Molecular characterization of *Cercospora beticola* and its relatives

Marizeth Groenewald

Promotoren:
Prof. dr. P.W. Crous
Hoogleraar in de Evolutionaire Fytopathologie
Wageningen Universiteit

Prof. dr. ir. P.J.G.M. de Wit
Hoogleraar in de Fytopathologie
Wageningen Universiteit

Promotie commissie:
Prof. dr. R.F. Hoekstra, Wageningen Universiteit
Dr. ir. G.H.J. Kema, Plant Research International
Prof. dr. Th.W. Kuyper, Wageningen Universiteit
Dr. ir. A.J. Termorshuizen, Wageningen Universiteit

Dit onderzoek is uitgevoerd binnen de onderzoekschool Experimental Plant Sciences

Molecular characterization of *Cercospora beticola* and its relatives

Marizeth Groenewald

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. dr. M.J. Kropff
in het openbaar te verdedigen
op maandag 19 februari 2007
des namiddags te 16:00 in de Aula

Marizeth Groenewald (2007)

Molecular characterization of *Cercospora beticola* and its relatives

PhD Thesis Wageningen University, Netherlands
With summaries in English, Dutch and Afrikaans

ISBN 90-8504-599-1

This thesis is dedicated to my dear husband and mother

CONTENTS

CHAPTER 1

General introduction and outline of thesis

THE GENUS *CERCOSPORA* WITH SPECIFIC REFERENCE TO THE *C. APII* COMPLEX

Fuckel (1863) collected a hyphomycete with hyaline conidia on celery (*Apium graveolens*), which he subsequently sent to Fresenius, who introduced the genus *Cercospora* to accommodate it along with *Passalora*-like species with pluriseptate conidia (in Fuckel, 1863). Ever since its introduction, the genus *Cercospora* has been a dumping ground for dematiaceous hyphomycetes with filiform conidia, making it one of the largest and most heterogeneous genera of hyphomycetes. This situation was recently addressed by Crous & Braun (2003), who monographed the genus.

Cercospora species are distributed worldwide, the majority being plant pathogens that cause leaf spots on a wide range of plant hosts (Crous & Braun, 2003). Since the description of the genus *Cercospora*, the taxonomy of this genus as well as the description of individual species within this group has proven cumbersome. Different characteristics, such as morphology, toxin production and host specificity were used through the years in an attempt to distinguish *Cercospora* species, but with little success. The morphological characters that were used to distinguish *Cercospora* species included the conidial shape and size (Fig. 1A) (Penzes, 1927), the presence or absence of external mycelium, and conidiophore morphology (Fig. 1B) (Solheim, 1930), but unfortunately they were not always reliable as too much intraspecific variation was found. Welles (1933) showed that the dimensions of the reproductive structures in *Cercospora* may vary significantly, and that the magnitude of variation for the same structure can range from six to ten times in dimension depending on the environmental conditions.

Many species of *Cercospora* produce a phytotoxic metabolite called cercosporin (Assante *et al*., 1977). It was initially thought that cercosporin production was associated only with "true" *Cercospora* species (Fajola, 1978), and that those species that did not produce this toxin belonged to other related genera. Later it became clear that the ability of a strain or isolate to produce cercosporin could be influenced dramatically by various environmental and nutritional conditions (Jenns *et al*., 1989). Because of the inconsistency in the production of

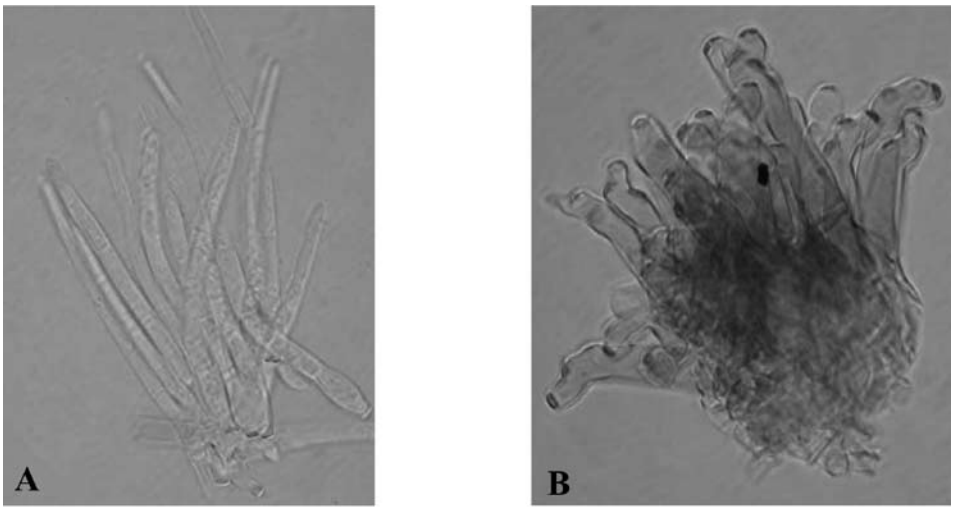


Fig. 1. A, Conidia and **B**, conidiophores of *Cercospora beticola*.

this toxin, as well as in the quantities produced, it proved impossible to apply this characteristic as a taxonomic feature within this genus.

