_09424	0	0	7.09E-43	0	72	82	71	78
STE7	1720	517	ATEG_08950	0	0	2.32113E-22	0	74	89	69	80
STE11	2851	892	ATEG_09389	0	0	1.00606E-28	0	73	82	71	80
STE12	2378	691	ATEG_09411	0	0	0	0	71	80	72	82
STE13	2848	915	ATEG_01673	3.0484E-41	0	3.62417E-19	0	72	71	67	71
STE14	812	252	ATEG_09679	1.86257E-21	0	1.68698E-9	0	70	71	68	73

STE16	2070	518	ATEG_00696	3.23457E-6	0	8.89708E-13	0	65	69	64	68
STE20	2834	838	ATEG_06035	0	0	0	0	73	75	73	79
STE23	3393	1063	ATEG_09820	9.08213E-26	0	0	0	73	82	72	78
STE24	1660	457	ATEG_05444	0	0	0	0	78	91	74	89
STE50	1551	481	ATEG_10030	6.6381E-41	0	5.06259E-20	0	73	84	71	77
StuA	1998	619	ATEG_09636	2.70689E-22	0	2.09685E-4	0	74	79	70	75
TubB	1876	453	ATEG_07846	0	0	0	0	76	94	72	91
VeA	1770	571	ATEG_00439	3.77731E-24	0	1.11521E-5	0	68	70	60	58
VelB	1238	357	ATEG_04893	9.0035E-12	0	8.7779E-9	0	70	79	66	69

[a]From The Broad Institute website.

[b]Nucleotide and amino acid identity is shown

NF indicates gene/protein not found by BLAST searching of the Broad Institute database.

4.4 Discussion

4.4.1 Presence of Complementary *MAT* Genes, and *MAT* Gene Expression and Distribution

Previous analyses of The Broad Institute genome sequencing data has shown the presence of apparently functional *MAT1-1-1* alpha-domain encoding genes in *A. clavatus* and *A. terreus*, lacking any obvious frameshift or stop codon mutations (Dyer 2007). The MAT1-1-1 proteins from both species also contain nuclear targeting signals, which is consistent with the suggested role of these proteins as transcriptional activators (Coppin *et al.* 1997; Jacobsen *et al.* 2002).

In this study complete *MAT1-2-1* genes have been identified and sequenced from other natural isolates of *A. clavatus* and *A. terreus*. These genes also appear to encode functional proteins and also possess nuclear targeting sequences and introns at conserved positions within ORFs. The *A. clavatus MAT1-2-1* gene shares greatest sequence identity to the *N. fumigata MAT1-2-1* gene, whereas the *A. terreus MAT1-2-1* gene shares greatest identity with the *A. flavus MAT1-2-1* gene (nucleotide alignments of the *MAT1-2-1* gene can be found in Appendix 2, Figure 1)

Aspergillus clavatus and *A. terreus* both have mating-type genes in a heterothallic-like arrangement (Figure 4.27), this arrangement has been found in other 'asexual' Aspergilli when the *MAT* genes have been investigated. Like all other asexual Aspergilli investigated, neither of these species appear to contain the putative *MAT1-2-4* gene.

The *MAT* genes within the MAT idiomorphs of *A. clavatus* and *A. terreus* have the same orientations as *A. oryzae*, *A. flavus*, *A. parasiticus*, *A. niger*, *P. marneffei* and *N. fumigata* (Figure 4.27) (Machida *et al.* 2005; Paoletti *et al.* 2005; Pel *et al.* 2007; Ramirez-Prado *et al.* 2008; Woo *et al.* 2006).

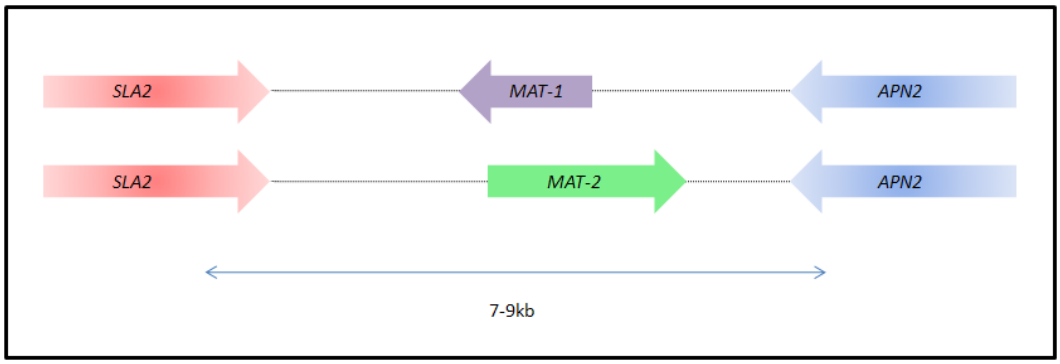

Figure 4.27: Idiomorph and *MAT* gene orientation of *A. terreus*, *A. clavatus, A. oryzae, A. niger, A. flavus*, *A. parasiticus, P. marneffei* and *N. fumigata*. [Adpated from Machida *et al*. (2005), Paoletti *et al*. (2005), Pel *et al*. (2007), Ramirez-Prado *et al*. (2008) and Woo *et al*. (2006).]

Whilst the *MAT1-2-1* gene was found in both *A. clavatus* and *A. terreus* species, expression was only induced in *A. clavatus*. The *MAT1-1-1* gene was expressed in *A. terreus* under the culture conditions outlined in section 2.2.13, in contrast to the *MAT1-2-1* gene whose expression could not be detected. MAT1-2 isolates of *A. terreus* were incubated for 5, 7, 10 and 14 days in sealed ACM plates in the dark at 28°C, MAT1-2 isolates were also incubated with MAT1-1 isolates to try and induce gene expression [Figure 2.1 (A) and (B) section 2.2.13]. RT-PCR was performed however, no gene expression was seen. A wider range of conditions will need to be assayed in order to see whether the *A. terreus MAT1-2-1* gene is expressed. *MAT* genes have been shown to be expressed in *A. flavus, A. parasiticus* and various other asexual *Fusarium* species (Kerényi *et al.* 2004; Ramirez-Prado *et al.* 2008; Turgeon *et al.* 1992; Yun *et al.* 2000).

The *MAT* gene survey of approximately 20 isolates of both *A. clavatus* and *A. terreus* revealed a distribution of *MAT* genes that does not vary significantly from a ratio of 1:1. Also, both mating types were found in the same geographical locations so a lack of mating partner does not seem to be the reason for a lack of observed sexual reproduction in either of these species.

Mating-type gene distribution has been investigated in both sexual and asexual species. Mating-type genes were found in equal distribution for *Fusarium* species, *Rhynchosporium secalis, Tapesia yallundae, Tapesia acuformis, N. fumigata* and *Mycospharella graminicola* (Dyer *et al.* 2001b; Linde *et al.* 2003; Paoletti *et al.* 2005; Yun *et al.* 2000; Zhan *et al.* 2002). Although only two isolates of *Cryptococcus neoformans*

var. *neoformans* out of 1500 isolates screened were found to possess a *MATa* gene, so a large mating-type gene skew was seen for this sexual species (Lengeler *et al.* 2000b).

4.4.2 Presence and Expression of Pheromone Response Pathway Genes

Examination of the genome data from The Broad Institute genome sequencing projects revealed the presence of an apparently functional α-factor pheromone precursor gene, *ppgA* and pheromone receptor genes homologous to *Saccharomyces cerevisiae ste2* and *ste3* in *A. clavatus, A. terreus* and *A. flavus*. The sequences of *ppgA* and the α-factor pheromone receptor encoding gene, *ste2* in *A. clavatus, A. terreus* and *A. flavus* seemed to encode functional proteins and lack any obvious frameshift or stop codon mutations. The **a**-factor pheromone receptor encoding gene, *ste3*, also appears to encode functional proteins in *A. terreus* and *A. flavus*. However, the *ste3* gene open reading frame of *A. clavatus* is suggested to encode an additional intron. Inclusion of this intron might result in a frameshift mutation resulting in premature stop codons. No **a**-factor *ppgB* pheromone precursor gene could be identified in *A. clavatus, A. flavus* or *A. terreus*. This gene has not been identified in any Aspergilli or Penicillia (Galagan *et al.* 2005; Nierman *et al.* 2005; Paoletti *et al.* 2005; Paoletti *et al.* 2007; Woo *et al.* 2006).

RT-PCR analysis of the *A. clavatus ste3* gene indicated that only two introns were present and were spliced, unlike the annotated genome sequence which indicates three introns. Sequencing of the RT-PCR products confirmed that the third intron was not spliced. According to The Broad Institute genome sequencing project, failure to removed this third intron would result in several premature stop codons and a frame-shift mutation causing amino acid identity to decrease dramatically after this intron. However, the sequencing of the *ste3* RT-PCR product in the present study revealed 17 nucleotide differences compared to The Broad Institute genome sequence. These nucleotide changes result in a longer STE3 protein sequence encoding a protein 439 amino acids in length without premature stop codons, which is comparable to other STE3 proteins. Amino acid sequence identity with *E. nidulans* and *N. fumigata* is increased for the gene with only two introns (but not greatly) compared to the 'three intron' genome sequence across whole protein (Table 4.23). It is possible that the genome sequencing and annotation is incorrect, as RT-PCR fragment sequencing was performed on one MAT1-1 and one MAT1-2 isolate and identical sequences were

obtained. *ste3* genes have been annotated in other Aspergilli (including from *A. terreus* and *A. flavus* genome sequences), these are all predicted to contain two introns not three. It is also conceivable that the *A. clavatus* isolate used for genome sequencing has been subcultured in the laboratory, and its sexual capacity has decreased and some genes may have mutated during vegetative propagation. Isolate degeneration or mutation after repeated subculturing has previously been shown for other fungal species (Horn and Dorner 2001; Mather and Jinks 1958; Ryan *et al.* 2002). In a parallel example, two isolates of *A. niger* have been genome sequenced, one an environmental isolate and one an isolate that has been subjected to specific selection pressure in the biotechnology industry, and sequence differences have been observed between these strains (Braumann *et al.* 2008a).

Expression of both *ste2* and *ste3* pheromone receptor genes was detected in *A. terreus*, with mRNA fragments produced of the predicted sizes. However, expression of the *ppgA* pheromone precursor gene was observed in either MAT1-1 or MAT1-2 isolates. The *ppgA* gene sequence does not show any significant sequence divergence when compared to other sexual or asexual *Aspergillus* species (Table 4.25). Likewise, no expression of the *MAT1-2-1* gene was detected despite incubation of MAT1-2 isolates for 4, 7, 10 and 14 days in sealed ACM plates (with MAT1-1 isolates), in the dark at 28°C. No significant sequence divergence in the *MAT1-2-1* gene was seen in either protein's amino acid sequence compared to other sexual and asexual Aspergilli (Tables 4.20 and 4.21). This lack of expression may be due to mutated promoter sequences controlling these genes. If so, it is unclear whether transcription of a *MAT* gene or pheromone pathway gene might have been first to be lost. The loss of expression of the *MAT1-2-1* gene would render a MAT1-2 isolate asexual, however the loss of *ppgA* gene expression renders a MAT1-1 isolate asexual as they are unable to attract a mate, whereas MAT1-2 isolates can secrete the elusive **a**-factor pheromone, PpgB.

Aspergillus flavus did not express either of the pheromone receptors or the α-factor pheromone precursor under the conditions assayed. This is surprising considering the putative *MAT* genes are known to be expressed (Ramirez-Prado *et al.* 2008). Ramirez-Prado *et al.* (2008) grew *A. flavus* isolates for 4 days in potato dextrose broth, not on ACM plates as was the case in this study. It may be that this species is more likely to

undergo its potential sexual cycle in potato dextrose broth instead of ACM. However, other *Aspergillus* species have been shown in the present study to express pheromone receptors and pheromone precursors under the conditions assayed (Paoletti *et al.* 2005; Paoletti *et al.* 2007). It is known that *P. alliaceus*, the closest sexual relative to *A. flavus,* takes a few months to develop and mature its ascospores, and it has subsequently been shown that *A. flavus* takes 6-11 months for fruiting bodies to form and re-named *Petromyces flavus* (Horn *et al.* 2009a). It was also shown in related studies that *A. parasiticus* also has a sexual cycle (*Petromyces parasiticus*), this species takes 6-9 months for fruiting bodies to form. Given the prolonged time these species take to undergo their sexual cycle, it does not seem surprising that gene expression was not seen. However, *N. fumigata* takes up to 6 months to produce ascospores, but pheromone gene expression is still seen after 5 days (O'Gorman *et al.* 2009; Paoletti *et al.* 2005).

Pheromone precursor and receptor genes' expression have been shown in various other sexual and asexual species. *N. fumigata* constitutively expresses pheromone precursor and receptor genes in a mating type-independent manner, whereas *E. nidulans* only expresses these genes under conditions inducive for sexual reproduction i.e. under the conditions used for RT-PCR analyses in this study (section 2.2.13) (Paoletti *et al.* 2005; Paoletti *et al.* 2007). *Neurospora crassa* expresses pheromone receptor genes constitutively and in mating type-independent manner, whereas the pheromone precursor genes are expressed in a mating type-dependent manner (Bobrowicz *et al.* 2002). *Cryphonectria parasitica*, *Magnoporthe grisea* and *Podospora anserina* also express their pheromone precursor genes in a mating type-dependent manner (Coppin *et al.* 2005).

4.4.3 Presence and Expression of VeA and NsdD Transcription Factors

Analysis of genome sequence data of the asexual Aspergilli revealed the presence of apparently functional VeA and NsdD transcription factors known to be involved in sexual reproduction (Calvo *et al.* 2004; Han *et al.* 2001). Neither of the encoding genes exhibited frameshift mutations or stop codons and encode apparently functional proteins. PSORT II analysis of the putative *A. clavatus* VeA protein revealed no clear nuclear targeting signals, but analysis of the putative *A. clavatus* NsdD protein revealed

two nuclear targeting signals (KKRR at position 424 and PDTKKRR at position 421). PSORT II analysis of the putative *A. terreus* VeA protein revealed 13 nuclear targeting signals (KRRR at position 672, KPRR at position 1048, PRRR at position 1214, RRRP at position 1215, KRKH at position 1233, PSLKRKS at position 1079, PCLRMRK at position 1179, PRRRPLQ at position 1214, PSPSKRK at position 1229, PSKRKHS at position 1231, KRPAGCSASSKNRGRRS at position 723, RRSEESDAVGVRRQKSR at position 915 and RKSANSRNVTPCLRMKK at position 1169) and analysis of the putative *A. terreus* NsdD protein revealed 4 nuclear targeting signals (PKKR at position 356, RKRR at position 357, PDPKKRR at position 354 and PKKRRGK at position 356). PSORT II analysis of the putative *A. flavus* VeA protein revealed no clear nuclear localisation signals, analysis of the putative *A. flavus* NsdD protein revealed two nuclear targeting signals (KKRR at position 380 and PDSKKRR at position 377). The detection of nuclear localisation signals in these proteins, support their proposed roles as transcriptional activators (Calvo 2008; Han *et al.* 2001; Kim *et al.* 2002).

The two transcription factors investigated in this study were found to be expressed by all of the species *A. clavatus, A. flavus* and *A. terreus*. This might appear surprising, especially as *A. terreus* does not express *MAT1-2-1* or *ppgA* genes, and *A. flavus* did not express any of the pheromone signaling genes. VeA acts to inhibit asexual reproduction and promote the sexual cycle in the dark. However, VeA also has a role as a key global metabolic regulator in the biosynthesis of cellular products e.g. secondary metabolites (Calvo 2008; Calvo *et al.* 2004; Cary *et al.* 2007; Duran *et al.* 2007; Kato *et al.* 2003). The expression of the *veA* gene may reflect its role in these other cellular processes, not an active role in sexual reproduction. NsdD does not appear to have other cellular roles, other than in sexual reproduction. However, Northern blot analysis in *E. nidulans* detected RNA transcripts during vegetative hyphal growth, with RNA transcript levels decreasing during the sexual cycle after an initial peak, but being detectable for most of the asexual cycle (Han *et al.* 2001).

4.4.4 Detection of Auxillary Sex-Related Genes by Bioinformatic Searching

Aspergillus clavatus, *A. flavus* and *A. terreus* possess all but one of the 61 genes searched for in this study. None of the remaining 60 genes investigated appear to have diverged significantly in any of these asexual species from either *E. nidulans* or *N.*

fumigata. An apparently functional set of 'sexual machinery' genes was also found in the asexual species *A. oryzae* and *A. niger* (Galagan *et al.* 2005; Pel *et al.* 2007). However, there is a mutation in the essential *pro1* gene in *A. niger*, this mutation results in a premature stop codon (Masloff *et al.* 2002; Pel *et al.* 2007). Also, various genes including *ppoB* and meiotic genes have not been identified and/or annotated in *N. fumigata* or *E. nidulans* (Galagan *et al.* 2005; Nierman *et al.* 2005). The 'sex-related' genes that have been found in this survey do not appear to have any mutations that would result in stop codons resulting in non-functional proteins. The nucleotide identity is low for some of the putative genes, however the amino acid homology was in general high. The putative *A. flavus ste24* gene lacked a complete gene sequence, which lowered the nucleotide and amino acid homology when compared to *E. nidulans* and *N. fumigata*. However, when annotation is completed for this species it may be that this sequencing fault is rectified.

Some 'sex-related' genes were not found during this study. In *A. clavatus*, sequence similar to the fatty acid oxygenase gene, *ppoB*, could not be found. However, this gene is not essential for sexual reproduction (Calvo *et al.* 2001). Meanwhile, in *A. flavus* sequence similar to the COP9 signalosome subunit 5 (*csnE*) gene could not be found. In *E. nidulans* deletion of *csn* genes blocks maturation of sexual fruiting bodies, therefore this gene is essential for sexual reproduction (Busch *et al.* 2003). As *A. flavus* is known to be sexual, this gene must either be present but diverged to a sequence that was not found in this analysis or *A. flavus* has evolved a pathway which bypasses this gene.

Meanwhile, *A. terreus* lacked sequence homologous to the *spo11* gene, which is essential for meiotic recombination (Borde 2007). It is conceivable that these genes may not be truly missing in these species and that they might be detected by means such as Sothern blot hybridisation. However, if these genes are genuinely missing from the genomes of *A. flavus, A. terreus* and *A. clavatus* then this may explain why some of these species are asexual.

4.4.5 Evolution of Reproductive Mode in the Aspergilli

It has been suggested that the sexual ancestor of the Aspergilli employed a homothallic sexual strategy, although this has been disputed (Butler 2007; Butler *et al.* 2004; Coppin

et al. 1997; Fraser *et al.* 2007b; Galagan *et al.* 2005; Kronstad and Staben 1997; Rydholm *et al.* 2007; Varga *et al.* 2000b; Yun *et al.* 1999).

Data from Chapter 3 gives possible scenarios of *MAT* gene evolution from either homothallic or heterothallic ancestry. The data from asexual species, either from this chapter or related studies suggests that a heterothallic gene arrangement is more common than previously thought (Galagan *et al.* 2005; Paoletti *et al.* 2005; Pel *et al.* 2007; Ramirez-Prado *et al.* 2008). Some 'heterothallic' species contain an additional putative gene within the MAT-2 idiomorph, the *MAT1-2-4* gene.

Table 4.26 shows the actual (from genome sequencing) or predicted MAT locus or idiomorph lengths for various sexual and asexual Aspergilli. Both *E. nidulans* MAT loci and the *N. ficheri* MAT-2 locus are smaller than the other Aspergilli as they are either split or surrounded by degenerated *SLA2* and *APN2* genes. Most MAT regions appear to be between 10,000 and 12,000 bp in size (including the full *SLA2* and *APN2* genes). The *A. flavus* MAT-2 idiomorph was predicted using the genome sequencing of the MAT-1 idiomorph and the *MAT1-2-1* gene size found by Ramirez-Prado *et al.* (2008). *A. terreus* has a MAT-1 idiomorph of comparable length, but the MAT-2 idiomorph is predicted to be smaller. Generally the MAT-2 idiomorphs are larger than the MAT-1, so the reason for the smaller predicted MAT-2 size in *A. terreus* is unknown and may be further evidence of the degeneration of the *MAT1-2-1* gene and the sexual reproductive cycle. *A. clavatus* idiomorphs are both of comparable lengths with other species.

Table 4.26: MAT idiomorph sizes (including full *SLA2* and *APN2* gene sequences), either actual from genome sequencing or approximated from gene sizes [*A. flavus* MAT-2 idiomorph predicted from Ramirez-Prado *et al*. (2008)].

Species	Idiomorph	Idiomorph length (bp)
N. fumigata	MAT-1	11519
	MAT-2	11729
N. fischeri	MAT-1	12082
	MAT-2	2747
E. nidulans	MAT-1	6506
	MAT-2	8021
A. flavus	MAT-1	10507
	MAT-2	~11000
A. oryzae	MAT-1	12594
A. clavatus	MAT-1	12057
	MAT-2	12000
A. terreus	MAT-1	11701

	MAT-2	~9300

Within their MAT-1 idiomorphs, *A. clavatus* contains a 343bp fragment of the *MAT1-2-1* gene from the 3' end of the gene (95.6% nucleotide identity with the *A. clavatus MAT1-2-1* gene over the 343bp fragment). *A. terreus* contains a 101bp fragment of the *MAT1-2-1* gene from the 3' end of the gene (93.1% nucleotide identity with the *A. terreus MAT1-2-1* gene over the 101bp fragment). *N. fumigata* also possesses a 356bp fragment of the *MAT1-2-1* gene from the 3' end of the gene (96.9% nucleotide identity with the *N. fumigata MAT-1-2-1* gene over the 356bp fragment) (Figure 4.28). *A. flavus, A. parasiticus, A. niger* and *A. oryzae* do not have these fragments (Machida *et al.* 2005; Paoletti *et al.* 2005; Pel *et al.* 2007; Ramirez-Prado *et al.* 2008).

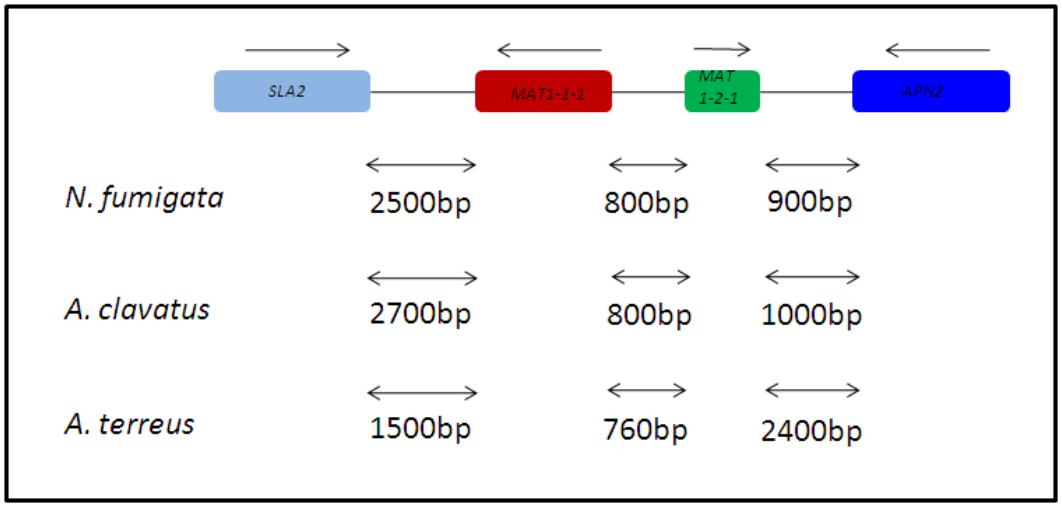

Figure 4.28: MAT-1 idiomorph gene organisation, including the partial *MAT1-2-1* gene fragment found in *A. clavatus*, *A. terreus* and *N. fumigata* [Adapted from Paoletti *et al*. (2005)].

4.4.6 Overall Findings

It was hoped that the genetic screens undertaken in the present study might identify a clear reason for asexuality in one or more of the study species. And in a screening of a limited number of isolates some possible causal factors were identified for *A. terreus*, although for *A. clavatus* the results instead suggested a latent potential for sexual reproduction. Reasons for an asexual lifestyle in *A. flavus* were suggested, but a sexual cycle has now been found in this species so alternative reasons for the missing *csn* gene were offered

Aspergillus terreus was found to be composed of isolates of both MAT1-1 and MAT1-2 genotype, and expressed pheromone receptor, *MAT1-1-1* and other transcription factor genes at the mRNA level. However, no expression of the *MAT1-2-1* or *ppgA* gene was detected. Thus, it would seem that asexuality might be due to the absence of MAT1-2-1 and PpgA proteins.

Aspergillus clavatus was also composed of isolates of both MAT1-1 and MAT1-2 genotype, and expressed all of the 'sex' genes assayed (including a pheromone receptor *ste3* gene that was spliced in a manner differing from that predicted from genome sequencing. Therefore, there are no obvious genetic reasons why *A. clavatus* is asexual. However, no sexual structures were produced after 6 months incubation under the various conditions outlined in section 2.2.19.

Finally, *A. flavus* did not express either of the putative pheromone receptor genes or the putative pheromone precursor gene under the conditions assayed in this study, which might explain the apparent asexuality in this species and possible the prolonged sexual cycle, despite the presence of both MAT1-1 and MAT1-2 isolates within natural field populations.

Bioinformatic searching revealed that all three species contain most of the auxillary genes needed for sex. However these genes may not be expressed in the species. This may be due to faulty promoter signals, or alternatively it may be due to genes being expressed under different conditions i.e. temporal or environmental coordination of the sexual cycle has been lost, rendering these species asexual.

Quantitative RT-PCR was not performed in this study due to time constraints. Therefore no information is available about the quantitative level of mRNA transcripts. However, bands on the agarose gels were approximately similar to the actin controls visualised on the same gel when similar amounts of mRNA was used in the RT-PCR. It has been suggested that for *nsdD* a minimum threshold level of transcripts must be reached for sexual reproduction to proceed (Große and Krappmann 2008). If this is the case for other 'sex' genes then a failure to reach a threshold level for some genes in *A. clavatus, A. terreus* and *A. flavus* may result in asexuality. There is evidence of low level

constitutive expression of *MAT* and pheromone genes in some species (Kerényi *et al.* 2004; Kim and Borkovich 2004; Paoletti *et al.* 2005; Pöggeler and Kück 2001).

Alternatively it may be that the correct conditions needed to initiate sexual reproduction in *A. clavatus* and *A. terreus* have not yet been found. Indeed, even after intensive research it was only in 2009 that the conditions necessary to induce sexual reproduction in *N. fumigata* were found (O'Gorman *et al.* 2009). *N. fumigata* takes up to 6 months to develop cleistothecia on OA, this is in contrast to its close relative *N. fischeri*. *N. fischeri* produces sexual structures on MEA and other commonly used growth media, and its ascospores mature within 2 weeks (Klich 2002; Raper and Fennell 1965; Rydholm *et al.* 2007).

Transformation of genes required for sexual development (usually *MAT* genes) from one species into another species have been performed involving ectopic or site-specific integration (Arnaise *et al.* 1993; Coppin *et al.* 2005; O'Shea *et al.* 1998; Pöggeler *et al.* 1997; Pyrzak *et al.* 2008). Replacement of key genes using site-specific integration could be performed to determine the potential functionality of the 'sex-related' genes identified from *A. clavatus* and *A. terreus*. Genes could be controlled by either the native or constitutive promoters as the native promoter may be defective in *A. terreus*, or be activated under different conditions as the conditions needed to induce sexual reproduction seem to be species specific.

Chapter 5 Mating-Type Genes in the Genus *Penicillium*

5.1 Introduction

5.1.1 The Genus *Penicillium*

The taxonomic placement of the genus *Penicillium* is as follows (Hibbett *et al.* 2007; James *et al.* 2006a).

Kingdom: Fungi
Subkingdom: Dikarya
Phylum: Ascomycota
Subphylum: Pezizomycotina
Class: Eurotiomycetes
Subclass: Eurotiomycetidae
Order: Eurotiales
Family: mitosporic Trichocoma
Genus: *Penicillium*

The genus *Penicillium* was first described by Link in 1809, and is now comprised of over 220 species split into four subgenera which are named; *Aspergilloides*, *Furcatum*, *Penicillium* and *Biverticillium* (Link 1809 cited in Pitt 1979; Peterson and Horn 2009; Pitt 1979; Pitt *et al.* 2000). Species within the genus *Penicillium* are haploid, filamentous, mitosporic, generally ubiquitous, opportunistic saprophytes (Pitt 1979; Raper and Thom 1949). *Penicillium* species are often food contaminants as well as being found on other decaying organic matter (Frisvad and Samson 2004; Pitt 1979; Raper and Thom 1949).

As already stated in section 1.6.2, species in this genus are characterised by the production of an asexual reproductive structure known as a penicillus (Figure 5.1).

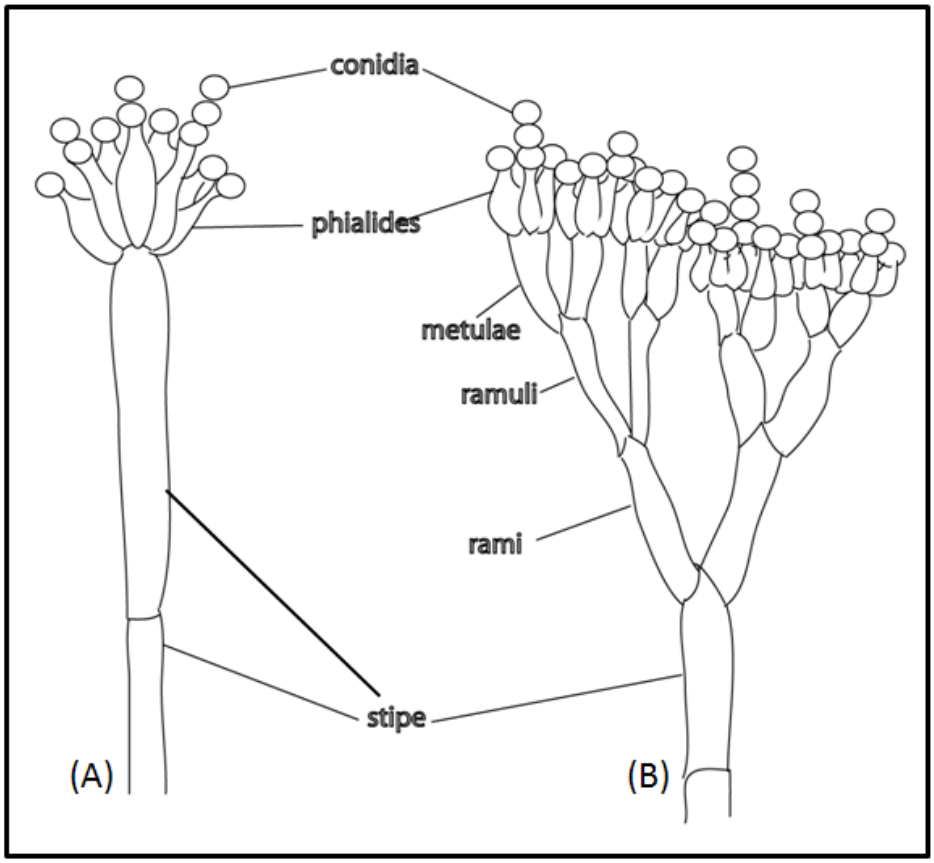

Figure 5.1: Structure of a penicillus. (A) is a monoverticillate penicillus, (B) is a quarterverticillate penicillus. [Adapted from Pitt (1979) and Raper and Thom (1949).]

Most of the species in this genus produce green or blue-green colonies (Figure 5.2), therefore colony colour cannot be used to define species or be used for phylogenetic analyses (Frisvad and Samson 2004; Pitt 1979; Raper and Thom 1949). Instead, species within the genus *Penicillium* are distinguished morphologically by the number of branch points in their penicilli. The penicillus in Figure 5.1 (A) has a single branch point between the conidium or phialide and the stipe and is known as a monoverticillate penicillus. The penicillus in Figure 5.1 (B) is a quarterverticillate penicillus, because it has four branch points (Pitt 1979; Raper and Thom 1949). A single isolate may contain a mixture of penicilli with different branch numbers. The first component of the penicillus is the stipe, a fertile hypha that is also known as the conidiophore. The stipe provides the support for the phialides and stipe length has been used for taxonomic analysis within subgenera (Pitt 1979; Raper and Thom 1949). There may then be further sub-branches of hyphae termed rami, ramuli and metulae [Figure 5.1 (B)]. Finally, the phialides, also

known as the sterigmata, bear the asexual spores, called conidia. The conidia are small (2-4µm), single-celled, often pigmented asexual spores. It has been suggested that pigmentation may protect the cell from potential damage caused by ultraviolet light (Pitt 1979; Raper and Thom 1949).

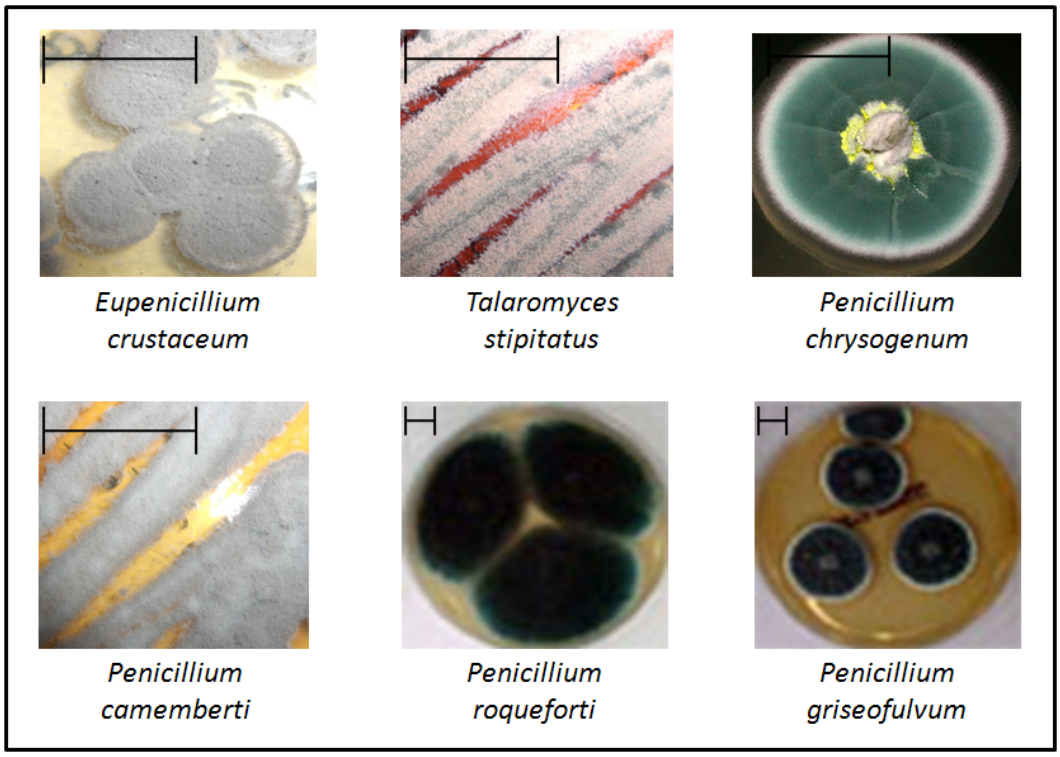

Figure 5.2: Culture morphology of four *Penicillium* species, one *Eupenicillium* species and one *Talaromyces* species. Cultures were grown for 7 days on MEA at 28°C in the light. Scale bar indicates approximately 1 cm.

All species within the genus *Penicillium* are, by definition, asexual as they are characterised by their asexual conidiation. The four *Penicillium* subgenera are often interspersed within one of two sexual genera, namely *Eupenicillium* and *Talaromyces* (Banke *et al.* 1997; Berbee *et al.* 1995; Peterson 2000b; Pitt 1995). *Eupenicillium* species only have *Penicillium* anamorphs, whereas *Talaromyces* species may have *Penicillium*, *Paecilomyces*, *Geosmithia* and *Merimbla* anamorphs (Berbee *et al.* 1995; Ogawa and Sugiyama 2000; Pitt 1979; Pitt 1993; Stolk 1965). Of the ~100 *Eupenicillium* and *Talaromyces* species that produce *Penicillium* anamorphs all are homothallic, except *T. derxii* (anamorph: *P. derxii*), which is a biallelic, heterothallic species (Takada and Udagawa 1988).

The four subgenera that *Penicillium* species are divided into were originally based on morphological features (i.e. penicillus branch length) and have since been shown, via ITS-5.8S rDNA sequencing, to be paraphyletic. Subgenera *Furcatum*, *Aspergilloides* and *Penicillium* form a monophyletic clade along with *Eupenicillium* species and appear to be closely related to *Aspergillus* species and their teleomorphs (Figure 5.3). *Talaromyces* and the subgenus *Biverticillium* form their own monophyletic clade which appears to have diverged from the other *Penicillium, Eupenicillium* and *Aspergillus* species before they underwent further evolutionary divergence from each other (Berbee and Taylor 1993; Berbee *et al.* 1995; LoBuglio *et al.* 1993; LoBuglio and Taylor 1993; Peterson 1993; Pitt 1995; Pitt and Cruickshank 1989). Indeed, based on fruiting body morphology, ascospore morphology and ITS-5.8S rDNA sequencing data *Talaromyces* species appear to be more closely related to *Byssochlamys* species rather than *Eupenicillium* species and species with *Aspergillus* anamorphs (Berbee *et al.* 1995; Pitt 1995).

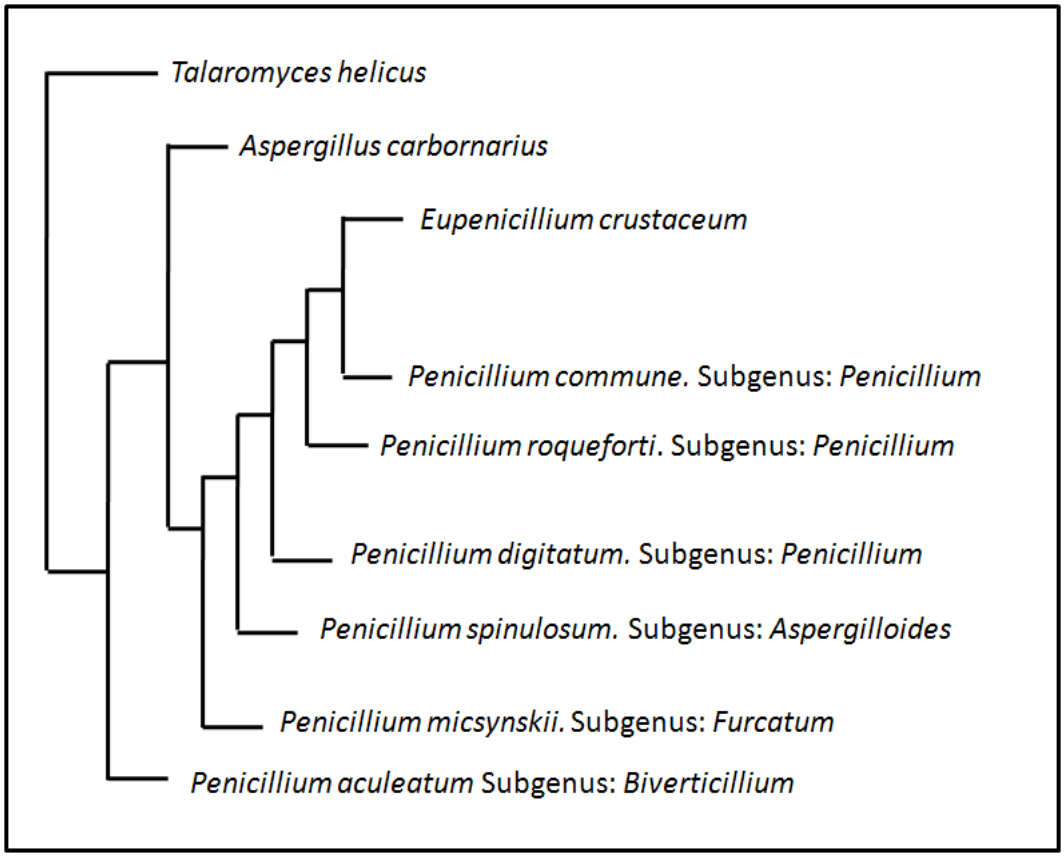

Figure 5.3: Phylogeny of the genera *Penicillium*, *Eupenicillium*, *Talaromyces* and *Aspergillus*. [Adapted from Peterson (1993).]

Thus, *Talaromyces* and *Eupenicillium* species appear to have diverged a comparatively long time ago, and although they share a common anamorphic state, in terms of their teleomorphic state they are morphologically very different, with *Talaromyces* species producing gymnothecia, instead of the cleistothecia produced by *Eupencillium* species (Figure 5.3, sections 5.1.2 and 5.1.3).

The taxonomy of *Penicillium* species has been plagued with the production of incongruent phylogenies. Colony morphology, isoenzyme profiles, RFLP patterns, penicillus structure, secondary metabolite production, electron mass spectrometry, 18S rDNA, ITS-5.8S rDNA and β-tubulin sequencing have all been used to try and determine species relationships. The relationships between individuals or small groups of species within either *Eupenicillium* or *Talaromyces* are relatively stable. However, the taxonomy of the genus *Penicillium* has been in constant flux (e.g. Banke *et al.* 1997; Berbee and Taylor 1993; Berbee *et al.* 1995; Bridge *et al.* 1989; Dupont *et al.* 2006; Frisvad 1981; Frisvad *et al.* 1990; Hong *et al.* 2000; LoBuglio *et al.* 1993; LoBuglio *et al.* 1994; LoBuglio and Taylor 1993; Malloch and Cain 1972a; Ogawa and Sugiyama 2000; Paterson *et al.* 1989; Peterson 2000b; Peterson 2006; Pitt 1989; Pitt 1993; Pitt and Cruickshank 1989; Samson *et al.* 2004; Skoube *et al.* 1999; Smedsgaard *et al.* 2004).

Whilst no sexual cycle has been demonstrated for any of the Penicillia, numerous species have been shown to possess a parasexual cycle (section 1.3.2.4). Species shown to have this capacity include *P. roqueforti* (Hong and Robbers 1985), *P. expansum* (Barron 1962), *P. italicum* (Strømnaes *et al.* 1964) and *P. chrysogenum* (Pontecorvo and Sermonti 1954). These species have only been shown to undergo a parasexual cycle in the laboratory. However, *P. cyclopium* has been found to undergo a parasexual cycle in nature and is able to produce stable heterokaryons, showing that a parasexual cycle may be present in other *Penicillium* species in nature (Jinks 1952a). Strømnaes *et al.* (1964) showed a parasexual cycle in *P. italicum*, but were not able to induce parasexuality in *P. digitatum*. This demonstrates that while a parasexual cycle may be present in some species it is not ubiquitous throughout the genus (Strømnaes *et al.* 1964).

Sexuality has been demonstrated in two genera, *Talaromyces* and *Eupenicillium* both of which have anamorphs within the genus *Penicillium*. These two sexual genera will now be discussed in more detail, followed by their related asexual taxa.

5.1.2 The Genus *Talaromyces*

Talaromyces species are thermophilic and do not produce cleistothecia as their sexual fruiting body, unlike *Eupenicillium* species and teleomorphic species with *Aspergillus* anamorphs. The genus *Talaromyces* was first introduced by Benjamin (1955) to classify sexual *Penicillium* species that instead produce a fruiting body consisting of tightly interwoven hyphae known as a gymnothecium (Benjamin 1955). The gymnothecia are usually yellow or white and may be considered as primitive or highly reduced 'naked' ascomata (Webster and Weber 2007). The asci in which the ascospores are produced, may be borne singly or in chains. The ascospores are heat resistant, usually ellipsoidal and may be hyaline, yellow or red (Pitt 1979; Pitt and Hocking 1997a; Takada and Udagawa 1988).

5.1.3 The Genus *Eupenicillium*

The genera *Eupenicillium* was first identified and described in 1892 (Pitt 1979). Species in this genus produce cleistothecia which are often macroscopic (>100µm in diameter) and range in colour from buff to orange-brown. Cleistothecia may require an extended period to reach full maturity (e.g. over three weeks) and are morphologically similar within the genus. Thus, making taxonomic analysis of this genus more difficult than that of the genus *Talaromyces*, which produce different coloured sexual fruiting structures (Frisvad *et al.* 1990; Pitt 1979; Pitt 1995; Raper and Thom 1949). Ascospores of *Eupenicillium* species are ellipsoidal or spherical and are either hyaline or yellow in colour (Pitt 1979). Most *Eupenicillium* species are soil-borne. However, their ascospores are able to survive the heat processing of foods and, like their *Penicillium* relatives, they are also able to produce mycotoxins. Both these factors make their occurrence in food and beverages of economic and health importance (Pitt and Hocking 1997a).

Currently, all known *Eupenicillium* species have a homothallic sexual reproductive strategy. The morphology of their cleistothecia show a close resemblance to *Hemicarpenteles* species, which have *Aspergillus* anamorphs (Pitt 1995). However, ITS-5.8S rDNA sequencing does not support a close relationship between these genera (Berbee *et al.* 1995).

The genera *Talaromyces* and *Eupenicillium* show taxonomic affinity to the asexual *Biverticillium* and *Furcatum*, *Aspergilloides* or *Penicillium* subgenera, respectively (Figure 5.3). Each of these asexual subgenera will now be considered individually, and species of economic importance will be highlighted.

5.1.4 The Subgenus *Biverticillium*

The subgenus *Biverticillium* is phylogenetically distinct from the other three subgenera within the genus *Penicillium*. Most species within this subgenus produce biverticillate penicilli although monoverticillate or terverticillate penicilli may also sometimes be present (Dupont *et al.* 2006; Pitt 1979; Raper and Thom 1949). All species produce clusters of slim metulae and acerose phialides (Raper and Thom 1949).

Most *Biverticillium* species are primarily associated with soil or decaying material, as well as mycotoxigenic species that can contaminate food, and other species that are of biotechnological importance being used to produce metabolites or enzymes and tester strains for biodegradation purposes (Dupont *et al.* 2006). There are six species that are commonly found in cereals, beans and nuts. Five of these species also produce various mycotoxins including the genotoxin, patulin (Table 5.1) (Pitt and Hocking 1997a). This subgenus also contains the dimorphic human pathogen *P. marneffei* (Disalvo *et al.* 1973), *P. piceum*, *P. purpurogenum* and *P. rugulosum* are also human pathogens (Dupont *et al.* 2006).

Table 5.1: Secondary metabolites produced by various *Penicillium* species in all subgenera and their detrimental human effects.

Secondary metabolite	Detrimental Effects on Human Health
Citrinin	Neurotoxin, nephropathy
Communesin B	Cytotoxin
Cyclopiazonic Acid	Neurotoxin

Griseofulvin	Headache, diarrhoea, fatigue, dizziness, fever, mouth sores
Mycophenolic Acid	Vomiting, diarrhoea, nausea, heartburn
Patulin	Genotoxin/carcinogen
Penicillin	Vomiting, diarrhoea, wheezing, hives, allergic reactions
PR-toxin	Carcinogen
Roquefortine	Vomiting, panting, muscle tremors, seizures

The subgenus *Biverticillium* is associated with the only heterothallic sexual species with a *Penicillium* anamorph, *T. derxii* (Takada and Udagawa 1988). The intergeneric relationship between *Talaromyces* and *Biverticillium* species has been examined. Analysis of both mtDNA and ITS-5.8S rDNA have shown that meiotic *Talaromyces* species are interspersed among mitotic *Penicillium* species (Dupont *et al.* 2006). It has therefore been suggested that one or more of the hundreds of genes involved in sexual reproduction might have been lost or mutated, resulting in multiple independent losses of sexual reproduction in *Talaromyces* species, thereby converting them to relatively short-lived anamorphic *Penicillium* species classified within the subgenus *Biverticillium* (Berbee and Taylor 1993; LoBuglio *et al.* 1993; LoBuglio and Taylor 1993; Peterson 2006; Pitt 1995).

Particular attention has focussed on the human pathogen, *P. marneffei*. This thermally dimorphic fungus can cause life-threatening respiratory, skin and systemic mycosis in AIDs patients (LoBuglio and Taylor 1995; Woo *et al.* 2006). In 2006 mating-type genes were found in *P. marneffei* with an arrangement similar to that observed in *N. fumigata* (anamorph: *A. fumigatus*) (Woo *et al.* 2006). This same study also found pheromone receptor genes and most of the associated response pathway genes in the sequenced genome (Yuen *et al.* 2003). However, no pheromone precursor genes could be found (Woo *et al.* 2006). Prior to the discovery of mating-type genes, population genetic analyses had already been performed on *P. marneffei* revealing low genetic variation and a fragmented population genetic structure (Fisher *et al.* 2005). Specific genotypes were found to localize to discrete geographical areas, which was suggested to have arisen from asexuality leading to the evolution of niche-adapted genotypes. Thus, there was no evidence from population studies for a recombining population or sexual cycle in *P. marneffei*, despite the presence of mating-type genes and other 'sex-related' genes (Fisher *et al.* 2005).

5.1.5 The Subgenus *Furcatum*

The subgenus *Furcatum* is believed to be the earliest diverged anamorphic lineage in the genus *Penicillium*. While no teleomorphic genera have been unequivocally associated with the subgenus *Furcatum*, ITS-5.8S rDNA sequencing has shown that some members of this subgenus have close phylogenetic relationships with *Eupenicillium* species (Peterson 2000b; Pitt 1995).

Species in this subgenus may produce uniform biverticillate, or a mixture of monoverticillate and biverticillate penicilli. While most species in this subgenus produce biverticillate penicilli, they are excluded from the subgenus *Biverticillium*, as species in the latter subgenus produce well characterised clusters of slim metulae and acerose phialides. The subgenus *Furcatum* was formed to include species that, via morphology, do not appear to belong to any of the other three *Penicillium* subgenera (Pitt 1979; Raper and Thom 1949). Most species in the subgenus *Furcatum* are found in the soil and seem to be relatively uncommon in other environments (Peterson 1993; Pitt 1979).