Host specificity has often been used in the past as criterium to distinguish *Cercospora* species from one another. Throughout the years various cross-inoculations were performed with different *Cercospora* species on different hosts (Welles, 1933; Johnson & Valleau, 1949; Berger & Hanson, 1963; Kaiser & Lukezic, 1965), which revealed that species of the *Cercospora apii* complex could infect an extremely wide range of hosts. Although *Cercospora* species are commonly associated with leaf spots, some of them can also cause necrotic lesions on flowers, fruits, bracts, seeds and pedicels of numerous hosts (Agrios, 1997). It has been shown that morphologically uniform *Cercospora* isolates can be found on a wide range of different host plant genera and families. Johnson & Valleau (1949) concluded that these morphologically indistinguishable *Cercospora* isolates represent a single species, and proposed reducing all of these to synonymy under *C. apii*. This conclusion was questioned by Chupp (1954), who stated that species of *Cercospora* were generally host-specific, and used this argument as base to formulate the concept of "one plant host genus/family equals one *Cercospora* species". Chupp's concept led to the description of a large number of species based on the host they were isolated from. Chupp (1954) accepted 1419 *Cercospora* species in his monograph, occurring on 2500 host genera from 155 different plant families. Presently the connection between a *Cercospora* isolate and the host plant from which it was isolated is still a major factor in the taxonomic description of most *Cercospora* species (Crous & Braun, 2003). Approximately 3,000 *Cercospora* species are known from literature (Pollack, 1987). In a recent revision by Crous & Braun (2003), 659 species were recognized as true species of *Cercospora*, with a further 281 being reduced to synonyms under *C. apii*, the latter being the oldest recognizable species name for this complex.

The *Cercospora apii* species complex

Cercospora apii is the oldest name for a large complex of variable, but morphologically indistinguishable *Cercospora* taxa (Johnson & Valleau, 1949), referred to as *C. apii sensu lato* by Crous & Braun (2003). Species belonging to the *C. apii* complex usually form definite leaf spots, but may also grow as saprobes on necrotic plant tissue. They have also been occasionally observed to occur as secondary invaders on leaf spots caused by other fungi. A species complex such as *C. apii* is taxonomically problematic. Three scenarios need to be considered when examining the host-species association of fungi belonging to the *C. apii* complex: (i) there is only one species of *Cercospora* that occurs on a wide host range, (ii) *Cercospora* species are highly host-specific, or (iii) some *Cercospora* species are host-specific, whereas others have wide host ranges that can occur intermingled on the same lesion. It is possible that anamorphs of *C. apii sensu lato* from different substrates are connected by a single, uniform teleomorph. This would support the idea that we are dealing with a single plurivorous species which might comprise races, varieties or *formae specialis* that are specific for different hosts. However, the possibility also exists that *C. apii sensu lato* is composed of different taxa, plurivorous as well as specialized, with morphologically differentiated teleomorphs, but morphologically indistinguishable anamorphs.

Recent genetic analyses of *Cercospora* species have relied mainly on DNA sequences of the internal transcribed spacers (ITS) and the 5.8S rRNA gene (Stewart *et al*., 1999; Goodwin *et al*., 2001; Pretorius *et al*., 2003). These studies have revealed that most species of *Cercospora*, in particular the members of the *C. apii* complex, are identical or very closely related. Judging from their morphological similarity, as well as their proven ability to cross-infect each other's hosts, it is probable that species in the *C. apii* complex could be considered synonymous. Tessmann *et al*. (2001) used elongation factor 1-α (EF1-α), β-tubulin, histone H3 and ITS to study *C. piaropi* and *C. rodmanii*, two species which cause leaf spot disease of water hyacinth and which can not be distinguished morphologically. The ITS region did not contain any differences compared to their outgroup sequence of *C. beticola*. Although Tessmann *et al*. (2001) found phylogenetic differences between isolates identified as *C. piaropi* and *C. rodmanii*, they chose to treat it as a single species in order to avoid identification and communication problems among scientists and quarantine officials.

Cercospora species causing Cercospora leaf spot on celery and sugar beet

Cercospora apii, which causes Cercospora leaf spot on *Apium graveolens* (celery, Fig. 2), was described from the region between the Netherlands and Germany (Fresenius, 1863), and is assumed to have originated in western Europe. Although Cercospora leaf spot is no longer considered the most destructive disease on celery (Koike *et al*., 2004), *C. apii* is still seen as a serious pathogen of this crop in some parts of the world, e.g. Florida (Kucharek, 2004).

Fig. 2. *Apium graveolens* (celery) leaf infected by *Cercospora apii*.

Cercospora beticola, causal agent of Cercospora leaf spot on *Beta vulgaris* (sugar beet, Fig. 3), was originally described by Saccardo (1876) and is assumed to have originated in central Europe and the Mediterranean area. Cercospora leaf spot on sugar beet is a serious problem wherever this crop is grown. It is one of the most common and destructive sugar beet diseases, affecting more than a third of all fields worldwide (Shane & Teng, 1992; Holtschulte, 2000). A whole sugar beet field can be destroyed by an outbreak of *C. beticola*, resulting in complete

Fig. 3. *Beta vulgaris* (sugar beet) plant infected by *Cercospora beticola*.

loss of the crop (Rossi *et al*., 2000; Weiland, 2004). *Cercospora beticola* is seen as part of the *C. apii* complex (Ellis, 1971; Crous & Braun, 2003), and the similarity in disease symptoms and pathogen morphology led Crous & Braun (2003) to conclude that *C. beticola* should be placed under *C. apii sensu lato.*

Chupp (1954) associated Cercospora leaf spot on sugar beet with infections of *C. beticola* and that on celery with *C. apii*. Although morphological characteristics are frequently used to identify newly isolated fungi, it is not possible to distinguish *C. apii* from *C. beticola* based solely on morphology. Ellis (1971) discussed the *C. apii sensu lato* isolates in detail and described a wide host range for this species, but he changed his opinion five years later and narrowed the host range of *C. apii* and *C. beticola* again to celery and sugar beet, respectively (Ellis, 1976). Crous & Braun (2003) linked 83 host genera to *C. apii* and nine host genera to *C. beticola* infections. Crous & Groenewald (2005) introduced the pogo stick hypothesis to explain the colonization of necrotic Mycosphaerella lesions by other species of *Mycosphaerella* that jump hosts in the process of reaching their real hosts. The possibility that this process of substrate colonization and host jumps also occurs in asexual *Mycosphaerella* species could explain the isolation of specific *Cercospora* species from "atypical" hosts.