The most important species in terms of economic significance in the subgenus are *P. citrinum*, *P. corylophilum*, *P. fellutanum* and *P. oxalicum*, all of which are involved in food spoilage (Pitt and Hocking 1997a).

5.1.6 The Subgenus *Aspergilloides*

Species in the subgenus *Aspergilloides* are most closely related to species in the subgenus *Penicillium*. A few species within this subgenus possess *Eupenicillium* teleomorphs (Peterson 2000b; Pitt 1993), including *E. cinnamopurpureum* (anamorph: *P. phoeniceum*) and *E. hirayamae* (anamorph: *P. hirayamae*) (Pitt and Hocking 1997a).

Species within this subgenus usually produce monoverticillate penicilli and seem to be relatively rare in the wild, and have so far only been found to occur in soils (Pitt 1979; Raper and Thom 1949).

5.1.7 The Subgenus *Penicillium*

5.1.7.1 Introduction

The fourth subgenus within the genus *Penicillium* is the subgenus *Penicillium*. This subgenus will be the focus of this chapter. The subgenus *Penicillium* contains 59 species, split into 6 sections and 17 series based on morphological and molecular grounds (Table 5.2). The most common form of sporulation involves production of terverticillate penicilli although biverticillate, quarterverticillate and even more complex forms have been found. Species in this subgenus are usually associated with human domestication, food spoilage, animal nutrition and excreta. They are able to grow in media with a low water potential and have a wider range of permissible growth temperatures (5-37°C) than other *Penicillium* species (Pitt 1979; Raper and Thom 1949).

Table 5.2: Sections and Series of the *Penicillium* subgenus *Penicillium*. Adapted from Frisvad and Samson (2004).

Section	Series	Species
Coronata	Olsonii	*P. bialowiezense* *P. brevicompactum* *P. olsonii*
Roqueforti	Roqueforti	*P. carneum* *P. paneum* *P. roqueforti*
Chrysogena	Chrysogena	*P. chrysogenum* *P. dipodomyis* *P. flavigenum* *P. nalgiovense*
	Mononematosa	*P. confertum* *P. mononematosum*
	Aethiopica	*P. aethiopicum*
	Persicina	*P. persicinum*
Penicillium	Expansa	*P. expansum* *P. marinum* *P. sclerotigenum*
	Urticicolae	*P. dipodomyicola* *P. griseofulvum*
	Claviformia	*P. clavigerum* *P. concentricum* *P. coprobium* *P. coprophilum* *P. formosanum* *P. glandicola* *P. vulpinum*

	Italica	*P. italicum* *P. ulaiense*
	Gladioli	*P. gladioli*
Digitata	Digitata	*P. digitatum*
Viridicata	Viridicata	*P. aurantiogriseum* *P. cyclopium* *P. freii* *P. melanoconidium* *P. neoechinulatum* *P. polonicum* *P. tricolor* *P. viridicatum*
	Corymbifera	*P. albocoremium* *P. allii* *P. hirsutum* *P. hordei* *P. radicicola* *P. tulipae* *P. venetum*
	Verrucosa	*P. nordicum* *P. thymicola* *P. verrucosum*
	Camemberti	*P. atramentosum* *P. camemberti* *P. caseifulvum* *P. commune* *P. crustosum* *P. palitans*
	Solita	*P. cavernicola* *P. discolor* *P. echinulatum* *P. solitum*

The type species of the subgenus *Penicillium* is *P. expansum*. *P. expansum* was discovered early in the 19th Century, and is commonly isolated from pomaceous fruits, especially apples, and from indoor air samples. It has also been found on wood, cereal crops, fabrics, nuts, dried meats and is common in or on organic material in contact with soil (Barron 1962; Link 1809 cited in Pitt 1979; Pitt 1979; Raper and Thom 1949; Samson *et al.* 2004). *P. expansum* produces various secondary metabolites many of which are mycotoxins including the nephrotoxic citrinin, patulin and the cytotoxic communesin B (Table 5.1) (El-Banna *et al.* 1987; Frisvad and Filtenborg 1983; Samson *et al.* 2004). The

ubiquitous saprobic nature of *P. expansum* coupled with an ability to produce a range of secondary metabolites is typical for the subgenus *Penicillium*.

Unlike many other fungal genera the ITS-5.8S rDNA sequences of species within the subgenus *Penicillium* are too similar to allow inter- and intra-subgenus phylogenetic analysis (Skoube *et al.* 1999). Instead, the β-tubulin gene, which exhibits greater variability, has been used to examine phylogenetic placement of the species within the subgenus *Penicillium* in relation to other *Penicillium* and *Aspergillus* species (Samson *et al.* 2004).

Figure 5.3 shows the broad phylogenetic nature of the subgenus and it also demonstrates the relationship between species in the subgenus *Penicillium* and the genus *Eupenicillium* (Peterson 1993). Anamorphs of *Eupenicillium* species are generally located in the subgenus *Penicillium*. Like *Talaromyces* species and the subgenus *Biverticillium*, the genus *Eupenicillium* and the subgenus *Penicillium* show close associations and there appear to have been multiple conversions through loss of sexuality in *Eupenicillium* species leading to a mitotic lifestyle in the subgenus *Penicillium* (Banke *et al.* 1997; Berbee *et al.* 1995; Peterson 2000b; Pitt 1995).

As already stated the ITS-5.8S rDNA sequences of *Eupenicillium* species and *Penicillium* species in the subgenus *Penicillium* are too similar to allow meaningful phylogenetic relationships to be determined. However, a range of secondary metabolites (approximately 20) are produced by these species (Frisvad and Filtenborg 1983). Metabolite profiles, isoenzyme patterns and β-tubulin sequence comparisons have all been used in an attempt to determine the taxonomic structure of this subgenus (Banke *et al.* 1997; Paterson *et al.* 1989; Pitt 1993; Samson *et al.* 2004). Unfortunately, phylogenetic analyses do not produce congruent trees, although there are similarities between all trees. β-tubulin sequence analysis appears to agree most often with isoenzyme, metabolite and morphological analyses (Figure 5.4).

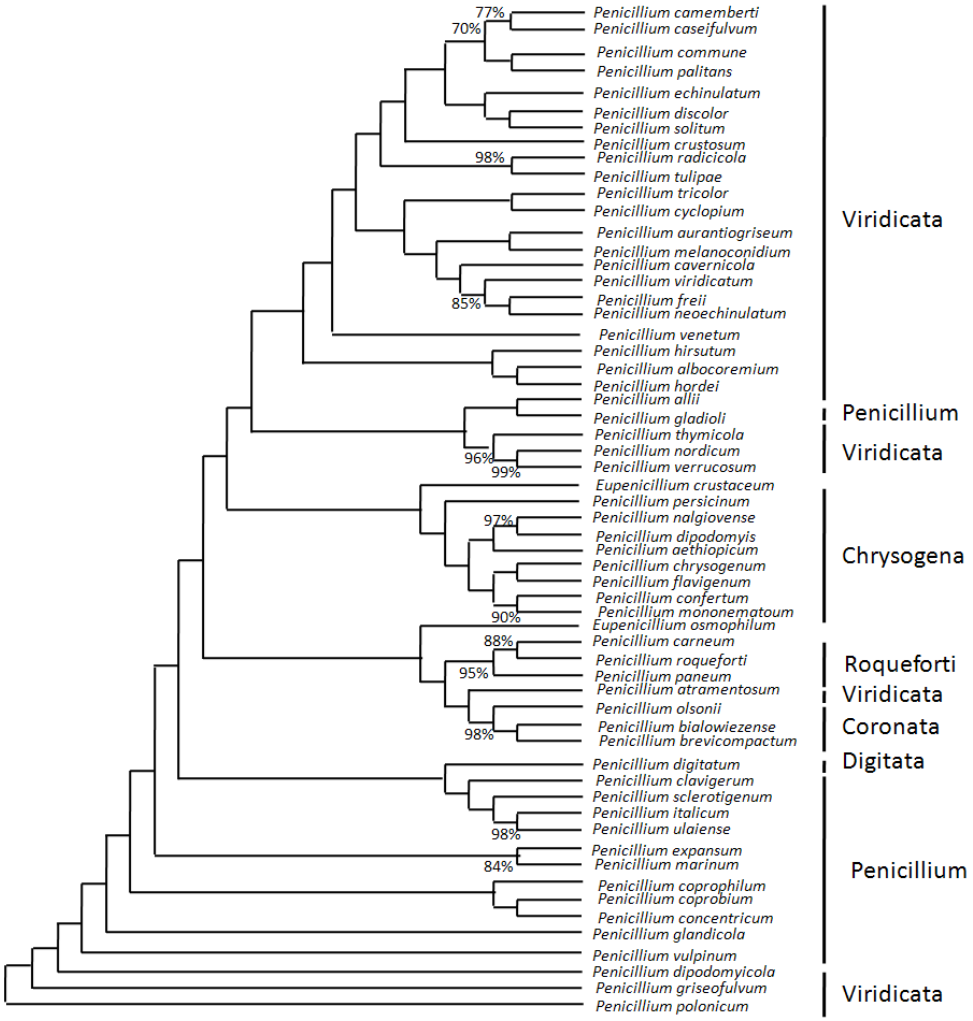

Figure 5.4: Phylogram of the subgenus *Penicillium* based on β-tubulin sequences. *Eupenicillium crustaceum* and *E. osmophilum* are included as representative *Eupenicillium* species. For culture identification codes and GenBank Accession numbers used to construct the phylogram see Appendix 4. Bootstrap values greater than 70% are shown, trees was constructed using MEGA version 4 program, using maximum parsimony analyses (under standard conditions with bootstrapping of 500 replicates and gaps excluded).

In this chapter the occurrence and distribution of mating-type genes was analysed for 58 species within the subgenus *Penicillium*. I also focussed on four 'key' species within this subgenus. These were firstly the penicillin producing species, *P. chrysogenum*. Secondly, *P. griseofulvum* a producer of another broad spectrum antibiotic, griseofulvin. And finally two species used in cheese production, *P. roqueforti* and *P. camemberti*. These species are described in more detail below. It was anticipated that analysis of mating-type gene sequence and distribution would help to further elucidate any potential for

sexual reproduction and also help determine phylogenetic relationships between species.

5.1.7.2 *Penicillium chrysogenum*

Penicillium chrysogenum first came to prominence in 1944 as a species able to produce high levels of penicillin (Raper *et al.* 1944). Penicillin is a broad spectrum β-lactam antibiotic, discovered by Alexander Fleming in 1928 (Fleming 1929). In 2007, 33,000 metric tonnes of penicillin was used worldwide, at a cost of $400 million (US) (Najafpour 2007).

Penicillium chrysogenum is the most ubiquitous *Penicillium* species, capable of growing in a range of habitats including deserts, dried foods and cheese and has a worldwide distribution. *P. chrysogenum* is classified within the subgenus *Penicillium*, section *Chrysogena*, series *Chrysogena* (Table 5.2) (Frisvad and Samson 2004). *P. chrysogenum* may produce biverticillate, terverticillate and quarterverticillate penicilli. Isolates resembling *P. chrysogenum* but having biverticillate penicilli were formerly classified as *P. griseoroseum*, but this classification is no longer used. *P. chrysogenum* produces blue-green conidia and is capable of growing at 5-37°C (Figure 5.2). The species produces a wide range of secondary metabolites and mycotoxins, in addition to penicillin such as the antibiotic xanthocillin X. Mycotoxins produced by *P. chrysogenum* include roquefortine C and D, PR-toxin as well as, some would argue, penicillin (Table 5.1) (El-Banna *et al.* 1987; Frisvad and Filtenborg 1983; Frisvad and Samson 2004; Lund *et al.* 1995).

5.1.7.3 *Penicillium griseofulvum*

Penicillium griseofulvum is classified within the subgenus *Penicillium*, section *Penicillium* series *Urticicolae* (Table 5.2). This species produces terverticillate or quinterverticillate penicilli bearing green-grey conidia (Figure 5.2). *P. griseofulvum* is relatively widespread worldwide and is common on grasses such as barley or wheat and their seeds. This species may also be found in pasta, bread and rotting vegetation (Frisvad and Samson 2004; Pitt 1979; Raper and Thom 1949). *P. griseofulvum* is of medical significance as the producer of the broad spectrum antibiotic griseofulvin, which is particularly effective against other fungal species. Griseofulvin was first used in horticulture to treat

Alternaria and *Botrytis* infections in crops but is now used in humans to treat infection against a range of dermatophytes with annual market sales in the USA of $38 million (US) (year ending March 2007) (MediLexicon 2008). Despite its broad spectrum activity and relatively low level of toxicity, griseofulvin can still cause vomiting, diarrhoea and dizziness in patients (Table 5.1) (De Carli and Larizza 1988). *P. griseofulvum* can also produce a range of mycotoxins including cyclopiazonic acid, patulin and roquefortine C (Table 5.1) (El-Banna *et al.* 1987; Frisvad and Filtenborg 1983; Frisvad and Samson 2004).

5.1.7.4 *Penicillium camemberti*

Penicillium camemberti was first described in 1906, and is believed to be the domesticated form of a related 'wild' species *P. commune*. *P. camemberti* is used in the manufacture of white mould cheeses, such as Brie and Camembert. *P. camemberti* has never been isolated outside white mould cheese or their production facilities and is therefore unknown in nature (Frisvad and Samson 2004; Pitt 1979; Raper and Thom 1949).

Penicillium camemberti is classified within the subgenus *Penicillium*, section *Viridicata*, section *Camemberti* (Table 5.2) (Frisvad and Samson 2004). This species produces terverticillate and quarterverticillate penicilli which are sometimes irregular. *P. camemberti* usually produces white conidia although they may be grey-green (Figure 5.2) (Frisvad and Samson 2004; Pitt 1979).

Penicillium camemberti produces cyclopiazonic acid, although the toxic effects of this compound are only seen after high levels are reached (Table 5.1). There are strains of *P. camemberti* that do not produce mycotoxins, which have been selected for use in cheese making (Frisvad and Filtenborg 1983; Frisvad and Samson 2004; Pitt *et al.* 1986).

5.1.7.5 *Penicillium roqueforti*

Penicillium roqueforti was first identified and described in 1906 and is used in the production of blue mould cheeses, such as Roquefort and Stilton (Pitt 1979). *P. roqueforti* produces terverticillate and occasionally quarterverticillate penicilli and produces green conidia (Figure 5.2) (Frisvad and Filtenborg 1983; Pitt 1979). *P. roqueforti* is classified within the subgenus *Penicillium*, section *Roqueforti*, series

Roqueforti (Table 5.2) and is part of the *P. roqueforti* species complex, also containing *P. carneum* and *P. paneum* (Frisvad and Samson 2004).

Penicillium roqueforti is a saprophytic species. As well as being present in cheese production facilities, it is also isolated from contaminated bread, wood, forest soil, and is of agricultural significance as it can cause spoilage of silage (Frisvad and Samson 2004; Lund *et al.* 1995; Lund *et al.* 1996; Pitt 1979). *P. roqueforti* is able to grow at 5°C, and is consequently a common cause of commercial and domestic spoilage of cold-stored foods (Pitt 1979). Unusually for species in the subgenus *Penicillium*, *P. roqueforti* is not found on cereal and cereal products, but is widespread in various other environments (Frisvad and Samson 2004; Pitt 1979).

Although this species has been used in food production for approximately 200 years, its presence can have detrimental health effects due to possible production of mycotoxins. *P. roqueforti* may produce PR-toxin, patulin, roquefortine C and mycophenolic acid (Table 5.1), although there are strains that do not produce these and these are primarily used in cheese making (El-Banna *et al.* 1987; Frisvad and Filtenborg 1983; Lafont *et al.* 1979). Mycophenolic acid is an immunosuppressant given to transplant patients to decrease organ rejection rates (Bhorade *et al.* 2006). However, this immunosuppressive quality may cause secondary mycotoxicosis.

5.1.8 Experimental Aims

This chapter will focus on species within the subgenus *Penicillium.* The subgenus as a whole is of major economic and medical importance due to widespread usage within the medical, food and biotechnological sectors. The aims of this chapter were to make a preliminary assessment of the possibility of sexual reproduction within the species of *Penicillium* currently only known to reproduce by asexual means. This was achieved by using a degenerate PCR primer approach to investigate the occurrence, distribution and possible functionality of mating-type genes within the subgenus *Penicillium* as a whole. A similar approach to locate and sequence *MAT* genes from both sexual and asexual Aspergilli, together with analysis of MAT1-1:MAT1-2 distribution, has already been used in Chapter 3 and 4, respectively. Further studies were then undertaken with four

particular species, namely *P. chrysogenum*, *P. griseofulvum*, *P. camemberti* and *P. roqueforti*. These studies focussed on determining the orientation of mating-type genes within the idiomorphic region, to give insights into the evolutionary origins of these MAT regions. Once the *MAT* genes had been sequenced their expression and potential functionality was investigated using Reverse Transcriptase (RT) PCR. Finally, laboratory crosses between potentially sexually compatible MAT1-1 and MAT1-2 isolates were set up for selected species.

5.2 Materials and Methods

5.2.1 Screening of the Subgenus *Penicillium* for Mating-Type Genes

A total of 301 isolates representing 58 *Penicillium* species within the subgenus *Penicillium* were initially screened for the presence of mating-type genes using the degenerate PCR protocol described in section 2.2.4. These isolates and species are listed below in Table 5.3. DNA from these isolates was obtained directly from J. Houbraken and R. Samson (CBS, Netherlands) as part of an ongoing research collaboration. These species were chosen to represent the broad spectrum of the subgenus *Penicillium*.

Table 5.3: *Penicillium* species and isolates used in screening.

Species	Culture Identification Code (CBS)	Source of Isolate
Penicillium aethiopicum	270.97	Locust bean gum flour, Denmark
	287.97	Soil from tropical room, Canada
	484.84	Grain of *Hordeum vulgare*, Ethiopia
	109574	*Zea mays*
	109575	Salami
	109576	Pearl millet
	109577	*Vitis* species fruit
	109602	Cassave chips
Penicillium albocoremium	472.84	Salami, Denmark
	109582	Cake factory, Denmark
	109583	Soil, salterns
	109584	Ginger
	109587	Unknown
	109614	Cake, Denmark
Penicillium allii	131.89	*Allium sativum*, Egypt
	188.88	Food, UK
	875.95	Garlic

	109578	Unknown
	109581	*Oryzae sativa*, Czech Republic
	117777	Bulb of *Tulipa* species
Penicillium antarcticum	100492	Soil scraping near nest site of Southern Fulmar
	116936	Unknown
	116937	Fungal growth in seawater bottled at a platform in the Atlantic Ocean
	116938	Salami
	116939	Beach sand
	116940	Indoor air, cabin near beach
Penicillium atramentosum	194.88	Pig feed
	243.73	Man, nasal excretion of patient with lung abscess
	291.48	French Camembert cheese, USA
	490.84	*Capsicum annuum*, imported
	109588	Soil
	109611	Soil
	109612	Cheese
Penicillium aurantiogriseum	324.89	Unknown, Belgium
	642.95	Chicken feed, Denmark
	792.95	Apple juice production plant, Denmark
	110327	Unknown
	110329	Soil
Penicillium bialowiezense	227.28	Soil under conifers, Poland
	110102	Bread
	110104	Seaweed, Denmark
	112477	Barley
	112478	Margarine
	113235	Soil under *Salix* species
	113236	Soil under *Nothofagus* species
Penicillium brevicompactum	257.29	Unknown
	317.59	Soil
	480.84	*Raphanus* species, Denmark
	110067	Soil under Junipers, USA
	110068	Soil, USA
	110069	Artificial maple syrup preserved with 650ppm benzoic acid, Australia
Penicillium camemberti	190.67	Camembert cheese, Netherlands
	112078	Cheese contaminant. Appenzella, Switzerland
	112325	Unknown
	112562	Brie
Penicillium carneum	449.78	Cheddar cheese

	466.95	Cured meat, Germany
	467.95	Water tank
	112297	Mould rye bread, Denmark
Penicillium caseifulvum	101134	Danablue cheese, Denmark
	108956	Cheese, Denmark
	111838	French cheese
	112324	German Montagnolo cheese
	112881	Food waste, (compost)
Penicillium cavernicola	100540	Unknown
	109556	Salami, Germany
	109557	Butter, Japan
	109558	Guacharo Cave, Venezuela
Penicillium chrysogenum	412.69	Soil, Syria
	478.84	Air, fruit store, Denmark
	775.95	Air in kitchen, Denmark
	776.95	Lechuguila cave, USA
	306.48	Cheese, ex-neotype strain, USA
Penicillium clavigerum	189.89	Soil
	255.94	Man, Canada
	112482	Gopher hair, Canada
	112564	Rodent survey
	113244	Unknown, USA
Penicillium commune	279.67	Roquefort cheese, Netherlands
	341.59	Cheese
	111835	Mummified bee larva
	112079	Feta cheese
Penicillium concentricum	185.89	Wheat flour
	191.88	Soil, USA
	285.36	Unknown
	477.75	Colon of deer, Germany
	101024	Soil
	110763	Dung from white tailed deer
Penicillium confertum	171.87	Cheek pouch of *Dipodomys spectabilis* (Kangaroo rat), USA
Penicillium coprobium	185.88	Pig feed
	280.97	Barley, Denmark
	561.90	Pig feed, Norway
	562.90	Unknown, Czech Republic
	110761	Grass root, mangrove
Penicillium coprophilum	186.89	*Andropogon sorghum*, Denmark
	473.75	*Zea mays*
	102444	Woodchip paper behind skirting board, Germany
	110757	*Styra cifolia* (liquid amber)
	110760	Rabbit dung, Netherlands
Penicillium crustosum	181.89	Soil with *Agaricus bisporus*

	471.84	Thymus species, Denmark
	101025	Cheese, Portugal
	110074	Lechuguila Cave
	110077	Deteriorating preserved wood stakes
	313.48	*Oryza sativa*
Penicillium cyclopium	144.45	Fruit, Norway
	349.59	Soil
	475.84	Grain of *Hordeum vulgare*
	477.84	Grain of *Hordeum vulgare*, Denmark
	101136	Harness, Saudi Arabia
	110331	*Hordeum vulgare*
	110335	Cereals
	110337	Malting barley
Penicillium digitatum	136.65	Fruit of *Citrus medica limonium*, Netherlands
	319.48	Fruit of Citrus species
	101026	Chili mix, Indonesia
	112082	Citrus lemon
	112322	Soil
Penicillium dipodomyicola	173.87	Cheek pouch of *Dipodomys spectabilis*, USA
	110421	Soil under *Artemisia tridentate* (sage bush), USA
	110422	*Oryza sativa*, Australia
	110423	Soil under *Artemisia tridentate*
	110424	*Dipodomys spectabilis*
Penicillium dipodomyis	170.87	Cheek pouch of *Dipodomys spectabilis*, USA
	110412	Cheek pouch of *Dipodomys spectabilis*, USA
	110413	Barley, USA
	110414	*Dipodomys spectabilis*
	110415	Harness
Penicillium discolor	271.97	Acorn, Denmark
	278.97	Dairy cooling device, Denmark
	474.84	*Raphanus sativus*, Israel
	112557	Lechuguila cave
	112568	Cereal
Penicillium echinulatum	317.48	Culture contaminant, Canada
	337.59	Soil, Japan
	101027	Air, Denmark
	112286	Lemon grass
	112287	Cheddar cheese
Penicillium expansum	281.97	Chilled food, Denmark

	481.84	*Brassica oleracea*, Denmark
	110402	Lime quarry
	110403	Spoiled margarine
	325.48	Fruit of *Malus sylvestris*, ex-neotype strain, USA
Penicillium flavigenum	419.89	Flour, Denmark
	110406	Soil under *Chrysothmnus nauseosus*, USA
	110407	White beans, USA
	110409	Mud from evacuation
	211.92	Soil, Taiwan
Penicillium freii	794.95	Chicken feed, Denmark
	796.95	*Hordeum vulgare*
	101486	Barley, South Africa
	112022	Barley
	112290	Wheat
	112291	Wheat
	112292	Barley, Denmark
	112293	Pig feed
Penicillium gladioli	214.28	*Gladiolus* species
	278.47	Corm of *Gladiolus* species, UK
	332.48	Corm of *Gladiolus* species, imported from the Netherlands, USA
	101029	Corm of *Gladiolus* species
Penicillium glandicola	333.48	Soil, USA
	498.75	Mouldy wine cork, Portugal
	111218	Soil, Switzerland
	111221	Wood
	112317	Unknown
Penicillium griseofulvum	485.84	Grain of *Hordeum vulgare*, Denmark
	110416	Malting barley
	110419	*Triticum aestivum*
	110420	*Zea mays* seed, Bulgaria
	185.27	Ex-neotype isolate, Belgium
Penicillium hirsutum	135.41	Aphid, green fly, Netherlands
	349.75	Bulb of *Tulipa* species, Netherlands
	437.92	External of *Tulipa* species
	110098	Butter
	110100	Root of horse radish, USA
	117774	Bulb of *Tulipa* species
Penicillium hordei	473.84	*Lycopersicon esculentum*
	559.90	Fern in greenhouse
	560.90	*Hordeum vulgare*
	704.68	Grain of *Hordeum vulgare*,

		Netherlands
	788.70	Cereal, UK
	701.68	Grain of *Hordeum vulgare*, ex-neotype strain, Denmark
Penicillium italicum	278.58	Fruit of *Citrus sinensis*, Netherlands
	339.48	Fruit of Citrus species, USA
	489.84	*Raphanus sativus*, Israel
Penicillium marinum	109545	Unknown
	109546	Unknown
	109547	Sandy soil, Tunisia
	109548	Unknown
	109549	Sandy soil, Tunisia
	109550	Sandy soil, Japan
Penicillium melanoconidium	218.90	*Hordeum vulgare*, Denmark
	640.95	*Panicum miliaceum* imported to Denmark, Unknown origin
	641.95	Mixed cereal feed for birds
	109542	Salami
	109603	Unknown
Penicillium mononematosum	172.87	Seed of *Amaranthus* species that was badly contaminated with mold, USA
	109616	Salt marsh soil
	112104	*Dipodomys spectabilis*, USA
	112105	Squash
	112106	Jerusalem artichoke
	112575	Salt marsh soil
Penicillium nalgiovense	297.97	Sandy soil
	318.92	Sausage, imported from Italy, Denmark
	352.48	Ellischauer cheese, Czech Republic
	109610	Salami
Penicillium neoechinulatum	1101135	Cheek pouch of *Dipodomys spectabilis*, USA
	101472	Cheek pouch of *Dipodomys spectabilis*
	110343	Seed cache*, Dipodomys spectabilis*, USA
Penicillium nordicum	109535	Serano ham
	109536	Air in meat packing plant
	109537	Jam, Japan
	109538	Fish feed, Denmark
	109541	Lumpsucker (*Cycloptenus lumpus* eggs), Denmark
	110770	Sausage, Germany
	110771	Sausage

	112565	Sausage
	112573	Salami, Italy
Penicillium olsonii	312.97	Forest soil
	349.61	Rubber life-raft, Netherlands
	381.75	*Fragaria* species, Netherlands
	833.88	Cactus pot soil, Denmark
	232.60	Root of *Picea abies*, ex-neotype strain, Austria
Penicillium palitans	101031	Cocoa, Japan
	111834	*Dipodomys spectabilis* mound
	112204	Unknown, Russia
	115507	Unknown, Japan
Penicillium paneum	464.95	Rye bread (non preserved), Denmark
	465.95	Mouldy baker's yeast, Denmark
	101032	Mouldy rye bread, Denmark
	112294	Unknown
	112296	Cassava chips
Penicillium persicinum	111235	Soil, China
Penicillium polonicum	294.48	Unknown
	690.77	Air, Spain
	793.95	*Hordeum vulgare*
	101479	Foods, Bulgaria
	110332	Unknown
	112560	Rhizosphere of garlic
	222.28	Soil, ex-neotype isolate, Poland
Penicillium radicicola	109551	Soil, near waterfall , Iceland
	109553	Unknown
	109554	Onion, Denmark
	112425	Carrot, Denmark
	112430	*Armoracia rusticana* root, Denmark
Penicillium roqueforti	135.67	Blue cheese, Germany
	234.38	Blue Cheshire cheese, Unknown
	479.84	Mouldy baker's yeast, Denmark
	498.73	Fruit of *Malus sylvestris*
	221.30	Roquefort cheese, ex-neotype isolate, USA
Penicillium sclerotigenum	306.97	*Dioscori cayenensis*, Jamaica
	307.97	Blue yams, Philippines
	101033	Rotting tuber of *Dioscorea batatas*, Japan
	112566	Unknown
Penicillium solitum	143.86	Mouldy salami
	146.86	Fruit of *Malus sylvestris*, Denmark
	147.86	Fruit of *Malus sylvestris*, Denmark
	160.42	Unknown

	424.89	Unknown, Germany
	500.73	*Malus sylvestris*
	109827	Waste
Penicillium thymicola	111223	Soil, under *Populus augustifolia*
	111224	South Europe
	111225	Thyme, South Europe
	111226	Air of archive, Czech Republic
	111227	*Sorghum*, Sudan
Penicillium tricolor	635.95	*Triticum aestivum*, Canada
	636.95	*Triticum aestivum*, Canada
	637.95	*Triticum aestivum*, Canada
	101488	*Triticum aestivum*
Penicillium tulipae	407.92	*Tulipa* species
	734.74	Bulb of *Tulipa* species
	109555	Bulb of *Tulipa* species, Denmark
	111217	Leaf of *Tulip*, Denmark
	117773	Bulb of *Tulipa* species
Penicillium ulaiense	136.41	Fruit of *Citrus medica limonium*
	210.92	Skin of decaying orange, Taiwan
	262.94	Grapefruit, USA
	314.97	Apricot
	322.92	Lemon
Penicillium venetum	201.57	Bulb of *Hyacinthus* species, UK
	253.96	Asparagus, Netherlands
	405.92	Iris species, South Korea
	502.75	*Ornithogalum* species
	110094	Metal polluted soil
Penicillium verrucosum	223.71	White bean
	321.90	*Triticum aestivum*
	112485	*Hordeum vulgare*
	112488	Soil in spruce forest
	112577	*Hordeum vulgare*
	603.74	Unknown, Ex-neotype isolate, Belgium
Penicillium viridicatum	390.48	Air, USA
	101034	Beans, Bulgaria
	101475	Piece of a branch
	109826	*Zea mays*, Bulgaria
	112052	Corn seed
Penicillium vulpinum	126.23	Unknown
	295.65	Soil
	305.65	Insect
	488.84	*Hordeum vulgare*
	101133	Melon
	110772	Soil, India

5.2.2 Screening *P. chrysogenum* for Mating-Type Genes

5.2.2.1 *MAT1-1-1* and *MAT1-2-1* Amplification and Screening

A PCR diagnostic was devised to determine whether isolates of *P. chrysogenum* were of MAT1-1 or MAT1-2 genotype, based on the presence of the *MAT1-1-1* alpha-domain or *MAT1-2-1* HMG-domain encoding genes, respectively. Initial screening of *P. chrysogenum* isolates was performed using *MAT* gene-specific degenerate primers (section 2.2.4). Eight microlitre aliquots of the resulting PCRs were resolved on 2% agarose gels and the gels were visualised by ethidium bromide staining as described in section 2.2.4. Bands of interest were then excised from gels and purified as per section 2.2.8. Finally, the gel extracted products were cloned into *E. coli* via ligation in plasmid pTOPO4 and sequenced using M13 Forward and M13 Reverse primers (sections 2.2.9 to 2.2.12). Detection of *MAT1-1-1* and *MAT1-2-1* amplicons allowed sequencing of the entire *MAT1-1-1* and *MAT1-2-1* genes (see results section 5.3.2.2). This gene sequencing was then used to design specific primers ChM1F, ChM1R, ChM2F, ChM2R and ChM2R2. Primers ChM1F with ChM1R and ChM2F with ChM2R2 were then used to screen the 97 isolates for mating type utilizing the PCR protocol described below.

PCRs were performed using Abgene RedHot Taq DNA Polymerase (Abgene, UK). Each 25µl reaction contained ~10ng genomic DNA, 2.5µl 10X PCR Buffer, 1.5µl (18mM) $MgCl_2$, 0.5µl (10mM each) dNTPs, 2.5µl (10µM) of specific *MAT* forward primer, 2.5µl (10µM) of specific *MAT* reverse primer, 0.2µl Abgene RedHot Taq DNA Polymerase and ~14.3µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler using the following cycling parameters: an initial denaturation step at 94°C for 5 min; 30 cycles consisting of 1 min at 94°C, 1 min at an appropriate annealing temperature and 1 min at 72°C; followed by a final extension step at 72°C for 5 min, all steps used a ramp rate of 70°C/min. For screening of the *MAT1-1-1* gene, the primer pair ChM1F and ChM1R was used (Table 5.4), at an annealing temperature of 53.5°C. For screening of the *MAT1-2-1* gene, the primer pair ChM2F and ChM2R2 was used at an annealing temperature of 50°C (Table 5.4). Resultant PCR products were resolved on 2% agarose gels and visualised by ethidium bromide staining (section 2.2.4).

The list of the 92 worldwide isolates of *P. chrysogenum* used in *MAT* screening is provided in Table 5.11, DNA from these isolates was kindly provided by D. Henk and M. Fischer (Imperial College, University of London) as part of an ongoing research collaboration. These isolates had previously been typed by DNA sequencing, RAPD-PCR and/or RFLP methods to ensure against the presence of clonal isolates (Henk, personal communication and data not shown, see section 2.2.6). These 92 isolates were used in addition to the 5 isolates already obtained from J. Houbraken and R. Samson (Table 5.3).

5.2.2.2 Cloning of Idiomorph Region and Idiomorph Orientation

Whole Idiomorphic Region

Species-specific *SLA2* and *APN2* primers [ChSLA2-1, ChSLA2-2, ChAPN2-1 and ChAPN2-2 (Table 5.4)] were designed from gene fragments amplified using the protocol described in section 2.2.4 and sequenced using the protocols described in sections 2.2.8 to 2.2.12. The idiomorph region of *P. chrysogenum* was amplified using the *SLA2-APN2* positional PCR strategy as described in section 2.2.5. Primers ChAPN2-1 and ChSLA2-1 (Table 5.4) were used to amplify the whole idiomorph region from both MAT1-1 and MAT1-2 isolates, in PCRs utilising Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~40ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) ChSLA2-1 primer, 2.5µl (10µM) ChAPN2-1 primer, 0.25µl DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler using the following cycling parameters: an initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 94°C, 20 sec at 58°C and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min. Resultant PCR products were resolved on 0.8% agarose gels and visualised by ethidium bromide staining (section 2.2.5).

MAT-1 and MAT-2 Idiomorph Orientation

To determine the orientation of the *MAT1-1-1* gene and the *MAT1-2-1* gene relative to genes bordering the MAT-1 and MAT-2 idiomorphs, the *SLA2-APN2* positional PCR strategy, described in section 2.2.5, was used.

PCRs to determine the *MAT* gene orientations were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~20ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5µM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) of respective *MAT* primer, 2.5µl (10µM) of respective flanking gene primer, 0.25µl DNA Polymerase and ~12.5µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler using the following cycling parameters: an initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 94°C, 20 sec at an appropriate annealing temperature and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min. However, different conditions were used for specific PCRs. Thus, for the amplification of the MAT-1 idiomorph, primer pair ChM1F and ChSLA2-1 was used (Table 5.4) at an annealing temperature of 51.6°C with inclusion of 1% DMSO and primer pair ChM1R and ChAPN2-1 was used at an annealing temperature of 53.5°C also with inclusion of 1% DMSO. Whilst for the amplification of the MAT-2 idiomorph, primer pairs ChM2F with ChSLA2-2 and ChM2R2 with ChAPN2-2 were used at an annealing temperature of 50°C. Resultant PCR products were visualised on 0.8% agarose gels and visualised by ethidium bromide staining (section 2.2.5).

Table 5.4: *P. chrysogenum* Primer List

Primer	Sequence 5' to 3'
ChM1F	CGACGACTGGATGTGTTGGGCA
ChM1R	CCCATCAGAGGCAGGCAAAGCT
ChM2F	AGTCTTCCTGCCTCGCAATG
ChM2R	TGGACGAGGAACTTTTGCTGG
ChM2R2	ATGCTTGATAGCGTGTTCC
ChSLA2-1	AGGCTCTTATGGAACTGGAGGAACTG
ChSLA2-2	TTCGATTTCTGCATACTGACCATGTTCC
ChAPN2-1	TAGGATACTCGGGTGTTGTGATTTACACG
ChAPN2-2	TTGCGGGATGACATGGTCTTGGTTC
Acl Actin Forward	TTCCATTGTCGGTCGTCC
Acl Actin Reverse	ATCTTCATCAGGTAATCCGTCAG

5.2.2.3 RT-PCR Analysis

RT-PCR analysis was performed to determine whether gene expression and intron splicing of the putative *MAT* genes occurred in *P. chrysogenum*. Species-specific, gene-specific primers were designed using the sequencing data obtained during this study.

Where possible primer pairs were designed to span putative introns to confirm intron splicing. Samples were grown, RNA extracted and DNase treated using the protocols described in sections 2.2.13 to 2.2.15. RT-PCR was performed using the protocols in sections 2.2.16 and 2.2.17 and bands were resolved on 1.5% agarose gels and visualised by ethidium bromide staining (section 2.2.18).

To analyse the putative *MAT1-1-1* gene primer pair ChM1F and ChM1R was used (Table 5.4). This was predicted to yield a 340bp fragment from unspliced genomic DNA, and after splicing of a 48bp intron, predicted to yield a 292bp fragment from processed mRNA To analyse the putative *MAT1-2-1* gene primer pair ChM2F and ChM2R was used (Table 5.4). This was predicted to yield a 341bp fragment from unspliced genomic DNA and, after splicing a 53bp intron, predicted to yield a 289bp fragment from processed mRNA.

RT-PCR analysis of the constitutively expressed actin gene was included as a control. Actin primers were designed using *A. clavatus* actin sequencing data obtained from the Broad Institute genome sequencing project (http://www.broad.mit.edu) (gene locus ID: ACLA_095800). Primer pair Acl Actin Forward and Acl Actin Reverse was predicted to yield an approximate 560bp fragment from unspliced genomic DNA and an approximate 480bp fragment from processed mRNA, after splicing of ~80bp intron (Figures 5.4 and 5.12).

5.2.3 Screening of *P. griseofulvum* for Mating-Type Genes

5.2.3.1 *MAT1-2-1* Gene Amplification and Screening

A PCR diagnostic was devised to determine whether isolates of *P. griseofulvum* were of MAT1-1 or MAT1-2 genotype, based on the presence of the *MAT1-1-1* alpha-domain or *MAT1-2-1* HMG-domain encoding genes, respectively. 17 *P. griseofulvum* isolates were screened (Tables 5.3 and 5.5), using the degenerate primer PCR approach utilising *MAT1-1-1* gene degenerate primers MAT5-6 and MAT3-4 and *MAT1-2-1* gene degenerate primers MAT5-7 and MAT3-5 using the protocol described in section 2.2.4. DNA from additional *P. griseofulvum* isolates was kindly provided by J. Houbraken and R. Samson. Eight microlitre aliquots of the resulting PCRs were resolved on 2% agarose gels

and gels visualised by ethidium bromide staining as described in section 2.2.4. Bands of interest were then excised from gels and purified as per section 2.2.8. Finally, the gel extracted products were cloned into *E. coli* via ligation into plasmid pTOPO4 and sequenced using M13 Forward and M13 Reverse primers (sections 2.2.9 to 2.2.12). The arising sequence data allowed the design of a set of *P. griseofulvum MAT1-2-1* specific diagnostic primers (Table 5.6). Detection of *MAT1-2-1* amplicons allowed sequencing of the entire *MAT1-2-1* gene (see results section 5.2.3.2). This gene sequence was then used to design specific primers GrM2F and GrM2R (Table 5.5). These primers were then used to screen all 17 *P. griseofulvum* isolates to confirm their mating type, using the PCR protocol described below.

PCRs were performed using Abgene RedHot Taq DNA Polymerase (Abgene, UK). Each 25µl reaction contained ~10ng genomic DNA, 2.5µl 10X PCR Buffer, 1.5µl (18mM) $MgCl_2$, 0.5µl (10mM each) dNTPs, 2.5µl (10µM) GrM2F primer, 2.5µl (10µM) GrM2R primer, 0.2µl Taq Polymerase and ~14.3µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler using the following cycling parameters: an initial denaturation step at 94°C for 5 min; 35 cycles consisting of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C; followed by a final extension step at 72°C for 5 min, all steps used a ramp rate of 70°C/min.

Table 5.5: *P. griseofulvum* isolate list. These isolates were used in addition to those in Table 5.3.

Isolate Identification Code	Source of Isolate
CBS 118882	Soil of pepper field, Korea
CBS 118868	Soil of pepper field, Korea
CBS 112298	Wyoming, USA
CBS 110418	Russia
CBS 110417	Cereal grain from field, Denmark
CBS 100233	Soil, Nepal
CBS 493.75	Contents of deer colon, Germany
CBS 746.70	Soil, Italy
CBS 315.63	Grain elevator, South Africa
CBS 124.14	Soil, UK
51-C6	Processed clay
13-E7	Flour for tortillas in silo

5.2.3.2 Idiomorph Orientation

MAT1-2-1 Gene Orientation

Species-specific *SLA2* and *APN2* primers [GrSLA2 and GrAPN2 (Table 5.6)] were designed from gene fragments amplified using the protocol described in section 2.2.4 and sequenced using the protocols described in sections 2.2.8 to 2.2.12. To determine the orientation of the putative *MAT1-2-1* gene relative to the genes bordering the MAT-2 idiomorph, the *SLA2-APN2* positional PCR strategy, as described in section 2.2.5, was used.

PCRs to determine the *MAT1-2-1* gene orientations were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~20ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) of respective *MAT* primer, 2.5µl (10µM) of respective flanking gene primer, 0.25µl DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler using the following cycling parameters: an initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 94°C, 20 sec at an appropriate annealing temperature and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min. However, different conditions were used for specific PCRs. Thus for the amplification of the MAT-2 idiomorph, the primer pair GrM2F and GrSLA2 was used at an annealing temperature of 53°C and primer pair GrM2R and GrAPN2 was used at an annealing temperature of 55.3°C. Resultant PCR products were resolved on 0.8% agarose gels and visualised using ethidium bromide staining (section 2.2.5).

Table 5.6: *P. griseofulvum* Primer List

Primer	Sequence 5' to 3'
GrM2F	TACCGACAGGCAAACCATCAC
GrM2R	CCTCGGGGGTCTCATTGTTCC
GrSLA2	CGCAACAATGACCGCCAGG
GrAPN2	TATGAAGGCTACTCTGGTGTGG
ATe Actin Forward	ATTGTCGGTCGTCCCCG
ATe Actin Reverse	TCTGGGTCATCTTCTCACGG

5.2.3.3 RT-PCR Analysis

RT-PCR analysis was performed to determine whether gene expression and intron splicing of the putative *MAT1-2-1* gene occurred in *P. griseofulvum*. Species-specific, *MAT1-2-1* gene-specifc primers were designed using the sequence data obtained during this study.

The *MAT1-2-1* gene-specific primer pair was designed to span the putative intron to confirm intron splicing. Samples were grown, RNA extracted and DNase treated using the protocols described in sections 2.2.13 to 2.2.15. RT-PCR was performed using the protocols in sections 2.2.16 and 2.2.17, and resultant bands were resolved on 1.5% agarose gels and visualised using ethidium bromide staining (section 2.2.18).

To analyse the putative *MAT1-2-1* gene, primer pair GrM2F and GrM2R was used (Table 5.6). This was predicted to yield a 157bp fragment from unspliced genomic DNA and, after splcing of a 51bp intron, predicted to yield a 106bp fragment from processed mRNA .

RT-PCR analysis of the constitutively expressed actin gene was included as a control. Actin primers were designed using *A. terreus* actin sequencing data obtained from The Broad Institute genome sequencing project (gene locus ID: ATEG_06973.1). Primer pair Ate Actin Forward and Ate Actin Reverse was used (Table 5.6). This was predicted to yield an approximate 330bp amplicon from unspliced genomic DNA and an approximate 265bp amplicon from processed mRNA (after splicing of a ~65bp intron) (Tables 5.5 and 5.13).

5.2.4 Screening of *P. camemberti* for Mating-Type Genes

5.2.4.1 *MAT* Gene Amplification and Sequencing

The four isolates of *P. camemberti* listed in Table 5.3 were screened for mating type using the degenerate PCR strategy described in section 2.2.4, which determined whether isolates were of MAT1-1 or MAT1-2 identity based on the presence of a *MAT1-1-1* alpha-domain or *MAT1-2-1* HMG-domain encoding genes, respectively. Eight microlitre aliquots of the resulting PCRs were resolved on 2% agarose gels and gels were

visualised by ethidium bromide staining as described in section 2.2.4. Bands of interest were then excised from gels and purified as per section 2.2.8. Finally, the gel extracted products were cloned into *E. coli* containing plasmid pTOPO4 and sequenced using M13 Forward and M13 Reverse primers, as described in sections 2.2.9 to 2.2.12. The resulting sequence data allowed specific primers to be designed [CaM1F, CaM1R, CaM2F and CaM2R (Table 5.7)] for further study of the putative *MAT1-1-1* and *MAT1-2-1* genes of *P. camemberti*.

5.2.4.2 Idiomorph Orientation

MAT-1 and MAT-2 Idiomorph Orientation

Specific *SLA2* and *APN2* primers [CaSLA2-1, CaSLA2-2, CaAPN2-1 and CaAPN2-2 (Table 5.7)] were designed from gene fragments amplified using the protocol described in section 2.2.4 and sequenced using the protocols described in sections 2.2.8 to 2.2.12. To determine the orientation of the putative *MAT1-1-1* gene and the *MAT1-2-1* gene relative to the genes bordering the MAT-1 and MAT-2 idiomorphs, the *SLA2-APN2* positional PCR strategy, as described in section 2.2.5, was used.

PCRs to determine *MAT* gene orientations were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~20ng genomic DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) of respective *MAT* primer, 2.5µl (10µM) of respective flanking gene primer, 0.25µl DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler using the following cycling parameters: an initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 94°C, 20 sec at an appropriate annealing temperature and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min. Resultant PCR products were resolved on 0.8% agarose gels and visualised by ethidium bromide staining (section 2.2.5). However, different conditions were used for specific PCRs. Thus for the amplification of the MAT-1 idiomorph primer pairs CaM1F with CaSLA2-1 and CaM1R with CaAPN2-1 were used at an annealing temperature of 50°C (Table 5.7). To amplify the MAT-2 idiomorph, primer pair CaM2F and CaSLA2-2 was used at an

annealing temperature of 50°C and primer pair CaM2R and CaAPN2-2 was used at an annealing temperature of 60.1°C (Table 5.7).

Table 5.7: *P. camemberti* Primer List

Primer	Sequence 5' to 3'
CaM1F	CGGCCGCTGAACTGGTTTATAG
CaM1R	GCCGGACTTCGCTTTCTGAG
CaM2F	TCTTTATCGGCAAGCACATCATCCA
CaM2R	TTCTCAGTTTCCGCGTTCCATCG
CaSLA2-1	TTGCTCAAGTTTGTGATTAATGCTG
CaSLA2-2	GCGAGAAGGACGAGGAGATTGAGG
CaAPN2-1	AGCGGAAAGATTTGCGGGATG
CaAPN2-2	TTGCGGGATGACATGGTCTTGGTTC
Acl Actin Forward	TTCCATTGTCGGTCGTCC
Acl Actin Reverse	ATCTTCATCAGGTAATCCGTCAG

5.2.4.3 RT-PCR Analysis

RT-PCR analysis was performed to determine whether gene expression and intron splicing of the putative *MAT* genes occurred in *P. camemberti*. Species-specific, gene-specific primers were designed using the sequence data obtained during this study.

Where possible primer pairs were designed to span introns to confirm intron splicing. Samples were grown, RNA extracted and DNase treated using the protocols described in sections 2.2.13 to 2.2.15. RT-PCR was performed using the protocols described in sections 2.2.16 and 2.2.17, and resultant bands were resolved on 1.5% agarose gels and visualised by ethidium bromide staining (section 2.2.18).

To analyse the putative *MAT1-1-1* gene, primer pair CaM1F and CaM1R was used (Table 5.7). This was predicted to yield a 132bp fragment from unspliced genomic DNA and, after splicing of 51bp intron, predicted to yield a 87bp fragment from processed mRNA To analyse the putative *MAT1-2-1* gene, primer pair CaM2F and CaM2R was used (Table 5.7). This was predicted to yield a 154bp fragment from unspliced genomic DNA and, after splicing of a 46bp intron, predicted to yield a 108bp fragment from processed mRNA

RT-PCR analysis of the constitutively expressed actin genes was included as a control. Actin primers were designed using *A. clavatus* actin sequencing data obtained from The

Broad Institute genome sequencing project (gene locus ID: ACLA_095800). Primer pair Acl Actin Forward and Acl Actin Reverse was predicted to yield an approximate 560bp fragment from unspliced genomic DNA and an approximate 480bp fragment from processed mRNA (after splicing of ~80bp intron) (Tables 5.7 and 14).

5.2.5 Screening of *P. roqueforti* for Mating-Type Genes

5.2.5.1 *MAT* Gene Amplification and Sequencing

The five isolates of *P. roqueforti* listed in Table 5.3 were screened for mating type using the degenerate PCR strategy described in section 2.2.4, which determined whether isolates were of MAT1-1 or MAT1-2 genotype, based on the presence of a *MAT1-1-1* alpha-domain or *MAT1-2-1* HMG-domain encoding gene, respectively. Eight microlitre aliquots of the resulting PCRs were resolved on 2% agarose gels and gels were visualised by ethidium bromide staining as described in section 2.2.4. Bands of interest were then excised from gels and purified as per section 2.2.8. Finally, the gel extracted products were cloned into *E. coli* via ligation into plasmid pTOPO4 and sequenced using M13 Forward and M13 Reverse primers, as described in sections 2.2.9 to 2.2.12. The resulting sequence data allowed specific primers to be designed [RoM1F, RoM1R, RoM2F and RoM2R (Table 5.8)] for further study of the putative *MAT1-1-1* and *MAT1-2-1* genes of *P. roqueforti*.