Despite of its wide distribution, no teleomorph has yet been described for species that are part of the *C. apii* complex (e.g. *C. apii* and *C. beticola*). Phylogenetic analyses using the ITS sequences of a number of *Cercospora* species have resolved *Cercospora* as a well-defined monophyletic clade within the teleomorph genus *Mycosphaerella* (Stewart *et al*., 1999; Crous *et al*., 2000, 2001, 2004; Goodwin *et al*., 2001; Pretorius *et al*., 2003). Based on these data it is clear that, if sexual states do exist for these species, they would reside in the genus *Mycosphaerella*. Finding the teleomorph(s) of fungi associated with the *C. apii* complex, along with molecular data analysis of the progeny, will help to improve the understanding and taxonomic treatment of this complex and could resolve the problems with species identity in the *C. apii* complex.

MATING TYPE (*MAT*) GENES

The existence of fungal mating types was first recognized in the genus *Rhizopus* by Blakeslee (1904), who was also the first to introduce the terms homothallism for self-fertile, and heterothallism for self-sterile individuals. In the early 1980s the first mating type loci were characterized from *Saccharomyces cerevisiae* (Astell *et al*., 1981), whereas the first filamentous ascomycete mating type genes that were identified from *Neurospora crassa* (Glass *et al*., 1988; Staben & Yanofsky, 1990). The development of new techniques allowed the isolation and characterization of mating type genes of additional economically important fungi (Turgeon *et al*., 1993; Arie *et al*., 1997, 2000; Waalwijk *et al*., 2002; Goodwin *et al*., 2003).

A standardized nomenclature that is used for mating type genes of filamentous ascomycetes was proposed by Turgeon & Yoder (2000). For most heterothallic species of ascomycetes studied thus far, only one mating type locus (*MAT1*) that contains two alternating alleles is present. The DNA sequences at this locus show no homology between strains of opposite mating type that belong to the same species, and therefore these regions have been termed idiomorphs and not alleles (Glass *et al*., 1988; Metzenberg & Glass, 1990). The two idiomorphs at *MAT1* were designated *MAT1-1* and *MAT1-2*, where each of these idiomorphs contains open reading frames that encode proteins with confirmed or putative DNA-binding motifs. The *MAT1-2* idiomorph contains the *MAT1-2-1* gene that encodes a regulatory protein with a high mobility group (HMG) domain. The *MAT1-1* idiomorph of all the heterothallic loculoascomycetes studied thus far contains the *MAT1-1-1* gene encoding a protein with an alpha helix domain (Turgeon *et al*., 1993). More than one gene can be present in the *MAT1-1* idiomorph of some heterothallic pyrenomycetes and discomycetes (Sing & Ashby, 1998, 1999; Yun *et al*., 2000; Pöggeler, 2001) that include the *MAT1-1-1,* the *MAT1-1-2* encoding an amphipathic alpha helix protein, the *MAT1-1-3* gene encoding a HMG protein and the *MAT1-1-4* gene encoding a metallothionein protein. The specific function(s) of these proteins have not yet been identified, but it is most likely that they are transcription factors that control pathways of cell speciation and sexual morphogenesis (Turgeon, 1998).

Mating type genes in the Mycosphaerellaceae

Different methods have been used to isolate *MAT* genes from a range of fungal species. Most of these genes have been isolated by heterologous hybridization, or by using PCR techniques based on sequence homologies found within the idiomorphs or regions that are flanking the idiomorphs (Turgeon *et al*., 1993; Arie *et al*., 1997, 2000; Waalwijk *et al*., 2002; Goodwin *et al*., 2003). The mating type genes of only two species of the Mycosphaerellaceae have been characterized thus far, namely *Mycosphaerella graminicola* and *Septoria passerinii*.

Both mating type loci of the heterothallic, sexually reproducing fungal species *M. graminicola* have been isolated and characterized by Waalwijk *et al*. (2002). Molecular data showed that either the *MAT1-1-1* or *MAT1-2* is present in a single strain, and therefore confirmed that *M. graminicola* is potentially heterothallic, as was already known from conventional crosses. The predicted coding region of the *MAT1-1-1* is 995 bp encoding a MAT1 protein of 297 amino acids. The coding region contains two potential introns of which one is located in the conserved alpha domain (Waalwijk *et al*., 2002). The predicted coding region of the *MAT1-2* is 1,233 bp

encoding a MAT2 protein of 394 amino acids. One intron is predicted within the coding region and is located in the conserved HMG domain.

Both mating type loci of the anamorph species *S. passerinii* have been isolated and characterized by Goodwin *et al*. (2003). This species is phylogenetically closely related to *M. graminicola.* Because the two *MAT* genes were never found to be present in the same strain, this species is classified as potentially heterothallic. The predicted coding region of the *MAT1-1-1* is 1,038 bp in length, and contains two introns of which one is located in the conserved alpha domain (Goodwin *et al*., 2003) and encodes a MAT1 protein of 310 amino acids. The predicted coding region of the *MAT1-2* is 754 bp contains one intron located in the conserved HMG domain, and encodes a MAT2 protein of 234 amino acids.