5.2.5.2 Idiomorph Orientation

MAT-1 and MAT-2 Idiomorph Orientation

Specific *SLA2* and *APN2* primers [RoSLA2-1, RoSLA2-2, RoAPN2-1 and RoAPN2-2 (Table 5.8)] were designed from gene fragments amplified using the protocol described in section 2.2.4 and sequenced using the protocols described in sections 2.2.8 to 2.2.12. To determine the orientation of the *MAT1-1-1* gene and the *MAT1-2-1* gene relative to the genes bordering the MAT-1 and MAT-2 idiomorphs, the *SLA2-APN2* positional PCR strategy, as described in section 2.2.5, was used.

PCRs to determine *MAT* gene orientations were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25µl reaction contained ~20ng genomic

DNA, 5µl 5X PCR Buffer (containing 7.5mM $MgCl_2$), 1µl (10mM each) dNTPs, 2.5µl (10µM) of respective *MAT* primer, 2.5µl (10µM) of respective flanking gene primer, 0.25µl DNA Polymerase and ~12.75µl H_2O. The amplification of fragments was performed on a Techne Genius thermal cycler using the following cycling parameters: an initial denaturation step at 98°C for 2 min; 30 cycles consisting of 10 sec at 94°C, 20 sec at an appropriate annealing temperature and 4 min at 72°C; followed by a final extension step at 72°C for 10 min, all steps used a ramp rate of 70°C/min. Resultant PCR products were resolved on 0.8% agarose gels and visualised by ethidium bromide staining (section 2.2.5). Different conditions were used for specific PCRs. Thus, for the amplification of the MAT-1 idiomorph, primer pair RoM1F and RoSLA2-1 was used at an annealing temperature of 50°C and primer pair RoM1R and RoAPN2-1 was used at an annealing temperature of 60.1°C (Table 5.8). To amplify the MAT-2 idiomorph, primer pair RoM2F and RoSLA2-2 was used at an annealing temperature of 51.6°C and primer pair RoM2R and RoAPN2-2 was used with inclusion of 5% DMSO at an annealing temperature of 50°C (Table 5.8).

Table 5.8: *P. roqueforti* Primer List

Primer	Sequence 5' to 3'
RoM1F	TGGAGGCCGTTGAACAGGTTC
RoM1R	GAGGATGCCGGACTTCGCT
RoM2F	CCGCCAACATCATCATCCTAAGC
RoM2R	GATTCTGCCTTCCACTGTTTCCCT
RoSLA2-1	TATGGAACAGGCTCTTATGGAACTGGAGG
RoSLA2-2	TTGCTGAGATGTGTGATTTATGCTG
RoAPN2-1	AGCGGAAGGATTTGCGGGATG
RoAPN2-2	TTGCGGGATGATATGGTCTTGGTTC
A. cl Actin Forward	TTCCATTGTCGGTCGTCC
A. cl Actin Reverse	ATCTTCATCAGGTAATCCGTCAG

5.2.5.3 RT-PCR Analysis

RT-PCR analysis was performed to determine whether gene expression and intron splicing of the putative *MAT* genes occurred in *P. roqueforti*. Species-specific, gene-specific primers were designed using the sequence data obtained in this study.

Where possible primer pairs were designed to span putative introns to confirm intron splicing. Samples were grown, RNA extracted and DNase treated using the protocols

described in sections 2.2.13 to 2.2.15. RT-PCR was performed using the protocols in sections 2.2.16 and 2.2.17 and resultant bands were resolved on 1.5% agarose gels and visualised by ethidium bromide staining (section 2.2.18).

To analyse the putative *MAT1-1-1* gene, primer pair RoM1F and RoM1R was used (Table 5.8). This was predicted to yield a 141bp fragment from unspliced genomic DNA and, after splicing a 51bp intron, predicted to yield a 90bp fragment from processed mRNA. To analyse the putative *MAT1-2-1* gene, primer pair RoM2F and RoM2R was used (Table 5.8). This was predicted to yield a 158bp fragment from unspliced genomic DNA and, after splicing of a 62bp intron, predicted to yield a 96bp fragment from processed mRNA.

RT-PCR analysis of the constitutively expressed actin gene was included as a control. Actin primers were designed using *A. clavatus* actin sequencing data obtained from The Broad Institute genome sequencing project (gene locus ID: ACLA_095800). Primer pair Acl Actin Forward and Acl Actin Reverse was predicted to yield an approximate 560bp fragment from unspliced genomic DNA and an approximate 480bp fragment from processed mRNA, after splicing of a ~80bp intron (Tables 5.8 to 5.15).

5.2.6 Attempted Induction of Sexual Reproduction in *Penicillium* Species

Attempts were made to induce a sexual cycle of *P. chrysogenum, P. camemberti* and *P. roqueforti* (Table 5.9) under conditions known to induce sex in *N. fumigata* (O'Gorman *et al.* 2009) and described in section 2.2.13, Figure 2.1 B and C.

Hoff *et al*. (2008) suggested that a reason for an apparent lack of a sexual cycle in *P. chrysogenum* was the use of repeatedly subcultured isolates with a consequent decrease in fertility. For this reason 'fresh' isolates that had not been subcultured (apart from initial culture into liquid media followed by inoculation of 'sexual' plates) were used. These were isolates 088B, 087B (both isolated in Lyon, France, February 2008), 0820 (isolated in Grenoble, France, February 2008) and 0816A (isolated in Toronto, Canada, February 2008), these isolates were provided by D. Henk and M. Fisher (Imperial College, University of London) as part of an ongoing research collaboration.

Table 5.9: *Penicillium* isolates used for investigation into the occurrence of the sexual cycle and method of crossing.

Species	MAT1-1 isolate used	MAT1-1 isolate used	MAT1-2 isolate used	MAT1-2 isolate used	Plugs or mixing of isolates
P. chrysogenum	088B	/	087B	/	Mixing
P. chrysogenum	0820A	/	0816A	/	Mixing
P. chrysogenum	B2	B8	CBS 775.95	CBS776.95	Plugs
P. chrysogenum	B11	G4	E10	F12	Plugs
P. chrysogenum	B2	/	CBS 775.95	/	Mixing
P. roqueforti	CBS 135.65	/	CBS 221.3	/	Plugs
P. roqueforti	CBS 135.65	/	CBS 221.3	/	Mixing
P. camemberti	CBS 190.67	/	CBS 122325	/	Plugs
P. camemberti	CBS 190.67	/	CBS 112325	/	Mixing

5.2.7 Phylogenetic Analyses

The nucleotide and amino acid sequences obtained in this study from four *Penicillium* species were subjected to phylogenetic analyses. Alignments of sequences were performed using Clustal W (Thompson *et al.* 1994). Maximum parsimony phylogenetic trees were generated using MEGA version 4 (default parameters were used, with 500 bootstrap replicates and gaps excluded, section 2.2.20) (Tamura *et al.* 2007).

5.3 Results

5.3.1 Screening of the Subgenus *Pencillium* for Mating-Type Genes

The degenerate primer sets MAT5-6 with MAT3-4 and MAT5-7 with MAT3-5 (section 2.2.4) were successfully able to amplify putative *MAT* gene fragments from 52 of the 58 test species of Penicillia (Table 5.9). *MAT1-1-1* amplicons were predicted to be ~150bp and the *MAT1-2-1* amplicons were predicted to be ~270bp in length. Forty four species produced only *MAT1-2-1* gene fragments, two species produced *MAT1-1-1* gene fragments only and three species (*P. camemberti, P. roqueforti* and *P. verucosum*) had isolates that produced either *MAT1-1-1* or *MAT1-2-1* amplicons i.e. the latter exhibited a 'heterothallic' organisation (Table 5.10). Intriguingly, the three remaining species (*P. atramentosum, P. expansum* and *P. olsonii*) had isolates that produced both *MAT1-1-1*

and *MAT1-2-1* amplicons, i.e. resembling a 'homothallic' organisation. The final 6 species failed to amplify *MAT1-1-1* or *MAT1-2-1* gene fragments. Representative results of the *MAT* PCR screening are shown in Figures 5.5 to 5.11.

Table 5.10: Results of *MAT* screening of the 301 isolates from 58 *Penicillium* species from the subgenus *Penicillium*. Figures in table indicate number of isolates, figures in brackets are percentages of total.

Species	MAT1-1 genotype[a]	MAT1-2 genotype[b]	No *MAT* gene detected	Homothallic or Heterothallic[c]
Penicillium aethiopicum		8 (100)		
Penicillium albocoremium			6 (100)	
Penicillium allii		6 (100)		
Penicillium antarcticum			6 (100)	
Penicillium atramentosum	7 (100)	7 (100)		Homothallic
Penicillium aurantiogriseum		5 (100)		
Penicillium bialowiezense			7 (100)	
Penicillium brevicompactum		6 (100)		
Penicillium camemberti	2 (50)	2 (50)		Heterothallic[d]
Penicillium carneum		4 (100)		
Penicillium caseifulvum		5 (100)		
Penicillium cavernicola		4 (100)		
Penicillium chrysogenum		5 (100)		
Penicillium clavigerum		5 (100)		
Penicillium commune		4 (100)		
Penicillium concentricum		6 (100)		
Penicillium confertum		1 (100)		
Penicillium coprobium		5 (100)		
Penicillium coprophilum		5 (100)		
Penicillium crustosum		6 (100)		
Penicillium cyclopium		8 (100)		
Penicillium digitatum	5 (100)			
Penicillium dipodomyicola		5 (100)		
Penicillium dipodomyis		5 (100)		
Penicillium discolor		5 (100)		
Penicillium echinulatum		5 (100)		
Penicillium expansum	5 (100)	5 (100)		Homothallic
Penicillium flavigenum		5 (100)		
Penicillium freii		8 (100)		
Penicillium gladioli	4 (100)			
Penicillium glandicola		5 (100)		
Penicillium griseofulvum		5 (100)		
Penicillium hirsutum		6 (100)		
Penicillium hordei		6 (100)		

Penicillium italicum			3 (100)	
Penicillium marinum		6 (100)		
Penicillium melanoconidium			5 (100)	
Penicillium mononematosum		6 (100)		
Penicillium nalgiovense		4 (100)		
Penicillium neoechinulatum			3 (100)	
Penicillium nordicum		9 (100)		
Penicillium olsonii	5 (100)	5 (100)		Homothallic
Penicillium palitans		4 (100)		
Penicillium paneum		5 (100)		
Penicillium persicinum		1 (100)		
Penicillium polonicum		7 (100)		
Penicillium radicicola		5 (100)		
Penicillium roqueforti	2 (40)	3 (60)		Heterothallic[e]
Penicillium sclerotigenum		4 (100)		
Penicillium solitum		7 (100)		
Penicillium thymicola		5 (100)		
Penicillium tricolor		4 (100)		
Penicillium tulipae		5 (100)		
Penicillium ulaiense		5 (100)		
Penicillium venetum		5 (100)		
Penicillium verrucosum	3 (50)	3 (50)		Heterothallic[f]
Penicillium viridicatum		5 (100)		
Penicillium vulpinum		6 (100)		

[a]Indicates *MAT1-1-1* ~150bp amplicon produced. [b]Indicates *MAT1-2-1* ~270bp amplicon produced. [c]Indicates whether species has a heterothallic (both *MAT* genes present in different isolates) or homothallic (both *MAT* genes present in the same isolate) *MAT* configuration. [d]*P. camemberti* isolates CBS 190.67 and CBS 112078 were of MAT1-1 genotype, isolates CBS 112325 and CBS 112562 were MAT1-2 genotype. [e]*P. roqueforti* isolates CBS 135.67 and CBS 239.38 were MAT1-1 genotype, whilst isolates CBS 221.30, CBS 479.84 and CBS 4987.73 were MAT1-2 genotype. [f]*P. verrucosum* isolates CBS 112577, CBS 112488 and CBS 603.74 were MAT1-1 genotype, whilst CBS 223.71, CBS 321.90 and CBS 112485 were MAT1-2 genotype.

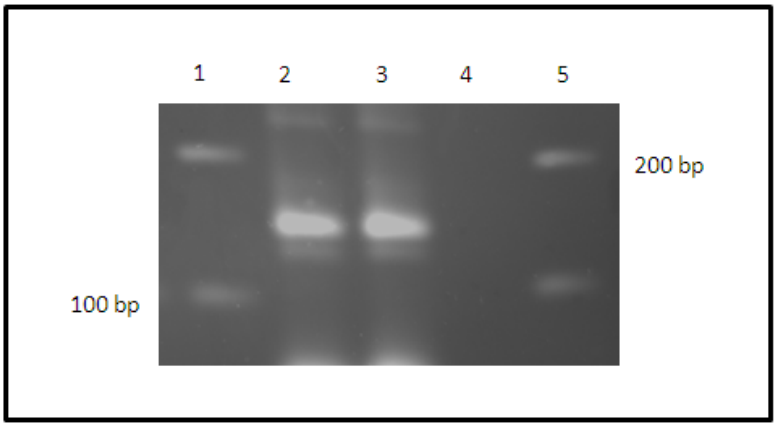

Figure 5.5: 2% agarose gel showing representative results of *MAT* PCR screening of *Penicillium* species that amplify only a *MAT1-1-1* gene fragment. Lanes 1 and 5: 100bp ladder. Lane 2: *P.*

digitatum CBS 136.65. Lane 3: *P. gladioli* CBS 214.28. Lane 4: Water control with MAT5-6 and MAT3-4 primers only.

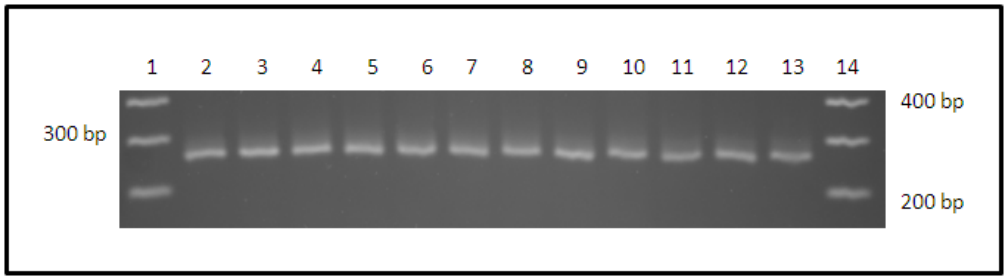

Figure 5.6: 2% agarose gel showing representative results of *MAT* PCR screening of *Penicillium* species that amplify only a *MAT1-2-1* gene fragment. Lanes 1 and 14: 100bp ladder. Lane 2: *P. albocoremium* CBS 109583. Lane 3: *P. allii* CBS 109578. Lane 4: *P. aurantiogriseum* CBS 792.95. Lane 5: *P. brevicompactum* CBS 317.59. Lane 6: *P. carneum* CBS 449.78. Lane 7: *P. caseifulvum* CBS 101134. Lane 8: *P. cavernicola* CBS 100540. Lane 9: *P. chrysogenum* CBS 776.95. Lane 10: *P. clavigerum* CBS 189.89. Lane 11: *P. commune* CBS 341.59. Lane 12: *P. concentricum* CBS 185.89. Lane 13: *P. confertum* CBS 171.87.

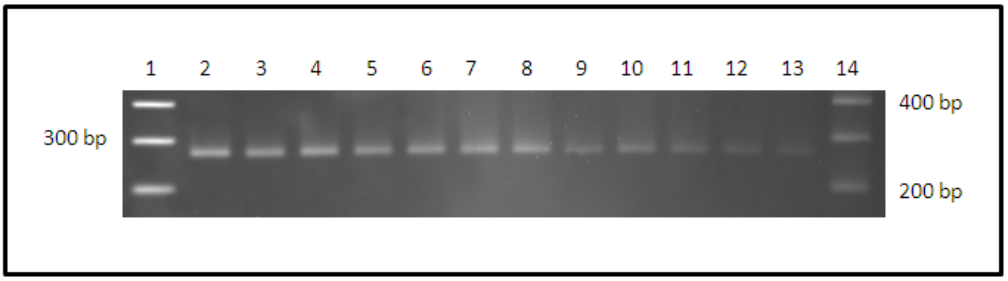

Figure 5.7: 2% agarose gel showing representative results of *MAT* PCR screening of *Penicillium* species that amplify only a *MAT1-2-1* gene fragment. Lanes 1 and 14: 100bp ladder. Lane 2: *P. coprobium* CBS 280.97. Lane 3: *P. coprophilum* CBS 186.89. Lane 4: *P. crustosum* CBS 10077. Lane 5: *P. cyclopium* CBS 101136. Lane 6: *P. dipodomyicola* CBS 173.87. Lane 7: *P. dipodomyis* CBS 110412. Lane 8: *P. discolor* CBS 271.97. Lane 9: *P. echinulatum* CBS 317.48. Lane 10: *P. flavigenum* CBS 110409. Lane 11: *P. formanosum* CBS 211.92. Lane 12: *P. freii* CBS 794.95. Lane 13: *P. glandicola* CBS 333.48.

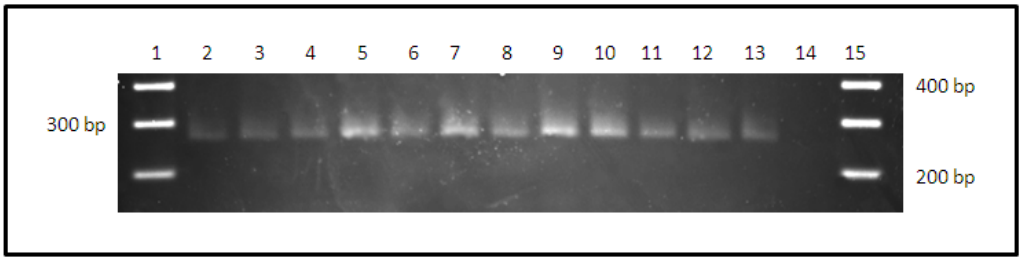

Figure 5.8: 2% agarose gel showing representative results of *MAT* PCR screening of *Penicillium* species that amplify only a *MAT1-2-1* gene fragment. Lanes 1 and 15: 100bp ladder. Lane 2: *P. griseofulvum* CBS 110420. Lane 3: *P. hirsutum* CBS 110098. Lane 4: *P. hordei* CBS 559.90. Lane 5: *P. marinum* CBS 109545. Lane 6: *P. mononematosum* CBS 172.87. Lane 7: *P. nalgiovense* CBS 318.92. Lane 8: *P. nordicum* CBS 109541. Lane 9: *P. palitans* CBS 111834. Lane 10: *P. paneum* CBS 465.95. Lane 11: *P. persicinum* 111235. Lane 12: *P. polonicum* CBS 793.95. Lane 13: *P. radicicola* CBS 109551. Lane 14: water control MAT5-7 and MAT3-5 primers only.

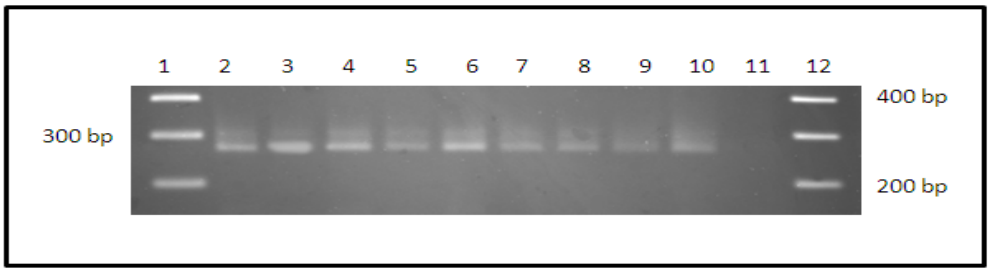

Figure 5.9: 2% agarose gel showing representative results of *MAT* PCR screening of *Penicillium* species that amplify only a *MAT1-2-1* gene fragment. Lanes 1 and 12: 100bp ladder. Lane 2: *P. sclerotigenum* CBS 306.97. Lane 3: *P. solitum* CBS 143.86. Lane 4: *P. thymicola* 111223. Lane 5: *P. tricolor* CBS 635.95. Lane 6: *P. tulipae* CBS 117773. Lane 7: *P. ulaiense* CBS 210.92. Lane 8: *P. venetum* CBS 201.57. Lane 9: *P. viridicatum* CBS 390.48. Lane 10: *P. vulpinum* CBS 110772. Lane 11: water control containing MAT5-7 and MAT3-5 primers only.

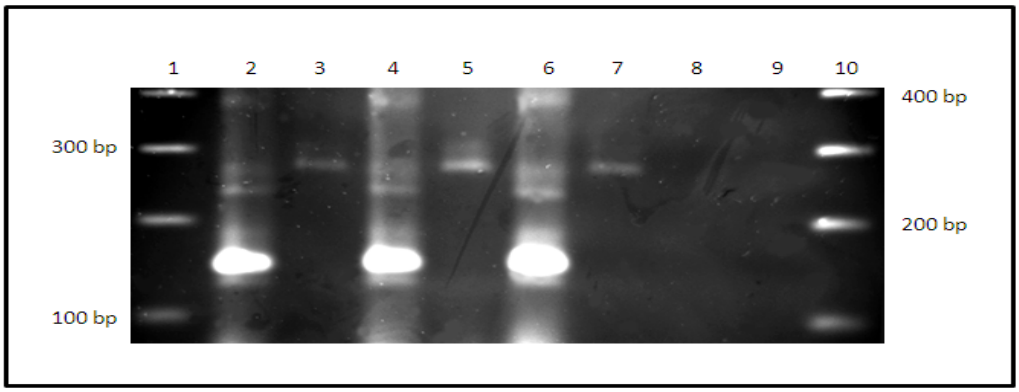

Figure 5.10: 2% agarose gel showing results of *MAT* PCR screening of *Penicillium* species that amplify both *MAT1-1-1* and *MAT1-2-1* gene fragments from the same isolate, i.e. homothallic arrangement. Lanes 1 and 10: 100bp ladder. Lane 2: *P. atramentosum* CBS 109612 *MAT1-1-1*. Lane 3: *P. atramentosum* CBS 109612 *MAT1-2-1*. Lane 4: *P. exspansum* CBS 110403 *MAT1-1-1*. Lane 5: *P. exspansum* CBS 110403 *MAT1-2-1*. Lane 6: *P. olsonii* CBS 232.60 *MAT1-1-1*. Lane 7: *P. olsonii* CBS 232.60 *MAT1-2-1*. Lane 8: Water control containing MAT5-6 and MAT3-4 primers only. Lane 9: Water control containing MAT5-7 and MAT3-5 primers only.

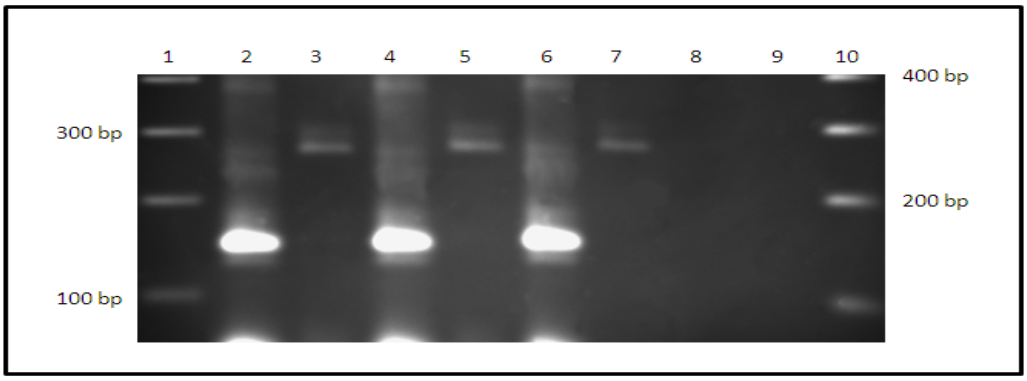

Figure 5.11: 2% agarose gel showing results of *MAT* screening of *Penicillium* species that amplify *MAT1-1-1* and *MAT1-2-1* genes in different isolates i.e. heterothallic arrangement. Lanes 1 and 10: 100bp ladder. Lane 2: *P. camemberti* CBS 190.67 *MAT1-1-1*. Lane 3: *P. camemberti* CBS

112325 *MAT1-2-1*. Lane 4: *P. roqueforti* CBS 135.67 *MAT1-1-1*. Lane 5: *P. roqueforti* CBS 221.30 *MAT1-2-1*. Lane 6: *P. verrucosum* CBS 112577 *MAT1-1-1*. Lane 7: *P. verrucosum* CBS 223.71 *MAT1-2-1*. Lane 8: Water control containing MAT5-6 and MAT3-4 primers only. Lane 9: Water control containing MAT5-7 and MAT3-5 primers only.

5.3.2 *Penicillium chrysogenum*

5.3.2.1 *MAT* Gene Screening

A 150bp putative *MAT1-1-1* gene fragment was amplified from certain isolates of *P. chrysogenum* using degenerate primers MAT5-6 and MAT3-4 (section 2.2.4). This allowed the isolation of the entire putative *MAT1-1-1* gene sequence (section 5.3.2.2). A 270bp putative *MAT1-2-1* gene fragments was successfully amplified from other isolates of *P. chrysogenum* using degenerate primers MAT5-7 and MAT3-5 (section 2.2.4). This allowed the isolation of the entire putative *MAT1-2-1* gene sequence (section 5.3.2.2). These genes' sequences allowed the design of a PCR diagnostic to determine the mating type of isolates (Table 5.11) (see section 5.2.2.1).

Of the 97 isolates screened, 51 isolates produced a *MAT1-1-1* gene fragment only, and 46 isolates produced a *MAT1-2-1* gene fragment only i.e. a ratio of 52.6% MAT1-1: 47.4% MAT1-2 (Tables 5.3, 5.10 and 5.11). These ratios are not significantly different (using Chi-squared, x^2=0.258, p=0.6117, 1 degree of freedom), therefore *P. chrysogenum* appears to have a 1:1 worldwide distribution and there was no apparent geographical clustering of the putative *MAT1-1-1* or *MAT1-2-1* genes.

Table 5.11: *P. chrysogenum* isolates used in *MAT* screening.

Culture Identification Code[a]	Source of Isolate	MAT genotype[b]
A1	Uraguay	1-2
A2	Arizona, USA	1-1
A3	France	1-1
A4	Arizona, USA	1-1
A5	Portugal	1-1
A6	Arizona, USA	1-1
A7	Canada	1-1
A8	Canada	1-1
A9	USA	1-1
A10	Canada	1-1
A11	Arizona, USA	1-1

B1	Argentina	1-1
B2	Ohio, USA	1-1
B3	Kentucky, USA	1-1
B4	Syria	1-1
B5	USA	1-1
B6	Arizona, USA	1-1
B7	Sweden	1-1
B8	Solomon Islands	1-1
B9	Pacific Ocean	1-1
B10	Pacific Ocean	1-1
B11	Arizona, USA	1-1
B12	Arizona, USA	1-1
C1	Arizona, USA	1-1
C2	UK	1-2
C3	Minesota, USA	1-1
C4	Ohio, USA	1-1
C5	Belgium	1-2
C6	Arizona, USA	1-1
C7	Florida, USA	1-1
C8	New Guinea	1-1
C9	Michigan, USA	1-2
C10	Unknown	1-2
C11	Denmark	1-2
C12	Atlantic Ocean	1-2
D1	Arizona, USA	1-1
D2	Arizona, USA	1-2
D3	Oregon, USA	1-2
D4	UK	1-2
D5	Portugal	1-2
D6	Arizona, USA	1-2
D7	Canada	1-2
D8	Canada	1-2
D9	Denmark	1-2
D10	Illinois	1-2
D11	Argentina	1-2
D12	Arizona, USA	1-2
E1	Massachusetts, USA	1-2
E2	Georgia, USA	1-2
E3	Argentina	1-1
E4	Arizona, USA	1-2
E5	Arizona, USA	1-1
E6	Oklahoma, USA	1-2
E8	Arizona, USA	1-1
E9	Denmark	1-2
E10	Pacific Ocean	1-2
E11	Arizona, USA	1-2

E12	Canada	1-2
F1	Ohio, USA	1-2
F2	Ohio, USA	1-1
F3	UK	1-1
F4	California, USA	1-2
F5	Arizona, USA	1-2
F6	Arizona, USA	1-2
F7	New Guinea	1-2
F8	Arizona, USA	1-2
F9	Arizona, USA	1-1
F10	Utah, USA	1-1
F11	Arizona, USA	1-2
F12	Utah, USA	1-2
G1	Ohio, USA	1-1
G2	Ohio, USA	1-1
G3	Illinois, USA	1-2
G4	California, USA	1-1
G5	Arizona, USA	1-2
G6	Arizona, USA	1-2
G7	Panama	1-1
G8	Florida, USA	1-1
G9	Arizona, USA	1-1
G10	Pacific Ocean	1-2
G11	Arizona, USA	1-1
G12	Arizona, USA	1-1
H1	Clinical Isolate, Unknown	1-2
H3	Norway	1-1
H4	Cameroon	1-1
H5	Arizona, USA	1-1
H6	Arizona, USA	1-1
H7	New Guinea	1-2
H8	Florida, USA	1-2
H10	Utah, USA	1-1
H11	Arizona, USA	1-1
H12	Arizona, USA	1-1

[a]Isolate identification used by D. Henk, and subsequently used for this study. [b]MAT genotype determined by the presence of a *MAT1-1-1* alpha domain (MAT1-1), or *MAT1-2-1* HMG domain (MAT1-2) encoding genes.

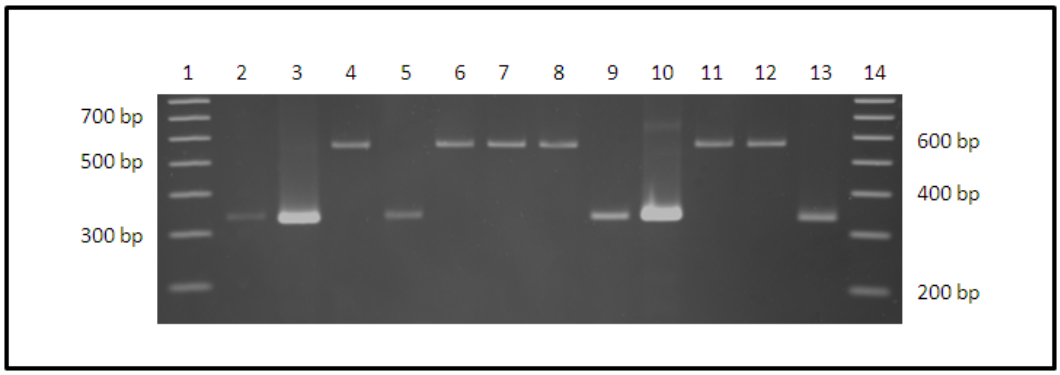

Figure 5.12: 2% agarose gel showing representative results of *MAT* PCR screening of *P. chrysogenum* isolates (Lanes 2-14). Lanes 1 and 14: 100bp ladder. Lane 2: B1. Lane 3: B2. Lane 4: D9. Lane 5: G4. Lane 6: F12. Lane 7: C11. Lane 8: F1. Lane 9: C8. Lane 10: A2. Lane 11: F5. Lane 12: F7. Lane 13: A7.

5.3.2.2 *MAT* Gene Isolation and Sequence Analysis

Using the *SLA2-APN2* positional PCR strategy (section 2.2.5), it was possible to clone and sequence the entire MAT-2 idiomorph region from *P. chrysogenum* isolate CBS 776.95. The MAT-1 idiomorph was sequenced until significant sequence homology (99-100%) to the MAT-2 idiomorph was reached, the MAT-1 idiomorph was sequenced from isolate B2. The primer pair PchAPN2 and PchSLA2 produced a PCR fragment of approximately 7kb in length (Figures 5.17 to 5.19). The idiomorphs were then analysed in order to determine whether complete *MAT* genes were present, including the alpha-domain and HMG-domain encoding sequences, necessary for function (for partial MAT-1 and full MAT-2 idiomorph sequences see Appendix 3, Figures 1 and 2).

Analysis of the MAT1-1-1 region from isolate B2 revealed a 1074bp open reading frame, which included one putative intron and was predicted to encode a 342 amino acid MAT1-1-1 protein (Figure 5.13). PSORT II (http://psort.nibb.ac.jp/) and TFSITESCAN (http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl) programs were used to interrogate resultant sequences for nuclear targeting and promoter region motifs. No clear nuclear targeting motifs were found in the putative MAT1-1-1 amino acid sequence, however a TATA-box motif was found 132bp upstream of the putative ATG translation start codon. The MAT1-1-1 translation start site could not be confirmed using the Kozak rule (Kozak 1986), although its position is consistent with other MAT1-1-1 proteins. A second possible ATG start codon was detected 19 amino acids inwards from the proposed

MAT1-1-1 start codon, which did obey the Kozak rule. However, this would have produced an unusually truncated MAT1-1-1 protein.

```
1     cgaaatcgtt ttttaatgag ccaccatgtt acgtcaaata tagtaagcat
51    tacacagaga agctaccagt tacgatctga attcccagcc tgaacatcca
101   ggtatacagt agtacctcgt tgtcgaaatg gtacatgatg ctgaagtacc
151   ttctgctgta gtcttatccc tccataattg actttctttg ttctaatggt
201   actattccta acaaggaaga cctttctagg ataaaaacat gtgattggtg
251   ttgttgctag gaaacaaaga aaatacgtgt ctcagctggc tctgtgattg
301   gtgttgccgc taaacaaaga aatcgcccac ctcgtcacat cacctaccgt
351   gttctgcagt cccaggtcct atatttgttt acccccccaca ttgtttcttt
401   cttttctttc ttctctcctc tccacccttc ttctttttt ttcacccgac
451   acctttttcat tcatattctc ttcgctctga acccttacta cattgttcaa
      |M||S||T|| S||L||D||A ||S||V||P| |P||G||Y|| G||P||A||H
501   atgtctacct ctcttgatgc ttcggtccct ccaggatatg gccctgctca
      ||M||E||M| |L||L||F|| R||Y||I||E ||T||L||S| |L||R||H||
551   tatggagatg cttttgtttc gatacattga aaccttgtcc ctccgccacg
      A||L||R||V ||L||E||R| |W||P||E|| D||S||P||V ||G||Q||Y|
601   cccttcgggt gctggaacgt tggcccgaag attctcctgt ggggcagtat
      |A||A||Q|| V||L||R||D ||I||P||A| |N||Y||F|| Q||Q||P||R
651   gcagctcaag ttctacgtga cattcctgca aactacttcc agcagccccg
      ||L||L||P| |T||G||P|| R||F||V||Y ||A||N||G| |V||L||E||
701   gcttttgccc actggaccac gcttcgtcta cgcaaatggt gtgctggagc
      L||K||R||I ||D||Q||A| |L||P||M|| Q||Q||L||H ||P||D||S|
751   tgaagcgtat tgatcaagcc cttcctatgc agcagcttca ccctgactcg
      |T||T||G|| C||V||G||H ||V||M||Q| |N||V||L|| S||S||P||L
801   acgactggat gtgttgggca tgtgatgcag aatgtactgt cctctccttt
      ||E||Q||R| |R||L||R|| P||L||N||S ||F||M||L| |F||R||
851   ggagcaacgt cgtcttcgtc cattgaactc ttttatgctg ttcagaagta
      S||F|
901   agtcgcatct ttatggccat tgattcgctt ctgacagtat cttaggcttt
      |C||A||P|| M||F||P||G ||I||P||Q| |K||V||K|| S||M||A||I
951   tgcgctccga tgttccctgg catcccgcaa aaagtcaagt ctatggccat
      ||S||E||M| |W||Q||D|| D||T||L||K ||S||H||W| |A||I||L||
1001  cagcgaaatg tggcaggatg ataccttgaa gtcccactgg gctattctcg
      A||K||A||Y ||T||I||I| |R||D||H|| F||D||V||D ||T||P||S|
1051  ccaaggctta cacaatcatc cgcgatcact tcgatgtcga caccccttca
      |L||S||T|| F||V||E||L ||C||L||P| |L||M||G|| H||L||D||R
1101  ctctctacct ttgtcgagct ttgcctgcct ctgatgggtc accttgatcg
      ||Q||Q||Y| |L||R||M|| A||G||W||D ||V||Q||P| |T||G||N||
1151  tcaacagtat ctcaggatgg ctggttggga tgtccagccc actggaaata
      S||L||S||L ||R||K||I| |G||L||S|| N||L||A||S ||L||S||V|
1201  gcctcagctt gcgcaagatc ggcttgtcga acttggctag tcttagcgtg
      |P||A||I|| A||V||D||Q ||V||V||K| |H||C||I|| D||N||N||Y
1251  ccggctattg ctgtcgacca agttgtgaag cactgcatcg ataacaacta
      ||A||Q||I| |R||N||E|| E||W||D||K ||H||I||L| |E||N||G||
1301  cgcccagatc cgcaacgaag agtgggacaa gcacattctc gaaaacggac
      Q||V||F||A ||V||D||P| |A||F||S|| A||T||V||R ||D||P||Q|
1351  aggtgttcgc cgtggatcca gccttttcag ccaccgttcg ggatcctcag
```

```
     |N||W||V|| F||N||N||V ||P||Q||W| |P||I||E|| E||F||E||V
1401 aactgggtgt tcaacaacgt tccccagtgg ccaattgaag aatttgaggt
     ||D||E||M| |Y||S||S|| L||D||T||E ||R||D||L| |G||L||P||
1451 tgatgagatg tactcctccc tcgataccga gcgtgacctt gggcttcctg
     V||I||Y||D ||P||N||K| |N||P||S|| V||A||A||T ||M||A||N|
1501 tcatctatga ccctaacaag aacccctctg tggctgccac catggcgaac
     |L||D||R|| I||F||G||H ||N|
1551 cttgaccgga tctttgggca caactagagc tttcacctgt ctgctcatcc
1601 ctaccgtgtt ctaaatgtcc cattcacaat ttcgactcgg ctcagcgaca
1651 tttgacatga tcattgccaa caaatcatga tcaagggttc cgttacccag
1701 ccctctcaat tcactgaccg accgactctg gtgataagtg gacgactcaa
1751 cgatcagaca tatttccatc tttacccaca aaagtcgaaa aaaaaaaaaa
1801 aaagaaaaag aaaaaaaaaa aaaaaaggaa aaaaggaaaa aagaaatttt
1851 actttctcat gttttgcgcc catctgtaca attagttgaa tctttctgcc
1901 gacttttcg gctttcaatt gttttgtttc cgctcatgac aagttctcag
1951 gaactgagct gactggcttt ctttcccaag gggttttcca gacgctcttt
2001 tcctgcaacg tgagcgcttt ttatgtactt tagtttgaaa ttccatttgc
2051 gtgtttcaca aaatgcttcc tttg
```

Figure 5.13: DNA sequence of MAT1-1-1 region of *P. chrysogenum* isolate 'B2'. The putative encoded amino acid sequence is indicated above the relevant base pair sequence. Primer ChM1F is highlighted red (forward), primer ChM1R is highlighted green (reverse) and putative introns are highlighted in yellow. Also included is 500bp upstream and downstream of the putative gene translation start site and end site.

Analysis of the MAT1-2-1 region from isolate CBS 776.95 revealed a 1012bp open reading frame, which included two putative introns and was predicted to encode a 302 amino acid MAT1-2-1 protein (Figure 5.14). PSORT II and TFSITESCAN analysis of the MAT1-2-1 amino acid sequence revealed two nuclear targeting signals [positions 208 (RKRR) and 205 (PSERKRR)] were found, this would support a role for this putative protein as a transriptional activator. A TATA-box motif was also found 148bp upstream of the ATG start codon. The MAT1-2-1 translation start site appeared to be consistent with the requirements of the Kozak rule (Kozak 1986).

```
1    tgagccacca tgttacgtca aatatagtaa gcattataca gagaagctac
51   cagttacgat ctgaatcccc agcctgaaca tccaggtata cagtagtacc
101  tcgttgtcga aatggtacat gatgctgaag taccttccgc tgtagtctta
151  tccctctata attgactttc tttgttcaaa tggtactatt cctaacaagg
201  aaaacctttt taggatgaaa acatgtgatt ggtgttgttt ctaggaaaca
251  aagaaatacg tgtctcagct ggctctgtga ttggtgttgc cgtcaaacaa
301  agaaatcgcc caccccgtca cgtcacctac cgtgctccgc agtcccaggt
351  cctatatttg tttacctccc aacacgtttc ttcttctctt tcttccctct
401  cctcttccaa ccccatcctc ttttccattc cctgcacacc cgaattcctt
451  tcttactgca ttccccagtt catttacctc ctcatttcat cttttacatg
     |M||A||K|| T||L||L||D ||F||V||G| |D||D||D|| R||V||T||P
501  atggcgaaaa ccctcttgga cttcgttggt gacgacgacc gcgttactcc
```

```
     ||R||R||S| |M||E||L|| L||W||A||D ||A||V||N| |H||L||P||
551  tcgtcgttcc atggagcttc tttgggcaga tgccgtcaat cacctgcctc
     Q||T||D||G ||E||V||F| |L||P||R|| N||V||V||D ||G||V||L|
601  agactgatgg tgaagtcttc ctgcctcgca atgttgtcga tggcgttctt
     |D||L||D|| H||L||K||A ||M||A||I| |R||W||
651  gacctggatc atctcaaggc gatggcgatt cgttggcgta agtgctgaac
          P||M| |L||L||T|| S||Y||N||R ||C||M||L|
701  cagtacctac ctgagccatg ttattaacaa gctacaacag atgcatgcta
     |N||K||K|| V||D||I||V ||L||D||Q| |S||I||D|| A||Y||R||M
751  aataagaagg ttgacatcgt cctcgaccaa tccatcgatg cttaccgcat
     ||F||P||K| |D||L||N|| A||E||H||D ||E||N||F| |W||A||D||
801  gttccctaag gacctcaacg ctgagcatga tgagaacttc tgggccgatc
     Q||H||G||L ||D||L||G| |D||H||L|| L||I||S||K ||S||I||H|
851  aacatggact tgacctcggt gatcaccttc taatttccaa gtctatccac
     |E||A||G|| D||S||V||K ||I||P||A| |R||P||A|| K||V||P||R
901  gaggctggcg attctgtcaa gatccctgct cgccagcaa aagttcctcg
     ||P||P||N| |C||F||I|| L||Y||R||Q ||A||N||H| |H||L||V||
951  tccacccaat tgcttcattc tttaccgtca ggccaaccac catttggtca
     K||D||A||N ||P||G||V| |S||N||N|| E||I||
1001 aggatgccaa ccccggtgtt tctaacaacg aaatttgtga gttatctccg
      S||R ||I||L||G|
1051 actacttgtg ttgtttctag ctaatgactg gattagctcg tatccttggt
     |A||R||W|| N||N||E||S ||P||E||V| |R||E||Q|| F||T||H||L
1101 gcacgctgga acaatgagag ccctgaagtt cgagagcagt tcacccacct
     ||A||D||E| |L||K||K|| E||H||A||I ||K||H||P| |D||Y||Q||
1151 ggctgatgaa ctcaagaagg aacacgctat caagcatcct gattaccaat
     Y||A||P||R ||R||P||S| |E||R||K|| R||R||T||P ||R||S||R|
1201 atgctcctcg tcgcccttct gagcgcaagc gccgcactcc ccgttcccgt
     |A||N||C|| L||P||F||V ||Q||A||D| |S||H||Y|| E||D||M||F
1251 gccaactgcc tgccttttgt tcaggctgat tcccattacg aggacatgtt
     ||D||D||A| |D||F||E|| D||R||T||I ||S||I||D| |D||N||F||
1301 cgatgacgct gatttcgaag accgaactat ttctatcgat gacaacttca
     I||T||E||L ||N||D||N| |G||L||L|| F||G||P||N ||G||V||E|
1351 tcaccgagct gaatgacaat ggtctacttt tcggtccgaa tggtgtcgag
     |P||L||S|| P||P||F||T ||H||E||E| |C||F||E|| M||S||N||D
1401 ccactctctc ccccttcac tcacgaggaa tgcttcgaaa tgagcaacga
     ||L||T||S| |G||N||S|| L||A||S||M ||E||F||L| |P||M||N||
1451 cctgacttcg ggcaacagcc ttgcctcaat ggagttcctc cctatgaaca
     S||V||F|
1501 gcgtgttcta aatgtcccat tcacaatttc gactcggctc agcgacattt
1551 gacatgatca ttgccaccaa atcatgatca acggttccgt tacccaacca
1601 tctcaattcc ccgaccgacc gtctctggtg ataagtggat gattctctac
1651 gatcaggcat atttttatct ttacccacaa aagccggaaa aaaaaaattg
1701 aaaacgaaaa taccgaaaaa aaaaatttac tttctcatgt ctcgcgccca
1751 tctgtacaat tagctgaatc tttctgccga ctttttcggc tttcaattgt
1801 tttgtttccg ctcatgacaa gttctcagga actgagctga ctggctttct
1851 ttcccaaggg gttttccaga cgctcttttc ctgcaacgtg agcgctttta
1901 tgtactttag tttgaaattc catttgcgtg tttcacaaaa tgcttccttt
1951 gtagcatata ggattgcaaa tggatatgta gagacgcatg gcgaggacac
2001 gatcgcat
```

Figure 5.14: DNA sequence of MAT1-2-1 region of *P. chrysogenum* isolate CBS 776.95. The putative encoded amino acid sequence is indicated above the relevant base pair sequence. Primer ChM2F is highlighted red (forward), primer ChM2F is highlighted in grey (reverse), primer ChM2R is highlighted green (reverse) and putative introns are highlighted in yellow. Also included is 500bp upstream and downstream of the putative translation start site and end site.

5.3.2.3 Idiomorph Orientation

A variety of PCR amplifications were attempted in order to determine the orientation of the *MAT1-1-1* and *MAT1-2-1* genesa relative to the flanking *SLA2* and *APN2* genes (section 2.2.5 for protocol).

Amplicons of approximately 4kb in size were produced by primers ChM1F and ChSLA2-1, 3kb by primers ChM1R and ChAPN2-1, 3.2kb by ChM2F and ChSLA2-2 and 2.5kb by primers ChM2R and ChAPN2-2 (Figures 5.15 and 5.16). This allowed the gene orientations of *MAT1-1-1* and *MAT1-2-1* to be deduced as shown in Figure 5.17. The species has a heterothallic-like MAT idiomorph arrangement, with both *MAT1-1-1* and *MAT1-2-1* orientated in the same direction as, and downstream of, the *APN2* gene (Figure 5.17).

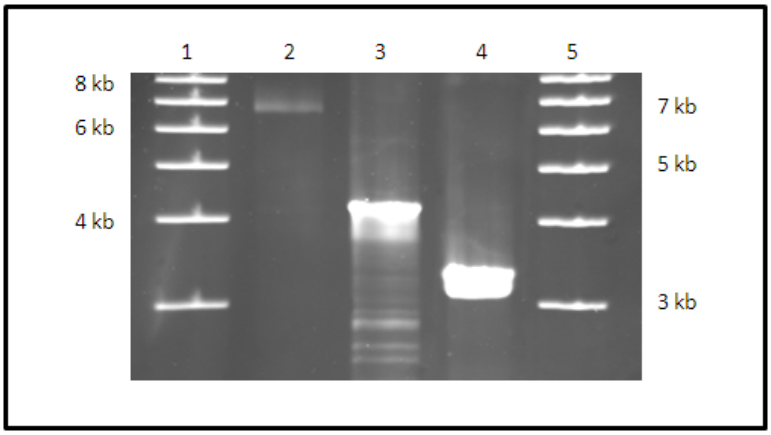

Figure 5.15: 0.8% agarose gel showing results of PCR amplifications to determine *MAT1-1-1* gene orientation within the MAT-1 idiomorph of *P. chrysogenum* isolate 'B2'. Lanes 1 and 5: 1kb ladder. Lane 2: Amplicons produced by primers ChSLA2-1 and ChAPN2-1. Lane 3: Amplicons produced by primers ChM1F and ChSLA2-1. Lane 4: Amplicons produced by primers ChM1R and ChAPN2-1.

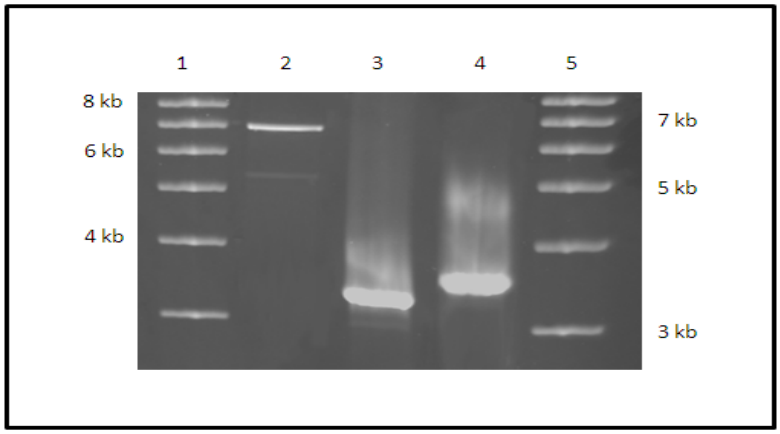

Figure 5.16: 0.8% agarose gel showing the results of PCR amplifications to determine *MAT1-2-1* gene orientation within the MAT-2 idiomorph of *P. chrysogenum* isolate CBS 776.95. Lanes 1 and 5: 1kb ladder. Lane 2: Amplicons produced by primers ChSLA2-1 and ChAPN2-1. Lane 3: Amplicons produced by primers ChM2F and ChSLA2-2. Lane 4: Amplicons produced by primers ChM2R and ChAPN2-2.