Measuring the sexual reproduction potential

Mating type genes play an important role in the biology and evolution of fungal species and knowledge of these genes can provide insight in the potential prevalence of sex in fungi. Although sexual reproduction is absent in a large number of filamentous ascomycetes, mating type sequences have been isolated from several apparently asexual fungi such as *Alternaria alternata* (Arie *et al*., 2000), *Rhynchosporium secalis* (Linde *et al*., 2003) and *S. passerinii* (Goodwin *et al*., 2003). This introduced the possibilities that (i) a sexual cycle may also be active in some of these supposedly asexual species but that the cycle has not yet been identified, or (ii) that asexual fungi which carry functional mating type genes may lack some other attributes that are required for mating (Sharon *et al*., 1996; Arie *et al*., 2000; Yun *et al*., 2000).

In the absence of a known sexual stage, a method to test for the possibility of sexual reproduction is by looking directly at the occurrence and frequency of the mating type genes (Milgroom, 1996). As mentioned above both mating types have been characterized for the solely asexually reproducing filamentous ascomycetes such as *A. alternata*, *Fusarium oxysporum*, *S. passerinii* and *R. secalis* (Arie *et al*., 1997, 2000; Goodwin *et al*., 2003; Linde *et al*., 2003) but the presence of the mating type idiomorphs in a given species alone is insufficient to prove that a sexual stage does exist. However, it is probable that sexual recombination does take place if the two mating types occur in approximately equal frequencies within a given population (Milgroom, 1996; Halliday *et al*., 1999; Waalwijk *et al*., 2002; Linde *et al*., 2003). Goodwin *et al*. (2003) found both mating types of *S. passerinii* to occur on the same leaf and showed that both mating types were present in approximately equal frequencies in field populations from North Dakota and Minnesota. They concluded that the equal distribution of the mating types and the high number of unique genotypes found on the same leaf are indications that *S. passerinii* undergoes regular cycles of sexual reproduction in the field. A recent study that managed to cross isolates of *S. passerinii* in culture, resulting in a *Mycosphaerella* state with viable ascospores, confirmed this hypothesis (Ware, 2006). Linde *et al*. (2003) found *R. secalis* isolates with opposite mating types co-existing in the same lesion. Detailed analyses have been reported on the distribution of the mating types of the sexually reproducing *M. graminicola* (Waalwijk *et al*., 2002; Zhan *et al*., 2002). Equal distribution of the mating types were found in most of the populations sampled from different geographical scales that included different plots within the same field, as well as different fields within the same region or country.

Another method that can be used to test for the possibility of sexual reproduction is by looking at the genotypic diversity within a given population. Populations that regularly undergo sexual reproduction should have many unique genotypes that result in higher levels of genotypic diversity compared to those with only asexual reproduction (Milgroom, 1996). A genotypically diverse type of genetic structure is seen in most populations of *M. graminicola* (Linde *et al*., 2002; Zhan *et al*., 2003; Zhan & McDonald, 2004).

MOLECULAR MARKERS

Various types of molecular techniques have been developed in an attempt to unravel some of the questions regarding diversity among species and populations (Milgroom, 2001). Some of these techniques allow a great number of polymorphic loci to be detected in individuals for direct assessment of genetic variation in populations and can thus be used to study the genetic variation at species and population levels with great reliability. The level of genetic variation that is present within a single population is measured in terms of genotype or gene diversity and may indicate how rapidly a pathogen can evolve and adapt to changing environments. It is possible to find a high level of genetic variation within the same genus and even species isolated from the same host (Brayford, 1990; Milgroom *et al*., 1992; Wang *et al*., 1997). Both somatic mutations and sexual recombination can lead to increased genetic diversity in a population. Sexual reproduction produces recombinant genotypes and frequent recombination causes random association of alleles at different loci and therefore sexual populations generally have greater genotypic diversity than asexual populations. If large numbers of unrelated genotypes are found, it may indicate that in addition to somatic mutations sexual recombination might occur (Milgroom *et al*., 1992; Wang *et al*., 1997; Kohli & Kohn, 1998).

Molecular markers are powerful tools but may differ with respect to important features such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and cost effectiveness. The choice of the most appropriate molecular marker technique depends on several factors such as the specific application, the presumed level of polymorphism, the presence of sufficient technical facilities, time constraints and financial limitation. Advances in molecular technology in the late 1980s and 1990s led to the development of robust molecular markers such as restriction fragment length polymorphisms (RFLPs) (Botstein *et al*., 1980), amplified fragment length polymorphisms (AFLPs) (Vos *et al*., 1995), microsatellites or simple sequence repeats (SSRs) (Litt & Luty, 1989; Weber & May, 1989), randomly amplified polymorphic DNAs (RAPDs) (Williams *et al*., 1990) and multilocus sequence typing (MLST) (Maiden *et al*., 1998). The availability of these techniques generates the possibility to study genetic variation in great deal and might improve identification and management of fungal diseases.

Amplified fragment length polymorphisms (AFLPs)

The AFLP technique is used to visualize numerous amplified DNA restriction fragments simultaneously (Vos *et al*., 1995). An AFLP protocol includes several steps: (i) the digestion of gDNA with two restriction endonucleases, (ii) the ligation of digested DNA to double-stranded

nucleotide adaptors, (iii) pre-selective amplification of genomic fragments containing an adaptor at each end and (iv) selective amplifications using primers with selective base extensions (Fig. 4). Only those fragments with complementary nucleotides extending beyond the restriction site will be amplified by the selective primers under stringent annealing conditions and this reduces the complexity of the mixture.

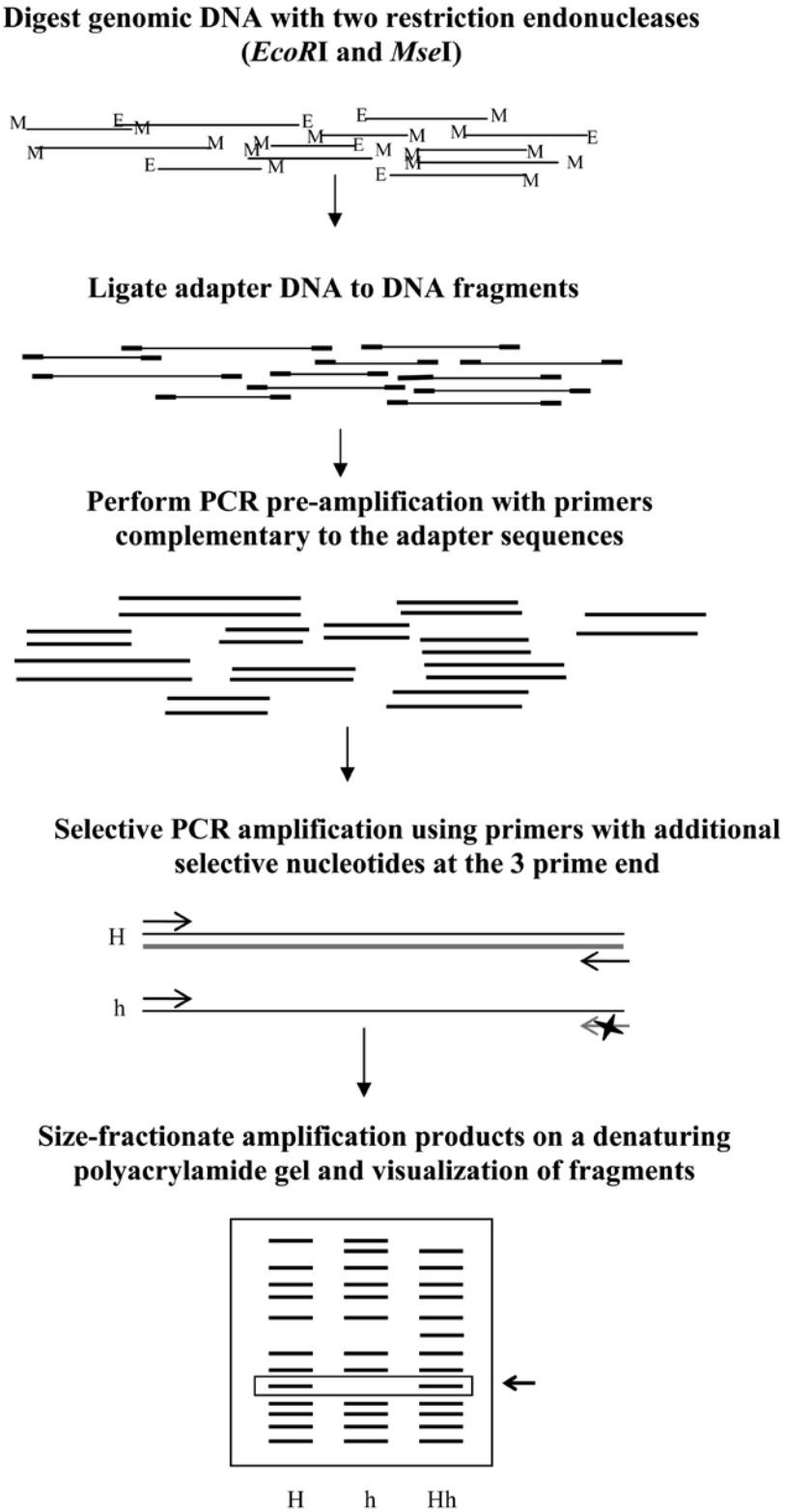


Fig. 4. Graphical representation of the amplified fragment length polymorphism (AFLP) marker system.

This technique has a number of advantages: (i) minimal amounts of gDNA are required, (ii) no prior genome sequence data is necessary for primer construction, (iii) markers are randomly distributed throughout the genome, and (iv) many bands are generated that potentially provide a large number of polymorphisms (Maheswaran *et al*., 1997; Mueller & Wolfenbarger, 1999). There are also some disadvantages to this technique: (i) it requires extremely pure, high molecular weight gDNA, (ii) co-migration of non-allelic fragments of the same size can occur (Mueller & Wolfenbarger, 1999), and (iii) AFLP markers are dominant and therefore it is not well possible to distinguish between the homozygous state of the dominant allele and the heterozygote.

High-throughput AFLPs have been used as a powerful tool to perform detailed analysis on the genetic diversity within populations of *Fusarium oxysporum* f. sp. *cubense* (Groenewald *et al*., 2006). The AFLP technique has also been used for fingerprinting in *Cercospora zeae-maydis* (Wang *et al*., 1998), *Cercospora sorghi* (Okori *et al*., 2004), *Cercospora* species associated with maize (Wang *et al*., 1998), and *Phaeosphaeria nodorum* (Bennet *et al*., 2004).