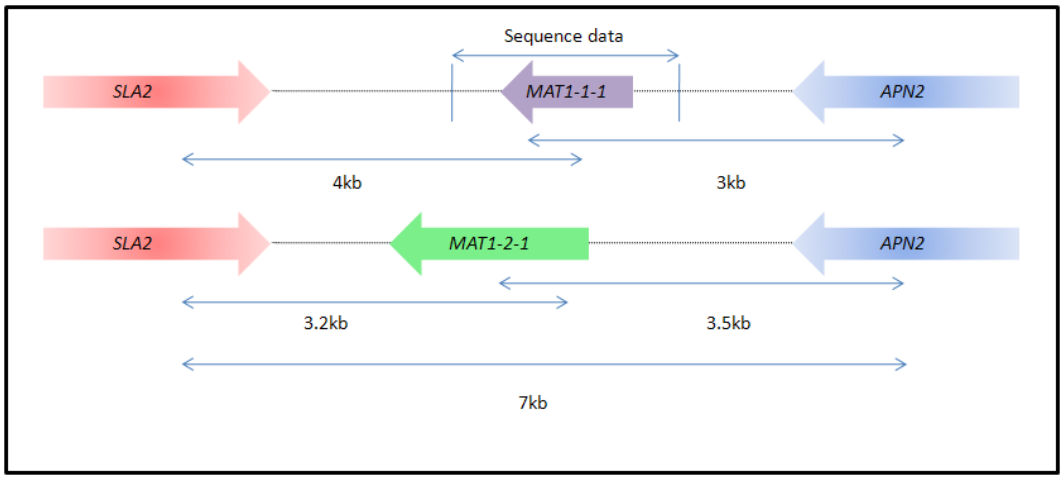

Figure 5.17: Schematic diagram showing the MAT-1 idiomorph (top) and the MAT-2 idiomorph (bottom) of *P. chrysogenum* (distances in kb).

5.3.2.4 RT-PCR Analysis

RT-PCR analysis provided clear evidence of expression of both the putative *MAT1-1-1* and *MAT1-2-1* genes of *P. chrysogenum*, and of the actin control gene. Amplicons of a smaller size were produced by RT-PCR, relative to genomic DNA controls (Figures 5.18 and 5.19 and Table 5.12). These corresponded to the predicted sizes of mRNA transcripts allowing for splicing of a 48bp intron from the *MAT1-1-1* gene and a 53bp intron from the *MAT1-2-1* gene, respectively.

Table 5.12: Primers used for *P. chrysogenum* RT-PCR analysis and predicted lengths of fragments.

Forward primer	Reverse primer	Genomic DNA fragment length (bp)	Processed mRNA fragment length (bp)
A. cl Actin Forward	A. cl Actin Reverse	~560	~480
ChM1F	ChM1R	340	292
ChM2F	ChM2R	342	289

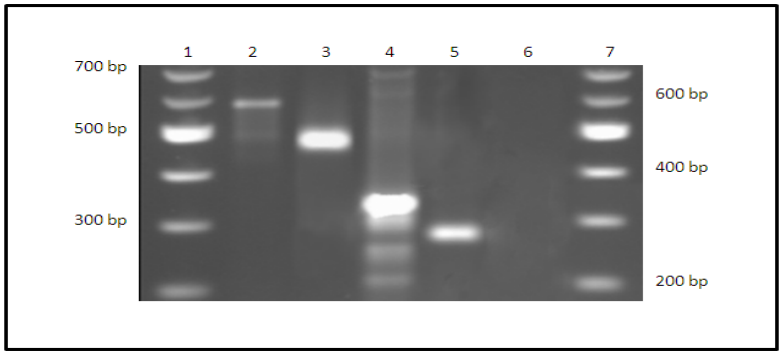

Figure 5.18: 1.5% agarose gel showing results of *P. chrysogenum* isolate 'B2' *MAT1-1-1* RT-PCR gene analysis. Lanes 1 and 7: 100bp ladder. Lane 2: Genomic DNA Actin control. Lane 3: RNA Actin control. Lanes 4 and 5: Amplifications with *MAT1-1-1* specific primers of genomic DNA and RNA extracts, respectively. Lane 6: Amplification with *MAT1-1-1* specific primers of water control with no RNA.

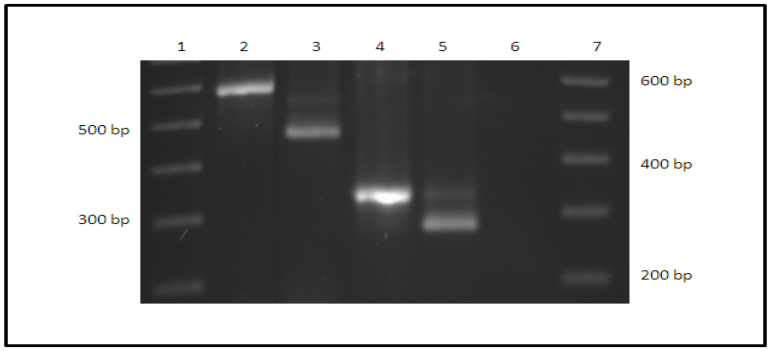

Figure 5.19: 1.5% agarose gel showing results of *P. chrysogenum* isolate CBS 776.95 *MAT1-2-1* RT-PCR gene analysis. Lanes 1 and 7: 100bp ladder. Lane 2: Genomic DNA Actin control. Lane 3: RNA Actin control. Lanes 4 and 5: Amplification with *MAT1-2-1* specific primers of genomic DNA and RNA extracts, respectively. Lane 6: Amplification with *MAT1-2-1* specific primers of water control with no RNA.

5.3.3 *Penicillium griseofulvum*

5.3.3.1 *MAT* Gene Screening

A 270bp putative *MAT1-2-1* gene fragment was successfully amplified from all isolates of *P. griseofulvum* screened using degenerate primers MAT5-7 and MAT3-5 (section 2.2.4). This allowed the isolation of the entire putative *MAT1-2-1* gene sequence (see section 5.3.3.2) and design of a PCR diagnostic to determine the mating type of isolates (section 5.2.3.1). The *MAT1-1-1* specific degenerate primers failed to produce any PCR product from any of the isolates i.e. the population screened was entirely of the MAT1-2 genotype.

The *MAT* PCR diagnostic using primer pair GrM2F and GrM2R successfully amplified putative *MAT1-2-1* gene fragments from all 17 worldwide isolates, confirming results of the degenerate PCR screening (Tables 5.3, 5.5 and 5.10). Primers were designed in such a way that the MAT1-2 genotype was predicted to produce a 160bp product (Figure 5.20). To ensure that all these were independent isolates i.e. not clonal, RAPD analysis was performed using the primers listed in Table 2.2 and the protocol described in section 2.2.6. Results showed all isolates to produce distinct RAPD-PCR fingerprints, and therefore none of the isolates were clonal (data not shown).

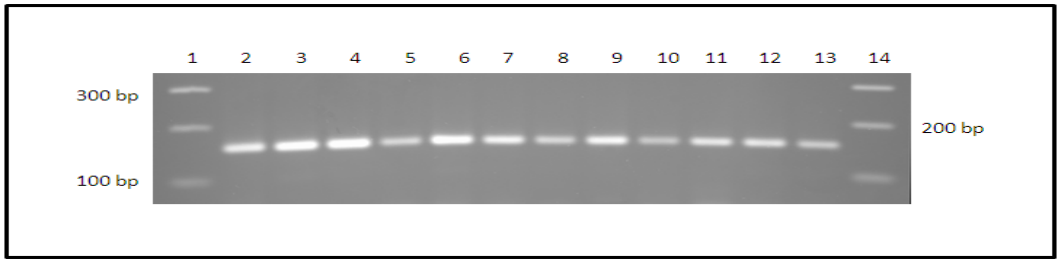

Figure 5.20: 2% agarose gel showing representative results of *MAT1-2-1* PCR screening of *P. griseofulvum* isolates, using primers GrM2F and GrM2R. Lanes and 1 and 14: 100bp ladder. Lane 2: Isolate CBS 118882. Lane 3: Isolate CBS 118868. Lane 4: Isolate CBS 112298. Lane 5: Isolate CBS 110418. Lane 6: Isolate CBS 110417. Lane 7: Isolate CBS 100233. Lane 8: Isolate CBS 493.75. Lane 9: Isolate CBS 110420. Lane 10: Isolate CBS 746.70. Lane 11: Isolate CBS 124.14. Lane 12: Isolate 57C6. Lane 13: Isolate 57E7.

5.3.3.2 *MAT* Gene Isolation and Sequence Analysis

Using the *SLA2-APN2* positional PCR strategy (section 2.2.5) it was possible to amplify fragments of the MAT-2 idiomorph region containing the putative *MAT1-2-1* gene from *P. griseofulvum* isolate CBS 110420. The idiomorph was then partially sequenced by PCR chromosome walking outwards from the *MAT1-2-1* gene fragment to determine whether the complete putative *MAT1-2-1* gene was present, including the HMG-domain encoding sequence necessary for function. In total 4115bp of the MAT1-2-1 region was sequenced from isolate CBS 110420 (see Appendix 3, Figure 4 for sequence) containing a 1031bp open reading frame, which included two putative introns and was predicted to encode a 305 amino acid MAT1-2-1 protein (Figure 5.21). PSORT II and TFSITESCAN programs were used to interrogate resultant sequences for nuclear targeting and promoter region motifs. Within the MAT1-2-1 amino acid sequence, three nuclear targeting signals [positions 206 (RRRR), 203 (PEDRRRR) and 21 (PWVKSRK)] were found, this would support a role for this putative protein as a transriptional activator. A GATA transcriptional activator motif was found 122bp upstream of the start codon. The putative ATG translation start codon could not be confirmed using the Kozak rule (Kozak 1986) but the length of the predicted protein was consistent with other members of the MAT1-2-1 family.

```
1     accctcctgg gatcgaacga tcgttgcaga ccgcatgcct atgcattcag
51    gtaacgctaa ggcttgcgta agcaacgttg agatagtaaa gtgctaccta
101   gctatagtcg tcgatctcgt atgcacttat ggcattgatc gtaccagcat
151   gtagtgtctg tcaagtctat gagtatcagc tagttggtcg tgagactagt
201   cgcacctggg atcgatcatc gttgcaaact gctcggatat cctcacagtc
251   tatccattgg atatctccat gtacatgttg gtcgacattt cgctgagctg
301   catatcgaac aaatggctgg tcgcagtgct aaaaagccaa gcatagccat
351   cattttccga tggatgaagg cggacttctg acggtcgcac gcataacccg
401   agtcgattat ctttgttctt gattagcaat gtcctcgcct tccgccttcg
451   cctatactta ctaatgttgt ttgcgagcac ttcccgacac ctcctccttt
501   ccagagcacg tttcctatta gctgggtttg atatgtcatg tagtcgaaag
551   ggctaagttt ggtagtctgc aactatatat tgtgtgcccc gtagataatt
601   cccccgtcga tccttgccga ggatggcccg ccacggaaat agaaacaggt
651   gacgaggctc atcacaaagt gggacccatc gtgggcggtt cgcctaggca
                    |M||T ||V||R||P| |L||N||N|| K||A||I||Y
701   gcggtgatca cacgtatgac tgtccggccg cttaataata aagctattta
      ||I||T||H| |R||N||I|| C||C||P||W |
751   tattacccac cgcaacatat gctgtccttg ggtaggtaaa ttcactcgaa
801   ggataagaaa ccattgaatt cgcagccagt atagttgaaa tgttacgaag
```

```
     |V||K||S|| R||K||E||T ||L||E||H| |F||Q||L|| L||K||S||T
851  gtcaaaagta gaaaagaaac attggaacat tttcaacttt tgaaatccac
     ||F||R||L| |I||P||L|| R||S||L||S ||N||L||M| |N||P||F||
901  atttcgctta atccccctta gaagtctgtc caacctcatg aatccctttt
     L||S||D||D ||H||I||F| |I||T||L|| A||F||F||H ||A||R||L|
951  tatctgatga ccacatcttc attactttag ccttctttca tgcccgcctt
     |I||A||D|| G||S||A||P ||S||L||A| |C||A||P|| R||W||A||S
1001 atcgccgatg ggagtgcccc cagtctggca tgcgcgccca ggtgggcctc
     ||P||A||S| |R||Q||A|| P||M||F||S ||P||G||R| |P||G||G||
1051 gccagcaagc cgacaggcac cgatgttctc accaggacgt ccaggcggca
     N||A||E||R ||D||Q||P| |R||L||S|| Q||F||C||N ||N||T||L|
1101 atgccgaacg ggatcagcct cgcctttcgc agttctgtaa caacacacta
     |S||I||N|| C||L||F||I ||A||R||P| |P||N||G|| F||I||L||Y
1151 tccataaatt gccttttttat agcgaggccg ccgaacgggt ttatcttgta
     ||R||Q||A| |N||H||H|| L||V||K||N ||A||N||P| |G||L||S||
1201 ccgacaggca aaccatcact tggtcaagaa tgccaaccct ggtctgtcaa
     N||N||E||I ||
1251 acaatgaaat ctgtgagtta tatcctctgt ttacgtttga tttttagcta
                  S||R||I ||L||G||A| |R||W||N|| N||E||T||P
1301 atgaatgtat cagcccgcat cctcggcgcg cgctggaaca atgagacccc
     ||E||V||R| |H||Q||F|| T||R||L||A ||N||D||L| |K||R||E||
1351 cgaggttcgt caccagttca cccgcttggc caacgatctg aagcgggaac
     H||A||I||K ||H||P||D| |Y||Q||Y|| A||P||R||R ||P||E||D|
1401 acgccattaa gcatcctgat taccaatatg cccctcgccg ccctgaggat
     |R||R||R|| R||T||R||P ||R||A||A| |A||A||I|| A||A||M||E
1451 cgcagacgcc gcactcgccc tcgtgccgct gccgctatcg ctgccatgga
     ||S||D||P| |T||E||E|| F||D||E||N ||L||I||P| |I||D||D||
1501 gtctgacccc actgaagagt ttgatgaaaa tctgatccct atcgacgacg
     E||F||M||S ||T||L||S| |D||T||D|| M||L||F||G ||P||N||G|
1551 aattcatgtc aactctgagc gacactgata tgctcttcgg tccgaacggt
     |A||E||P|| I||P||A||P ||W||P||Y| |Y||D||R|| M||E||I||S
1601 gctgagccta ttccggctcc gtggccctac tatgatcgca tggaaatcag
     ||N||E||L| |A||S||G|| K||R||F||K ||S||M||E| |Y||I||P||
1651 caacgaacta gcttctggca aacgtttcaa atcaatggaa tacatcccca
     M||P||F||D ||S||D||E| |S||S||L|| P||M||P||S ||V||F|
1701 tgccatttga ttccgacgaa tccagcctcc ctatgccctc cgtcttctaa
1751 tgatttcttc tcgatcttga ttcgattcag ttacatgact tgacaacaat
1801 ctatcaacaa tcaataaccc ccacatcaat aactttattt cctcgtcatg
1851 tcctcgctga tcgatcgtcc caatcatgtt gaattaccca ttgagtttcg
1901 tcctttcgtc ttttctttca gttcgcccca aaaacacgaa aaaccaaaaa
1951 aaaaaaacaa aacaaaaaca aaaaacaaag aaaaacaaac caaaacaaaa
2001 aaaaatattt gtttttttgtc tttcatgcga ccaatactcc tgtacgaata
2051 gtttgatttt tttttttttgt tttcgctcat ggcaagttct caggaactga
2101 gctgactggc tttctttccc acggggtttt ccagacgctc atttcctgcc
2151 atttgagctt tcatgtactt aagtgcaatt gaattttgct ccaactgctt
2201 cctctgtaat cgtagtcgct ttctactctg tcgtctcgat atacttgcag
```

Figure 5.21: DNA sequence of MAT1-2-1 region of *P. griseofulvum* isolate CBS 110420. The putative encoded amino acid sequence is indicated above the relevant base pair. Primer GrM2F is highlighted in red (forward), primer GrM2R is highlighted in green (reverse) and putaitve intron is highlighted in yellow. Also included is 715bp upstream of the putative ATG translation start site and 500bp downstream of the finish site.

5.3.3.3 Idiomorph Orientation

Amplicons of approximately 2.3kb in size were produced by primers GrM2F and GrSLA2, and of 3.5kb by primer pair GrM2R and GrAPN2 (Figure 5.22). This allowed the gene orientation of the *MAT1-2-1* gene fragment to be deduced, as shown in Figure 5.23. The *MAT1-2-1* gene was orientated in the same direction as, and downstream of, the *APN2* gene. Note that for reasons unknown, it was not possible to amplify the entire MAT idiomorph of *P. griseofulvum* as a single product. Therefore the overall size of the idiomorph was derived from the *SLA2* to *MAT1-2-1*, and the *APN2* to *MAT1-2-1* sub-region amplifications (Figure 5.23).

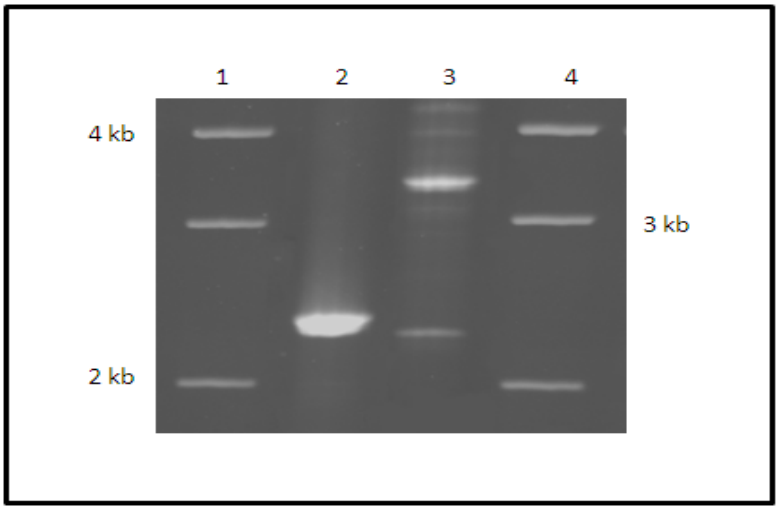

Figure 5.22: 0.8% agarose gel showing the results of PCR amplifications to determine *MAT-1-2-1* gene orientation within the MAT-2 idiomorph in *P. griseofulvum* isolate CBS 110420. Lanes 1 and 5: 1kb ladder. Lane 2: Amplification with primers GrM2F and GrSLA2. Lane 3: Amplification with primers GrM2R and GrAPN2.

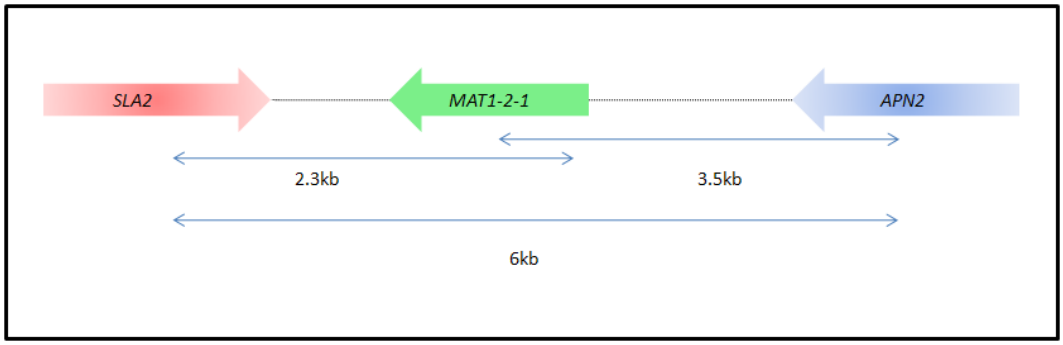

Figure 5.23: Schematic showing the MAT-2 idiomorph of *P. griseofulvum* (distances in kb).

5.3.3.4 RT-PCR Analysis

RT-PCR analysis showed evidence of expression and splicing of the actin control gene (Figure 5.24). However, although the genomic control was successfully amplified with the primer set GrM2F and GrM2R, there was no evidence of expression or splicing of the *MAT1-2-1* mRNA transcript. This suggests the *MAT1-2-1* gene in *P. griseofulvum* is not expressed under the conditions assayed in this study.

Table 5.13: Primers used for *P. griseofulvum* RT-PCR and predicted lengths of fragments.

Forward primer	Reverse primer	Genomic DNA fragment length (bp)	Processed mRNA fragment length (bp)
A. te Actin Forward	A. te Actin Reverse	~330	~265
GrM2F	GrM2R	157	106

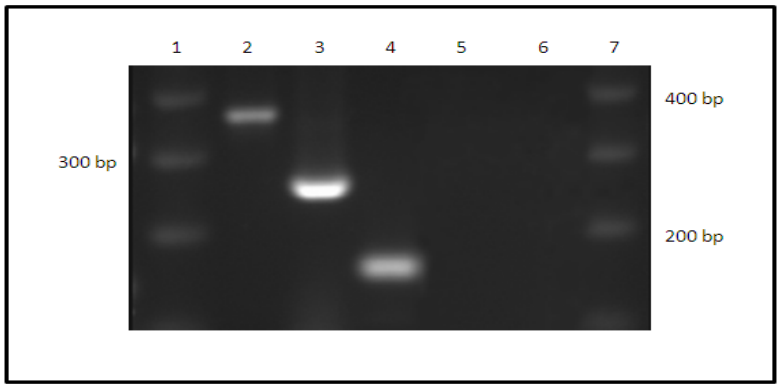

Figure 5.24: 1.5% agarose gel showing results of *P. griseofulvum* isolate CBS 110420 *MAT1-2-1* RT-PCR gene analysis. Lanes 1 and 7: 100 bp ladder. Lane 2: Genomic DNA Actin control. Lane 3: RNA Actin control. Lanes 4 and 5: Amplification with *MAT1-2-1* specific primers of genomic DNA controls and RNA extracts, respectively. Lane 6: Amplification with *MAT1-2-1* specific primers of water control with no RNA.

5.3.4 *Penicillium camemberti*

5.3.4.1 *MAT* Gene Isolation and Sequence Analysis

Initial screening of *P. camemberti* had revealed the presence of two MAT1-1 type isolates (CBS 190.67 and CBS 112078) and two MAT1-2 type isolates (CBS 112325 and CBS 112562) (Table 5.10). To confirm that the amplicons were indeed fragments of true *MAT* genes, PCR products from CBS 190.67 and CBS 112325 were cloned and sequenced.

This revealed the presence of 149bp and 273bp sequences encoding fragments of a characteristic MAT1-1-1 alpha domain protein, and MAT1-2-1 HMG-domain protein, respectively (Figures 5.25 and 5.26).

```
     |W||R||P|| L||N||W||F ||I||A||F| |R||
1    tggcggccgc tgaactggtt tatagctttc agaagtattc tatacacttc
                                        S||Y| |Y||S||V||
51   catctcatgc atgtacatcg gctaatgatt attaggttat tattcagtca
     M||F||P||D ||L||T||Q| |K||A||K|| S||G||I||L ||R||F|
101  tgtttccaga ccttactcag aaagcgaagt ccggcatcct ccgcttcct
```

Figure 5.25: Partial *MAT1-1-1* gene sequence of *P. camemberti* isolate CBS 190.67. Encoded putative amino acid sequence is indicated above the relevant base pair sequence. Primer CaM1F is highlighted in red (forward), primer CaM1R is highlighted in green (reverse) and putative intron is highlighted in yellow.

```
     |P||R||P| |P||T||G|| F||I||L||Y ||R||Q||A| |H||H||P||
1    tccccggccc cccaccgggt ttattcttta tcggcaagca catcatccac
     L||I||K||E ||N||H||P| |G||L||S|| N||N||E||I ||
51   taattaaaga aaaccacccc ggattatcca ataatgagat ttgtgagtta
                                       S| |L||M||L||
101  tctgcttaat gcaagcactg ctgcgagata atgactagca ctaatgcttg
     G||K||R||W ||N||A||E| |T||E||N|| V||R||V||H ||F||W||H|
151  gtaaacgatg gaacgcggaa actgagaacg tcagggtcca tttctggcac
     |L||A||E|| D||L||K||K ||K||H||A| |E||A||H|| P||N||Y||R
201  ctagctgagg atctcaagaa gaaacatgcg gaagctcacc ccaattaccg
     ||Y||P||P| |R|
251  ctacccccc cgca
```

Figure 5.26: Partial *MAT1-2-1* gene sequence of *P. camemberti* isolate CBS 112325. Encoded putative amino acid sequence is indicated above the relevant base pair sequence. Primer CaM2F is highlighted in red (forward), primer CaM2R is highlighted in green (reverse) and putative intron is highlighted in yellow.

5.3.4.2 Idiomorph Orientation

Amplicons of approximately 3.2kb in size were produced by primers CaM1F and CaSLA2-1, 4.5kb by CaM1R and CaAPN2-1, 5.5kb by CaM2F and CaSLA2-2 and 2.3kb by CaM2R and CaAPN2-2 (Figures 5.27 and 5.28). This allowed the gene orientation of *MAT1-1-1* and *MAT1-2-1* to be deduced, as shown in Figure 5.29. As observed with *P. chrysogenum* (section 5.3.2.3), the species has a heterothallic-like MAT idiomorph arrangement, with both *MAT1-1-1* and *MAT1-2-1* genes orientated in the same direction as, and downstream of, the *APN2* gene (Figure 5.29). For unknown reasons, it was not possible to amplify the whole idiomorphic region of *P. camemberti* as a single product, but its size (~7kb) can be predicted from the constituent fragment lengths.

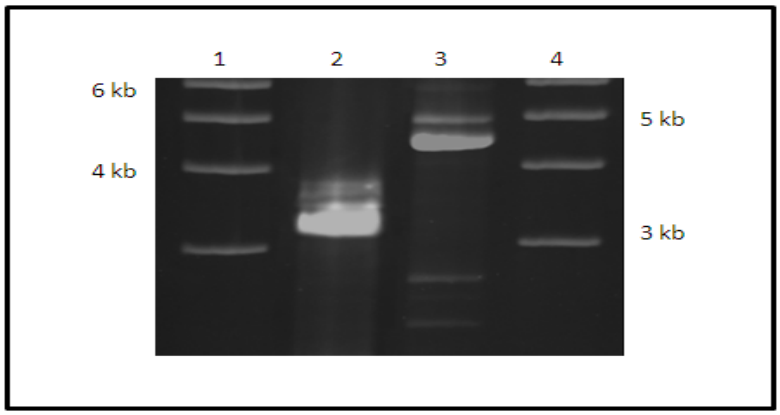

Figure 5.27: 0.8% agarose gel showing the results of PCR amplifications to determine *MAT1-1-1* gene orientation within the MAT-1 idiomorph in *P. camemberti* isolate CBS 190.67. Lanes 1 and 4: 1kb ladder. Lane 2: Amplicons produced by primers CaM1F and CaSLA2-1. Lane 3: Amplicons produced by primers CaM1R and CaAPN2-1.

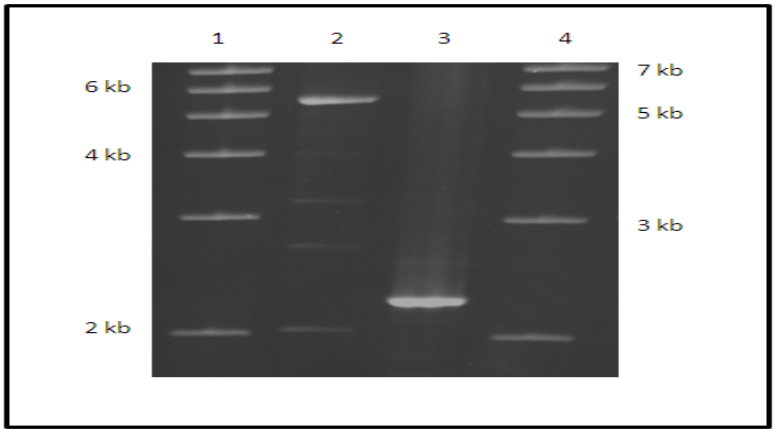

Figure 5.28: 0.8% agarose gel showing results of PCR amplifications to determine *MAT1-2-1* gene orientation within the MAT-2 idiomorph in *P. camemberti* isolate CBS 112325. Lanes 1 and 4: 1kb ladder. Lane 2: Amplicons produced by primers CaM2F and CaSLA2-2. Lane 3: Amplicons produced by primers CaM2R and CaAPN2-2.

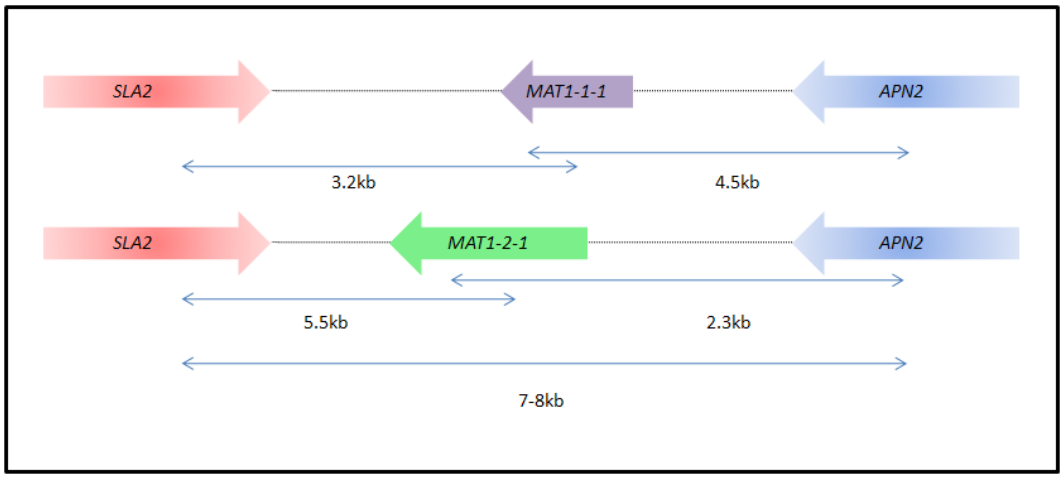

Figure 5.29: Schematic showing the MAT-1 idiomorph (top) and the MAT-2 idiomorph (bottom) of *P. camemberti* (distances in kb).

5.3.4.3 RT-PCR Analysis

As with *P. chrysogenum* (section 5.3.2.3), RT-PCR analysis provided clear evidence of expression of both putative *MAT1-1-1* and *MAT1-2-1* genes, together with the actin control. Amplicons of smaller size, relative to genomic DNA, were produced by RT-PCR analysis (Figures 5.30 and 5.31). These corresponded to the predicted sizes of mRNA transcripts, allowing for splicing of a 51bp intron from the *MAT1-1-1* gene and a 46bp intron from the *MAT1-2-1* gene (Table 5.14).

Table 5.14: Primers used for *P. camemberti* RT-PCR analysis and predicted lengths of fragments.

Forward primer	Reverse primer	Genomic DNA fragment length (bp)	Processed mRNA fragment length (bp)
A.cl Actin Forward	A. cl Actin Reverse	~560	~480
CaM1F	CaM1R	132	87
CaM2F	CaM2R	154	108

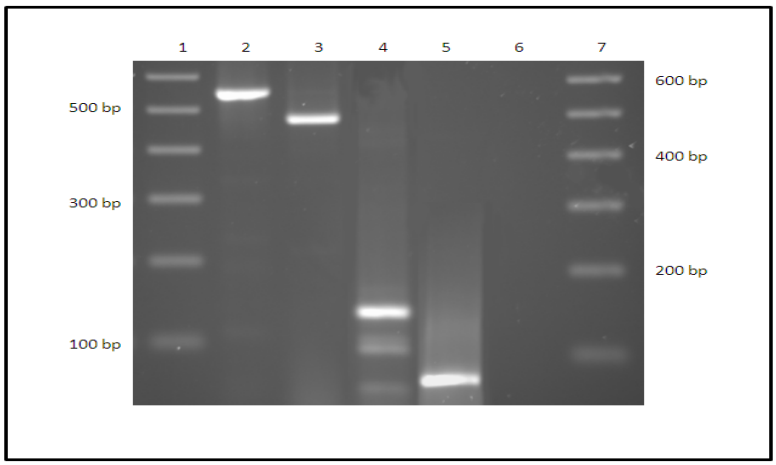

Figure 5.30: 1.5% agarose gel showing results of *P. camemberti* isolate CBS 190.67 *MAT1-1-1* RT-PCR gene analysis. Lanes 1 and 7: 100 bp ladder. Lane 2: Genomic DNA Actin control. Lane 3: RNA Actin control. Lanes 4 and 5: Amplification with *MAT1-1-1* specific primers of genomic DNA controls and RNA extracts, respectively. Lane 6: Amplification with *MAT1-1-1* specific primers of water control with no RNA.

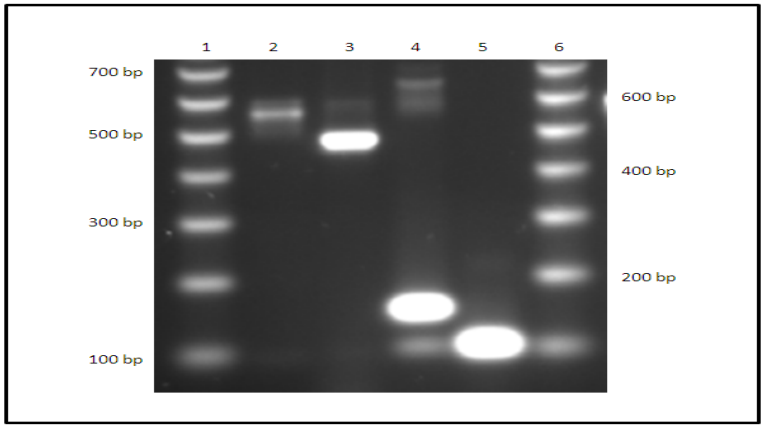

Figure 5.31: 1.5% agarose gel showing results of *P. camemberti* isolate CBS 112325 *MAT1-2-1* RT-PCR gene analysis. Lanes 1 and 6: 100 bp ladder. Lane 2: Genomic DNA Actin control. Lane 3: RNA Actin control. Lanes 4 and 5: Amplification with *MAT1-2-1* specific primers of genomic DNA controls and RNA extracts, respectively.

5.3.5 *Penicillium roqueforti*

5.3.5.1 *MAT* Gene Isolation and Sequence Analysis

Initial screening of *P. roqueforti* had revealed the presence of two MAT1-1 type isolates (CBS 135.67 and CBS 234.38) and three MAT1-2 type isolates (CBS 479.84, CBS 498.73 and CBS 221.30) (Tables 5.3 and 5.9). To confirm that the amplicons were indeed fragments of true *MAT* genes, PCR products from CBS 135.67 and CBS 221.30 were cloned and sequenced. This revealed the presence of 149bp and 268bp sequences encoding fragments of a charactertistic MAT1-1-1 alpha domain protein and MAT1-2-1 HMG domain protein, respectively (Figures 5.32 and 5.33).

```
     |W||R||P|| L||N||R||F  ||I||A||F| |R||
1    tggaggccgt tgaacaggtt catcgctttc agaagtattc tatacacttc
                                          S||Y| |Y||S||V||
51   catctcatgc atgtacatcg gctaatgatt attaggttat tattcagtca
     M||F||P||D ||L||T||Q| |K||A||K|| S||G||I||L ||R||F|
101  tgtttccaga ccttactcag aaagcgaagt ccggcatcct ccgcttcct
```

Figure 5.32: Partial *MAT1-1-1* gene sequence of *P. roqueforti* isolate CBS 135.67. Encoded putative amino acid sequence is indicated above relevant base pair sequences. Primer RoM1F is highlighted in red (forward), primer RoM1R is highlighted in green (reverse) and putative intron is highlighted in yellow.

```
      |A||R||P| |P||N||G|| F||I||L||Y ||R||G||H| |H||H||P||
1    tgcgaggccg ccgaacgggt tcatcctgta ccgccaacat catcatccta
     K||L||K||E ||A||H||P| |N||L||S|| N||N||E||I ||
51   agctcaagga ggctcacccg aatctttcaa ataacgagat ttgtgagttt
                                                S||V||I|
101  ccactactgt gcatacgttg gataatcgac taatttcccc agctgttatt
     |L||G||K|| Q||W||K||A ||E||S||E| |N||I||R|| V||E||F||R
151  ctagggaaac agtggaaggc agaatccgag aatatcaggg tcgagttcag
     |A||L||A|| D||E||L||K ||R||K||H| |A||E||A|| H||P||N||Y
201  agccctggcc gatgagttga agcggaagca tgcagaagct catcctaact
     ||R||F||P| |P||R|
251  accgcttccc cccccgca
```

Figure 5.33: Partial *MAT1-2-1* gene sequence of *P. roqueforti* isolate CBS 221.30. Putative encoded amino acid sequence is indicated above relevant base pair sequence. Primer RoM2F is highlighted in red (forward), primer RoM2R is highlighted in green (reverse) and putative intron is highlighted in yellow.

5.3.5.2 Idiomorph Orientation

Amplicons of approximately 2.6kb in length were produced by primer RoM1F and RoSLA2-1, 4.2kb by primers RoM1R and RoAPN2-1, 3.8kb by RoM2F and RoSLA2-2 and 2.4kb by primers RoM2R and RoAPN2-2 (Figures 5.34 and 5.35). This allowed the gene orientation of *MAT1-1-1* and *MAT1-2-1* within the idiomorphic region to be deduced as shown in Figure 5.36. As observed with *P. chrysogenum* and *P. camemberti*, this species has a heterothallic-like MAT idiomorph arrangement with both *MAT1-1-1* and *MAT1-2-1* orientated in the same direction as, and downstream of, the *APN2* gene (Figure 5.36). For unknown reasons it was not possible to amplify the entire MAT idiomorph in one step from either MAT1-1 or MAT1-2 isolates. MAT idiomorph length was therefore determined using the sub-region amplifications (6-7kb).

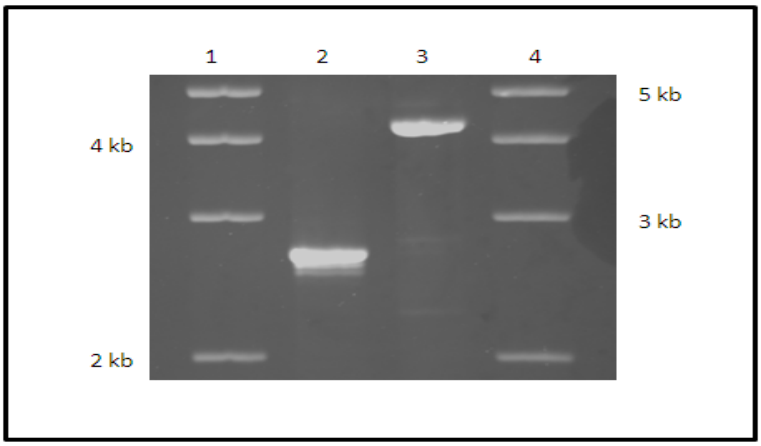

Figure 5.34: 0.8% agarose gel showing results of PCR amplifications to determine *MAT1-1-1* gene orientation within the MAT-1 idiomorph in the *P. roqueforti* isolate CBS 135.67. Lanes 1 and 4: 1kb ladder. Lane 2: Amplicons produced by primers RoM1F and RoSLA2-1. Lane 3: Amplicons produced by primers RoM1R and RoAPN2-1.

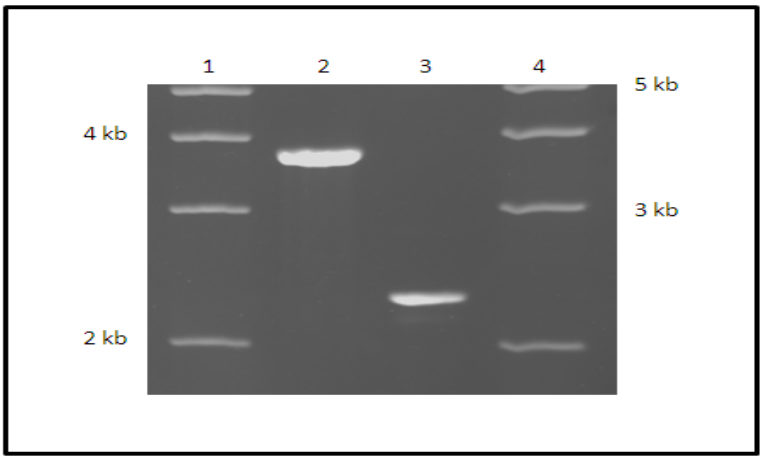

Figure 5.35: 0.8% agarose gel showing results of PCR amplification to determine *MAT1-2-1* gene orientation within the MAT-2 idiomorph in the *P. roqueforti* isolate CBS 221.30. Lanes 1 and 4: 1kb ladder. Lane 2: Amplicons produced by primers RoM2F and RoSLA2-2. Lane 3: Amplicons produced by primers RoM2R and RoAPN2-2.

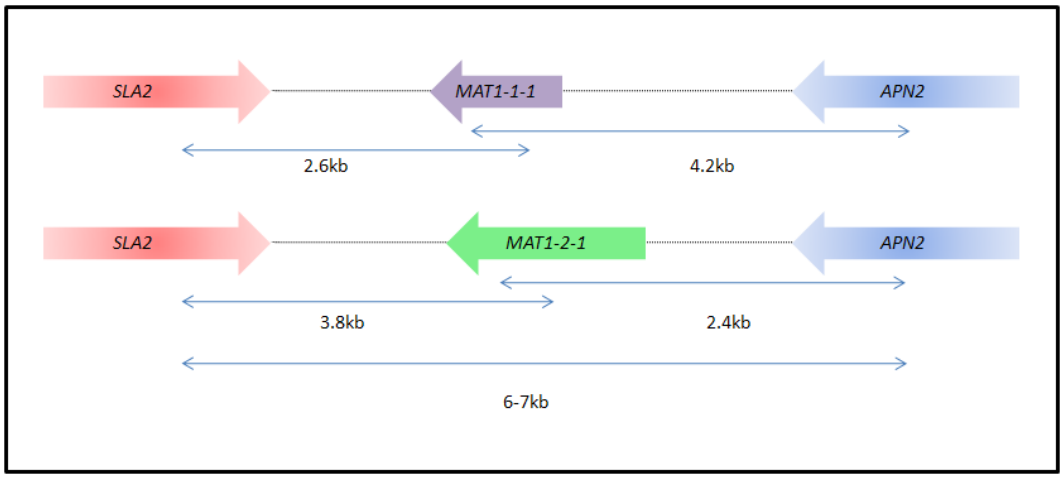

Figure 5.36: Schematic diagram showing the MAT-1 idiomorph (top) and the MAT-2 idiomorph (bottom) of *P. roqueforti* (distances in kb).

5.3.5.3 RT-PCR Analysis

As with *P. chrysogenum* and *P. camemberti*, RT-PCR analysis provided clear evidence of expression of both the putative *MAT1-1-1* and *MAT1-2-1* genes, together with the actin control. Amplicons of a smaller size were produced by RT-PCR relative to genomic DNA controls (Figures 5.37 and 5.38). These corresponded to the predicted size of mRNA transcripts, allowing for splicing of a 51bp intron from the *MAT1-1-1* gene and a 62bp intron from the *MAT1-2-1* gene (Table 5.14).

Table 5.15: Primers used for *P. roqueforti* RT-PCR analysis and predicted fragment lengths.

Forward primer	Reverse primer	Genomic DNA fragment length (bp)	Processed mRNA fragment length (bp)
A. cl Actin Forward	A. cl Actin Reverse	~560	~480
RoM1F	RoM1R	141	90
RoM2F	RoM2R	158	96

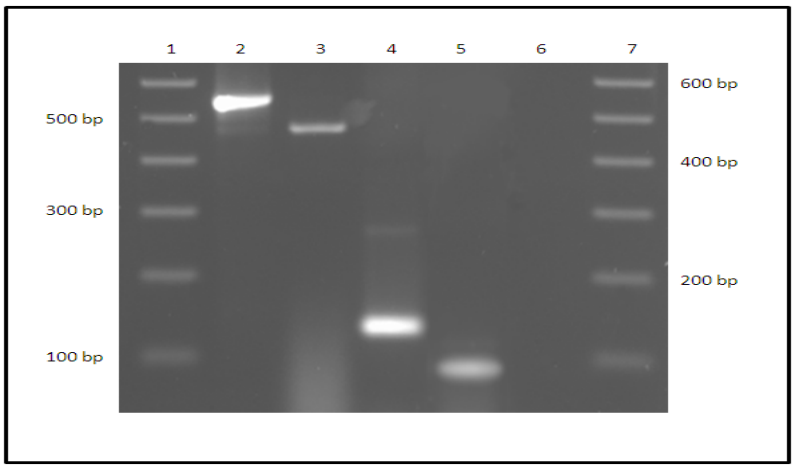

Figure 5.37: 1.5% agarose gel showing results of *P. roqueforti* isolate CBS 135.67 *MAT1-1-1* RT-PCR gene analysis. Lanes 1 and 7: 100bp ladder. Lane 2: Genomic DNA Actin control. Lane 3: RNA Actin control. Lanes 4 and 5: Amplification with *MAT1-1-1* specific primers of genomic DNA controls and RNA extracts, respectively. Lane 6: Amplification with *MAT1-1-1* specific primers of water control with no RNA.

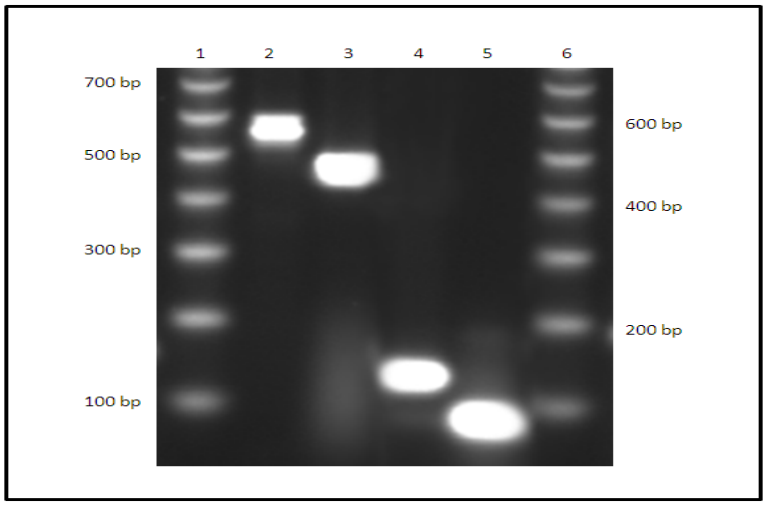

Figure 5.38: 1.5% agarose gel showing results of *P. roqueforti* isolate CBS 221.30 *MAT1-2-1* RT-PCR gene analysis. Lanes 1 and 6: 100 bp ladder. Lane 2: Genomic DNA Actin control. Lane 3: RNA Actin control. Lanes 4 and 5: Amplification with *MAT1-2-1* specific primers of genomic DNA controls and RNA extracts, respectively.

5.3.6 Attempted Induction of Sexual Cycle in *Penicillium* Species

Crossed cultures between MAT1-1 and MAT1-2 isolates of the various *Penicillium* species were incubated for 6 months under conditions described in section 2.2.19. Unfortunately, after this time no visible cleistothecia were produced by any of the isolates tested. However, cultures were not inspected microscopically for earlier stages of sexual development such as formation of ascogonial coils.

5.3.7 Phylogenetic Analysis of Mating-Type Gene Sequences

Multiple alignments (Figures 5.39 and 5.40) were made between putative MAT1-1-1 and MAT1-2-1 proteins of *P. chrysogenum* and *P. griseofulvum* and known MAT proteins from other Eurotiomycete fungi.

5.3.7.1 *MAT1-1-1* Family Alpha-Domain Gene Analysis

Alignment of the MAT1-1-1 protein sequences revealed 21-47% identity between *P. chrysogenum, N. fumigata, E. nidulans* and *P. marneffei* (Figures 5.16 and 5.39). A maximum parsimony phylogenetic tree (Figure 5.40) was constructed using the MEGA4 program (using default conditions with 500 bootstrap replicates and gaps excluded). This phylogeny revealed that *P. chrysogenum* was the most divergent of all Eurotiomycete species analysed, and was basal to the *P. marneffei, N. fumigata* and *E. nidulans* clade (Figure 5.40). Nucleotide alignments can be found in Appendix 3, Figure 5.

Table 5.16: Percentage amino acid identity between the MAT1-1-1 alpha domain proteins of *P. chrysogenum*, *N. fumigata*, *E. nidulans* and *P. marneffei*.

	N. fumigata	*E. nidulans*	*P. marneffei*
P. chrysogenum	21	21	22
N. fumigata		47	37
E. nidulans			31

```
P. chrysogenum  --MSTSLDAS VPPGYGPAHM EMLLFRYIET LSLRHALRVL ERWPEDSPVG
N. fumigata     ------MEAA ISPLERAFNT FLMTMPPEQL EELLQYLQDT KAQENNGLQL
E. nidulans     ------MENA LSPLQRAFNA FLLSMPPQQL DDLVKHIQDV KAQEQKPPVF
P. marneffei    MTTGLFPTAD LNPLQRAFNL FLLGLPSSDL NHLVNFVRHS DEVERLSYQD

P. chrysogenum  QYAAQVLRDI PANYFQQPRL LPTGPRFVYA NGVLELKRID QALPMQQLHP
N. fumigata     PNATPATTAN NALDNHHGAA VPV------- ---------- ----------
E. nidulans     RNEIPAIRAN TTQDAHHTFP TFP------- ---------- ----------
P. marneffei    DFMRQTTDVV TAISTEEQVP SSP------- ---------- ----------

P. chrysogenum  DSTTGCVGHV MQNVLSSPLE QRRLRPLNSF MLFRSFCAPM FPGIPQKVKS
N. fumigata     ----AATPRP LVTR-AKRTQ EGKKRPLNSF IAFRSFYSVI FPDLTQKAKS
E. nidulans     ----SSKHRP ASSR-GRRVH DGKRRPLNSF IAFRSFYSAI FPDITQKSKS
P. marneffei    ----ASSVGS TRTIRGKQTG EKKLRPLNSF IAYRSFYSTM FPEVTQKTKS

P. chrysogenum  MAISEMWQDD TLKSHWAILA KAYTIIRDHF DVDTPSLSTF VELCLPLMGH
N. fumigata     GTLRFLWQND PFKAKWAILA KAYSIIRDDH ESEVS-LDQF LEITAKFIGL
E. nidulans     GILRFLWQND PFKAKWTILA KAYSIIRDKH DDEVS-LESF LTLNAELIGV
P. marneffei    GIIKDLWQAD PYKGKWAILA KAYSIIRDDH RTEVS-LDTF LELTVPFIGL

P. chrysogenum  LDRQQYLRMA GWDVQPTGN- SLSLRKIGLS NLASLSVP-A IAVDQVVKHC
N. fumigata     FEPARYLDAM GWQLNFDDQQ QYTMAKVKIT TIPEADVSTN YSVGDIVKHC
E. nidulans     TQPDRYLDAM GWELTLNDQQ QYTMARVKSP VATEAQLSTH FSVDDLIKHC
P. marneffei    IQPEDYLGII GCQLVKIDD- QYIIQKISPA RHNLSEVATN YSVEDVLNYC

P. chrysogenum  IDNNYAQIRN EEWD--KHIL ENGQVFAVDP AFSATVRDPQ NWVFNNVPQW
N. fumigata     YDTGYVSEKP GKHTGSNGNN TSTMAFAAQP TFVVKAENGI QITGDDAIVT
E. nidulans     YATGYVTEDK RKKE-IRGHN APVMTFATQP ALVIHKNNSL QISGNHTVVS
P. marneffei    YERGYVDVQH TDHS----ET TSQVSFAAQP NSNIRTDHGA IVLDNVNRLM

P. chrysogenum  PIEEFEVDEM YSSLDTERDL GLPVIYDPNK NPSVAATMAN LDRIFGHN--
N. fumigata     DDAFATPEVD FPTPEETDGT QTPNP-VEAE PVVNNHPYAF MDVPGVPGGQ
E. nidulans     TNGSESVTKE TPAFEPTEAT ELPYPSDIVS PVTGDTSFES TDATRIYQ-R
P. marneffei    QYVPVQNSAE HCQVSTSALL GVRIPTQTQG DLLKNINIKV ADLRQHNN--

P. chrysogenum  ---------- ---------- ---------- ---------- ----------
N. fumigata     QLELELFQGN DFDLNNMQLP IIDALPFDLA VADAFPLNYD PLEEPPFGAF
E. nidulans     PQSRTSLAEN YLDMANMQFH TWDDQ----- T-ALLPYNTG PLMQESFDAL
P. marneffei    ---------G DPDLYAPFNP TVQGFP---- ------T-YD PMAHDPFDAF

P. chrysogenum  --------
N. fumigata     DIDQYINV
E. nidulans     DFKPFLNI
P. marneffei    NITDMPY-
```

Figure 5.39: Amino acid alignment of putative MAT1-1-1 proteins of *P. chrysogenum, N. fumigata, E. nidulans* and *P. marneffei*. Green highlighting indicates where amino acids are conserved between all species. Red highlighting indicates where amino acids are conserved between three species. For culture identification codes and GenBank accession numbers used in alignments, see Appendix 4.

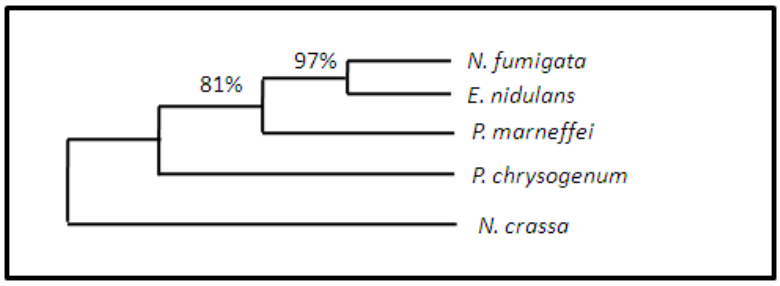

Figure 5.40: Phylogram showing genetic relatedness between the MAT1-1-1 amino acid sequences of *N. fumigata, E. nidulans*, *P. marneffei* and *P. chrysogenum*. *N. crassa* MATA is included as an outgroup. Bootstrap values greater than 70% are shown. Maximum parsimony analysis was performed using MEGA version 4 program with 500 bootstrap replicates and excluding gaps (section 2.2.20) (Tamura *et al.* 2007). For culture identification codes and GenBank accession numbers used to generate phylogeny, see Appendix 4.

5.3.7.2 *MAT1-2-1* Family HMG-Domain Gene Analysis

Alignment of the MAT1-2-1 protein sequences revealed 19-53% identity between *P. chrysogenum, P. griseofulvum, N. fumigata, E. nidulans* and *P. marneffei* (Table 5.17 and Figure 5.41). A maximum parsimony phylogenetic tree (Figure 5.42) was constructed using the MEGA4 program (using default settings with 500 bootstrap replicates and gaps excluded, section 2.2.20). This revealed that *P. griseofulvum* and *P. chrysogenum* were divergent from all other Eurotiomycete species analysed and formed their own distinct clade (Figure 5.42). Nulceotide alignments can be found in Appendix 3, Figure 6.

Table 5.17: Percentage amino acid identity between the HMG-domain MAT1-2-1 proteins of *P. chrysogenum*, *P. griseofulvum*, *N. fumigata*, *E. nidulans* and *P. marneffei*.

	P.griseofulvum	*N. fumigata*	*E. nidulans*	*P. marneffei*
P.chrysogenum	37	31	32	24
P. griseofulvum		21	22	19
N. fumigata			53	31
E. nidulans				33

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P. chrysogenum  MAKTLLDFVG DDDRVTPRRS MELLWADAVN HLPQTDGEVF LPRNVVDGVL
P. griseofulvum ---------- MTVRPLNNKA IYITHRNICC PWVKSRKETL EHFQLLKSTF
N. fumigata     --MATVPIAM KPAAESTDTL TELLWQDALR HLESTNNEVL LPINVTD-MI
E. nidulans     --MAAVSIAM KSPTQSPDSI TELLWKDALR HLGSTNDEVL LPTNVVD-II
P. marneffei    ----MESHSV EPGAGIATSA AELIWNKAVM NFHLTDSEIL LPLNITA-II

P. chrysogenum  DLDHLKAMAI RLACMLNKKV DIVLDQSIDA YRMFPKDLNA EHDENFWADQ
P. griseofulvum RLIPLRSLSN LMNPFLSDDH IFITLAFFHA RLIADGSAPS LACAPRWASP
N. fumigata     GQDNVDKIKT RLGALIGAPV VAFVDETIKA LRVMRTPAFS GTAVSVASHG
E. nidulans     GQDNVEKIKS RLSALLGAPV VSFVDESINA LRVLRTPTFS GSSISVASPS
P. marneffei    GSSNIERLKS QLSNFLRRPC VAFVDESLGC VRILSRPEFK GLVSAADNMV
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P. chrysogenum  HGLDLGDHL- -----LISKS IHEAGDS--V KIPARPAKVP RPPNCFILYR
P. griseofulvum ASRQAPMFSP GRPGGNAERD QPRLSQFCNN TLSINCLFIA RPPNGFILYR
N. fumigata     EAVKTNKVT- -----VTESF APRGKPV--- -GPLKAPKVP RPPNAFILYR
E. nidulans     RALDSWPSE- -----PPNKP RP-------- -ASMKPAKIP RPPNAFILYR
P. marneffei    LAQTSNRKQ- -----FVLNG RPVGSPEKMD KKAEKRPKVP RPPNAFILYR

P. chrysogenum  QANHHLVKDA NPGVSNNEIS RILGARWNNE SPEVREQFTH LADELKKEHA
P. griseofulvum QANHHLVKNA NPGLSNNEIS RILGARWNNE TPEVRHQFTR LANDLKREHA
N. fumigata     QHHHPKIKEA YPDYSNNDIS VMLGKQWKDE NEEIKTQFRN LAEELKKKHA
E. nidulans     QHHYPKVKEA RPDLSNNEIS VIIGKKWRAE PEEGKLHFKN LAEEFKKKHA
P. marneffei    KHYHTILKGR DPNMHNNDIS VTVGSQWNNE SEEVKSHFRA LAAEAKRQHA

P. chrysogenum  IKHPDYQYAP RRPSERKRRT ---------- --------P- -------RSR
P. griseofulvum IKHPDYQYAP RRPEDRRRRT ---------- ---------- ----------
N. fumigata     EDHPDYHYTP RKPSERKRRT SSRQFSKNTK PAALRDTPAS MNISS-DVST
E. nidulans     EEYPDYQYTP RKPSEKKRRA ASRISPKNSK RTVALENPGS MTAPSSNVFT
P. marneffei    QKYPNYQYTP RKPCEKKRRN -SRRATETSD LDAFTEDEEE ISLQTPCESP

P. chrysogenum  ANCLPFVQAD SHYEDMFDDA DFEDRTISID DNFITELNDN GLLFGPNGVE
P. griseofulvum ---RPRAAAA IAAMESDPTE EFDENLIPID DEFMSTLSDT DMLFGPNGAE
N. fumigata     PAMLEGMPV- GEIDFNAAFE DVPGINAIMT SNSILK--NQ QYHFEPNAFD
E. nidulans     PQMYPGIQN- GQLAGAGYIG YLDGLNSMVN TGGLT---DE PTNFGTNAFN
P. marneffei    VNPSPSTEVS GQDQDEFTEQ TMEELMSFVA VPSPPPEAYS FSDFDATEYN