Microsatellites

Microsatellites, also known as short simple repeats (SSRs), are short segments of DNA that contain a core motif which consists of a few nucleotides that is normally 1–6 bp in length. This core motif can be repeated up to a maximum of 60 times in tandem (Litt & Luty, 1989; Weber & May, 1989; Field & Wills, 1996). These regions are dispersed throughout the entire genome, both in protein-coding and noncoding regions although it has been shown that microsatellites are less abundant in coding than in noncoding regions (Hancock, 1995). The number of tandem repeats at a specific locus can vary between two individuals and it is possible to screen for these differences using primers that anneal to the flanking regions of the specific microsatellite region (Fig. 5). The generated PCR products can be size-fractionated on a polyacrylamide gel, allowing the discrimination of alleles with a size difference of up to one bp (Weber & May, 1989). Because of their high mutability, microsatellites are thought to play a significant role in genome evolution by creating and maintaining genetic variation.

Microsatellite markers have several advantages that make them excellent molecular markers to use: (i) low quantities of template gDNA are necessary, (ii) it is usually highly polymorphic, (iii) it is highly reproducible, (iv) the possibility exists to score more than one microsatellite at the same time by performing a multiplex PCR, and (v) it represent co-dominant markers (Litt & Luty, 1989; Weber & May, 1989; Gruis *et al*., 1993; Wu *et al*., 1994).

Unfortunately there are also a few disadvantages: (i) an allele can be absent ("null allele") due to deletions or mutations in the flanking DNA that contains the primer binding site (Callen *et al*., 1993), and (ii) the identification and development of these markers are expensive and time-consuming.

Microsatellite markers have been used in various types of studies, but especially in studying the population structures of *M. graminicola* (Owen *et al.*, 1998), *M. fijiensis* (Müller *et al*., 1997), *Phialophora gregata* (Chen *et al.*, 2002), *Diplodia pinea* (Burgess *et al*., 2001, 2004) and *Aspergillus fumigatus* (Rosehart *et al*., 2002).

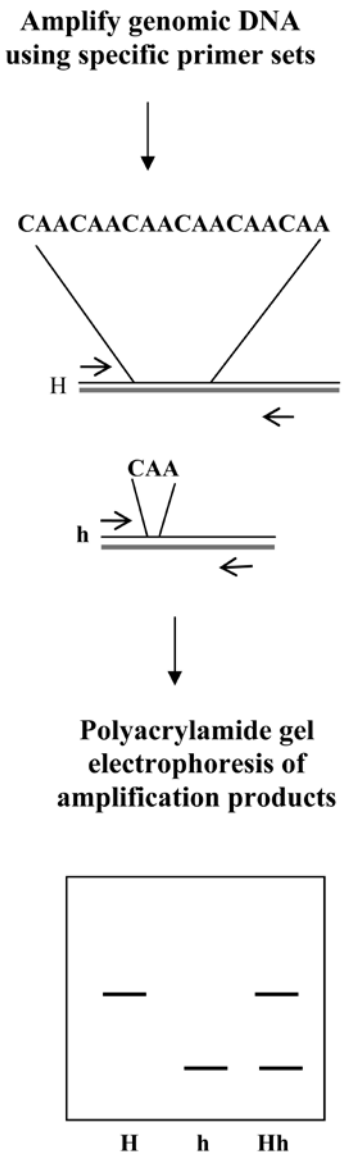


Fig. 5. Graphical representation of the steps of the microsatellite marker system.

Restriction fragment length polymorphisms (RFLPs)

Restriction fragment length polymorphism markers were the first DNA-based marker to be developed (Botstein *et al*., 1980; Beckman & Soller, 1983). The original method (Fig. 6) includes several steps: (i) digestion of gDNA with one or more restriction endonucleases, (ii) separation of digested fragments on high percentage agarose gels, (iii) fixation of the digested DNA onto a membrane (Southern blot technology - Southern, 1975), (iv) hybridization with a fluorescently or radioactively labeled fragment of which the sequence correlates with a region of interest within the genome, and (v) the detection of the bands of interest. Differences within restriction endonuclease recognition sites between the genomes of two or more individuals can be scored as the presence/absence as well as size differences of the fragments which are homologous to the probe used.

A variant of the hybridization-based method is the polymerase chain reaction/RFLP (PCR/RFLP)-based method also known as cleaved amplified polymorphic sequence (CAPS) (Fig. 7) (Deng, 1988). This method is much easier and faster and is based on the amplification of a specific locus in a genome, followed by the restriction endonuclease digestion of the amplicon using a specific restriction enzyme. Agarose or polyacrylamide gel electrophoresis can be used to reveal differences in fragment sizes after staining the gel with ethidium bromide or silver nitrate (Avise, 1994). Similar to the original technique, the fragment differences result from