P. chrysogenum  PLS--P-PFT HEECFEMSND LTSGNSLASM ---------- -EFLPMNSVF
P. griseofulvum PIP--A-PWP YYDRMEISNE LASGKRFKSM EYIPMPFDSD ESSLPMPSVF
N. fumigata     LMN-QVQNDY NKTALYQQLS LPEGQIGENF ---------- EFTDFISDCF
E. nidulans     SLFQQPQSDY GRTALFPQLE FAGPSLGDSL ---------- EFPEFAADYF
P. marneffei    SWVNNANTAQ RMAAVIQYNL RAQAQAHVQA ---------- RAQKCNVTTF
```

Figure 5.41: Amino acid alignment of putative MAT1-2-1 proteins of *P. chrysogenum, P. griseofulvum, N. fumigata, E. nidulans* and *P. marneffei*. Green highlighting indicates where amino acids are conserved between all species. Red highlighting indicates where amino acids are conserved between three or more species. For culture identification codes and GenBank accession numbers used to generate alignments, see Appendix 4.

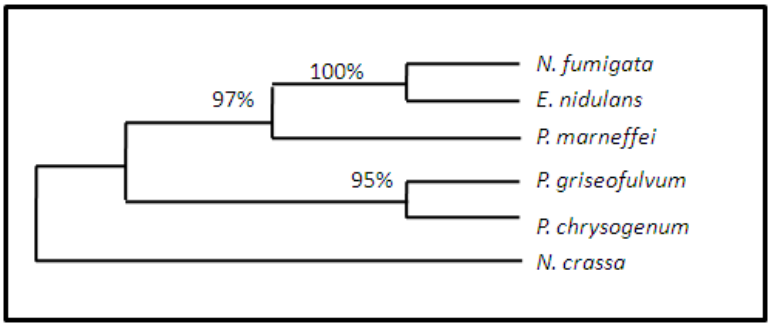


Figure 5.42: Phylogram showing genetic relatedness between the MAT1-2-1 amino acid sequences of *N. fumigata, E. nidulans*, *P.griseofulvum, P. marneffei* and *P. chrysogenum. N. crassa* MATA is included as an outgroup. Bootstrap values greater than 70% are shown. Maximum parsimony analysis was performed using MEGA version 4 program, with 500 bootstrap replicates and excluding gaps (section 2.2.20) (Tamura *et al.* 2007). For culture identification codes and GenBank accession numbers used to generate phylogeny see Appendix 4.

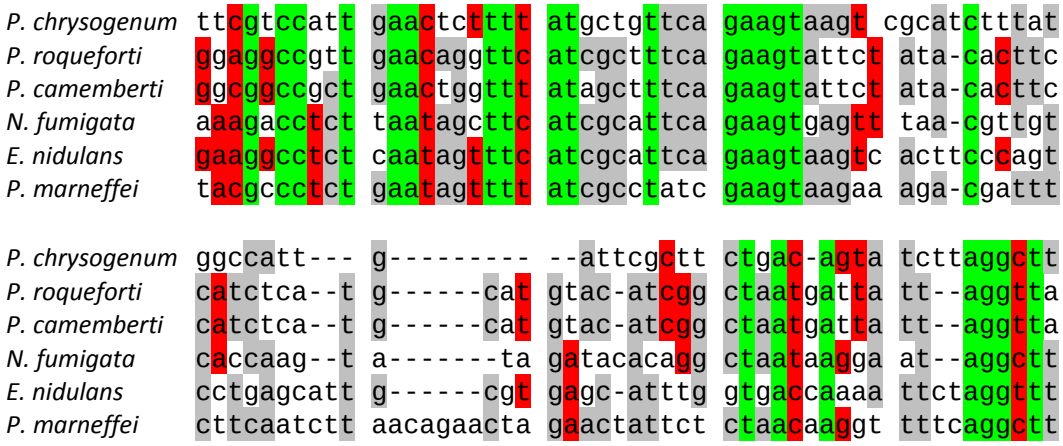
5.3.7.3 Multiple Alignment of Alpha and HMG-domain Core Regions

Multiple alignments were made between the DNA sequences and predicted amino acid sequences (Figures 5.44 and 5.46) obtained from cloning of putative fragments of alpha-domain and HMG-domain encoding genes of *P. chrysogenum, P. roqueforti, P. camemberti* and *P. griseofulvum*. Known DNA and amino acid sequences of *MAT* genes and proteins from *P. marneffei, E. nidulans* and *N. fumigata* were also included in the alignments.

Comparison of *MAT1-1-1* DNA sequence (Table 5.18 and Figure 5.43) revealed highest similarity between *P. roqueforti* and *P. camemberti* (93%) and between 52-70% sequence identity elsewhere. This was reflected in the amino acid alignment (Table 5.19 and Figure 5.44), which revealed highest identity between *P. roqueforti* and *P. camemberti* (97%) and lower values (21-84%) elsewhere. Phylogenetic analysis showed *P. chrysogenum* to be the most divergent of the test species based on nucleotide and amino acid sequence alignments (Figure 5.47).

Table 5.18: Percentage nucleotide identity between the *MAT1-1-1* alpha-domain genes of *P. chrysogenum*, *P. roqueforti*, *P. camemberti*, *N. fumigata*, *E. nidulans* and *P. marneffei*.

	P. roqueforti	*P. camemberti*	*N. fumigata*	*E. nidulans*	*P. marneffei*
P. chrysogenum	51	52	53	54	56
P. roqueforti		93	65	68	61
P. camemberti			63	66	62
N. fumigata				70	64
E. nidulans					61



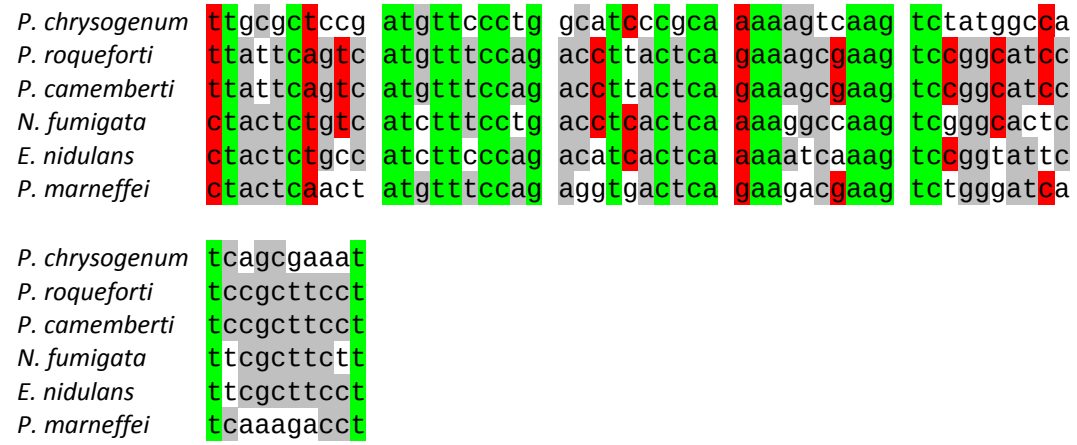


Figure 5.43: Nucleotide alignment of the alpha-domain region within the putative *MAT1-1-1* genes of *P. chrysogenum*, *P.roqueforti*, *P. camemberti*, *N. fumigata*, *E. nidulans* and *P. marneffei.* Green highlighting indicates where nucleotides are conserved between all species. Grey highlighting indicates where nucleotides are conserved between four or more species. Red highlighting indicates where nucleotides are conserved between three species. For culture identification codes and GenBank accession number used for alignments see Appendix 4.

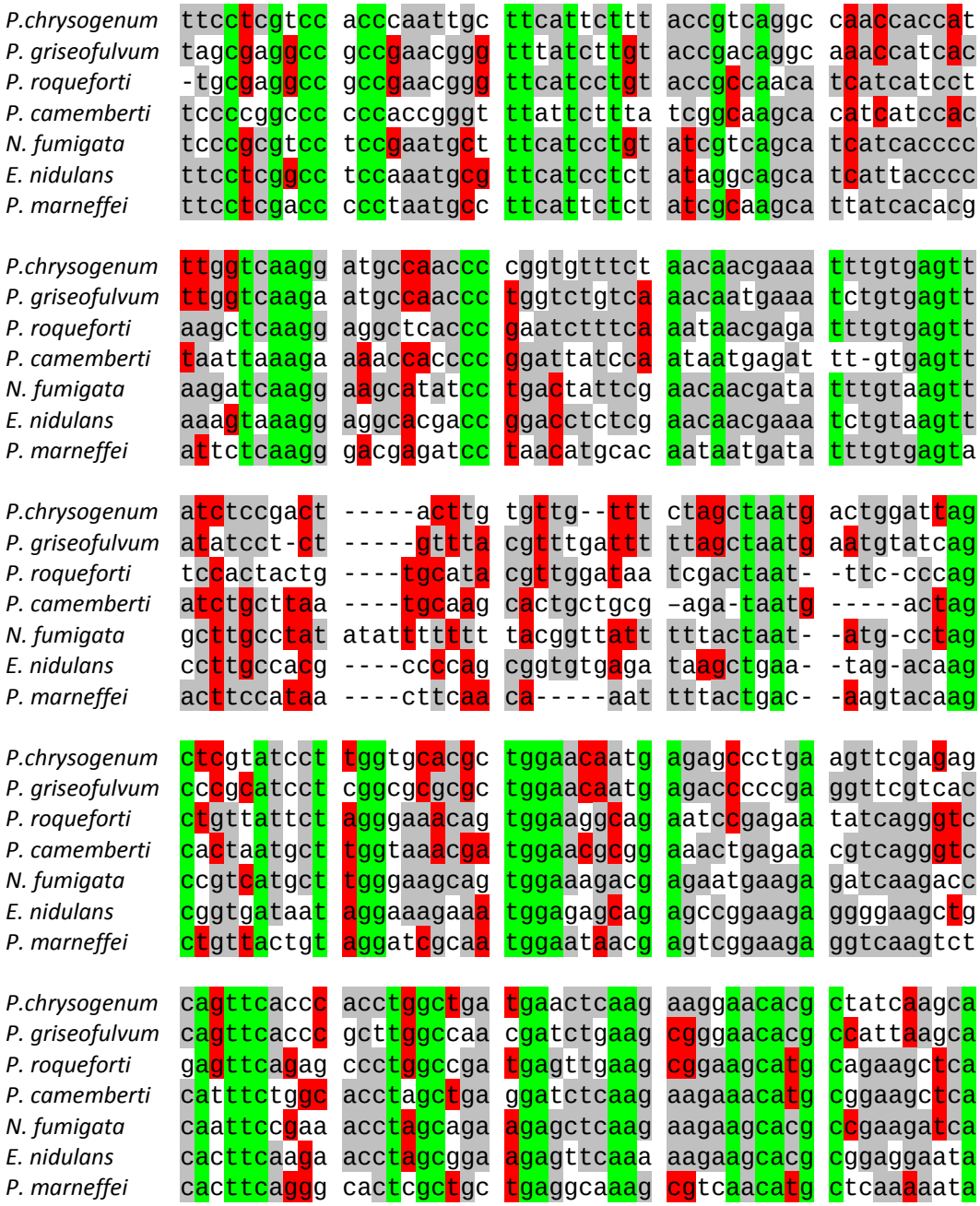
Table 5.19: Percentage amino acid identity between the alpha-domain MAT1-1-1 proteins of *P. chrysogenum*, *P. roqueforti*, *P. camemberti*, *N. fumigata*, *E. nidulans* and *P. marneffei.*

	P. roqueforti	*P. camemberti*	*N. fumigata*	*E. nidulans*	*P. marneffei*
P. chrysogenum	48	47	24	21	22
P. roqueforti		97	84	77	68
P. camemberti			84	78	66
N. fumigata				47	38
E. nidulans					32

Figure 5.44: Amino acid alignment of of the alpha-domain region within the MAT1-1-1 proteins of *P. chrysogenum*, *P.roqueforti*, *P. camemberti*, *N. fumigata*, *E. nidulans* and *P. marneffei.* Green highlighting indicates where amino acids are conserved between all species. Grey highlighting indicates where amino acids are conserved between four or more species. Red highlighting indicates where amino acids are conserved between three or more species. For culture identification codes and GenBank accession numbers used for alignments see Appendix 4.

Comparison of the *MAT1-2-1* DNA sequences revealed highest similarity between *P. roqueforti* and *P. camemberti* (64%) and between 39-64% identity elsewhere (Figure 5.45 and Table 5.20). This was reflected in the amino acid alignment (Figure 5,46 and Table 5.21), which revealed highest identity between *P. griseofulvum* and *P. chrysogenum* (83%), 72% identity was shared between *P. roqueforti* and *P. camemberti*, and 23-65% identity elsewhere. Phylogenetic analysis of DNA nucleotide sequence

showed that *P. griseofulvum, P. chrysogenum, P. camemberti* and *P. roqueforti* form a clade that is distinct form the *E. nidulans, N. fumigata* and *P. marneffei* clade (Figure 5.47). This relationship is also reflected in phylogenetic analysis of amino acid sequences (data not shown).



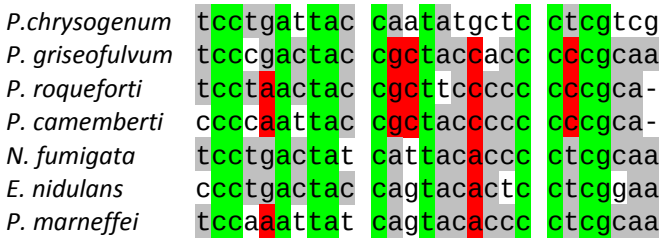


Figure 5.45: Nucleotide alignment of the HMG box domain region within the *MAT1-2-1* genes of *P. chrysogenum*, *P. griseofulvum*, *P.roqueforti*, *P. camemberti*, *N. fumigata*, *E. nidulans* and *P. marneffei.* Green highlighting indicates where nucleotides are conserved between all species. Grey highlighting indicates where nucleotides are conserved between four or more species. Red highlighting indicates where nucleotides are conserved between three species. For culture identification codes and GenBank accession numbers used for alignments see Appenidx 4.

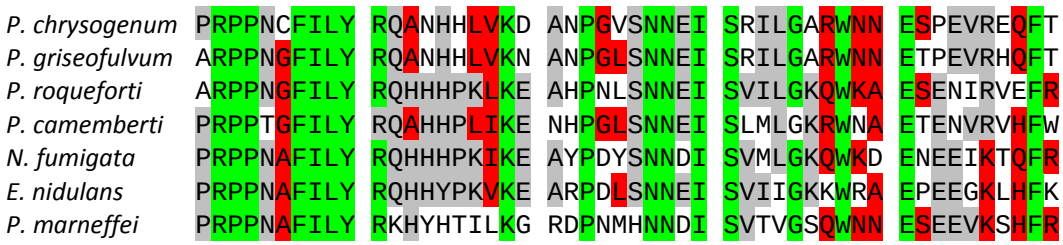


Figure 5.46: Amino acid alignment of the HMG box domain region within the MAT1-2-1 proteins of *P. chrysogenum*, *P. griseofulvum*, *P.roqueforti*, *P. camemberti*, *N. fumigata*, *E. nidulans* and *P. marneffei.* Green highlighting indicates where amino acids are conserved between all species. Grey highlighting indicates where nucleotides are conserved between four or more species. Red highlighting indicates where amino acids are conserved between three species. For culture identification codes and GenBank accession numbers used for alignments see Appenidx 4.

Table 5.20: Percentage nucleotide identity between the *MAT1-2-1* HMG box domain genes of *P. chrysogenum, P. griseofulvum*, *P. roqueforti*, *P. camemberti*, *N. fumigata*, *E. nidulans* and *P. marneffei*.

	P. griseofulvum	*P. roqueforti*	*P. camemberti*	*N. fumigata*	*E. nidulans*	*P. marneffei*
P. chrysogenum	61	57	60	46	45	45
P. griseofulvum		59	55	42	39	39
P. roqueforti			64	66	61	59
P. camemberti				61	58	56
N. fumigata					64	50
E. nidulans						48

Table 5.21: Percentage amino acid identity between the MAT1-2-1 HMG box domain proteins of *P. chrysogenum, P. griseofulvum*, *P. roqueforti*, *P. camemberti*, *N. fumigata*, *E. nidulans* and *P. marneffei*.

	P. griseofulvum	*P. roqueforti*	*P. camemberti*	*N. fumigata*	*E. nidulans*	*P. marneffei*
P. chrysogenum	83	57	62	31	32	23
P. griseofulvum		60	65	54	53	50
P. roqueforti			72	69	64	57
P. camemberti				64	61	49
N. fumigata					53	30
E. nidulans						32

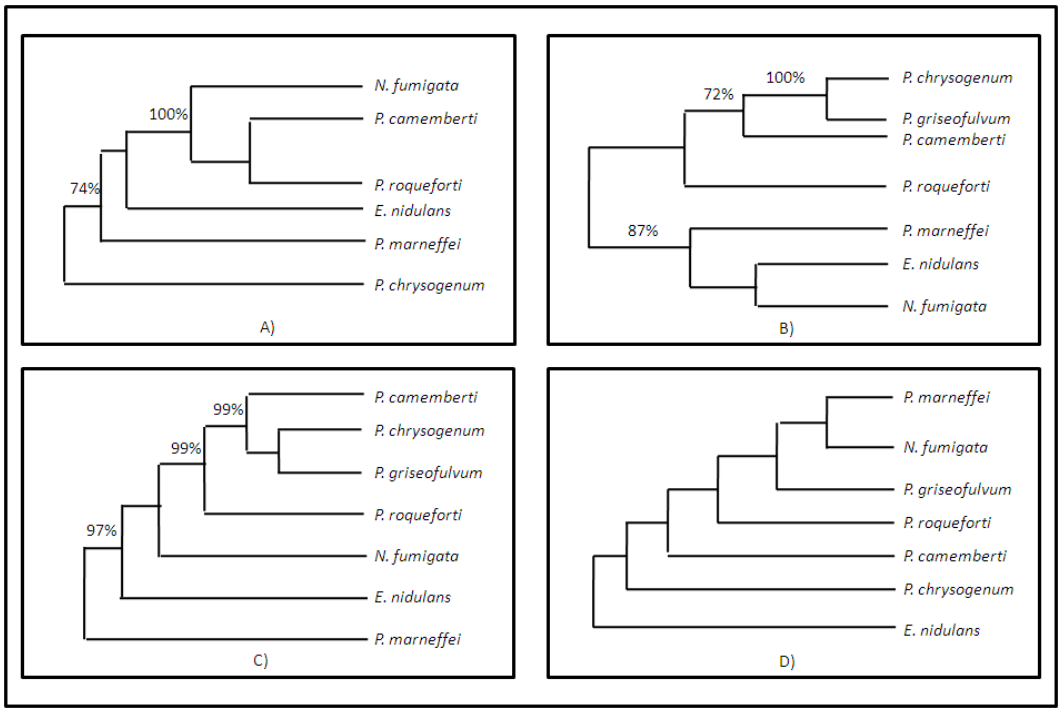


Figure 5.47: Phylograms showing genetic relatedness between certain *Penicillium* and *Aspergillus* species based on alignments of a variety of nucleotide sequences. A) *MAT1-1-1* alpha-domain encoding nucleotide sequences. B) *MAT1-2-1* HMG-domain encoding nucleotide sequences. C) ITS-5.8S rRNA DNA sequences. D) β-tubulin sequences. Bootstrap values greater than 70% are shown. Maximum parsimony analysis was performed using MEGA version 4 program, with 500 bootstrap replicates and excluding gaps (section 2.2.20) (Tamura *et al.* 2007)For culture identification codes and GenBank accession numbers see Appendix 4.

5.4 Discussion

Species within the genus *Penicillium* are of great economic and medical importance to mankind. When studies commenced, this was to be the first investigation made of the occurrence of *MAT* genes in the Penicillia, and although two papers have been published during the course of this study (Woo *et al*. 2006 and Hoff *et al*. 2008), the results obtained are the most comprehensive known to date. The results provide various insights into the possible evolution of asexuality within this group and the possibility of sexual reproduction in supposedly 'asexual' *Penicillium* species, focussing specifically on the subgenus *Penicillium*

5.4.1 *MAT* Gene Occurrence in *Penicillium* Species

Fifty eight of the 59 species included in the subgenus *Penicillium* were screened for the presence of *MAT* genes during the course of this study. Most species were found to be composed of isolates of only one mating type (Table 5.10). A lack of a compatible mating partner would be one key factor rendering these species asexual, as has been observed for the phytopathogen *Hypomyces solani* (also known as *Nectria haematococca*) which is only composed of one mating type in most worldwide locations (Snyder *et al.* 1975).

As can be seen in Figures 5.6 to 5.9, the vast majority of *Penicillium* species tested in the subgenus *Penicillium* produced a fragment of a *MAT1-2-1* HMG-domain gene during PCR screening. Two species amplified only a fragment of an alpha-domain gene, whilst three species produced both alpha- and HMG-domain encoding genes i.e. an apparently homothallic organisation. Finally, four species (including *P. chrysogenum* from additional data) are heterothallic in the sense that isolates were present that amplified either alpha- or HMG-domain fragments.

Figure 5.48 shows the accepted phylogeny of Penicillia based on β-tubulin sequences (Figure 5.3), and also includes details of the organisation of *MAT* gene loci. Although the presence of *MAT* genes can be seen as evidence of sexual potential, other interpretations are also possible, particularly with respect to the presence of a *MAT1-2-1* HMG-domain encoding gene. It has previously been shown that the HMG-box domain alone is sufficient for DNA binding and may have retained this function whilst being subverted for other cellular purposes even if its roles as a sex protein have ceased (Kronstad and Staben 1997). This is contrast to the alpha-domain protein encoded by the *MAT1-1-1* gene, which seems to only have a role in mating with no other cellular roles having been reported (Kronstad and Staben 1997). It has also been suggested that the HMG-domain proteins were ancestral sex determinants in fungi (Idnurm *et al.* 2008). It is therefore not surprising that the *MAT1-2-1* gene is widespread throughout this subgenus. For some species tested it was not possible to amplify either *MAT1-1-1* or *MAT1-2-1* genes, this could mean that these *MAT* genes are missing or have mutated. To test for this, Southern blot analysis would need to be performed to confirm the

presence or absence of these genes, as they may have significant sequence divergence preventing annealing of the degenerate primers used in this study.

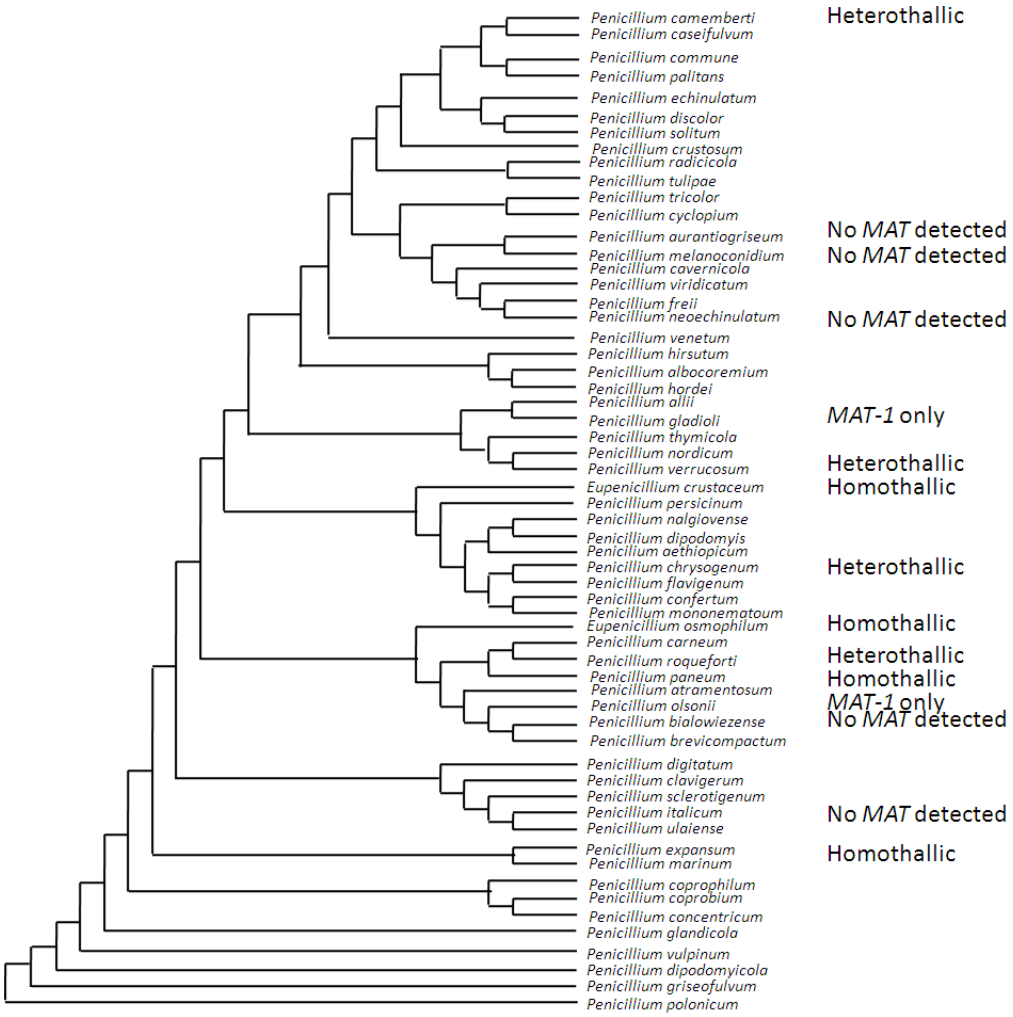


Figure 5.48: Phylogenetic relationships within the *Penicillium* subgenus *Penicillium* determined using β-tubulin sequencing. Organisation of *MAT* genes are as indicated. Unless otherwise stated, species possessed only a *MAT1-2-1* gene. Homothallic = both *MAT1-1-1* and *MAT1-2-1* gene fragments amplified from same isolate. Heterothallic = *MAT1-1-1* and *MAT1-2-1* gene fragments amplified from different isolates.

It should be noted that in this study on average 5 isolates were screened for most *Penicillium* species under examination but in some species only a single isolate was investigated. This means that the presence of isolates of complementary mating type might have been overlooked, due to the low number of isolates screened. Indeed, screening of *P. chrysogenum* from the CBS collection revealed only MAT1-2 genotypes, whereas a more extensive screen of 97 isolates revealed not only the presence of a

MAT1-1-1 gene fragment, but also that there is a 1:1 *MAT* gene distribution. The 1:1 distribution of *MAT1-1-1* to *MAT1-2-1* genes is highly significant as this suggests that this species is either still sexual or has recently lost its sexual capacity, because a drift away from a 1:1 ratio would be expected in a purely asexual population (Dyer and Paoletti 2005). A recent report by Hoff *et al.* (2008) also reported a 1:1 *MAT* distribution in *P. chrysogenum*, albeit with a much lower sample number. Results for *P. chrysogenum* parallel those for *N. fumigata*, where a 1:1 distribution of mating types was found during population survey work (Paoletti *et al.* 2005), before a sexual teleomorph stage was finally discovered a few years later following intensive laboratory studies involving crossing of 'fresh' field isolates (O'Gorman *et al.* 2009).

Thus it is possible that other *Penicillium* species might also be composed of populations containing both MAT1-1 and MAT1-2 isolates, but that further screening work is needed to identify the complementary mating type. For example, screening of 17 isolates of *P. griseofulvum* revealed that all were of MAT1-2 genotype, indicating a lack of MAT1-1 type isolates in nature. However, for *Cryptococcus neoformans*, a known sexual species, it was necessary to screen over 1500 isolates in order to find two MATa isolates. One of these MATa isolates has a defective pheromone gene and is unable to mate (Lengeler *et al.* 2000b; Nielsen *et al.* 2003). If a similar *MAT* gene skew is present in *Penicillium* species, then many more isolates will need to be surveyed in order to find compatible partners. It is noted that no screening was performed in this study to confirm that *Penicillium* isolates used were not clonal because evidence for variation between isolates had previously been obtained from analysis of β-tubulin sequences (Samson *et al.* 2004). Also, due to time and monetary constraints in this study only a small selection of *MAT* gene fragments amplified during screening were cloned and sequenced. For the rest of the species screened band size alone was used to determine mating type. Positive and negative controls were performed for each screen, and there was no evidence of PCR contamination, but it is impossible to entirely rule out the possibility of a PCR artefact of the same or similar size to that desired.

5.4.2 Evolution of Reproductive Strategy

It has been suggested that homothallism is the ancestral state of the *Aspergillus* and *Penicillium* clade (Fraser *et al.* 2007b; Galagan *et al.* 2005; Geiser *et al.* 1998a; Varga *et al.* 2003). If homothallism is the ancestral state of the genus *Penicillium* then multiple losses of the *MAT1-1-1* gene and a few *MAT1-2-1* gene losses would need to have occurred to reach the current distribution. Multiple losses of sex in the subgenus *Biverticillium* and *Talaromyces* clade has already been suggested, but *MAT* gene distribution in this clade is not known (LoBuglio *et al.* 1993; LoBuglio and Taylor 1993). As already stated the MAT1-1-1 protein is only used as a sex determinant, whereas the MAT1-2-1 protein also has the possibility to be used as a DNA binding factor for use in other pathways (Kronstad and Staben 1997). It therefore seems intuitive that, if species were to lose sex, a *MAT* gene used purely in sexual reproduction would be lost in preference to an alternative *MAT* gene that could be retained for use in other pathways. This seems to be the case in the subgenus *Penicillium* resulting in the *MAT* gene distribution currently seen (Figure 5.48).

There is accumulating evidence that heterothallism is the ancestral strategy of many Eurotiomycete fungi (e.g. Fraser *et al.* 2007b and current study). In this case, the distribution of *MAT* genes detected in the Penicillia can be explained by frequent loss of MAT1-1 mating partners, and occasional evolution of homothallic species by incorporation of *MAT1-1-1* or *MAT1-2-1* genes within the same genome (Paoletti *et al.* 2007; Turgeon 1998).

Three species in the subgenus *Penicillium* were found to have *MAT* genes in a 'homothallic' arrangement in the present study i.e. both *MAT1-1-1* and *MAT1-2-1* gene fragments were detected. This included the economically important pathogen *P. expansum*. This is potentially a very exciting discovery as there have not been any previous reports of 'asexual' species with the presence of both *MAT1-1-1* and *MAT1-2-1* genes; instead, all asexual species to date have a heterothallic-like *MAT* gene arrangement (e.g. Arie *et al.* 1997; Foster and Fitt 2004; Kerényi *et al.* 2004; Linde *et al.* 2003; Paoletti *et al.* 2005; Sharon *et al.* 1996; Yun *et al.* 2000). Due to time constraints it was not possible to investigate further the arrangement of *MAT* genes in these

'homothallic asexual' Penicillia. But it is known that approximately 40 homothallic *Eupenicillium* species are distributed throughout the subgenus *Penicillium*, consistent with sexual potential in the *Penicillium* grouping. Of these, the MAT locus arrangement of *E. crustaceum* (anamorph: *P. crustaceum*) is known (Figure 5.50) (Hoff personal communication), and is very similar to that of *N. fischeri*, but whether this arrangement is the same as that found in *Penicillium* species is unknown, but unlikely given the inversion of the *MAT1-2-1* gene in *E. crustaceum* compared to the *MAT1-2-1* gene in the *Penicillium* species investigated in this study (Figures 5.49 and 5.50).

5.4.3 *MAT* Gene Functionality

In order to gain insights into *MAT* gene functionality and potential sexuality, further studies were undertaken in *Penicillium* species. Fragments of *MAT* genes or entire idiomorph regions were sequenced from three species with *MAT* genes in a heterothallic-like arrangement namely, *P. chrysogenum, P. roqueforti* and *P. camemberti*, together with the species *P. griseofulvum* that only possessed a putative *MAT1-2-1* gene. Results showed the following.

The first key result was that for all the species under examination, it was confirmed that the putative *MAT* amplicons were indeed fragments of genuine mating-type genes, as judged by sequence conservation. Also that there were no apparent mutations, such as stop codons or frameshift mutations in these fragments, which would cause the genes to be non functional. Indeed for *P. chrysogenum* and *P. griseofulvum* it was possible to clone and sequence the entire *MAT1-1-1* and *MAT1-2-1* genes, and again there was no evidence of the presense of any mutation which would lead to a non-functional protein. During the course of this study Hoff *et al*. (2008) published a report in which they also described the isolation and sequencing of putative *MAT1-1-1* and *MAT1-2-1* genes from *P. chrysogenum*. When these sequences are compared to those obtained from *P. chrysogenum* isolate B2 from this study no nucleotide or amino acid polymorphisms were found within the putative *MAT1-1-1* gene. However, when the Hoff *et al*. (2008) putative *MAT1-2-1* gene sequence is compared to that obtained from CBS 776.95, 3/1012 nucleotide and 2/303 amino acid polymorphisms are seen. A putative *MAT1-2-1* gene was sequenced from an additional *P. chrysogenum* MAT1-2 isolate (CBS 775.95, for

sequence see Appendix 3, Figure 3). When the Hoff *et al.* putative *MAT1-2-1* gene sequence is compared to that obtained from CBS 775.95 9/1012 nucleotide and 8/303 amino acid polymorphisms are seen. Meanwhile, CBS 775.95 and CBS 776.95 exhibit 6/1012 nucleotide and 6/303 amino acid polymorphisms.

A full phylogenetic analysis of the *MAT* gene and protein sequences was undertaken, as shown in Tables 5.18 to 5.21 and Figures 5.39 to 5.41. Important features to note were that conservation of *MAT* gene sequence between species was restricted mainly to the alpha- and HMG-domain functional regions. Also that the phylogenies produced by the *MAT* genes were incongruent with other established taxonomic relations derived from ITS 5.8S rDNA and β-tubulin sequencing (see Figure 5.47 A to D). The reason for this incongruence is unclear, but might be due to the relatively small regions used for *MAT* gene analysis, meaning that their use for taxonomic analyses is limited. It may also be that the *MAT* genes have different evolutionary histories which may have resulted from gene insertions during the evolution of these species.

It has been suggested that *MAT* genes may be useful phylogenetic markers due to their relatively rapid evolutionary rate when compared to ITS-5.8S rDNA sequences (Turgeon 1998). A reliable phylogenetic marker would be especially useful in the subgenus *Penicillium* where ITS-5.8S rDNA sequences are not divergent enough to be used for phylogenetic analyses (Skoube *et al.* 1999). However, more complete *MAT* genes would have to be sequenced for this taxonomy to be viable. Also repeat sequences would have to be obtained as *P. chrysogenum* shows polymorphisms between isolates, CBS775.95 and CBS 776.95 have been shown to be closely related (Appendix 3, Figures 2 and 3) (Samson *et al.* 2004).

A second key result concerned the organisation of *MAT* genes within the idiomorph. The orientation of *MAT* genes was determined by the *SLA2-APN2* strategy for the three heterothallic species *P. chrysogenum, P. camemberti* and *P. roqueforti*, together with *P. griseofulvum* which exhibited only the MAT1-2 genotype. Figure 5.49 gives a diagrammatic summary of the idiomorphic arrangements found in this study. The *MAT* gene orientations within the idiomorphic region are conserved for all three species containing both MAT-1 and MAT-2 idiomorphs. The *P. griseofulvum* MAT-2 idiomorph

also has the same arrangement as the other MAT-2 idiomorphs examined. The predicted idiomorph lengths (6-7kb) are similar for all four species, as are the relative gene positions within the idiomorphs. Unfortunately, no '*MAT1-1-1* only' species were analysed so it cannot be known if the MAT-1 idiomorphs in these species are also conserved, or whether it is a coincidence that the *MAT1-1-1* genes were substituted/recombined into the idiomorph in the same orientation.

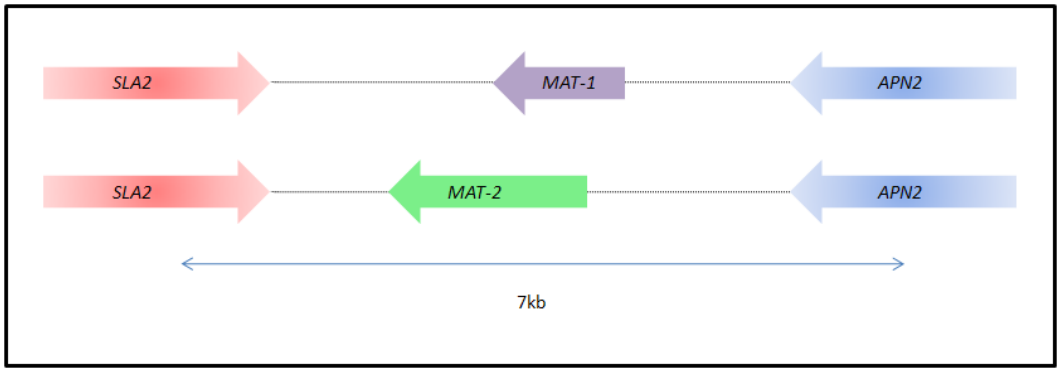


Figure 5.49: Summary of *MAT* gene orientations found during this study.

Figure 5.50 shows the idiomorphic arrangements of *P. marneffei*, *N. fumigata* and *E. crustaceum*. The *MAT1-1-1* genes have the same orientation relative to the flanking *SLA2* and *APN2* genes in all four *Penicillium* species, *E. crustaceum* and *N. fumigata*. However, while the *MAT1-2-1* gene has the same orientation between the four *Penicillium* species in the subgenus *Penicillium*, this is not the same orientation as that of *P. marneffei*, *N. fumigata*, *N. fischeri*, *E. nidulans* and *E. crustaceum*. The fact that the orientation of the *MAT1-2-1* gene in the idiomorph region of all the *Penicillium* species differed from that seen in the Aspergilli and most other ascomycete fungi (Debuchy and Turgeon 2006) suggests that there was an inversion event in an ancestral *Penicillium* species of the subgenus *Penicillium*. Whether this might have any functional consequence is unclear, but unlikely as the same *MAT1-2-1* gene orientation is seen in *N. fennelliae* (see Chapter 3). The species *P. marneffei*, *N. fumigata* and *N. fischeri* also possess an additional, putative gene within the MAT-2 idiomorph. The *MAT1-2-4* gene is not present in *E. nidulans*, nor is it present in other sexual Aspergilli (see Chapter 3), so is it not an ubiquitous gene in the genus *Aspergillus* and it does not seem necessary for sexual reproduction. Its presence in *E. crustaceum* is unknown. The whole MAT-2 idiomorph was sequenced for *P. chrysogenum* and the region between the putative

MAT1-2-1 and *SLA2* in *P. griseofulvum* was also sequenced (Appendix 3, Figures 2 and 4). Bioinformatic analyses of these regions revealed no evidence for the presence of a *MAT1-2-4* gene. *P. camemberti* and *P. roqueforti* were also tested for the presence of the *MAT1-2-4* gene by PCR using the *MAT1-2-4* specific primers developed in Chapter 3. However, no fragments were amplified for these species. Whilst the presence or absence of the *MAT1-2-4* gene has no repercussions on sexual potential in species, it may be a useful marker for the study of *MAT* gene evolution in these genera.

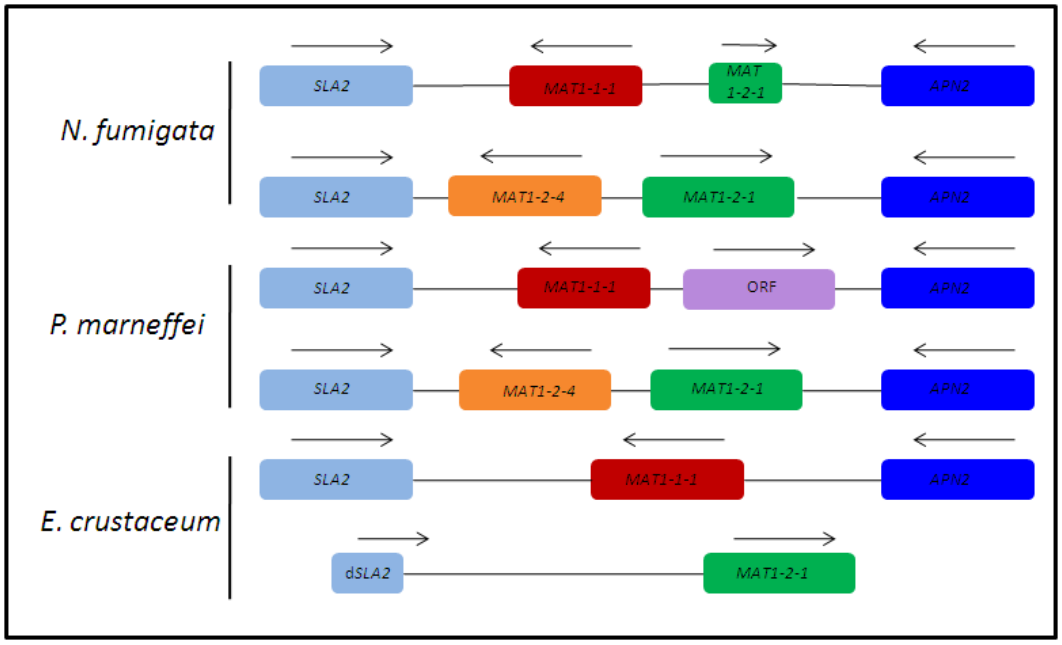


Figure 5.50: Mating-type gene orientation for *N. fumigata, P. marneffei* and *E. crustaceum* [Paoletti *et al*. (2005), Woo *et al*. (2006) and Hoff (personal communication).]

A third key result concerned the findings of RT-PCR analysis. For all three *Penicillium* species with a 'heterothallic' arrangement of *MAT* genes (i.e. *P. chrysogenum, P. camemberti* and *P. roqueforti*) it was possible to demonstrate expression of *MAT1-1-1* and *MAT1-2-1* genes, with processing of the mRNA to remove introns under conditions which induce sexual reproduction in *E. nidulans* (Paoletti *et al.* 2007). This indicated that the genes might have a functional role. However, the putative *MAT1-2-1* gene of *P. griseofulvum* was not expressed under the conditions assayed. Numerous asexual species have previously been shown to express *MAT* genes under the conditions used in this study or similar conditions (Galagan *et al.* 2005; Kerényi *et al.* 2004; Paoletti *et al.*

2005; Ramirez-Prado *et al.* 2008; Sharon *et al.* 1996; Woo *et al.* 2006; Yun *et al.* 2000). It does appear that *P. griseofulvum* has lost the ability to reproduce sexually as no *MAT1-1-1* isolate could be found and the putative *MAT1-2-1* gene is not expressed under the conditions used in this study. This is comparable to the *MAT1-1-1* gene found in *A. niger* for which no expression could be detected under the growth conditions assayed (Pel *et al.* 2007).

It is noted that expression of *MAT1-1-1* and *MAT1-2-1* genes from *P. chrysogenum* was also reported by Hoff *et al*. (2008). They were also able to demonstrate expression of putative pheromone precursor and receptor genes, again consistent with latent or cryptic sexuality in this species.

Finally, crossed cultures including MAT1-1 and MAT1-2 isolates were set up to try and induce sexual reproduction for *P. chrysogenum, P. roqueforti* and *P. camemberti.* However, after 6 months of incubation no visible sexual structures were seen, therefore no sexual reproduction had occurred. Sexual reproduction may be induced under different conditions as in *Aspergillus* species where sexual reproduction has been studied to a greater extent compared to *Penicillium* species. Within this clade there are many different variables that need to be considered and controlled to induce sex even in known sexual species, which might explain the failure to induce sex.

In parallel work, Hoff *et al.* (2008) showed *MAT* and pheromone gene expression occurred when isolates of *P. chrysogenum* of opposing mating-type were incubated together for ten weeks. However, no sexual reproduction was seen. It was suggested that lack of sexual reproduction may be due to decreased sexual potential when isolates are kept for a long time and repeatedly subcultured. Decreased culture viability, sexual potential and alteration in secondary metabolite production has been shown in other species subject to long-term subculture (Horn and Dorner 2001; Ryan *et al.* 2002). Consequently, I used isolates of *P. chrysogenum* that were recently collected from the environment, which were then screened to confirm possession of *MAT* genes and then compatible isolates were placed on plates together within 1 month of isolation and the incubation period was started. Although 'fresh' *P. chrysogenum* isolates were used to ensure that sexual ability had not been lost via subculturing, no sexual cycle was seen

during this study. Despite this result, population genetic studies suggest a recombining population structure with possible cryptic speciation (Henk, personal communication).

5.4.4 Conclusions

It is possible that loss of sexuality in *Penicillium* species may be correlated with their ecological niche. All *Penicillium* species are saprophytes. It has been suggested that in an environment where the substrate is constantly changing due to decay, sexual reproduction may not be the best reproductive strategy. The parasexual cycle allows faster adaptation in the short-term than the 'full' sexual cycle (Jinks 1952b). The parasexual cycle has been demonstrated in numerous *Penicillium* species, and might be the only possibility for recombination given that most species surveyed in the present study were comprised of isolates of only one mating type (Barron 1962; Hong and Robbers 1985; Jinks 1952a; Pontecorvo and Sermonti 1954; Strømnaes *et al.* 1964). In the present study species such as *P. roqueforti*, *P. chrysogenum* and *P. expansum* were found to possess both *MAT* genes (either homothallic or heterothallic configurations) and therefore might retain the capacity for sexual reproduction. Two of these species have also retained the parasexual cycle (Hong and Robbers 1985; Pontecorvo and Sermonti 1954). These species may switch from an asexual reproductive lifestyle, allowing rapid replication and little recombination, to sexual reproduction in order to increase genetic variation.

Although *P. camemberti* has the apparent potential for sexual reproduction, there is as yet no morphological evidence for a teleomorph. Given that this species is only isolated from cheese or cheese making facilities then one factor limiting sex might be lack of compatible mating types in a particular factory setting. The same is not true for *P. roqueforti* where mixing could occur in the external environment as this species occurs as a saprotroph in nature (Pitt 1979; Raper and Thom 1949)

The majority of species screened possessed only a *MAT1-2-1* gene, and no sexual reproduction could be demonstrated for the species where both *MAT* genes are present. The *MAT* genes sequenced have not significantly diverged from known functional *MAT* genes. A more extensive screen using more isolates may result in the

discovery of the complementary mating-type genes as was the case for *P. chrysogenum* in this study. The analysis of *MAT* genes for other *Eupenicillium* species may help to determine the origin of the *MAT* genes within this genus.

In conclusion, the work described in this chapter has demonstrated some potential for sexual reproduction in supposedly 'asexual' *Penicillium* species. However, much work remains to be undertaken to determine the true potential for sex. Many other genes are required for sex to occur, in addition to the *MAT* genes screened in the present work. There is some promise as recent sequencing of the *P. chrysogenum* genome will allow screening for a host of other 'sex-related' genes (van den Berg *et al.* 2008). Also future gene transformation experiments will be needed to assess the functionality of *Penicillium MAT* genes, such as functional assays for *N. fumigata MAT* genes performed in *E. nidulans* (Pyrzak *et al.* 2008).

Chapter 6 General Discussion

Research undertaken in the present study had an overall aim of gaining knowledge pertinent to the evolution of sexuality and asexuality in the genera *Penicillium* and *Aspergillus* with anticipation that this might provide insights into the potential for sexual reproduction for certain 'asexual' species. Data from studies of both *Aspergillus* and *Penicillium* species will now be discussed.

6.1 Identification of *MAT* and Other Genes Required for Sexual Reproduction

The present study focussed particularly on the presence or absence of functional mating-type (*MAT*) genes as an indication of potential for sex. Putative *MAT1-1-1* and *MAT1-2-1* genes were successfully found in all species in which they were sought, except for six *Penicillium* species for which it was not possible to amplify either an alpha- or HMG-domain PCR product. Where genes were partly or wholly sequenced, inspection of the resulting sequences showed introns to be present at conserved positions, predicted from previous genetic analyses. There were no mutations resulting in premature stop codons or a frameshift(s) in the amino acid sequences, and that overall lengths of entire MAT proteins were similar to those previously reported from euascomycete fungi (Debuchy and Turgeon 2006). Thus, absence, or mutation, of *MAT* genes did not provide an explanation for asexuality for the vast majority of species examined.

Meanwhile, screening of the genomes of *Aspergillus flavus, A. clavatus* and *A. terreus* was undertaken for 61 genes known to be involved in the sexual cycles of filamentous fungi, including mating-type and pheromone precursor and receptor genes. Genes identified from this screen were compared to those present in *Emericella nidulans* (anamorph: *A. nidulans*) and *Neosartorya fumigata* (anamorph: *A. fumigatus*), and no significant sequence divergence was seen for any of the putative 'sex-related' genes. In *A. clavatus* sequence similar to the fatty acid oxygenase gene, *ppoB* could not be found, but this gene is not essential for sexual reproduction (Tsitsigiannis *et al.* 2005). In *A. flavus* sequence similar to the COP9 Signalosome subunit 5 (*csnE*) gene could not be

found, but it has been shown in *E. nidulans* that deletion of *csn* genes does not disrupt early sexual processes, although maturation of sexual fruit bodies is blocked (Busch *et al.* 2003). The lack of a *csnE* gene may be a possible reason for the apparent asexuality and delayed fruiting body formation seen in *A. flavus*, however, this gene may yet be found in the genome of *A. flavus*, but with sequence divergence beyond the similarity cut off values used in this study (E<0.001). In *A. terreus* a sequence similar to the recombination gene *spo11* could not be found. This gene is essential for recombination (Borde 2007; Sasanuma *et al.* 2008) and recombination is essential during meiosis, so this may be a possible reason for asexuality in *A. terreus*. However, this does not explain the lack of initial stages of sexual development in *A. terreus*. As genomic screening was not performed for the *Penicillium* species investigated in this study, it is not possible to know whether genetic mutations have resulted in asexuality in these species.

6.1.1 *MAT* Gene Distribution

The genetic studies conducted to determine worldwide *MAT* gene distribution of *Penicillium chrysogenum, A. clavatus* and *A. terreus* showed distributions not significantly divergent from a 1:1 ratio of MAT1-1 to MAT1-2 genotypes. *A. flavus* and *A. parasiticus* have also previously been shown to have 1:1 ratio of *MAT1-1-1* to *MAT1-2-1* worldwide gene distribution (Ramirez-Prado *et al.* 2008). These surveys provide evidence that a teleomorphic state may be found for all of these species in future studies.

6.1.2 *MAT* and Pheromone Gene Expression

Expression of the putative *MAT1-2-1* genes of *A. terreus* and *P. griseofulvum* could not be detected during this study. Also, no expression of the pheromone precursor or pheromone receptor and precursor genes of *A. terreus* and *A. flavus*, respectively, could be detected under the conditions assayed in this study. In contrast, expression of both *MAT* and pheromone precursor and receptor genes was found for all other species investigated during this study. There is no obvious genetic reason for the disparity of

gene expression seen between species, therefore the reasons for lack of expression in only some species are unknown.

Pheromone and transcription factor gene analysis was not conducted for the *Penicillium* species as none had genome sequence available to allow identification of target genes. However, Hoff *et al.* (2008) did investigate expression of pheromone precursor and pheromone receptor genes in *P. chrysogenum*, and showed these to be expressed. No other studies to date have investigated expression of pheromone precursor and pheromone receptor genes in *Penicillium* species.

6.2 Phylogenetic Analyses and Evolutionary Implications

Figure 6.1 shows the phylogeny of various *Aspergillus* and *Penicillium* species as well as *Ajellomyces capsulatus* and *Coccioides immitis*. The suggested ancestor of this group had a heterothallic MAT idiomorph, with a gene arrangement similar to that seen in *N. fumigata*. This ancestor contains the *MAT1-2-4* gene and evidence for multiple losses of this gene has been presented in Chapter 3, Figure 3.29. It is conceivable that the *MAT1-2-4* gene might have been retained by the eight species where it is now present as it conferred some particular evolutionary or survival advantage in these species, such as increased fertility, but this is unclear. Phylograms produced using the *MAT* genes' nucleotide and amino acid sequences are congruent (data not shown).

Various gene insertions and gene inversions have been seen. In *E. repens* the entire MAT-1 idiomorph may have been inserted into a MAT1-2 isolate rendering this species homothallic. Insertion of either partial or full MAT-2 idiomorphs has occurred as two separate events in both *N. fischeri* and *E. crustaceum*. *MAT1-2-1* gene inversions have occurred in both *N. fennelliae* and in *Penicillium* species in the subgenus *Penicillium*.

MAT and β-tubulin gene analyses clearly produce phylogenetic trees that have 'asexual' species interspersed with sexual species. This may support the 'multiple losses of meiotic reproduction' theory suggested by LoBuglio *et al*. (1993) or may mean that many of the 'asexual' spcecies shown have latent or cryptic sexual cycles. Further work now needs to be undertaken, involving identification and sequencing of complete MAT idiomorph regions from additional *Eupenicillium, Talaromyces* and sexual species with

Aspergillus anamorphs, to help increase our understanding of the sexual status of many 'asexual' species.

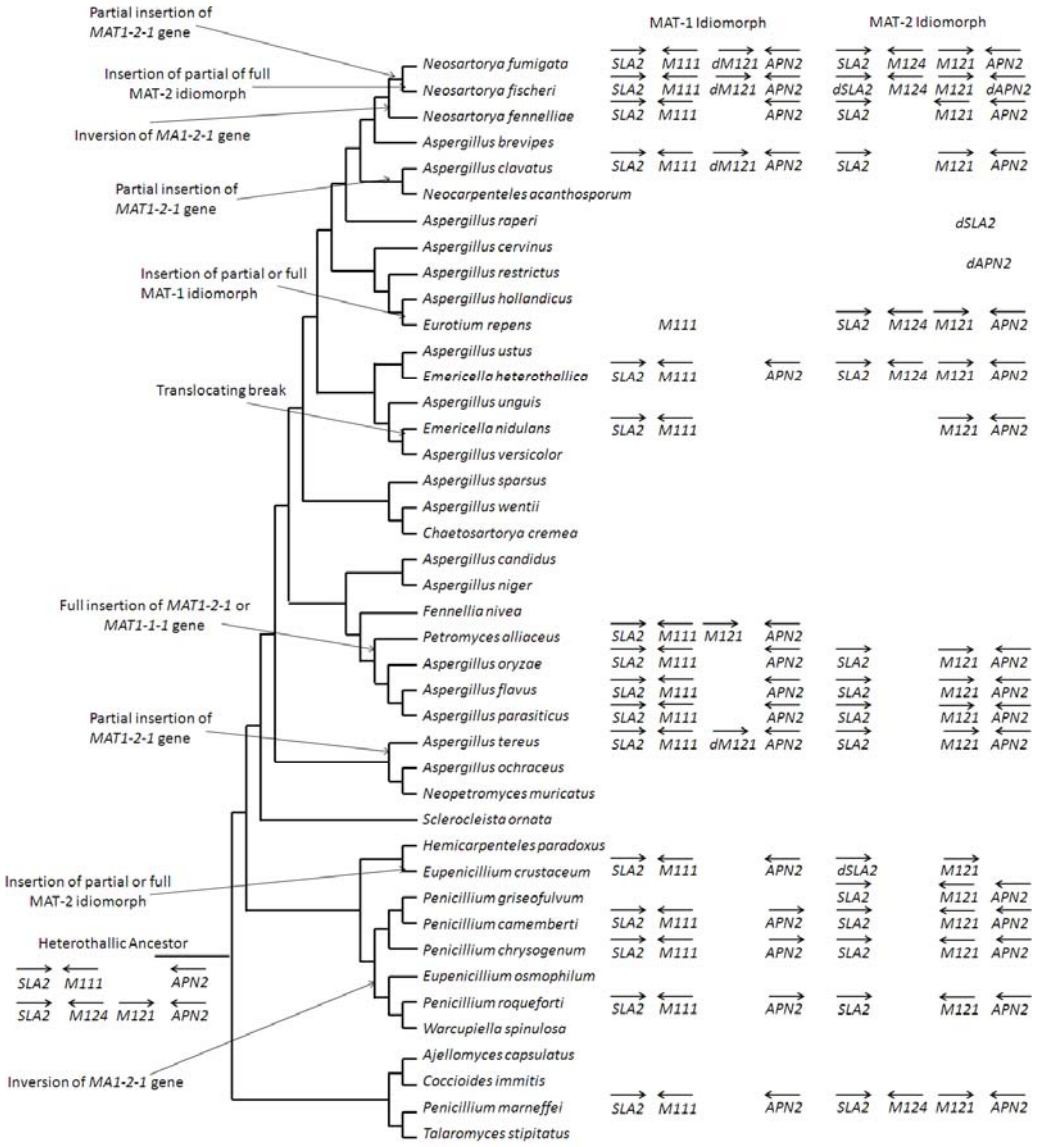


Figure 6.1: Phylogeny derived from β-tubulin sequencing data for various *Aspergillus* and *Penicillium* species. The *MAT* gene arrangements are shown for both MAT-1 (left hand side) and MAT-2 (right hand side) where appropriate and the suggested evolutionary events leading to these arrangements are indicated (note that *MAT1-1-1* and *MAT1-2-1* are abbreviated to *MAT111* and *MAT121*, respectively).

6.3 Evolution of Reproductive States

The evolutionary origins of fungal sexuality and heterothallic and/or homothallic breeding systems have been the subject of much debate (Metzenberg and Glass 1990; Whitehouse 1949). Knowledge gained from both sequencing of MAT regions and whole genome studies over the past decade has now provided key insights into these evolutionary questions. These have revealed how reproductive states have evolved in the following taxa.

6.3.1 Evolution of Reproductive State in Zygomycetes

The mating-type genes of two zygomycete species have been investigated, these are *Phycomyces blakeleeanus* and *Rhizopus oryzae*. In *P. blakesleeanus* two mating types are known, '+' and '-'. These mating types are characterised by the presence of a *sexP* (plus) or *sexM* (minus) gene, in *R. oryzae* only the *sexP* gene has been found (Idnurm *et al.* 2008). Both the *sexM* and *sexP* genes encode HMG-domain proteins similar to the MAT1-2-1 HMG-domain protein found in filamentous ascomycetes. No alpha-domain encoding gene has been found (Idnurm *et al.* 2008). The microsporidia species *Encephalitozoon cuniculi* and *Antonspora locustae* were also found to contain HMG-domain encoding regions as their sex-determining genes. An alpha-domain encoding gene was not found in these species either (Lee *et al.* 2008).

Phylogenetic analyses of the sex genes have suggested *sexM* to be ancestral to the ascomycete and basidiomycete *MAT* genes, with the *sexP* gene lost in the evolutionary history of the Dikarya as the Zygomycota diverged from the Dikarya 600-1,400 MYA (Idnurm *et al.* 2008). Idnurm *et al*. (2008) also suggested that the HMG-domain encoding gene has been replaced or lost in the Dikarya in a gradual process. Evidence for this derived from an additional HMG-domain encoding gene located close to the alpha-domain encoding gene in the MAT locus of some Sordariomycete species i.e. *Neurospora crassa* and *Podospora anserina*. However, this extra HMG-domain protein has a role later in mating compared to the 'main' HMG-domain protein located on the MAT-2 idiomorph or locus of these species (Coppin and Debuchy 2000; Debuchy and Turgeon 2006).

6.3.2 Evolution of Reproductive State in Yeasts

It has been suggested that the ancestor of all extant ascomycete yeast species may have had a heterothallic mating strategy (Butler 2007; Herskowitz 1989). However, it is also conceivable that the ancestor of this species may have been homothallic. The possible arrangement of *MAT* genes of the heterothallic ancestor may have been similar to *Yarrowia lipolytica* (Muller *et al.* 2007). Evolution from a species similar to *Y. lipolytica* then occurred, involving the ascquisition of silent cassettes to produce species such as *Kluyveromyces lactis*. Species like *Candida glabrata* and *Saccharomyces cerevisiae* were then produced following the integration of a *HO* endonuclease gene and a whole genome duplication event (Figure 6.2) (Butler 2007; Herskowitz 1989).

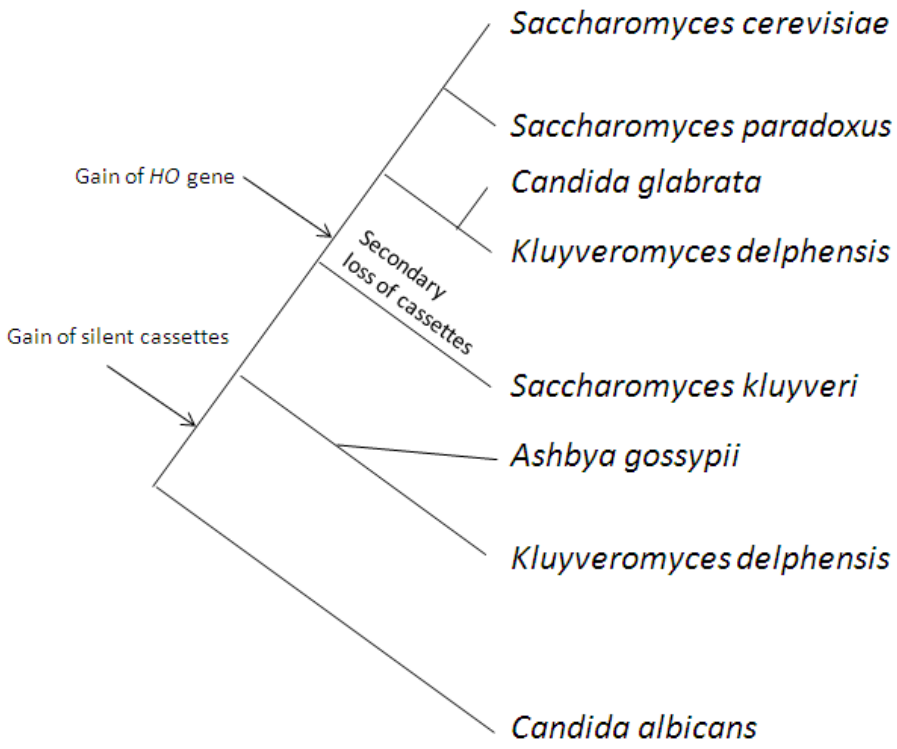


Figure 6.2: Phylogeny of the acquisition of the HO endonuclease gene, and silent cassettes in yeast species. [Adapted from Butler (2007) and Herskowitz (1989)].

The budding yeast, *S. cerevisiae* is a functionally homothallic species that undergoes mating-type switching. The fission yeast *Schizosaccharomyces pombe* also undergoes mating-type switching but this is by a different mechanism, which is believed to have evolved independently of the mechanism seen in *S. cerevisiae* (Nielsen and Egel 2007).

Homothallic species such as *Debaromyces hansenii* and *Pichia augusta* may have arisen by recombination of the α- and **a**-loci within a diploid cell making these species self-compatible (Muller *et al.* 2007). Thus, within yeast species as a whole, the evolution from an ancestral heterothallic state appears most likely.

6.3.3 Evolution of Reproductive State in Basidiomycetes

There are three mating strategies exhibited by basidiomycete species: tetrapolar heterothallism (two multi- or biallelic, unlinked loci control compatibility), bipolar heterothallism (one multi- or biallelic locus controls compatibility) and homothallism. It is suggested that tetrapolar heterothallism is the ancestral mating strategy. Due to the complexity of this multiallelic strategy, which greatly decreases inbreeding, it is suggested that tetrapolar heterothallism evolved only once (Figure 6.3). The bipolar heterothallic mating strategy is believed to have evolved several times independently and, consequently, bipolar heterothallic species are interspersed throughout genera (Figure 6.3).

Bipolar heterothallism may have arisen by fusion of the homeodomain proteins in the A locus or mutation of the pheromone genes, making them self-compatible so only the A locus confers sexual compatibility. Both of these mutations have been found in the homobasidiomycete *Coprinopsis cinereus* (Kües *et al.* 1994; Olesnicky *et al.* 2000). Chromosomal translocation could have moved either the pheromone genes or homeodomain genes into non-recombining regions of the genome. This would mean that these genes are no longer responsible for self-incompatibility. This is seen for the pheromone genes in *Phiolota nameko* and *Coprinopsis disseminatus*, but the homeodomain genes of these species are still multiallelic (Aimi *et al.* 2005; James *et al.* 2006b). *Ustilago hordei* and *Cryptococcus neoformans* have a single locus that contains the pheromone and homeodomain genes rendering these species bipolar (Bakkeren and Kronstad 1993; Lengeler *et al.* 2002). Homothallism, as seen in *Agaricus subfloccossus* may have arisen by acquisition of a compatible pair of *A* genes, or a fusion of A loci derived from compatible species (Casselton and Kües 2007).

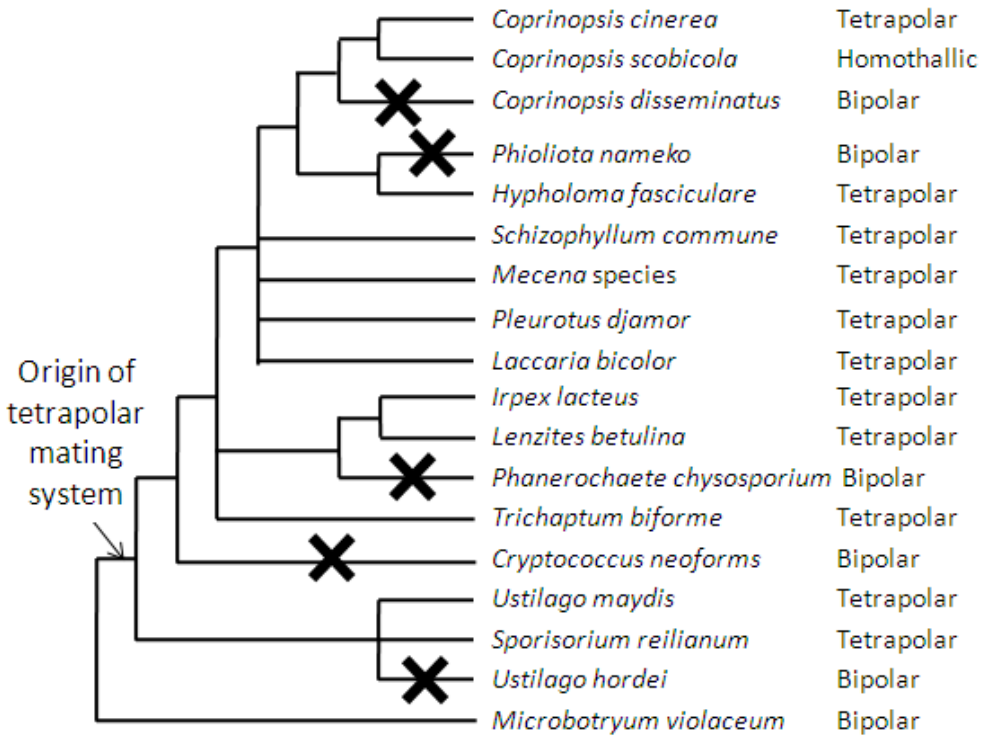


Figure 6.3: Phylogeny of homobasidiomycetes and heterobasidiomycetes derived from the Tree of Life Web project (http://tolweb.org/tree/phylogeny.html). Crosses indicate where species have converted to a bipolar heterothallic lifestyle. [Adapted from James (2007).]

Only 10% of homobasidiomycetes are homothallic, 25-35% are bipolar heterothallic, but the remainder, and majority, are tetrapolar heterothallic (Casselton and Kües 2007). This distribution makes intuitive sense if tetrapolar heterothallism is the ancestral state with a bipolar heterothallic system being derived from it, acting as an intermediate step towards homothallism.

6.3.4 Evolution of Reproductive State in Filamentous Ascomycetes

Investigations into the ancestral sexual state of fungal groups other than the Aspergilli have suggested heterothallism to be the ancestral reproductive strategy (Butler 2007; Butler *et al.* 2004; Coppin *et al.* 1997; Fraser *et al.* 2007a; Kronstad and Staben 1997; Rydholm *et al.* 2007; Yun *et al.* 1999). A clear example of this is the mating-type gene arrangements seen in *Cochliobolus* species (Chapter 1, Figures 1.4 and 1.6) (Yun *et al.* 1999). In this genus, heterothallic species have conserved *MAT* gene arrangements, whereas homothallic species vary in their *MAT* gene arrangements, with gene orders

and orientations, when compared to flanking genes, varying between species (Yun *et al.* 1999). There is also evidence of sequential *MAT1-2-1* gene insertions in *C. kusanoi* to confer homothallism in this species (Chapter 1, Figure 1.4). Idnurm *et al*. (2008) also found a conserved HMG-domain gene in ancestral heterothallic Zygomycete species, but no alpha-domain encoding gene. As the phylum Zygomycota diverged 600-1,400 million years ago from the Dikarya (containing phyla Ascomycota and Basidiomycota) the HMG-domain encoding gene is believe to be the ancestral sex determining gene (Idnurm *et al.* 2008).

It is possible that the Eurotiomycetes are unusual in that they may have been derived from a homothallic ancestor, which was itself originally derived from a heterothallic species (Galagan *et al.* 2005; Geiser *et al.* 1998a; Varga *et al.* 2003). Therefore, in the *Aspergillus* and *Penicillium* genera heterothallism may have re-evolved from a homothallic ancestor, which could explain the comparative rarity of heterothallism in these genera (Fraser *et al.* 2007b; Galagan *et al.* 2005).

The evidence for homothallism being the ancestral strategy of the Eurotiomycete is not, on balance taking into account the present findings, as likely as the reverse situation i.e. that heterothallism is ancestral (Figure 6.1). Homothallic species are more widespread within the Eurotiales compared to heterothallic species. However, this situation is reversed when looking at other orders, especially Onygenales and Ascosphaerales (Geiser *et al.* 1998a). Within the *Aspergillus* clade containing nearly 300 accepted species, there are approximately 70 homothallic species compared to 6 heterothallic species in their 10 related teleomorphic genera. There are approximately 100 teleomorphic species in the genera *Eupenicillium* and *Talaromyces* that have *Penicillium* anamorphs, of these only 1 is heterothallic and the rest are homothallic (Galagan *et al.* 2005; Kwon and Kim 1974; O'Gorman *et al.* 2009; Pitt *et al.* 2000; Takada and Udagawa 1985; Udagawa and Uchiyama 2002). I believe the ancestor of the *Aspergillus*/*Penicillium* clade was heterothallic with a *MAT1-2-4* gene in the MAT-2 idiomorph (Figure 6.1). The reasons for this are as follows.

Firstly, the heterothallic *MAT* gene arrangement is conserved in many sexual and asexual *Aspergillus* and *Penicillium* species. The *MAT* gene arrangement of *N. fumgiata*

seems typical, although it should be noted that some species lack the putative *MAT1-2-4* gene (Galagan *et al.* 2005; Machida *et al.* 2005; Payne *et al.* 2008; Ramirez-Prado *et al.* 2008). The *N. fumigata* gene arrangement is seen in heterothallic species *E. heterothallica*, but also in homothallic species *E. repens* and *N. fischeri* and asexual species *P. marneffei* (Rydholm *et al.* 2007; Woo *et al.* 2006).

Secondly, if homothallism is the ancestral reproductive sexual strategy, it would be expected that most homothallic species would have the same, or very similar, *MAT* gene arrangements. However, there is so far no evidence of a conserved homothallic *MAT* gene arrangement. It has been suggested that the possible ancestral homothallic *MAT* gene arrangement may resemble that seen in *P. alliaceus* (Figure 6.1) (Galagan *et al.* 2005). However, no other homothallic *Aspergillus* or *Penicillium* species have an arrangement like *P. alliaceus*. However, the homothallic species *E. nidulans, N. fischeri* and *E. repens* and *Eupenicilliumi crustaceum* all have different MAT locus organizations. It is possible that the flanking regions surrounding the putative *MAT1-1-1* gene in *E.repens* have degenerated. A similar situation is seen at the MAT-2 locus of *N. fischeri* (Figure 6.1). However, the exact location of the *MAT1-1-1* gene within the *E. repens* genome is unknown as further chromosome walking was not performed.

Finally, the orientation and location of the mating-type genes within the idiomorph is generally conserved. The *MAT1-1-1* gene has the same orientation across all sexual and asexual species investigated so far (Machida *et al.* 2005; Paoletti *et al.* 2005; Paoletti *et al.* 2007; Payne *et al.* 2008; Ramirez-Prado *et al.* 2008; Rydholm *et al.* 2007). The *MAT1-2-1* gene orientation id conserved between most species examined. The *MAT1-2-1* gene is inverted in *N. fennelliae* and *Penicillium* species in the subgenus *Penicillium*, when compared to other *Aspergillus* and *Penicillium* species (Figure 6.1). Gene inversions within MAT idiomorphs are not uncommon and have been recorded between *Coccidioides* species and their close relative *Uncinocarpus* species (Fraser *et al.* 2007b; Mandel *et al.* 2007) and between *Cochilobolus* species (Yun *et al.* 1999).

The conservation of the heterothallic *MAT* gene arrangement between heterothallic species, coupled with the variability of the *MAT* gene arrangement in the loci of

homothallic species supports a heterothallic sexual reproductive strategy being ancestral.

The *MAT1-2-1* gene fragment seen in *N. fumigata, A. clavatus* and other species (Figure 6.1) could have arisen from recombination between the MAT-1 and MAT-2 idiomorphs resulting in a fragment of the *MAT1-2-1* gene within these species. This fragment may not be enough to confer homothallism. A similar situation is seen in *C. kusanoi*, where as partial *MAT1-1-1* gene fragment has been inserted adjacent to the *MAT1-2-1* gene, but this was not enough to confer homothallism, so a second recombination/translocation event occurred transferring a complete *MAT1-1-1* gene resulting in homothallism in this species (Yun *et al.* 1999).

Ascospores that arise from self-fertilisation and outcrossing seem more resistant to environmental stresses that asexual conidia (Baggerman and Samson 1988; Dijksterhuis and Teunissen 2004; Eicher and Ludwig 2002; King and Halbrook 1987; Obeta and Ugwuanyi 1997). Ascospores may also be more longlived when compared to conidia, this may allow for more effective dissemination of ascospores (Anderson and Kohn 1998; Barve *et al.* 2003; Clarkson *et al.* 2003; Cozijnsen and Howlett 2003; Metzenberg and Glass 1990; Nelson 1996). Homothallic species do not have to find a compatible mate to benefit from all the advantages that ascospores convey, unlike self-sterile heterothallic species. Homothallic species also retain the ability to outcross. This means that, like heterothallic species, these homothallic species are able to benefit from recombination of genetic attributes from two different individuals either by combining advantageous mutations together or by disrupting deleterious mutations combining advantageous mutations together or by disrupting deleterious mutations (Barton and Charlesworth 1998; Charlesworth *et al.* 1993; Felsenstein 1974; Goddard 2007; Hurst and Peck 1996; Zeyl and Bell 1997). It would therefore seem likely that a homothallic species is more likely to survive and spread in a heterothallic population than vice versa (Coppin *et al.* 1997).

6.4 Future work

Whilst extensive study of *Aspergillus* and *Penicillium* species was performed in this study, there are still many unanswered questions regarding the sexual potential of these

species. Below are some suggestions of possible future avenues of work, which may help to further determine the sexual potential of the 'asexual' *Aspergillus* and *Penicillium* species.

Publication of the genome sequence of *P. chrysogenum* (van den Berg *et al.* 2008) will allow bioinformatic analyses to be undertaken of genes known to be involved in sexual reproduction, in addition to the *MAT* and pheromone precursor and receptor genes (Hoff *et al.* 2008 and current study). *P. marneffei* has also had its genome sequenced (Yuen *et al.* 2003), when the NCBI genome sequence data becomes publicly available, bioinformatic analyses of various genes for this species could be performed to determine the presence and possible functionality of these genes, i.e. presence of premature stop codons or frameshift mutations.

In this present study of 60 'asexual' *Aspergillus* and *Penicillium* species, three of them showed potential homothallic *MAT* gene distributions i.e. produced electrophoresis gel products of *MAT1-1-1* and *MAT1-2-1* genes from a single isolate. These gel products were not sequenced so no more conclusions can be made regarding a potential homothallic mating strategy in these species i.e. to check whether the genes are mutated to render them non-functional.

Of the species where the *MAT* genes were either partially or fully sequenced, the functionality of the putative *MAT* genes has yet to be proven, although expression has been demonstrated for many of the genes investigated. Integration of the putative *MAT* genes into known sexual species where one or both *MAT* genes removed would give clues as to the functionality of the *MAT* genes (Lee *et al.* 2003; Paoletti *et al.* 2007). Similar transformation experiments have previously been used to show *MAT* gene functionality in other species including *N. fumigata* (Pyrzak *et al.* 2008), *Coprinopsis cinereus* (O'Shea *et al.* 1998), *Sordaria macrospora* (Pöggeler *et al.* 1997) *Bipolaris sacchari* (Sharon *et al.* 1996) and *Podospora anserina* (*MAT* and pheromone genes) (Arnaise *et al.* 1993; Coppin *et al.* 2005). Transformation experiments could be performed using the *MAT1-2-1* and pheromone precursor genes of *A. terreus* as these are not expressed under the conditions assayed for in this study, this may be due to a mutation in the promoter sequence. Thus, if the native and *A. terreus* promoter were

used in transformation studies the functionality of the genes and promoter regions could be examined.

Intriguingly 6 of the 58 *Penicillium* species have no apparent *MAT* gene signal. Southern blot hybridisation experiments have previously identified mating-type and pheromone precursor and receptor genes in a variety of species including *Tapesia yallundae* (Pöggeler 2001), *Coprinopsis cinereus* (Halsall *et al.* 2000), *Cryptococcus neorformans* (Chaturvedi *et al.* 2002) and *Candida parapsilosis* (Logue *et al.* 2005). Probes could be designed using the *MAT* gene sequencing obtained from *Penicillium* species during this study. This may enable *MAT* genes to be located in these six species.

6.5 Concluding Remarks

Sexual reproduction is beneficial for many species, especially in a changing environment. Rapid evolution, via natural selection, can be seen in sexual species as favourable mutations can be brought together within an individual organism much faster than asexual reproduction, where mutations must arise sequentially (Felsenstein 1974; Goddard 2007; Goddard *et al.* 2005; Hurst and Peck 1996; Zeyl and Bell 1997). This can lead to faster acquisition of drug resistance, or increase infectivity (Jenczmionka *et al.* 2003).

A key aim of the present study was to help elucidate the evolution of *MAT* gene organisation and ancestral sexual strategy of economically and medically important *Penicillium* and *Aspergillus* species.

Figure 6.2 shows the possible evolutionary pathways of mating strategy and also mating-type genes in the Eurotiomycetes. *Aspergillus* and *Penicillium* species have a common sexual ancestor, which is reflected in the organisation of MAT loci in extant species. There is a shared arrangement of *MAT* genes seen in *N. fennelliae* and species within the *Penicillium* subgenus *Penicillium*. A shared arrangement of *MAT* genes is also seen in *N. fumigata, E. heterothallica*, *E. repens* and *P. marneffei*. Also homothallic *Eupenicillium crustaceum* (anamorph: *P. crustaceum*) has a degraded *SLA2* gene (and possibly an *APN2* gene) surrounding its *MAT1-2-1* gene (Hoff personal communiation), which is comparable to the gene arrangement of *N. fischeri*. Prior to these results for *E.*

crustaceum, three different homothallic *MAT* gene arrangements had been found in the Aspergilli from the three different species examined. *E. crustaceum* is the first example of a similar homothallic *MAT* gene arrangement being found for this group of species.

If heterothallism is the overall ancestral strategy of the *Aspergillus* and *Penicillium* clade it would be expected to be more widespread within sexual species with *Aspergillus* and *Penicillium* species anamorphs. Many homothallic *Aspergillus* and *Penicillium* species have not been sequenced and their *MAT* regions have not been investigated. However, heterothallism does appear to be widespread within other fungal orders and groups, including yeasts and basidiomycetes (Whitehouse 1949).

As can be seen in basidiomycetes, yeast and filamentous ascomcyetes many changes in mating strategy have occurred and conversions to an asexual lifestyle many times have been suggested. The overall conclusion must be that fungi are extremely flexible in their reproductive strategies, with evolution selecting particular genome arrangements that are best suited for particular species.

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Appendix 1

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1          atcgggaaat ggcggatctg gaggaggcat tgagggtaag tcatgaaagc
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101        tcgtgcccta gaggaataca gctccaggaa cgagatgcga caaggagacc
151        acgatactgc tctccgagaa aaggatgagg aaattgaggt ttataaatcc
201        gccatggagc aagctctaga agaacttgag gagctaaaac tggtgcgttt
251        gaggagcgaa gaaaattgtt agtcactaaa ctgacatgca ccacagagtc
301        aaggcgatgc cgacaacgca ctggacaccc aaattgacca cgtcttacag
351        ggcactgtct ccaaaatcaa tgacattatt gactctgtgc tccagactgg
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1051       caattgcaga gctgatcaaa gcagcaaccg catctcagca agagatcgtt
1101       cgagagggga gaggcagttc gtcccgtacg gctttttata agaagaacaa
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1201       ccaataccct tatcgaaact gccgatggcg tgatctctgg ccgcaactcg
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1501       tggagcaaca ggtatttcag tttttccacc ctgttaccca attttacgtg
1551       ggcgcccttt cgaaaagttg gttcagttga ctcgtccctt ttttttcttt
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1701       agctgactgc tgaatcgatc ctgatgctag ctgaatcgat gctgatcgat
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1851       gatcgcagcg ctgatcgact gtagcatgat cgagtgatgc atgctatcga
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9201        ttccgttg
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Figure 1: 9208bp of the MAT-2 idiomorph DNA sequence from *E. heterothallica* isolate 50-3. Incomplete *SLA2* DNA sequence, 1-1699b (direct strand). Complete *MAT1-2-4* DNA sequence, 2892-4665bp (complementary strand). Complete *MAT1-2-1* DNA sequence, 5175-6249bp (direct strand). Incomplete *APN2* DNA sequence, 7370-9208bp (complementary strand). Primer EhSLA2-1 (forward) is highlighted in purple, primer EhSLA2-2 (forward) is highlighted in red and primer EhAPN2-1 (reverse) is highlighted in green.

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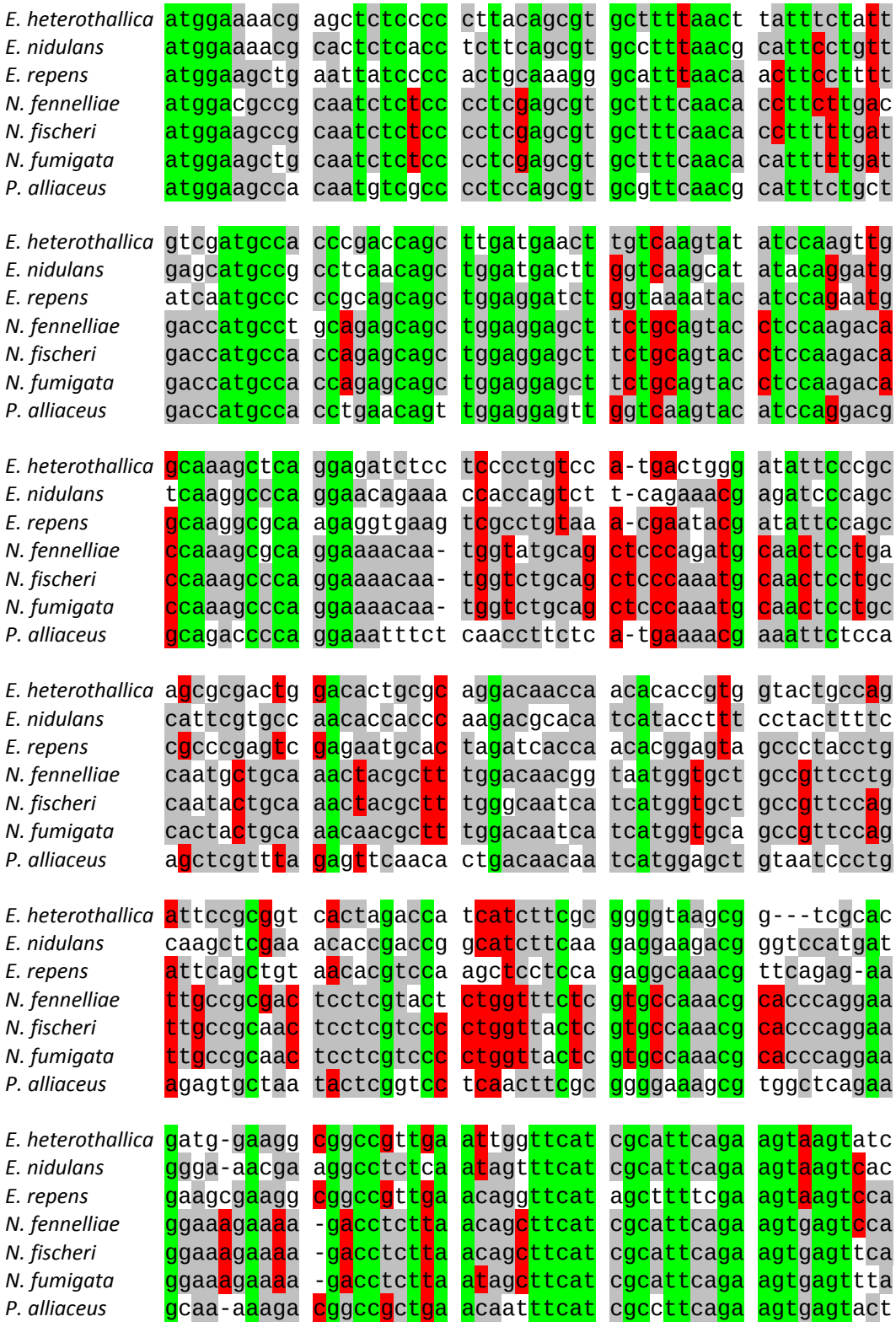
Figure 2: 9437bp of the MAT-2 idiomorph DNA sequence from *E. repens* isolate 51-1 and 51-2. Incomplete *SLA2* DNA sequence, 1-1662bp (direct strand). Complete *MAT1-2-4* DNA sequence, 4216-4989bp(complementary strand). Complete *MAT1-2-1* DNA sequence, 5518-6598bp (direct strand). Incomplete *APN2* DNA sequence, 7567-9437bp (complementary strand). Flanking regions were sequenced from isolate 51-1, *MAT* genes were sequenced from isolate 51-2. Nucleotides in emboldened fint indicate where the sequencing obtained from both isolates is identical (3615-4252bp and 6499-7068bp). Primer ErSLA2 (forward) is highlighted in red and primer ErAPN2 (reverse) is highlighted in green.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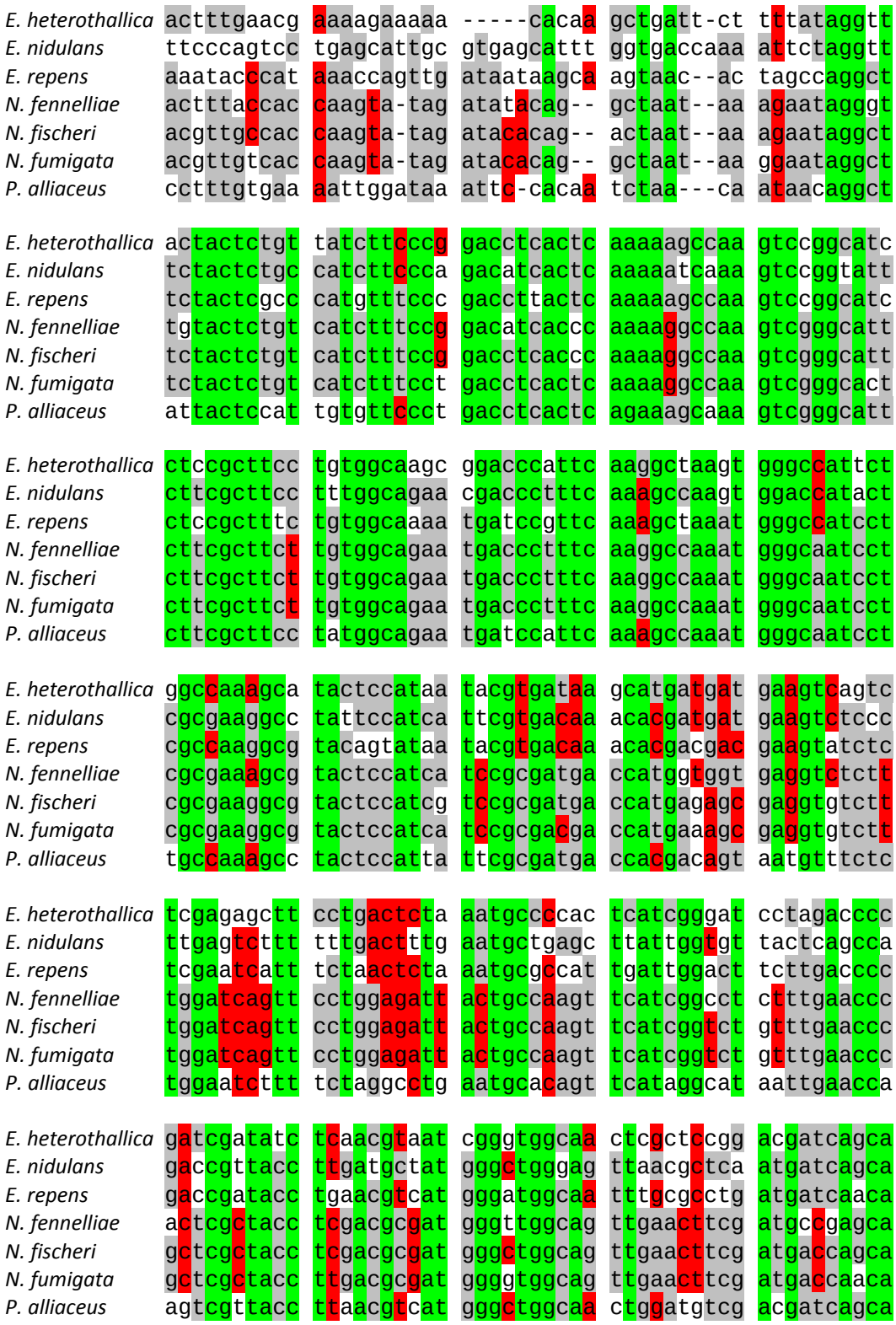
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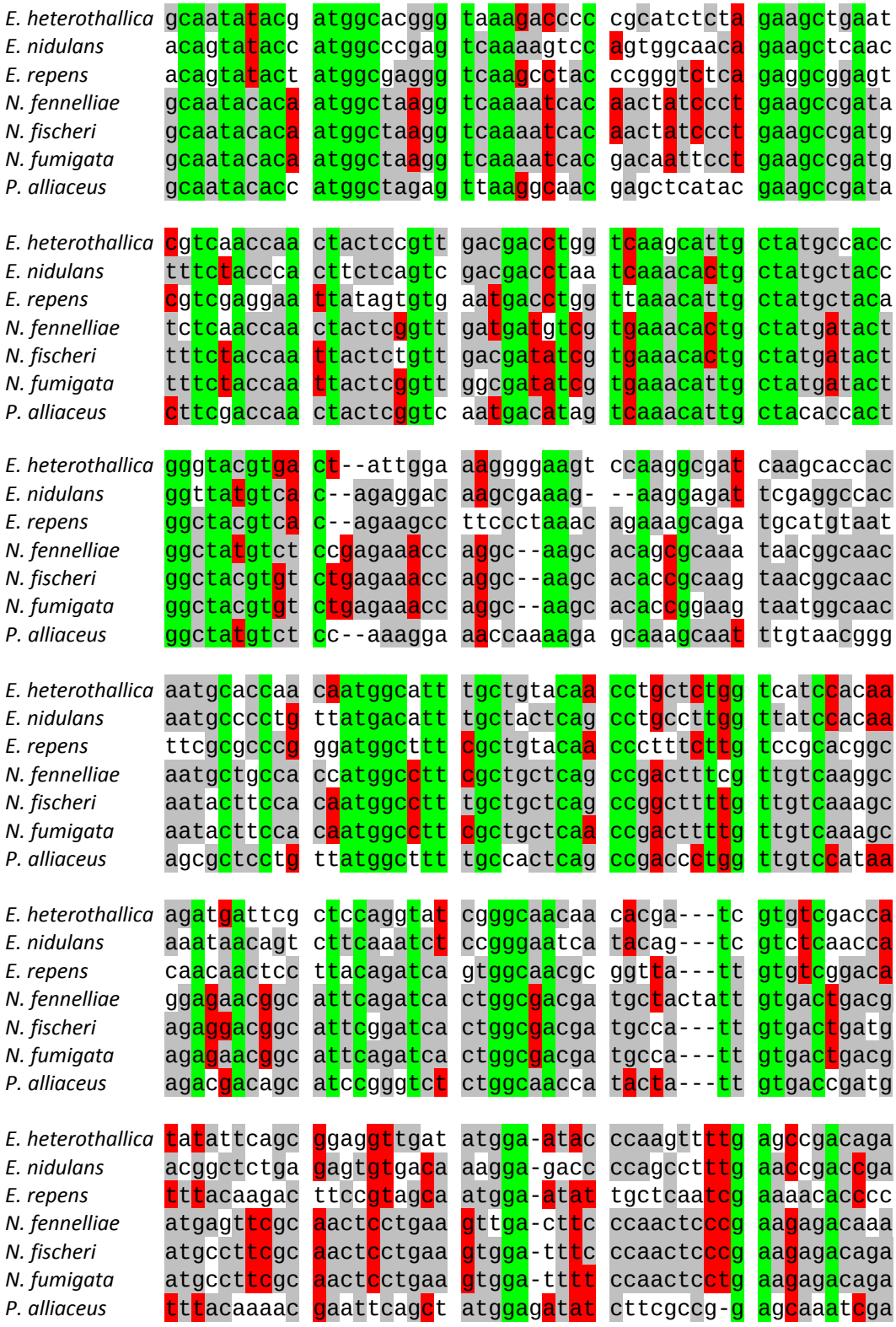
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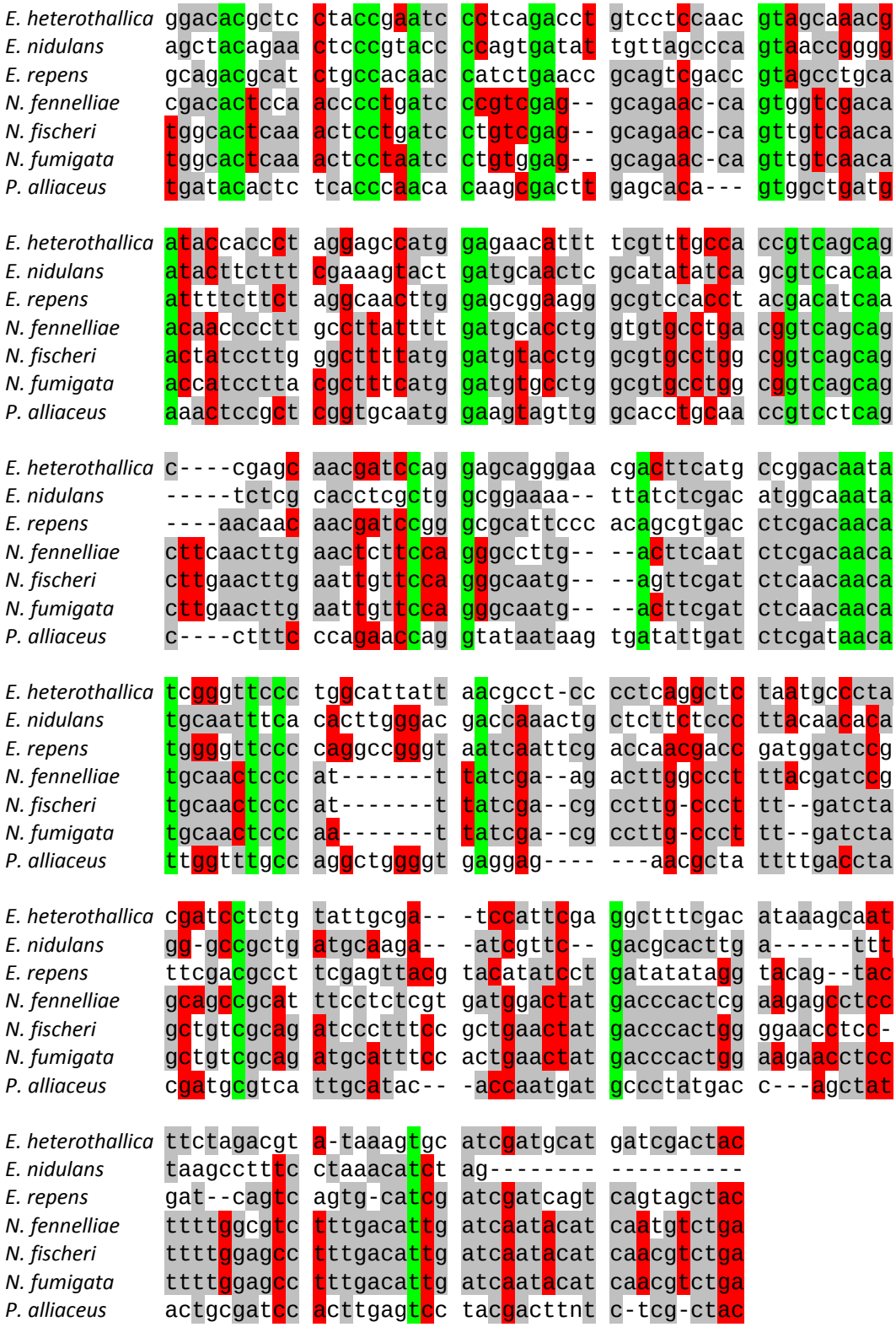
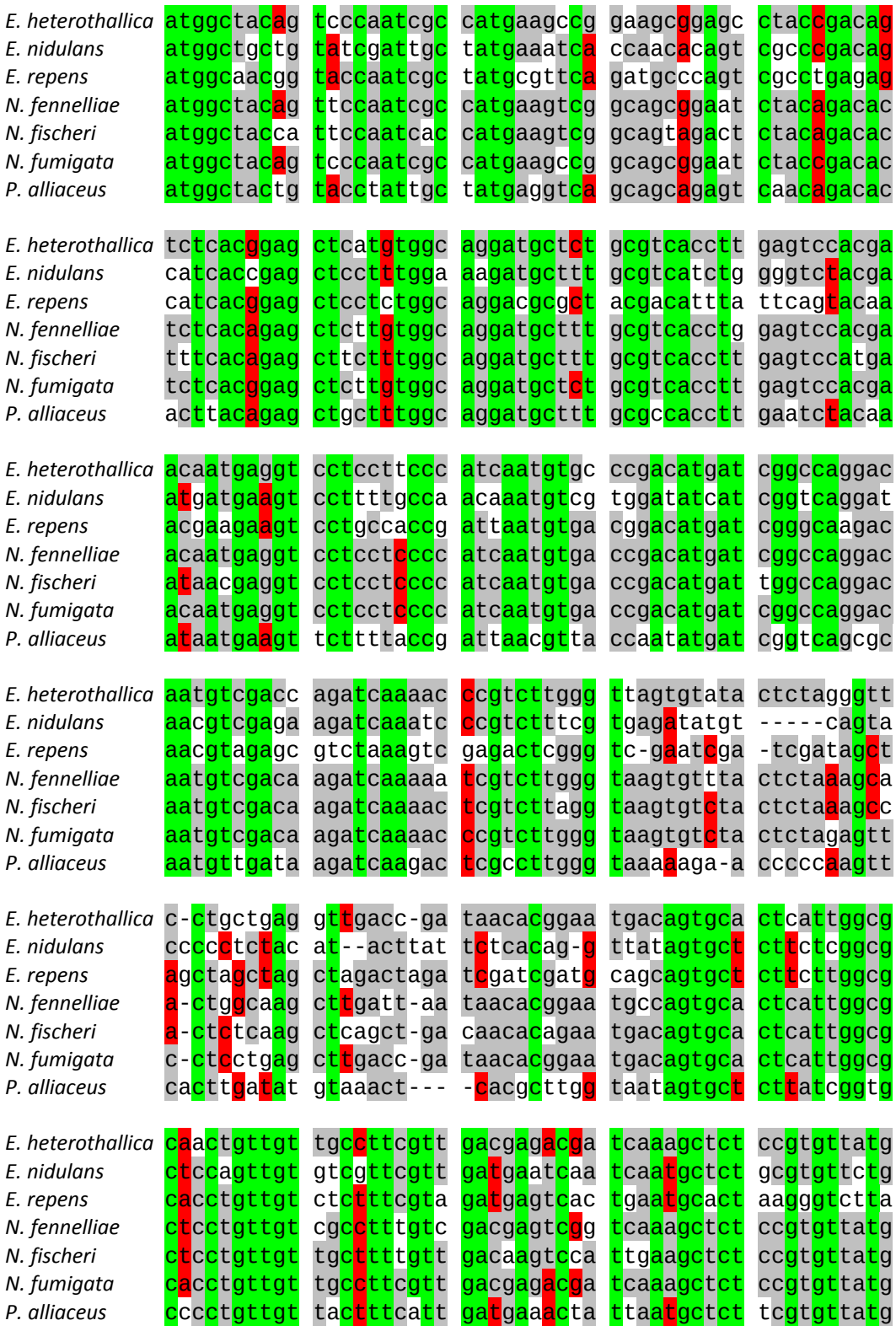
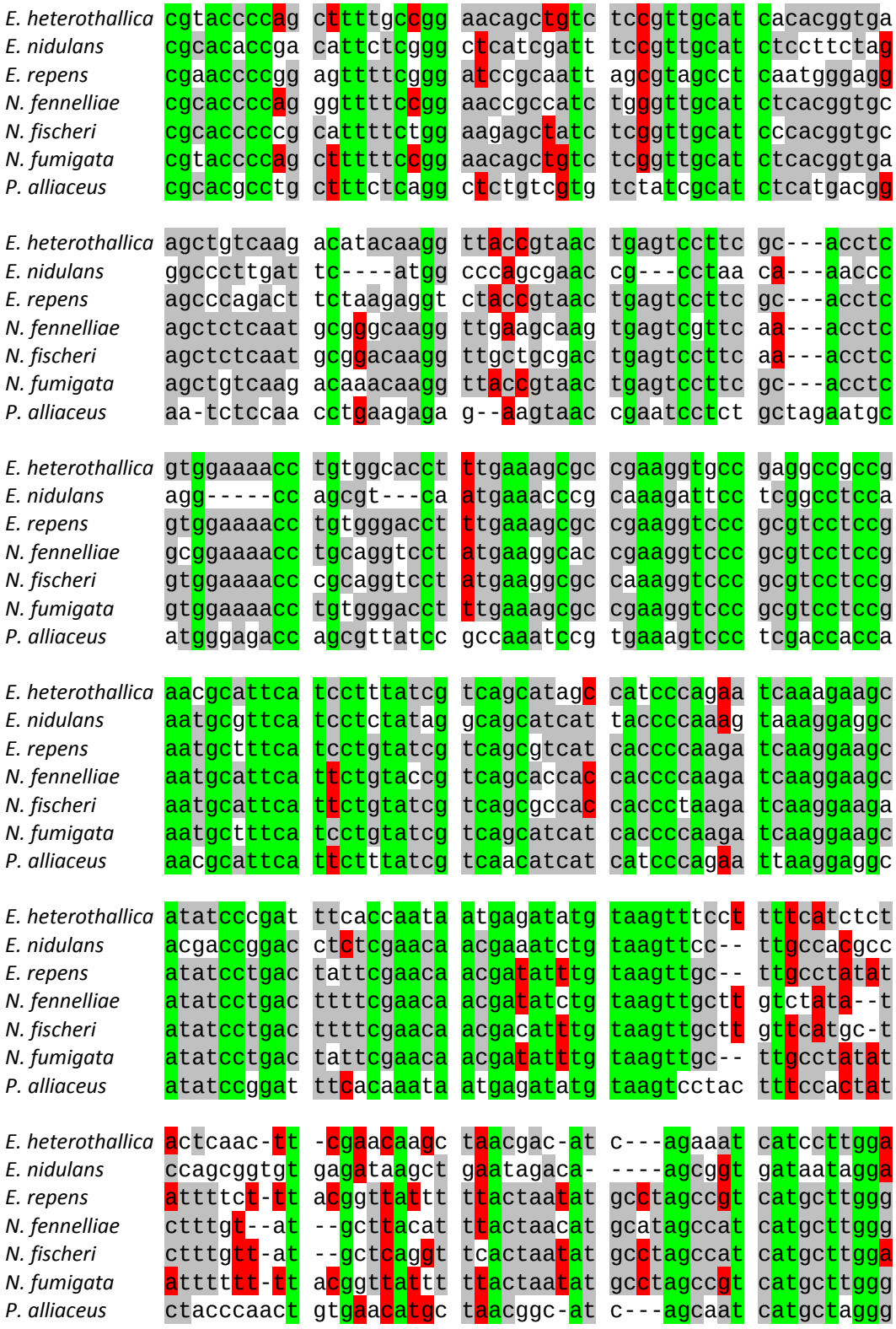


Figure 3: Nucleotide alignments of *MAT1-1-1* DNA sequences comparing *E. nidulans, E. repens, N. fennelliae, N. fischeri, N. fumigata* and *P. alliaceus*. Green highlighting indicates where nucleotides are conserved between all species. Grey highlighting indicates where nucleotides are conserved between four and six species. Red highlighting indicates where nuceltoides are

conserved between three species. For culture identification codes and GenBank accession numbers used for alignments see Appendix 4.

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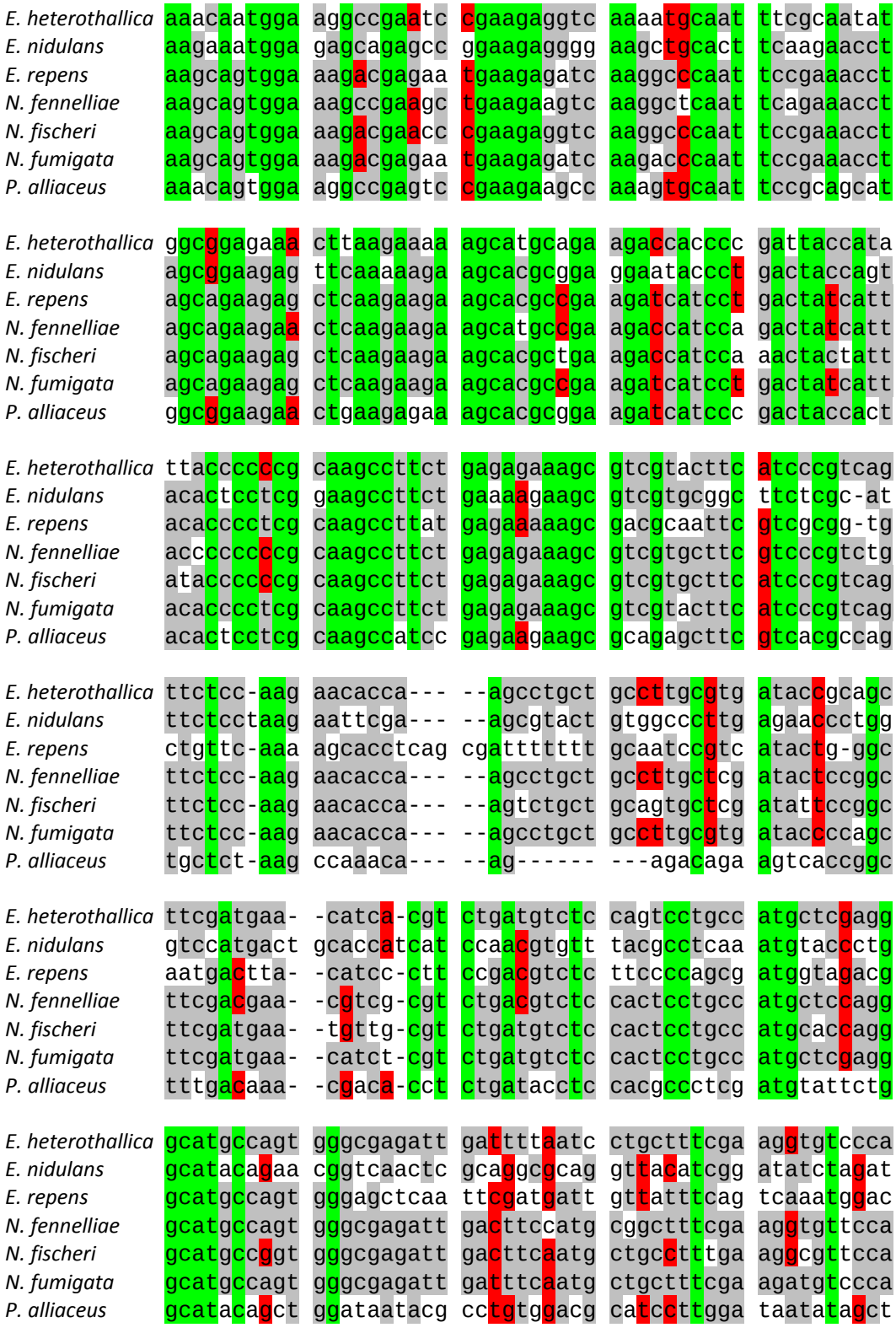
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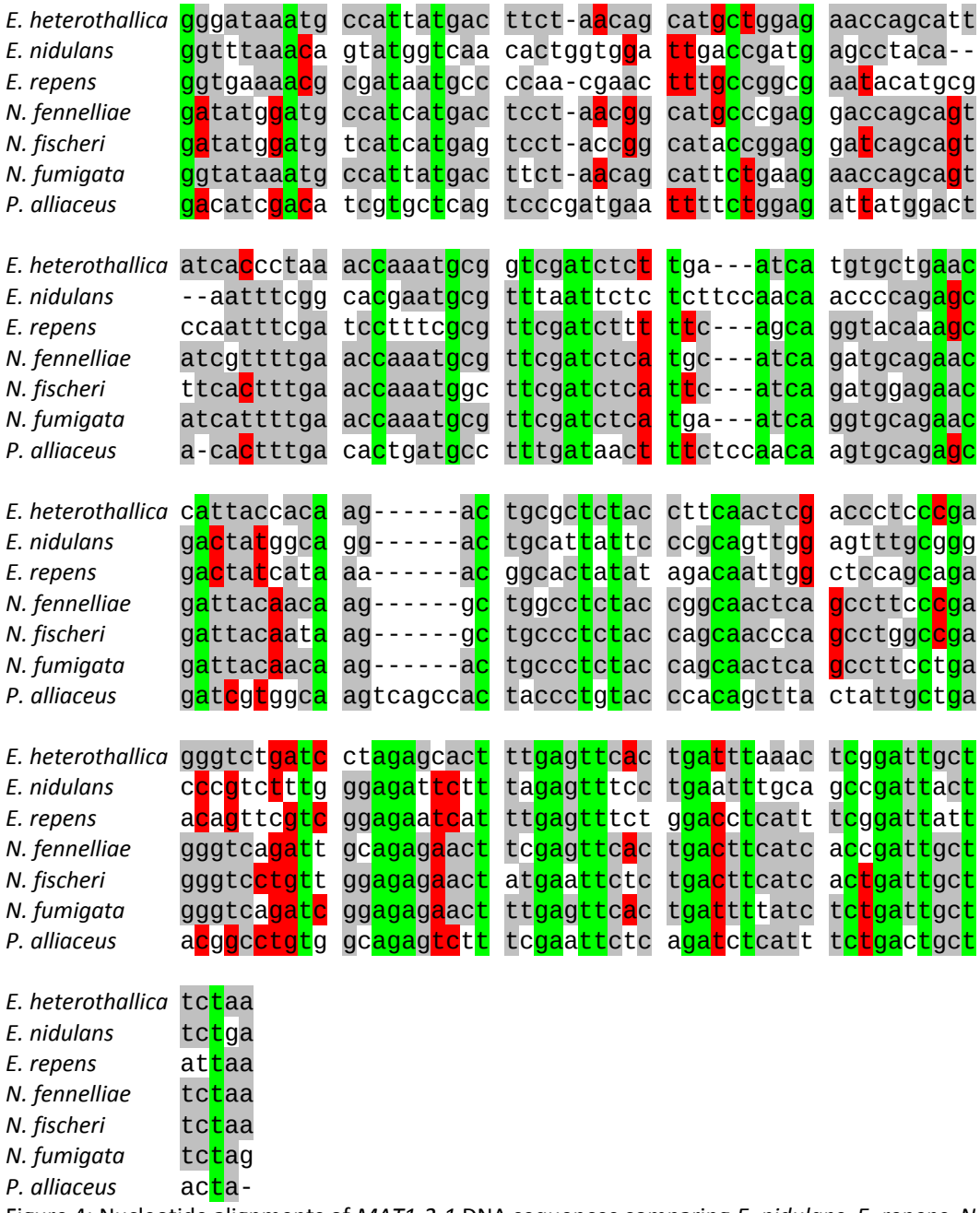
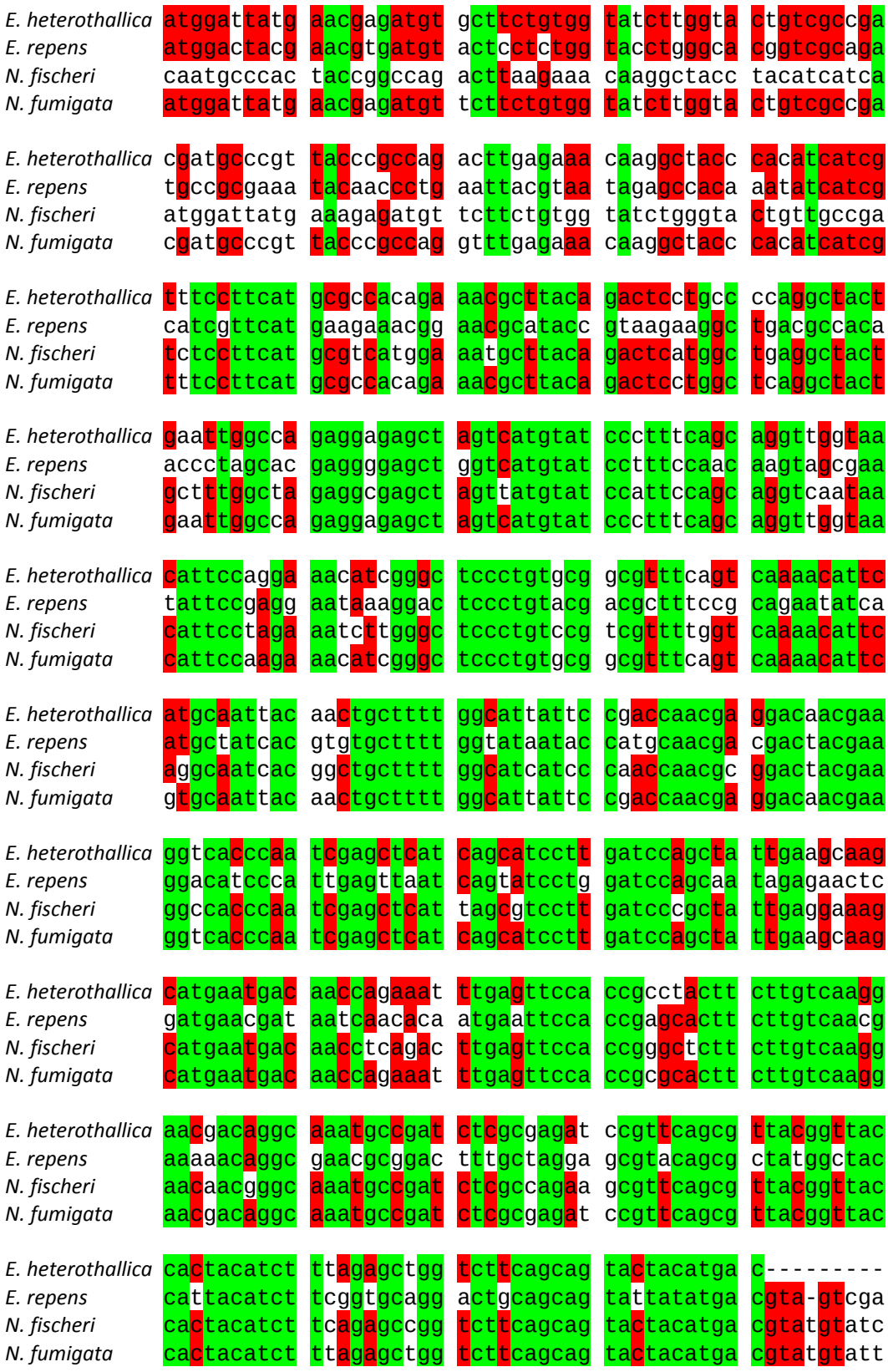


Figure 4: Nucleotide alignments of *MAT1-2-1* DNA sequences comparing *E. nidulans, E. repens, N. fennelliae, N. fischeri, N. fumigata* and *P. alliaceus*. Green highlighting indicates where nucleotides are conserved between all species. Grey highlighting indicates where nucleotides are conserved between four and six species. Red highlighting indicates where nuceltoides are conserved between three species. For culture identification codes and GenBank accession numbers used for alignments see Appendix 4.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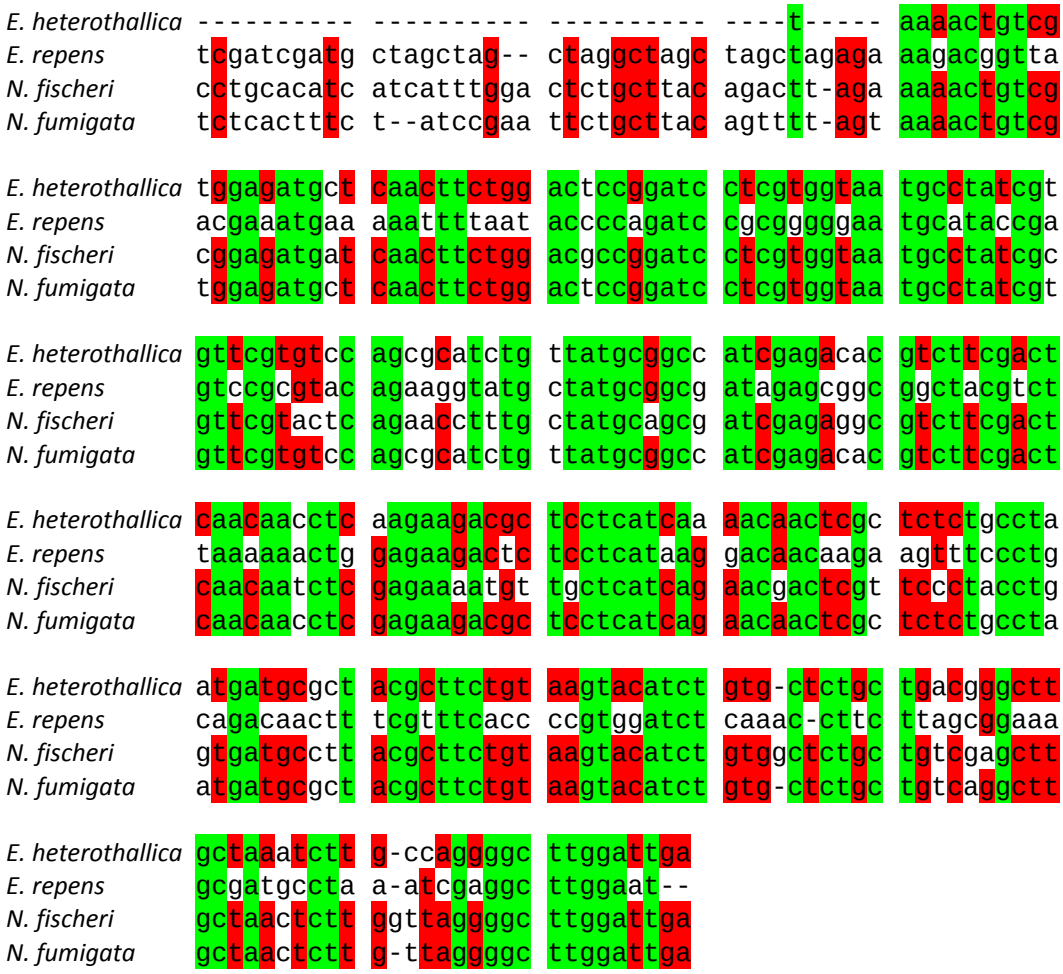
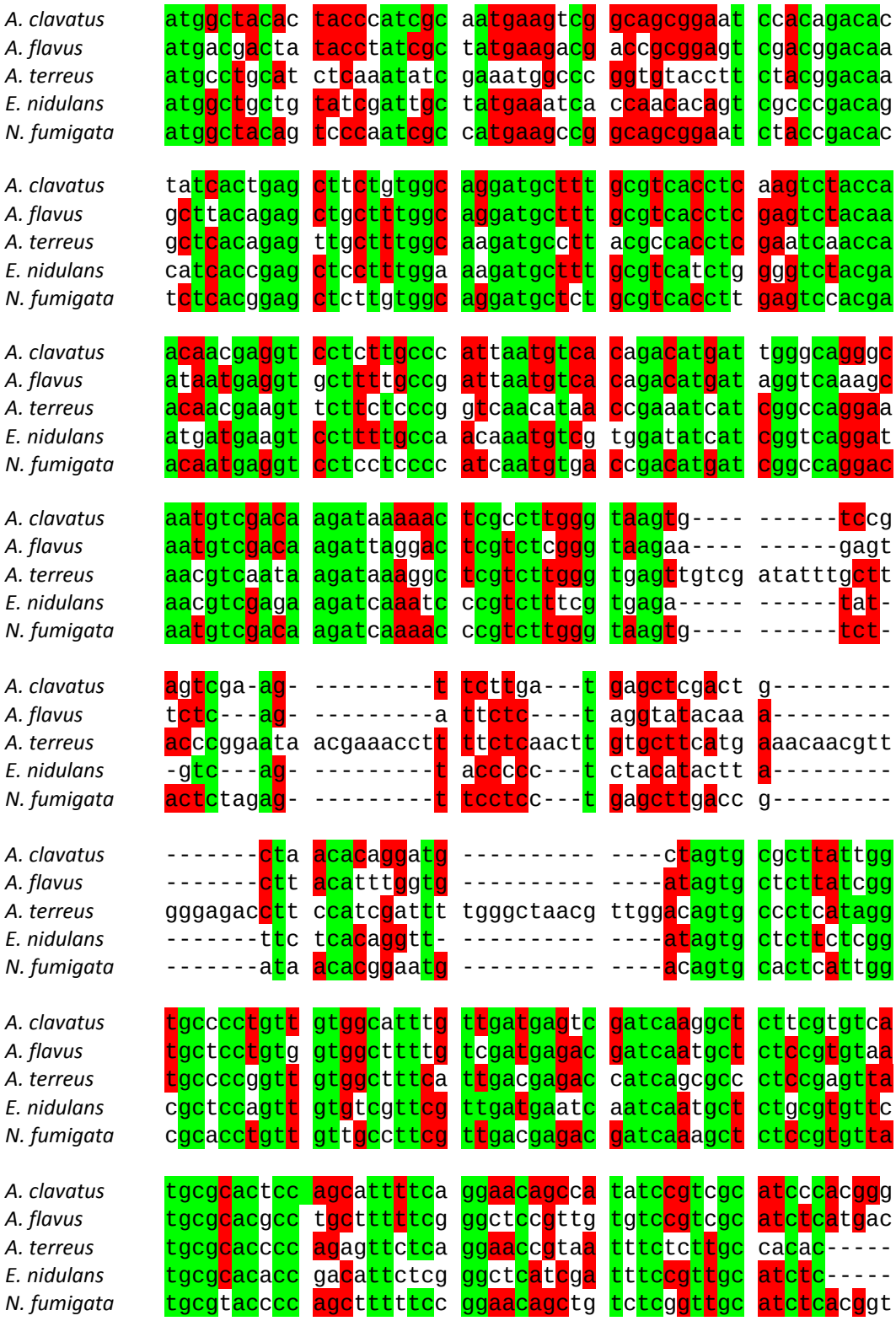


Figure 5: Nucleotide alignment of *MAT1-2-4* DNA sequences comparing *E. heterothallica, E. repens, N. fischeri* and *N. fumigata*. Green highlighting indicates where nucleotides are conserved between all species. Red highlighting indicates where nuceltoides are conserved between three species. (Sequencing was obtained from the Broad Institute genome sequencing project. (http://www.broad.mit.edu), *N. fumigata MAT1-2-4*, gene locus ID: Afu3g06160 , *N. fischeri MAT1-2-4*, gene locus ID: NFIA_024400.)

Appendix 2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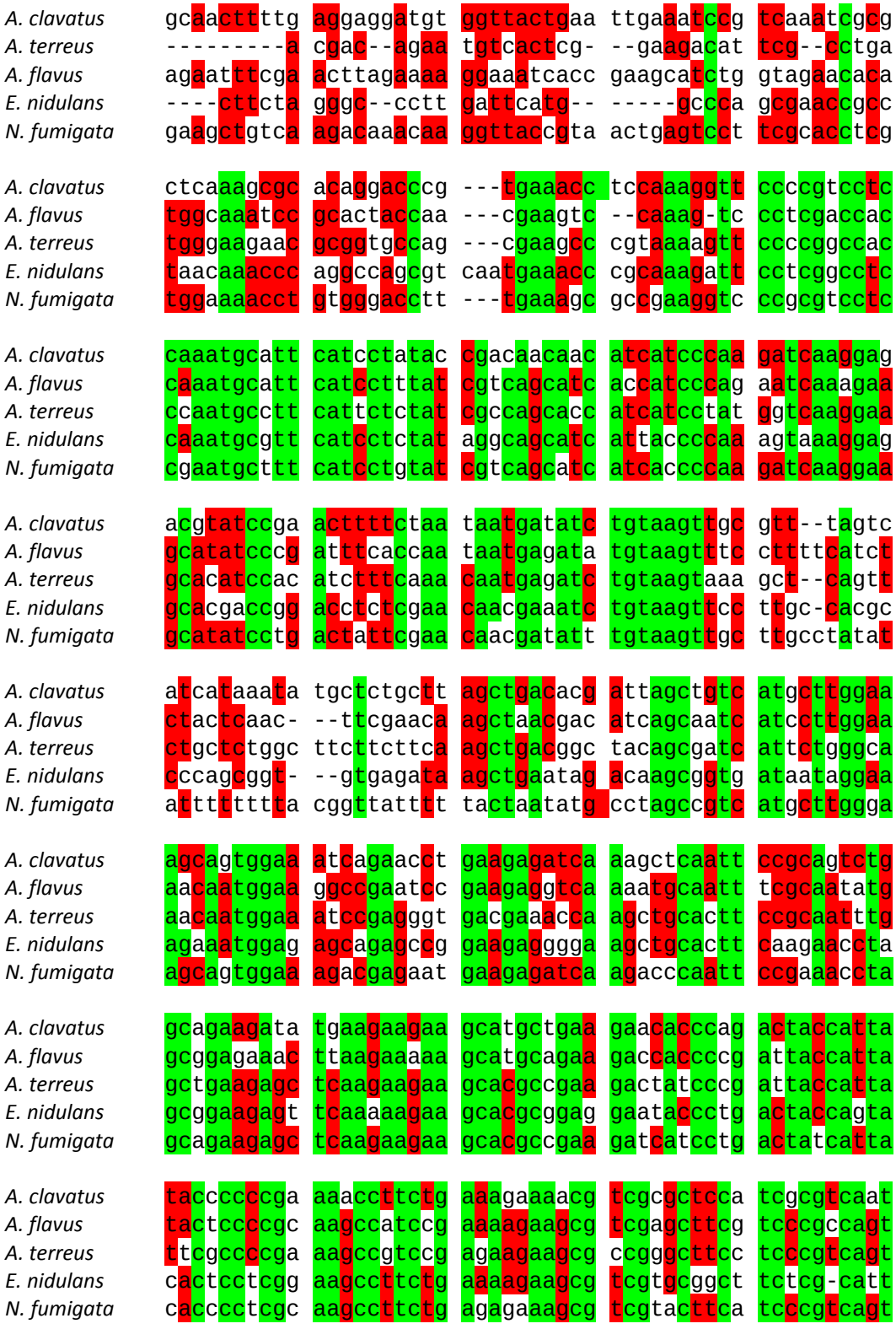
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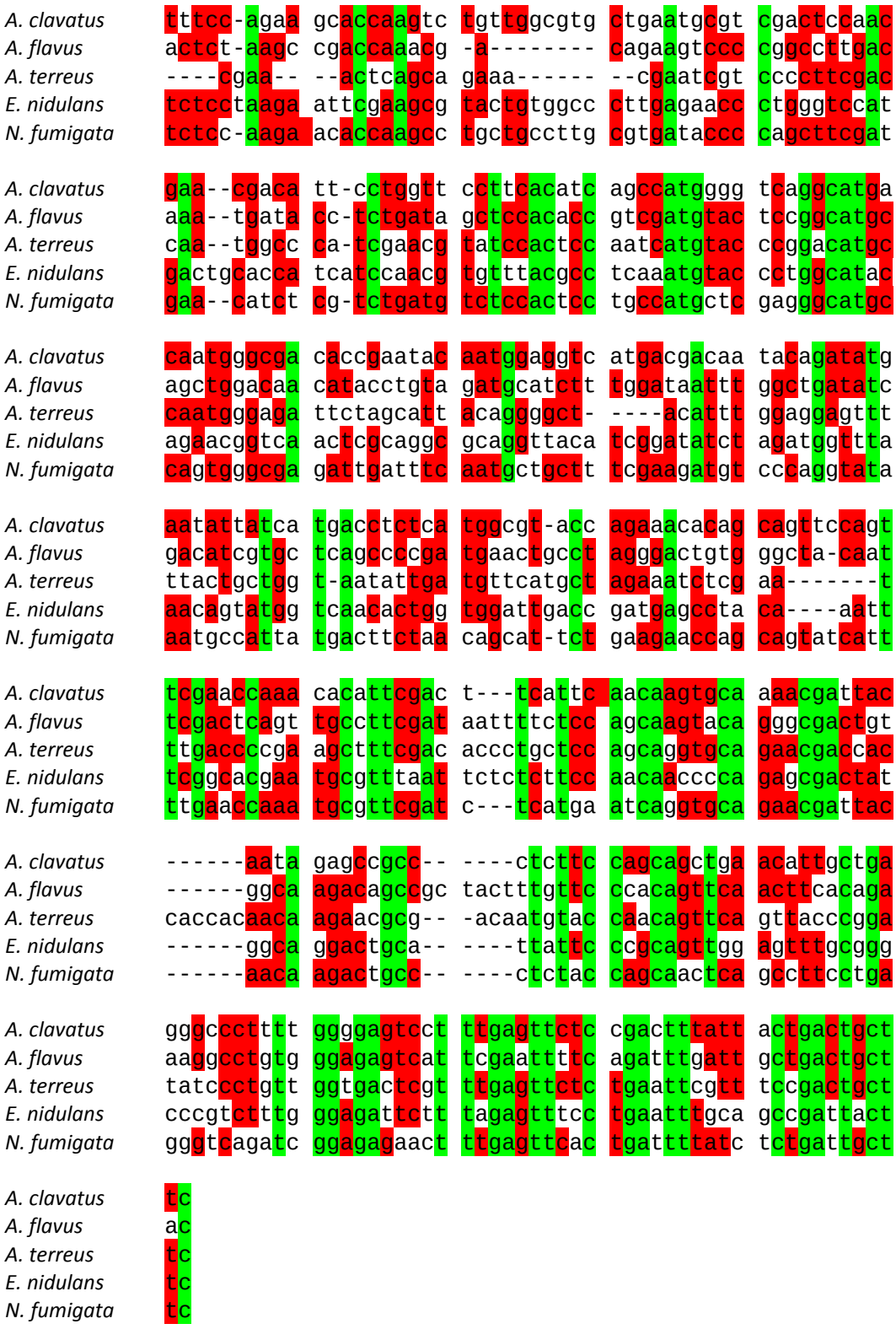

Figure 1: Nucleotide alignment of the *MAT1-2-1* gene region of *A. clavatus*, *A. terreus, N. fumigata* *E. nidulans* and *A. flavus.* Green highlighting indicates where nucleotides are conserved between all species. Red highlighting indicates where nucleotides are conserved

between three or four species. For culture identification codes and GenBank accession numbers used for alignments see Appendix 4.

Appendix 3

```
1          ttctccccaa cctccgtggt tagacttgtt ttgatttccc aatagcgaat
51         tcaagtaact tacccagttt tctgaataat tctggtgcat agtcccaatc
101        acctgtatcc tgcccatgta ccgtctccgg tagacaagcc tgtagagcgc
151        aagattgcac ggtcgcacag tcgtatggtc gtatggtcgc acagtcggga
201        tgcacggctc gcctccggca tgtcccctgc tcgactctgc atgccaagcc
251        ttgtctccat acctgactgg tggaaccatg gtacgaagcg catgccgatc
301        cgcgggtgga gccgactagc ggcggctgta aaggaagagt atcgagacgt
351        catctcatgc acgaagacaa agaaatgagt ttctgggcgt cactgcgtgg
401        ccggagcttc gagaacaaag agacgtagag gcccaatggt atttcgagcg
451        aggcattggg aaggtcgagt tcgtaattcg gaaaacatgg gggttgccga
501        tggttggttg tcgaactgaa tccccatcat gctaaagatg cgatcgtgtc
551        ctcgccatgc gcctctacat atccatttac aatcctatat gctacaaagg
601        aagcattttg tgaaacacgc aaatggaatt tcaaactaaa gtacataaaa
651        agcgctcacg ttgcaggaaa agagcgtctg gaaaacccct tgggaaagaa
701        agccagtcag ctcagttcct gagaacttgt catgagcgga aacaaaacaa
751        ttgaaagccg aaaaagtcgg cagaaagatt caactaattg tacagatggg
801        cgcaaaacat gagaaagtaa aatttctttt ttccttttct ccttttttct
851        ttttttttct ttttctttct ttttttttct tcgactttg tgggtaaaga
901        tggaaatatg tctgatcgtt gagtcgtcca cttatcacca gagtcggtcg
951        gtcagtgaat tgagagggct gggtaacgga acccttgatc atgatttgtt
1001       ggcaatgatc atgtcaaatg tcgctgagcc gagtcgaaat tgtgaatggg
1051       acatttagaa cacggtaggg atgagcagac aggtgaaagc tctagttgtg
1101       cccaaagatc cggtcaaggt tcgccatggt ggcagccaca gaggggttct
1151       tgttagggtc atagatgaca ggaagcccaa ggtcacgctc ggtatcgagg
1201       gaggagtaca tctcatcaac ctcaaattct tcaattggcc actggggaac
1251       gttgttgaac acccagttct gaggatcccg aacggtggct gaaaaggctg
1301       gatccacggc gaacacctgt ccgttttcga gaatgtgctt gtcccactct
1351       tcgttgcgga tctgggcgta gttgttatcg atgcagtgct tcacaacttg
1401       gtcgacagca atagccggca cgctaagact agccaagttc gacaagccga
1451       tcttgcgcaa gctgaggcta tttccagtgg gctggacatc ccaaccagcc
1501       atcctgagat actgttgacg atcaaggtga cccatcagag gcaggcaaag
1551       ctcgacaaag gtagagagtg aaggggtgtc gacatcgaag tgatcgcgga
1601       tgattgtgta agccttggcg agaatagccc agtgggactt caaggtatca
1651       tcctgccaca tttcgctgat ggccatagac ttgacttttt gcgggatgcc
1701       agggaacatc ggagcgcaaa agcctaagat actgtcagaa gcgaatcaat
1751       ggccataaag atgcgactta cttctgaaca gcataaaaga gttcaatgga
1801       cgaagacgac gttgctccaa aggagaggac agtacattct gcatcacatg
1851       cccaacacat ccagtcgtcg agtcagggtg aagctgctgc ataggaaggg
1901       cttgatcaat acgcttcagc tccagcacac catttgcgta gacgaagcgt
1951       ggtccagtgg gcaaaagccg gggctgctgg aagtagtttg caggaatgtc
2001       acgtagaact tgagctgcat actgccccac aggagaatct tcgggccaac
2051       gttccagcac ccgaagggcg tggcggaggg acaaggtttc aatgtatcga
2101       aacaaaagca tctccatatg agcagggcca tatcctggag ggaccgaagc
2151       atcaagagag gtagacatgt tgaacaatgt agtaagggtt cagagcgaag
2201       agaatatgaa tgaaaaggtg tcgggtgaaa aaaaaagaag aagggtggag
2251       aggagagaag aaagaaaaga aagaaacaat gtgggggtta aacaaatata
2301       ggacctggga ctgcagaaca cggtaggtga tgtgacgagg tgggcgattt
2351       ctttgtttag cggcaacacc aatcacagag ccagctgaga cacgtatttt
2401       ctttgtttcc tagcaacaac accaatcaca tgtttttatc ctagaaaggt
2451       cttccttgtt aggaatagta ccattagaac aaagaaagtc aattatggag
2501       ggataagact acagcagaag gtacttcagc atcatgtacc atttcgacaa
2551       cgaggtacta ctgtatacct ggatgttcag gctgggaatt cagatcgtaa
2601       ctggtagctt ctctgtgtaa tgcttactat atttgacgta acatggtggc
```

2651 **tcattaaaaa acgatttcga tctgtgctta gatctgtgct actcacgtat**
2701 **gcatgcacac aggtcgggag agtggatctc ccaccccacc acgtggcact**
2751 **gcggatctgg gggttagcgc ccgcacccgc acctaacctt aaaaagatat**
2801 **caaagcttac acaatatagc ttcaaattac ataccgagtc tgattccgat**
2851 **tgtgtaaaca gtctcagtct ctatgaaata taatcaaaaa agctatggta**
2901 **aagaacctaa tacaattttg tccattagag taccagcaac cttggaaggt**
2951 **acctgctcat atgtcgttac gtgaaagtcg ccatagcaac aaaaagcaaa**
3001 **cgaagtatgc aaacgatcgc ctgaccaata cgtatgagaa aaatcaaaca**
3051 **aagaaaatca aacaaagaat ccaagaaagg catcagacaa tcaataaata**
3101 **aattaaaaac aatgctaaaa taacacaaag gggacaataa cgacaaagaa**
3151 **aacggcagcc aatccaaaaa tggaaccagg gaattggaaa ttggcaaatg**
3201 **gtaagctgct aggcttcacc taaggcagcc atggacctag agcctgttcc**
3251 **atgccgttac tt**

Figure 1: 3262 bp of the MAT-1 Idiomorph DNA sequence from *P. chrysogenum* isolate 'B2'. Complete *MAT1-1-1* DNA sequence, 1095-2168bp (complementary strand). Nucleotides highlighted in emboldened font indicate where there is very high nucleotide homology (99-100%) between the idiomorphs sequenced from *P. chrysogenum* isolates CBS776.95 and 'B2' (1-821bp and 2282-3262bp).

1 accgggagat ggcggacttg gaggagtccc ttcgtgtgag taactgtcca
51 cttaggtgtg tgattcatgc tgaccttata gaacaaaacg cgcgcattgg
101 acgaacattc tagtcgcaat aatgaccgtc aggaagacct cgacctctct
151 cttcgcgaga aggacgagga gattgaggtg tacaagtccg gtatggaaca
201 ggctcttatg gaactggagg aactgaaact ggtaagtggt gatattgaca
251 acgttcgatt tctgcatact gaccatgttc cactgtagag ccaaggcgat
301 gtcgaccatg cgctggacag ccagattgac actgtcctcc acggcacagt
351 cgccaagatt aacgatatta tcgattccgt gctgcagact ggtgtgcagc
401 gtgtcgacga tgccctgtat gaactagact cgtccatgca ggcgggtaac
451 caaaatgcct ctcctcctta tgtcctttca cagattgaaa aggcgtcggc
501 ttctgcaacc gagttctcaa cagctttcaa caactttgtc gccgatggac
551 caaacagtcc ccatgctgag attatccgca cagtgtccat tttctctgga
601 tctgtctcgg atgtgttgag caacaccaag ggtctcatcc gcttcgccac
651 tgacgataag agctccgatc acctcattaa tgctgcacgc aagtctgctc
701 aggcgactgt gcggttttc cgtggcttgc agtcgttccg cctcgaaggt
751 ctggaaccac tgcagaagac tgatgttgtt atcaacaaca atcttgaagt
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851 agcataccaa gatcagcacc agtgtgatct gggagacctt gtcgatcagg
901 agctgctcaa ggctgccgat gccattgacg cagctgccca gcgtctggcc
951 aagctcaaga acaagccccg cgatgggtac tcgacctacg agctgcgcat
1001 caacgatgtc atccttgctg cggccattgc tgtcaccaac gcaatctcag
1051 agctgatcaa ggccgccaca gagacccagc aggagatcgt tcgtgaaggc
1101 cgtggtagct cgtctcgcac tgccttctac aagaagaaca accgctggac
1151 agagggcttg atctctgccg ccaaagcagt ggcctcttcg accaacacat
1201 tgatcgagac tgccgacggt gtaatctcgg gccgtaactc gccagagcaa
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1301 agccagtcgt gtcaaggcaa ccttcatgag caagacccag gaccgcctgg
1351 agaccgccag caaggctgtt ggtgctgctt gtcgtgccct cgtgcgacag
1401 gtgcaggaca tcatcaagga gaagaatcgc gatggagacg agggagagga
1451 ttatggaaag ttgagctcgc acgaattcaa ggtccgcgag atggagcagc
1501 aggtatgcga cgtccccttc cccttgtatc ccatccatcc aatgctaata
1551 tgtatcctct cgacaggtcg aaatcctcca actcgagaac ggtcttgctc
1601 gcgcccgcca gcgtctgggc gaaatgcgca agatctcgta tcaggaagaa
1651 tagtgtatct ttccctcttg gctgtctgta tattccgcgt tccaatctcg
1701 agcagtttga tattgtccgt gtcgcacttt gcgctcttcg gcctgggcct
1751 **ttctccccaa cctccgtggt tagacttgtt ttgatttccc aatagcgaat**
1801 **tcaagtaact tacccagttt tctgaataat tctggtgcat agtcccaatc**

1851 **acctgtatcc tgcccatgta ccgtctccgg tagggaagcc tgtagagcgc**
1901 **aagattgcac ggtcgcacag tcgcatggtc gtatggtcgc acagtcggga**
1951 **tgcacggctc gcctccggcc tgtcccctgc tcgactcttc atgccaagcc**
2001 **ttgtctccat acctgactgg tggaaccatg gtacgaagcg catgccgatc**
2051 **cgcgggtgga gccgactagc ggcggctgta aaggaagagt atcgagacgt**
2101 **catctcatgc acgaagacaa agaaatgagt ttctgggcgt cactgcgtgg**
2151 **ccggagcttc gagaacaaag agacgtagag gcccaatgat atttcgagcg**
2201 **aggcattggg acgtcgagtt cgtaattcgg aaaacatggg ggttgccgat**
2251 **ggttggttgt cgaactgaat ccccatgatg ctaaagatgc gatcgtgtcc**
2301 **tcgccatgcg tctctacata tccatttgca atcctatatg ctacaaagga**
2351 **agcattttgt gaaacacgca aatggaattt caaactaaag tacataaaag**
2401 **cgctcacgtt gcaggaaaag agcgtctgga aaaccccttg ggaaagaaag**
2451 **ccagtcagct cagttcctga gaacttgtca tgagcggaaa caaaacaatt**
2501 **gaaagccgaa aaagtcggca gaaagattca gctaattgta cagatgggcg**
2551 **cgagacatga gaaagtaaat** ttttttttc ggtattttcg ttttcaattt
2601 ttttttccg gcttttgtgg gtaaagataa aaatatgcct gatcgtagag
2651 aatcatccac ttatcaccag agacggtcgg tcggggaatt gagatggttg
2701 ggtaacggaa ccgttgatca tgatttggtg gcaatgatca tgtcaaatgt
2751 cgctgagccg agtcgaaatt gtgaatggga catttagaac acgctgttca
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3751 aacgcggtcg tcgtcaccaa cgaagtccaa gagggtttc gccatcatgt
3801 aaaagatgaa atgaggaggt aaatgaactg gggaatgcag taagaaagga
3851 attcgggtgt gcagggaatg gaaaagagga tggggttgga agaggagagg
3901 gaagaaagag aagaagaaac gtgt**tgggag gtaaacaaat ataggacctg**
3951 **ggactgcgga gcacggtagg tgacgtgacg gggtgggcga tttctttgtt**
4001 **tgacggcaac accaatcaca gagccagctg agacacgtat ttctttgttt**
4051 **cctagaaaca acaccaatca catgttttca tcctaaaaag gttttccttg**
4101 **ttaggaatag taccatttga acaaagaaag tcaattatag agggataaga**
4151 **ctacagcgga aggtacttca gcatcatgta ccatttcgac aacgaggtac**
4201 **tactgtatac ctggatgttc aggctgggga ttcagatcgt aactggtagc**
4251 **ttctctgtat aatgcttact atatttgacg taacatggtg gctcattaaa**
4301 **aaacgatttc gatctgtgct tagatctgtg ctactaacgt atgcatgcac**
4351 **acaggtcggg tgagtggatc tcccactcca ccacgtggca ctgcggatct**
4401 **gggggttagc gcccgcaccc gcacctaacc ttaaaaagat atcaaagctt**
4451 **acacaatata gcttcaaatt acataccgag tctgattccg attgtgtaaa**
4501 **cggtctcagt ctctatgaaa tataattaaa aaagctatgg taaaggacct**
4551 **gatacaattt tgtccattag agtaccagca accttggaag gtacctgctc**
4601 **atatgtcgtt acgtgaaagt cgccatagca acaaaaagca aacgaagtat**

4651 **gcaaacgatc gcctgaccaa tacatatgag aaaaatcaaa caaagaatcc**
4701 **aagaaaggca tcagacaaag aagtcaaaga gtaaaagtaa aaattcaata**
4751 **ataaattaa aaacaatgct aaaataacac aaaggggaca ataacgacaa**
4801 **agaaaacggc agccaatcca aaaatggaac cagggaattg gaaattggca**
4851 **aatggtaagc tgctaggctt cacctaaggc agccatggac ctagagcctg**
4901 **ttccatgccg ttactt**cgtt cggtacatgg cctcttaaat agaattacac
4951 agtgcgttgc ttgttgacag atgtattttg ttcagacaag acctcaagaa
5001 gtctattggt catatatgcg tatgtttaat atcggaaagt atcacatccg
5051 tggctctccg tgctcaatta accacgctgt ataaatatca gcaataataa
5101 ttcaaaccga atactcggct gtaaaatgtt caataaatta ttgactacgg
5151 agcgatatcc cttcctggaa catgtatttc attactcttg cgccgcaact
5201 agtgagttcc actcgctggc ccagatgaaa gtgggacatc gccattgcgt
5251 ccctttttcc ttttctccgc taggtcccaa tggccgggga cagatccaaa
5301 aggagcggcc acgattcatt ccgggtttct tcgtggtcaa gctgatacac
5351 ggctcttgat gtccttcgca cgttggaacg ggcttcttgg tgaagatttt
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5501 atgtcctctt gggaccccgg aggctgcggg ctgattcctg aagttttccc
5551 tttctcttgt ggaccttccg atagtttggg ggtgagtgac ggtgttgaag
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5751 agcgttttcg agaagccgtc cgagagagtc gatttccctc ggttatagat
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6001 agcgggcaat gcgcactcgt tgagtattcc tgttgacgct tgccacagtt
6051 aaacattccc ggtgggttga gaatatcccg gatgtttact tcgccttcgg
6101 gtcggtttac agattccttg atgacagcat atacaggaca atggtccgat
6151 ccctataaag cagtcaataa acgattcaag gcaaaggaga aaagtccata
6201 ccatcaatcc ctcttgaatg tttgagtcgg agaaccagtc ctgcatatgc
6251 aagctgcaca acacatagtc aattcgtgat ccgtaattcc ctggtcgagc
6301 attgagtttc tgatcccagc atgtatacat gcctgtacga tgtggatgaa
6351 atgacctgca gagatcgagg agaacaggat cctccctgcc tttgtcacgt
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6651 aggcagggca atagacacca atgaggacaa atgcaggaaa ctccagaatg
6701 acgcagcgcc cttcggagtc gagagttggt gcatctattg tggcatcggg
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6851 tgctcttctg gaaggctacg gaaagatgtt ggagatttcg gtggacagag
6901 aactccagta atgccctctt ctgcccttat tggagaacat gtggcatttc
6951 gcgtgtaaat cacaacaccc gagtatccta gaagttgtta ctatcctgta
7001 tggacaactc tggatagtag cttacctttc ttaacccggg ggagactgaa
7051 ataacagtcc catcctggaa ccaagaccat gtcatcccgc aaatccttcc
7101 gctg

Figure 2: 7104bp of the MAT-2 Idiomorph DNA sequence from *P. chrysogenum* isolate CBS776.95. Incomplete *SLA2* DNA sequence, 1-1653b (direct strand). Complete *MAT1-2-1* DNA sequence, 2784-3795bp (complementary strand). Incomplete *APN2* DNA sequence, 5203-7104bp (complementary strand). Nucleotides highlighted in bold indicate where there is very high nucleotide homology between the idiomorphs sequenced from *P. chrysogenum* isolates CBS776.95 and 'B2' (1751-2570bp and 3924-4916bp). Primer ChSLA2-1 (forward) is highlighted in

red, primer ChSLA2-2 (forward) is highlighted in purple, primer ChAPN2-1 (reverse) is highlighted in blue and primer ChAPN2-2 (reverse) is highlighted in green.