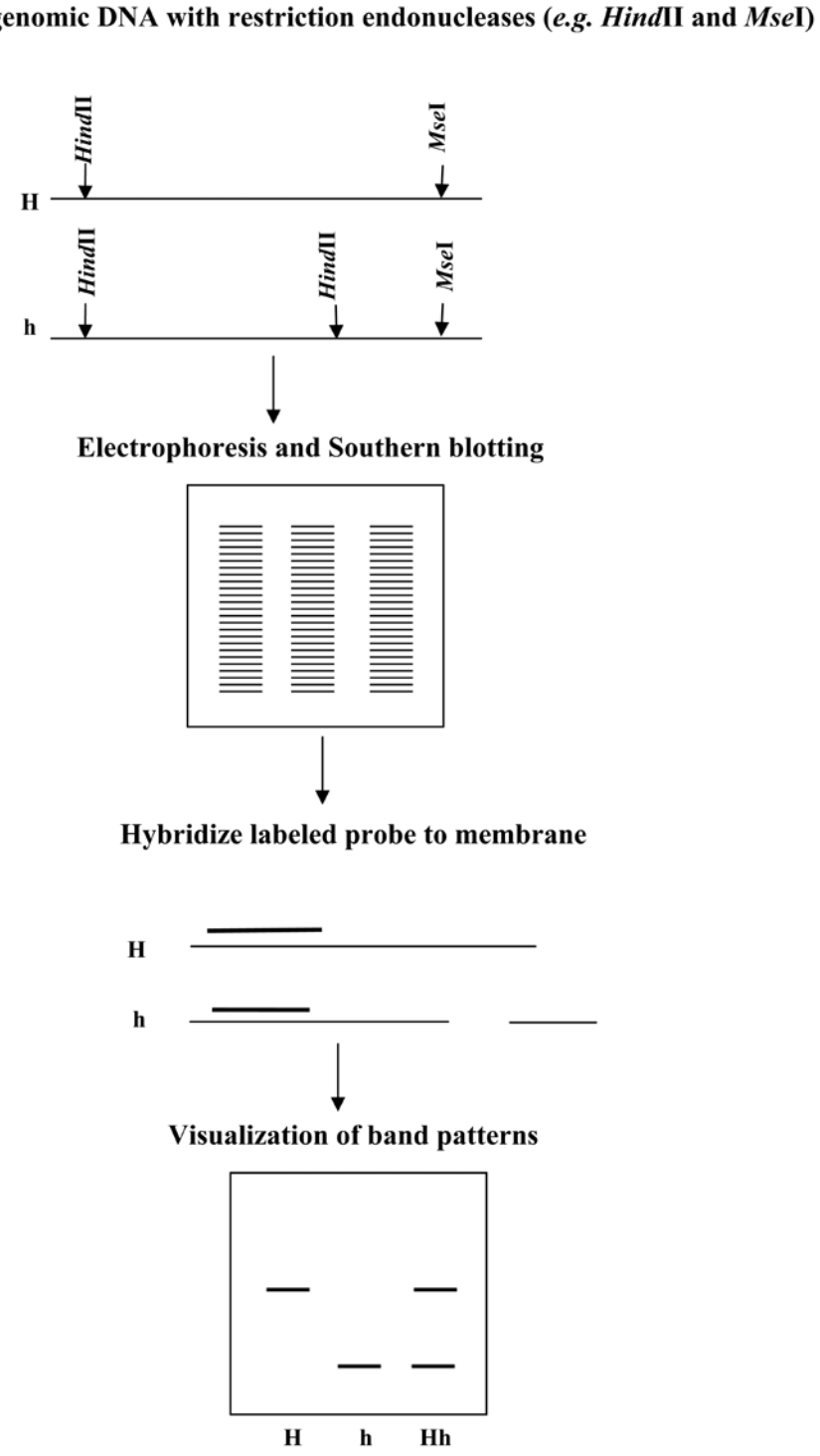


Fig. 6. Graphical representation of the procedure for the original restriction fragment length polymorphism (RFLP) marker system.

mutations of nucleotides within the restriction enzyme recognition sequence(s). This method is generally less laborious than the original one and provides results faster. A negative aspect of the PCR/RFLP method is that it requires the presence of a mutation(s) which can be recognized by a restriction endonuclease for at least one allele, and the availability of PCR primers for the amplification reaction. Unfortunately, many point mutations do not result in a restriction site or abolish it and the polymorphisms can therefore not be detected by this approach.

Both RFLP techniques have been successfully used to study the population structure of several important fungal plant pathogens, including *M. graminicola* (Linde *et al*., 2002; Zhan *et al*., 2003; Zhan & McDonald, 2004), *M. musicola* (Hayden *et al*., 2003a), *M. fijiensis* (Hayden *et al*., 2003b; Rivas *et al*., 2004), *R. secalis* (McDonald *et al*., 1999; Salamati *et al*., 2000), *Cercospora caricis* (Inglis *et al*., 2001) and *Armillaria* species (Mweje *et al*., 2003).