```
1           tagggtactc tttaccaata gccttttttat caatgcatag agactgaggg
51          atcgttacac aattcggatt caggactcgg gtatgtactg agctatattg
101         tggtaagctt ggatattctt tttaaggtag gtgcgggtgg cgggcgctaa
151         ccccccagat ccgcagtgcc acgtgggtgg agtggggaga tcccactcac
201         ccgacctgtg tgcatgcata cgttagtagc acagatctaa gcacagatcg
251         aaatcgtttt ttaatgagcc accatgttac gtcaaatata gtaagcatta
301         tacagagaag ctaccagtta cgatctgaat ccccagcctg aacatccagg
351         tatacagtag tacctcgttg tcgaaatggt acatgatgct gaagtacctt
401         ccgctgtagt cttatccctc tataattgac tttctttgtt caaatggtac
451         tattcctaac aaggaaaacc tttttaggat gaaaacatgt gattggtgtt
501         gtttctagga aacaaagaaa tacgtgtctc agctggctct gtgattggtg
551         ttgccgtcaa acaaagaaat cgcccacccc gtcacgtcac ctaccgtgct
601         ccgcagtccc aggtcctata tttgtttacc tcccaacacg tttcttcttc
651         tctttcttcc ctctcctctt ccaaccccat cctcttttcc attccctgca
701         cacccgaatt cctttcttac tgcattcccc agttcattta cctcctcatt
                              |M||A| |K||T||L|| L||D||F||F ||G||D||D|
751         tcatctttta catgatggcg aaaaccctct tggacttctt tggtgacgac
            |D||R||V|| T||A||R||R ||S||M||E| |L||L||W|| A||D||A||V
801         gaccgcgtta ctgctcgtcg ttccatggag cttctttggg cagatgccgt
            ||N||H||L| |P||Q||T|| D||G||E||V ||F||L||P| |R||N||V||
851         caatcacctg cctcagactg atggtgaagt cttcctgcct cgcaatgttg
            V||Y||G||V ||L||E||L| |V||H||H|| K||A||M||A ||I||R||L|
901         tctatggcgt tcttgagctg gttcatcaca aggcgatggc gattcgtttg
            |A
951         gcgtaagtgc tgaaccagta cctacctgag ccatgttatt aacaagctac
                 ||C|| M||L||N||K ||K||V||D| |I||V||L|| D||Q||S||I
1001        aacagatgca tgctaaataa gaaggttgac atcgtcctcg accaatccat
            ||D||A||Y| |R||M||F|| P||K||D||L ||N||A||E| |H||D||E||
1051        cgatgcttac cgcatgttcc ctaaggacct caacgctgag catgatgaga
            N||F||W||A ||D||Q||H| |G||L||D|| L||G||D||H ||L||L||I|
1101        acttctgggc cgatcaacat ggacttgacc tcggtgatca ccttctaatt
            |S||K||S|| I||H||E||A ||G||D||S| |V||K||I|| P||A||R||P
1151        tccaagtcta tccacgaggc tggcgattct gtcaagatcc ctgctcgccc
            ||A||K||V| |P||R||P|| P||N||C||F ||I||L||Y| |R||Q||A||
1201        agcaaaagtt cctcgtccac ccaattgctt cattctttac cgtcaggcca
            N||H||H||L ||V||K||D| |A||N||P|| G||V||S||N ||N||E||I|
1251        accaccattt ggtcaaggat gccaaccccg gtgtttctaa caacgaaatt
            |
1301        tgtgagttat ctccgactac ttgtgttgtt tctagctaat gactggatta
             S||R||I|| L||G||A||R ||W||N||N| |E||S||P|| E||V||R||E
1351        gctcgtatcc ttggtgcacg ctggaacaat gagagccctg aagttcgaga
            ||Q||F||T| |H||L||A|| D||E||L||K ||K||E||H| |A||I||K||
1401        gcagttcacc cacctggctg atgaactcaa gaaggaacac gctatcaagc
            H||P||D||Y ||Q||Y||A| |P||R||R|| P||S||E||R ||K||R||R|
1451        atcctgatta ccaatatgct cctcgtcgcc cttctgagcg caagcgccgc
            |T||P||R|| S||R||A||N ||C||L||P| |F||V||Q|| A||D||S||H
1501        actccccgtt cccgtgccaa ctgcctgcct tttgttcagg ctgattccca
            ||Y||E||D| |M||F||D|| D||A||D||F ||E||D||R| |T||I||S||
1551        ttacgaggac atgttcgatg acgctgattt cgaagaccga actatttcta

            I||D||D||N ||F||I||T| |E||L||N|| D||N||G||L ||L||F||G|
1601        tcgatgacaa cttcatcacc gagctgaatg acaatggtct acttttcggt
            |P||N||G|| V||E||P||L ||S||P||P| |F||T||H|| E||E||C||F
```

```
1651        ccgaatggtg tcgagccact ctctcccccc ttcactcacg aggaatgctt
            ||E||M||S| |N||D||L|| T||S||G||N ||S||L||A| |S||M||E||
1701        cgaaatgagc aacgacctga cttcgggcaa cagccttgcc tcaatggagt
            F||L||P||M ||N||S||V| |F|
1751        tcctccctat gaacagcgtg ttctaatgtc ccattcacaa tttcgactcg
1801        gctcagcgac atttgacatg atcattgcca ccaaatcatg atcaacggtt
1851        ccgttaccca accatctcaa ttccccgacc gaccgtctct ggtgataagt
1901        ggatgattct ctacgatcag gcatattttt atctttaccc acaaaagccg
1951        gaaaaaaaaa attgaaaacg aaaataccga aaaaaaaaaa tttacttctc
2001        attctcgcgc ccatcgcatg
```

Figure 3: DNA sequence of MAT1-2-1 region of *P. chrysogenum* isolate CBS 775.95. The putative encoded amino acid sequence is indicated about the relevant base pair sequence. Putative introns are highlighted in yellow. Also included is 764bp upstream of the putative ATG start site and 245bp downstream of the finish site.

```
1           accgggaaat ggcggatttg gaagagtctc ttcgagtgag tagctgttcg
51          ctttgcttag gtgtgtgatc actgctgacc ttgtagatta aaacacgcgc
101         attggacgaa cattccagtc gcaacaatga ccgccaggaa gacctcgacc
151         tgtctcttcg cgagaaggac gaggaaattg aggtgtacaa gtctggcatg
201         gaacaggctc ttatggaact ggaggagctg agactggtaa gtggtgatct
251         tgacaacatt cgatacctgc atactaactg tgctcccctg tagagccaag
301         gcgatgttga ccatgcgctg gacagccaga ttgacactgt gctccacggc
351         gcagtcgcga agattaacga cattatcgac tccgtgctgc agactggtgt
401         gcagcgtgtc gacgatgctc tgtatgaact ggactcgtcc atgcaggctg
451         gtaaccaaaa tgcctctcct ccatatgtgc tttcgcagat tgaaaaggcg
501         tcggcttctg ccaccgagtt ctcaacagct ttcaacaact ttatcgccga
551         tggccctaac agtccccatg ctgagattat ccgcacagtg tctgtcttct
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651         gccaccgacg acaagagctc cgatcatctg gtcaacgctg ctcgaaagtc
701         tgctcaggcg accgtgcggt tcttccgtgg cttgcagtca ttccgtctcg
751         aaggtctgga accactgcag aagactgatg tggttatcaa caacaacctc
801         gaagtccagc gtgatctgca ggcgctgtcg aagttggttg actcgttcgc
851         ccccaagcat accaagatca gcaccaatgg tgatctgggt gaccttgtcg
901         accaggagct gctcaaggct gccgatgcca ttgacgcagc tgcccagcgt
951         ttggccaagc tcaagaacaa gccccgtgac gggtactcga cttacgagct
1001        gcgcatcaac gatgtcatcc ttgctgcagc cattgctgtc accaacgcaa
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1201        acacattgat cgagactgcc gacggtgtaa tctcgggtcg taactcgcca
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1601        gcgcacgcca gcgtctgggc gaaatgcgca agatctccta ccaggaggat
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1951        catgaaagct caaatggcag gaaatgagcg tctggaaaac cccgtgggaa
2001        agaaagccag tcagctcagt tcctgagaac ttgccatgag cgaaaacaaa
```

```
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2301        attgattgtt gatagattgt tgtcaagtca tgtaactgaa tcgaatcaag
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4001        atcgacgact atagctaggt agcactttac tatctcaacg ttgcttacgc
4051        aagccttagc gttacctgaa tgcataggca tgcggtctgc aacgatcgtt
4101        cgatcccagg agggt
```

Figure 4: 4115 bp of the MAT-2 Idiomorph DNA sequence from *P. griseofulvum* isolate CBS 110420. *MAT1-2-1* DNA sequence, 2366-3400bp (complementary strand). Primer GrSLA2 (forward) is highlighted in red.

```
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E. nidulans     -tgcccttat tcagtgt-tc ttc----ctt -tctcttcgg agcaaagact
N. fumigata     ctacacttgt ctctcgtctg ttcat--ctt -cagctttg attcaaggt

P. chrysogenum  atatggagat gc----tttt gtttcgatac attgaaacct tgtccc-tcc
P. marneffei    ctctcgtgat tcgtcaaatg tctttcaaa atgacgaccg gccttttcc
E. nidulans     ag-tgcat-t tc-----att gctccaagat agaacgccaa tccccagcc
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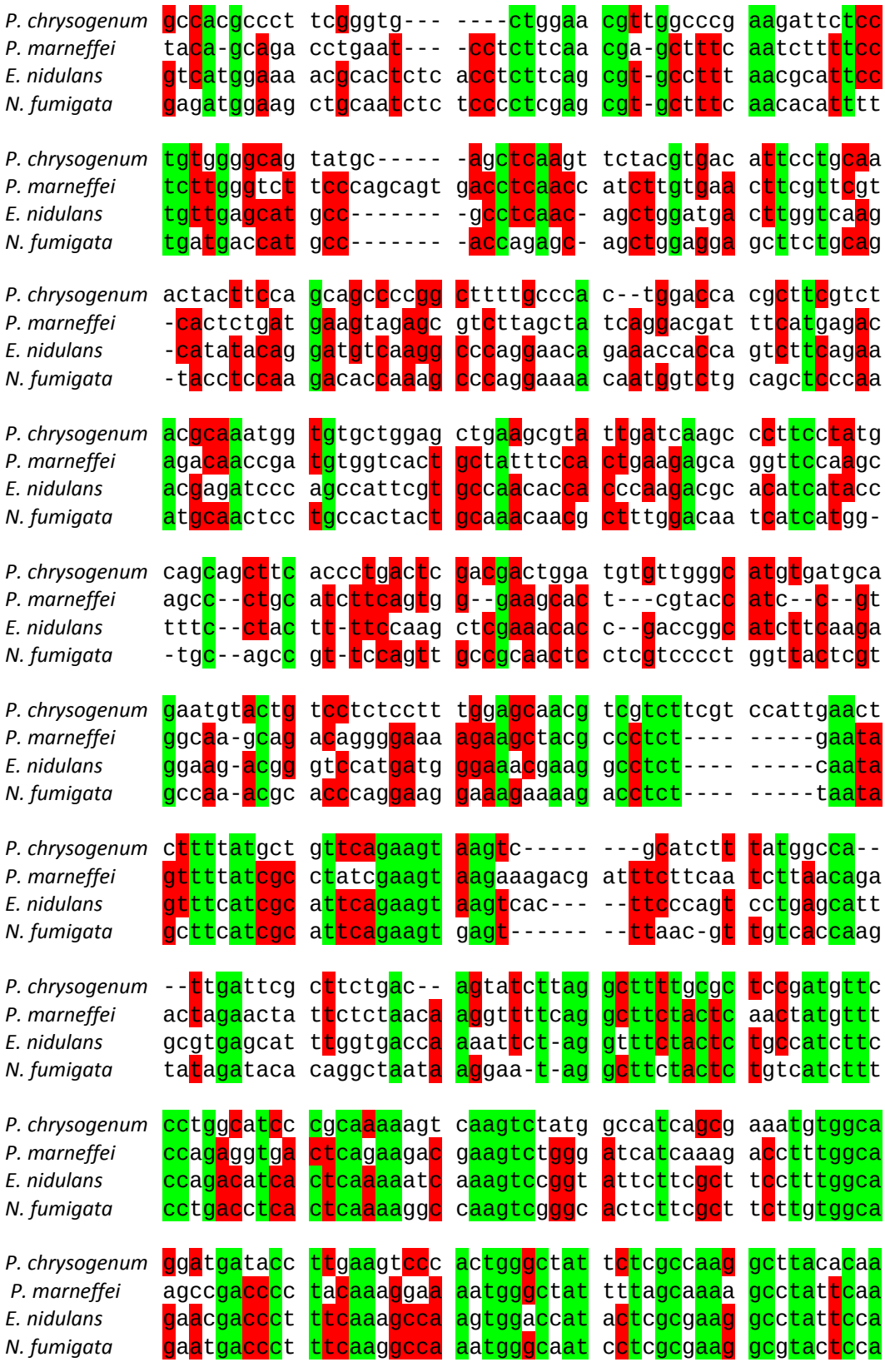
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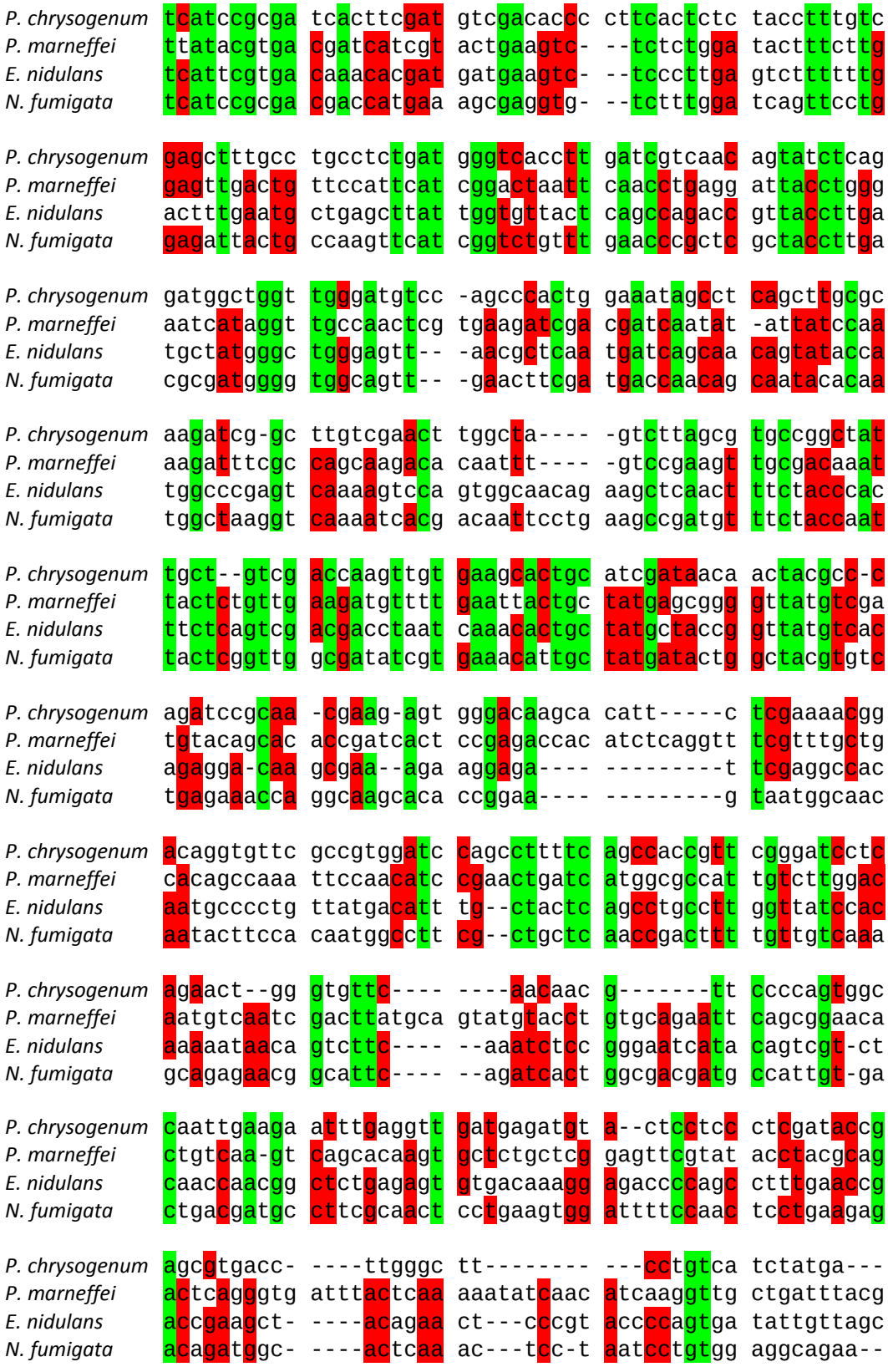
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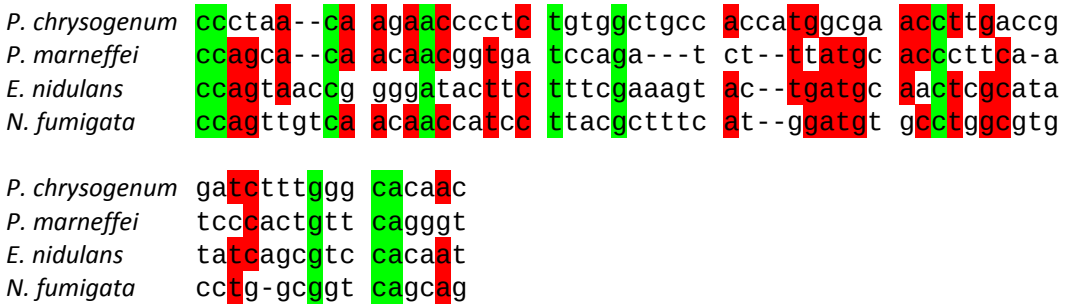
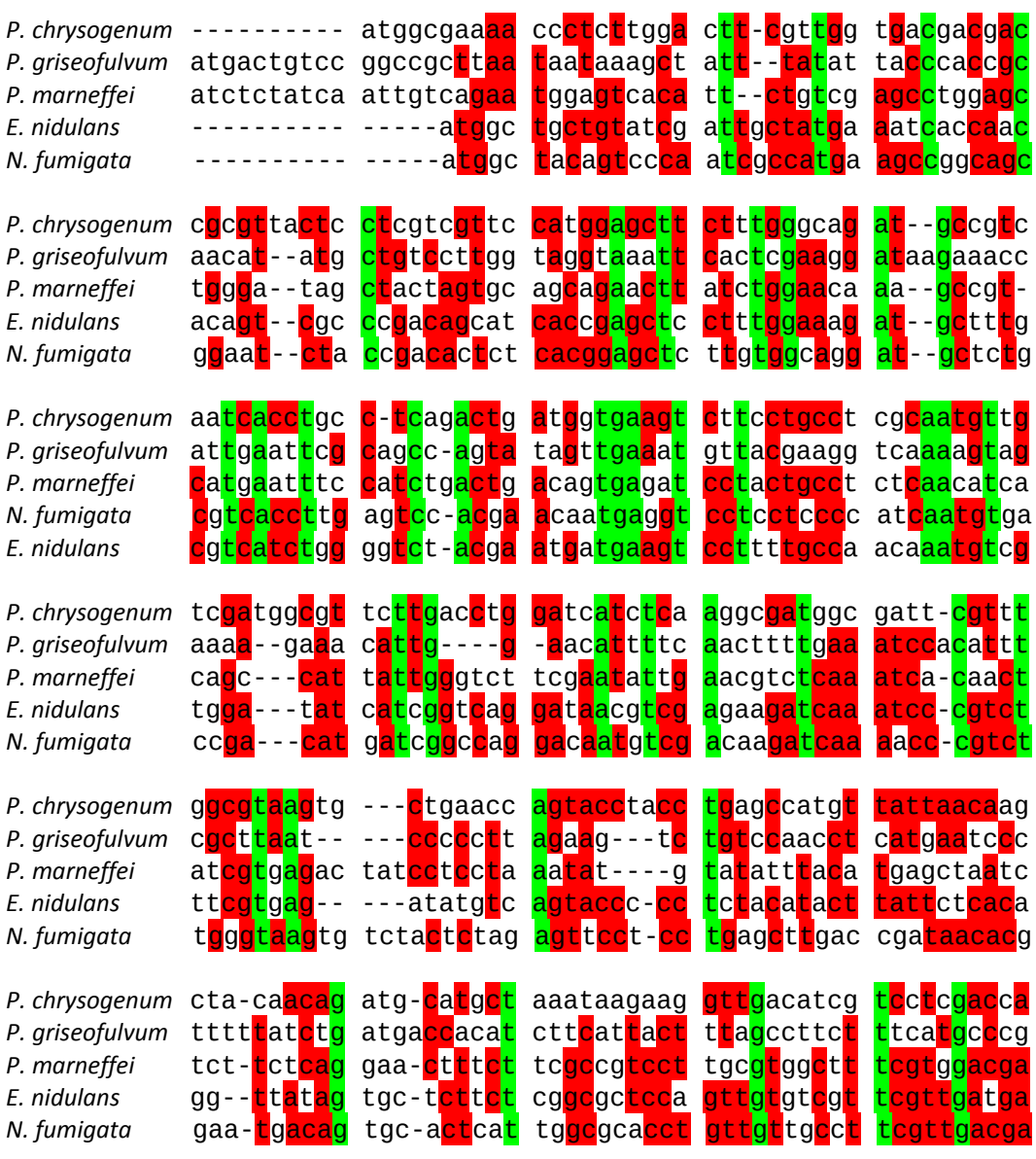
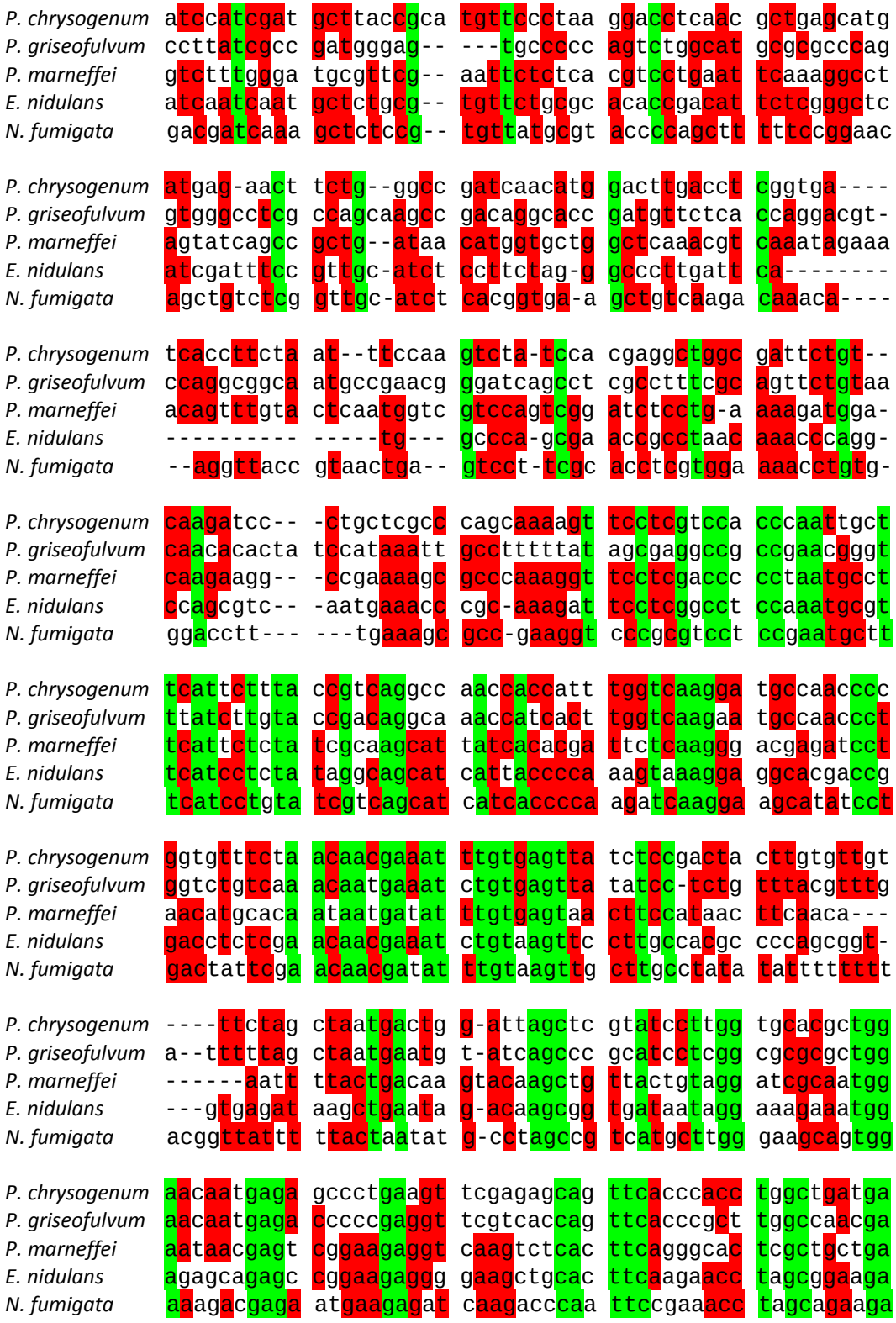


Figure 5: Nucleotide alignment of the *MAT1-1-1* gene region of *P. griseofulvum*, *P. chrysogenum*, *N. fumigata*, *E. nidulans* and *P. marneffei*. Green highlighting indicates where nucleotides are conserved between all species. Red highlighting indicates where nucleotides are conserved between three species. For culture identification codes and GenBank accession number used for alignments see Appendix 4.

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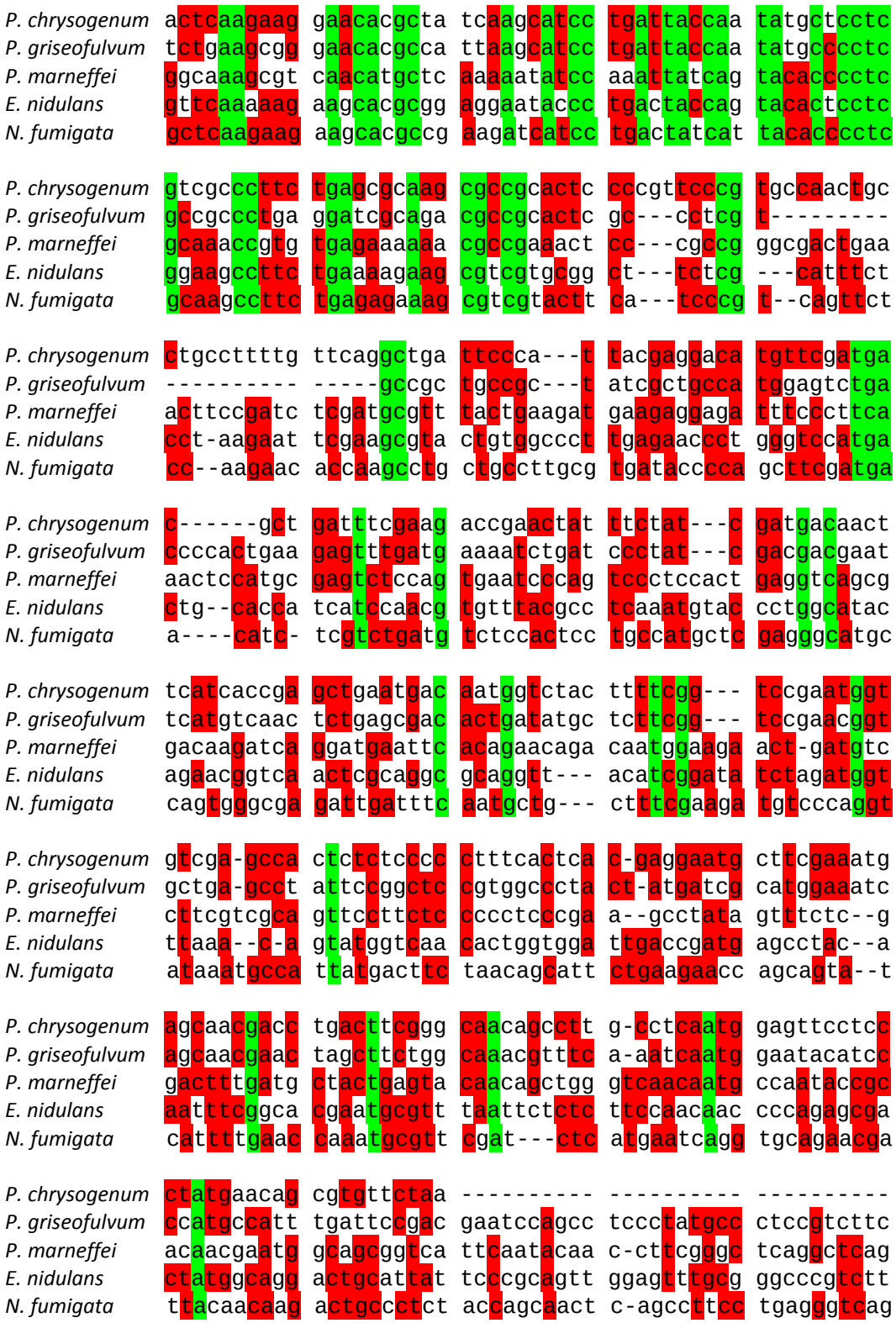
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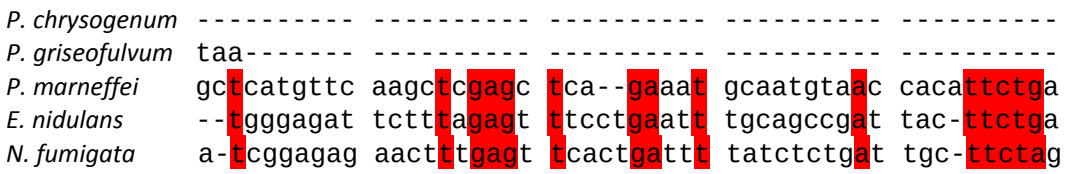

Figure 6: Nucleotide alignment of the *MAT1-2-1* gene region of *P. griseofulvum*, *P. chrysogenum*, *N. fumigata*, *E. nidulans* and *P. marneffei*. Green highlighting indicates where nucleotides are conserved between all species. Red highlighting indicates where nucleotides are conserved between three or four species. For culture identification codes and GenBank accession numbers used for alignments see Appendix 4.

Appendix 4

List of gene sequences used in phylogenetic analyses in the present study.

Species	Culture Idenitifcation Code	Gene	GenBank Accession Number[a] or Gene Locus ID[b]
Ajellomyces capsulatus	WU24	β-tubulin	HCBG_03593.2
Aspergillus brevipes	NRRL 2439	β-tubulin	EF669812
Aspergillus candidus	NRRL 313	β-tubulin	EU014093
Aspergillus cervinus	NRRL 5025	β-tubulin	EF661251
Aspergillus clavatus	NRRL 4097	β-tubulin	EF669830
Aspergillus flavus	NRRL 1957	β-tubulin	AY017536
Aspergillus hollandicus	NRRL 25850	β-tubulin	EF651900
Aspergillus ochraceus	NRRL 398	β-tubulin	EF661322
Aspergillus oryzae	NRRL 447	β-tubulin	EF661483
Aspergillus parasiticus	NRRL 502	β-tubulin	AY017537
Aspergillus raperi	NRRL 2641	β-tubulin	EF652278
Aspergillus restrictus	NRRL 154	β-tubulin	EF651880
Aspergillus sparsus	NRRL 1937	β-tubulin	EF661126
Aspergillus terreus	NRRL 1913	β-tubulin	EF669518
Aspergillus unguis	NRRL 6328	β-tubulin	EF652333
Aspergillus ustus	CNM-CM4036	β-tubulin	FJ624463
Aspergillus versicolor	Gr206	β-tubulin	FJ904899
Aspergillus wentii	NRRL 35068	β-tubulin	EF652112
Chaetosartorya cremea	NRRL 5081	β-tubulin	EF652120
Coccidioides immitis	RMSCC 2394	β-tubulin	CIMG_06928.3
Emericella heterothallica	NRRL 5097	β-tubulin	EF652324
Emericella nidulans	IFM 54206	β-tubulin	AB243109
Eupenicillium crustaceum	CBS 581.67	β-tubulin	AY674446
Eupenicillium osmophilum	CBS 462.72	β-tubulin	AY674376
Eurotium repens	NRRL 17	β-tubulin	EF65916
Fennellia nivea	NRRL 5502	β-tubulin	EU014099
Hemicarpenteles paradoxus	NRRL 5162	β-tubulin	EF669685
Neurospora crassa	OR74A	β-tubulin	AY974799
Neocarpenteles acanthosporum	NRRL 5293	β-tubulin	EF669848
Neopetromyces muricatus	NRRL 35674	β-tubulin	EF661356
Neosartorya fennelliae	NRRL 5534	β-tubulin	EU014108
Neosartroya fischeri	NRRL 4585	β-tubulin	EF669839
Neosartorya fumigata	CBS 109032	β-tubulin	AY685166
Penicillium aethiopicum	CBS 270.97	β-tubulin	AY495984
Penicillium albocoremium	CBS 109582	β-tubulin	AY674327
Penicillium allii	CBS 109581	β-tubulin	AY674332
Penicillium atramentosum	CBS 291.48	β-tubulin	AY674402
Penicillium aurantiogriseum	CBS 642.95	β-tubulin	AY674297

Penicillium bialowiezense	CBS 110104	β-tubulin	AY674440
Penicillium brevicompactum	CBS 110067	β-tubulin	AY674438
Penicillium camemberti	CBS 190.67	β-tubulin	AY674369
Penicillium carneum	CBS 449.78	β-tubulin	AY674384
Penicillium caseifulvum	CBS 108956	β-tubulin	AY674371
Penicillium cavernicola	CBS 109556	β-tubulin	AY674338
Penicillium chrysogenum	CBS 306.48	β-tubulin	AY495981
Penicillium clavigerum	CBS 112482	β-tubulin	AY674428
Penicillium commune	CBS 279.67	β-tubulin	AY674361
Penicillium concentricum	CBS 191.88	β-tubulin	AY674412
Penicillium confertum	CBS 171.87	β-tubulin	AY674373
Penicillium coprobium	CBS 280.97	β-tubulin	AY674423
Penicillium coprophilum	CBS 102444	β-tubulin	AY674422
Penicillium crustosum	CBS 101025	β-tubulin	AY674351
Penicillium cyclopium	CBS 477.84	β-tubulin	AY674309
Penicillium digitatum	CBS 136.65	β-tubulin	AY674404
Penicillium dipodomyicola	CBS 110421	β-tubulin	AY674411
Penicillium dipodomyis	CBS 110412	β-tubulin	AY495991
Penicillium discolour	CBS 271.97	β-tubulin	AY674350
Penicillium echinulatum	CBS 317.48	β-tubulin	AY674341
Penicillium expansum	CBS 281.97	β-tubulin	AY674401
Penicillium flavigenum	CBS 110406	β-tubulin	AY495994
Penicillium freii	CBS 101486	β-tubulin	AY674291
Penicillium gladioli	CBS 815.70	β-tubulin	AY674289
Penicillium glandicola	CBS 111218	β-tubulin	AY674414
Penicillium griseofulvum	CBS 110420	β-tubulin	AY674431
Penicillium hirsutum	CBS 110100	β-tubulin	AY674330
Penicillium hordei	CBS 701.68	β-tubulin	AF003240
Penicillium italicum	CBS 489.84	β-tubulin	AY674396
Penicillium marinum	CBS 109547	β-tubulin	AY674390
Penicillium marneffei	ATCC 18224	β-tubulin	XM_002151381
Penicillium melanoconidium	CBS 112104	β-tubulin	AY674302
Penicillium mononematosum	CBS 112104	β-tubulin	AY495998
Penicillium naligiovense	CBS 318.92	β-tubulin	AY496000
Penicillium neoechinulatum	CBS 110343	β-tubulin	AY674300
Penicillium nordicum	CBS 606.68	β-tubulin	AY674319
Penicillium olsonii	CBS 232.60	β-tubulin	AY674445
Penicillium pailitans	CBS 491.84	β-tubulin	AY674363
Penicillium paneum	CBS 101032	β-tubulin	AY674387
Penicillium persicinum	CBS 111235	β-tubulin	AY495982
Penicillium polonicum	188P	β-tubulin	EU128563
Penicillium radicicola	CBS 109551	β-tubulin	AY674359
Penicillium roqueforti	CBS 479.84	β-tubulin	AY674382
Penicillium sclerotigenum	CBS 306.97	β-tubulin	AY674395
Penicillium solitum	CBS 146.86	β-tubulin	AY674356
Penicillium thymicola	CBS 111227	β-tubulin	AY674322

Penicillium tricolor	CBS 636.95	β-tubulin	AY674311
Penicillium tulipae	CBS 109555	β-tubulin	AY674344
Penicillium ulaiense	CBS 210.92	β-tubulin	AY674408
Penicillium venetum	CBS 201.57	β-tubulin	AY674335
Penicillium verrucosum	NRRL 965	β-tubulin	AF001205
Penicillium viridicatum	CBS 109826	β-tubulin	AY674294
Penicillium vulpinum	CBS 305.63	β-tubulin	AY674418
Petromyces alliaceus	NRRL 4181	β-tubulin	AY160978
Sclerocleista ornata	NRRL 2291	β-tubulin	EF669677
Talaromyces stipitatus	ATCC 10500	β-tubulin	XM_002341495
Warcupiella spinulosa	NRRL 4376	β-tubulin	EF669680
Aspergillus clavatus	NRRL 1	ITS-5.8 sRNA	EF669942
Aspergillus flavus	A1.7	ITS-5.8 sRNA	EU833206
Aspergillus oryzae	IFO 30113	ITS-5.8 sRNA	D84355
Aspergillus parasiticus	NRRL 424	ITS-5.8 sRNA	EF661557
Aspergillus terreus	NRRL 1913	ITS-5.8 sRNA	EF669579
Emericella heterothallica	222	ITS-5.8 sRNA	L76743
Emericella nidulans	NHRC-FE064-2	ITS-5.8 sRNA	AJ937756
Eurotium repens	YQ5-1	ITS-5.8 sRNA	DQ411545
Neosartorya fennelliae	NRRL 5535	ITS-5.8 sRNA	EF669995
Neosartorya fischeri	083402	ITS-5.8 sRNA	DQ401533
Neosartorya fumigata	ATCC 16907	ITS-5.8 sRNA	AY214446
Neurospora crassa	OR74A	ITS-5.8 sRNA	M13906
Penicillium camemberti	944	ITS-5.8 sRNA	DQ681327
Penicillium chrysogenum	CBS 306.48	ITS-5.8 sRNA	AY213669
Penicillium griseofulvum	CBS185.27	ITS-5.8 sRNA	AF033468
Penicillium marneffei	IFM 51932	ITS-5.8 sRNA	AB353906
Penicillium roqueforti	NS190	ITS-5.8 sRNA	DQ068990
Petromyces alliaceus	FRR 4340	ITS-5.8 sRNA	AF149754
Aspergillus clavatus	NRRL 1	MAT1-1-1	XM_001270633
Aspergillus flavus	NRRL 29518	MAT1-1-1	EU357934
Aspergillus oryzae	RIB40/ATCC 42149	MAT1-1-1	AO090020000089
Aspergillus parasiticus	NRRL 29610	MAT1-1-1	EU357935
Aspergillus terreus	NIH 2624	MAT1-1-1	ATEG_08812.1
Emericella nidulans	FGSC A4	MAT1-1-1	AN2755
Neosartorya fischeri	NRRL 181	MAT1-1-1	XM_001263835
Neosartorya fumigata	Af250	MAT1-1-1	AY898661
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Penicillium marneffei	PM1	MAT1-1-1	DQ340761
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Aspergillus flavus	NRRL 29509	MAT1-2-1	EU357936
Aspergillus oryzae		MAT1-2-1	Paoletti *et al*. Unpublished data
Aspergillus parasiticus	ARRL 29606	MAT1-2-1	EU357937
Emericella nidulans	FGSC A4	MAT1-2-1	AN4734.3
Neosartorya fischeri	NRRL 181	MAT1-2-1	XM_001263956

Neosartorya fumigata	Af293	MAT1-2-1	XM_749896
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Penicillium marneffei	PM27	MAT1-2-1	DQ340762
Petromyces alliaceus	NRRL 4181	MAT1-2-1	EU591676

[a]GenBank Accession Number obtained from NCBI database (http://www.ncbi.nlm.nih.gov/sites/entrez).

[b]Gene Locus obtained from the Broad Insitutte Genome Sequencing Projects (http://www.broad.mit.edu/node/304).