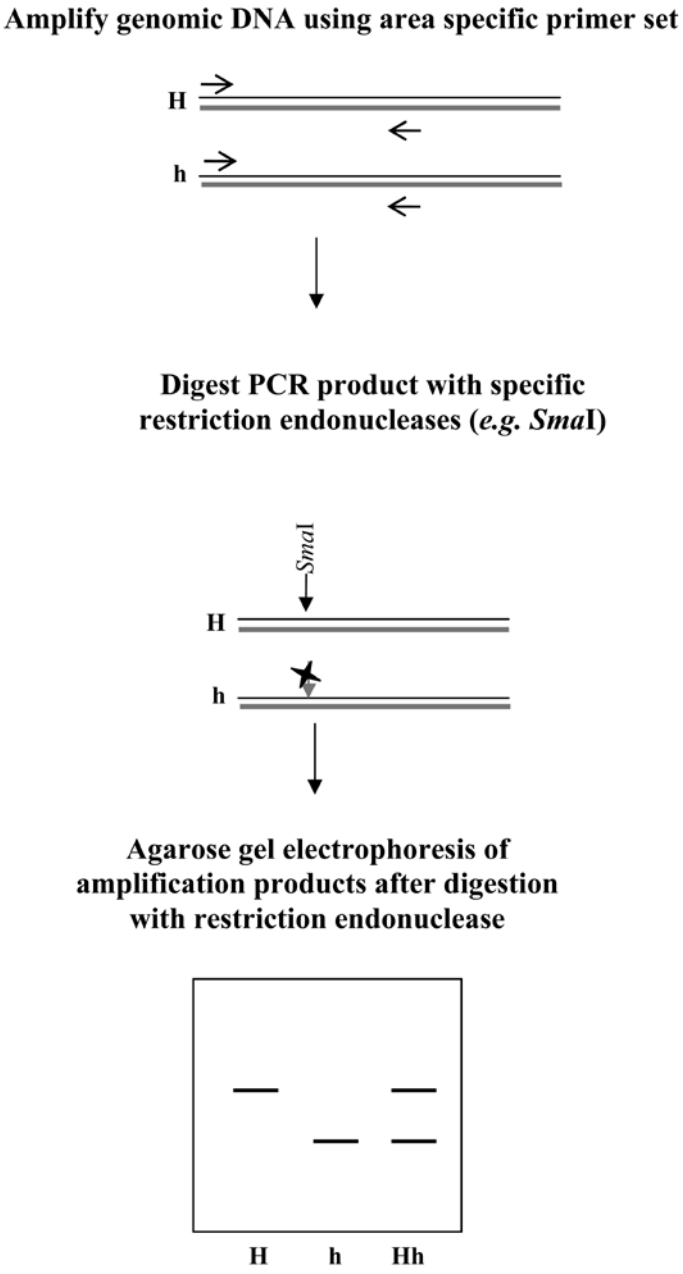


Fig. 7. Graphical representation of the rapid procedure of the polymerase chain reaction/RFLP (PCR/RFLP)-based method.

Randomly amplified polymorphic DNAs (RAPDs) and inter sequence simple repeats (ISSRs)

The randomly amplified polymorphic DNA technique (Williams *et al*., 1990; Welsh & McClelland, 1990) is a PCR-based method that involves the binding of a short single arbitrary primer of approximately 10 bp to arbitrary sequences in the genome under relatively low stringency conditions with annealing temperatures as low as 36 ^{o}C (Fig. 8). A fragment(s) is amplified when the primer binds on both of the gDNA strands, but in opposite orientation and with an amplifiable distance between the two primer binding sites. The variation in band patterns between different isolates results from sequence differences in the primer binding sites or sometimes from deletions and insertions within the specific regions between the primer binding sites. These differences are visible as presence or absence of particular bands or differences in band sizes (Fig. 8). The amplified products can be size-fractionated on either agarose or polyacrylamide gels.

This marker system has the advantage that: (i) low quantities of template gDNA are required, (ii) prior knowledge of the genome sequence is not necessary, (iii) markers are usually highly abundant within genomes and can reveal a number of polymorphisms at the same time, (iv) it is an easy, and (v) cheap method (Sosinski & Douches, 1996). Unfortunately this technique also has some disadvantages: (i) it is very sensitive to reaction conditions, (ii) it has a low reproducibility, (iii) the primers are able to amplify DNA fragments from any type of genome and it is therefore essential to avoid contamination with undesired gDNA, (iv) primers

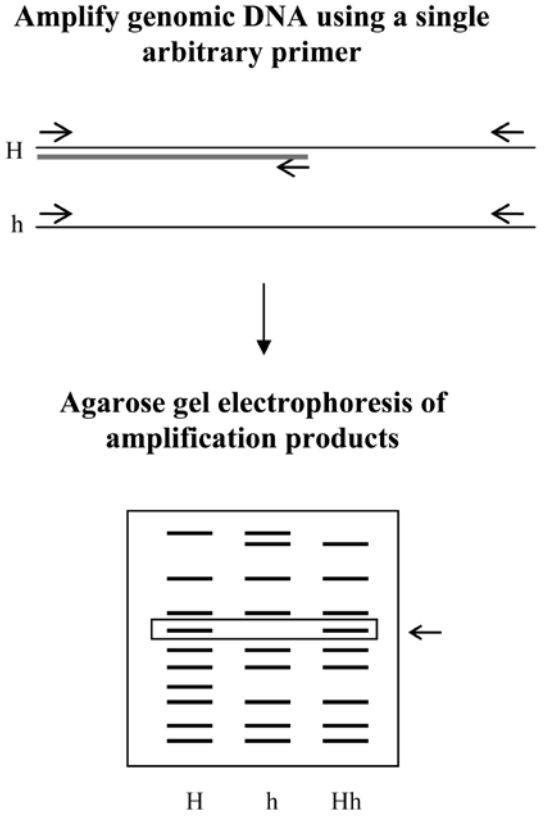


Fig. 8. Graphical representation of the reactions included in the randomly amplified polymorphic DNA (RAPD) marker system.

bind competitively to the template DNA and will therefore bind more often to repetitive DNA sequences than low-copy ones, (v) similar to AFLPs it is a dominant marker system, and (vi) similar-sized fragments may not be homologous (Nair *et al*., 1995; Jones *et al*., 1997).

An alternative to the RAPD method is the inter-simple sequence repeats (ISSRs) method which has been developed as an arbitrary, RAPDs-like approach that assesses variation in the numerous microsatellite regions dispersed throughout the genome (Wu *et al*., 1994; Zietkiewicz *et al*., 1994). The special RAPD-like primers contain microsatellite repeat sequences and often an arbitrary pair of bases at the 3-prime or 5-prime end. Areas within the genome flanked by microsatellite regions homologous to the specific ISSR primer sequence will be amplified. Due to the high abundance of microsatellite-rich areas in the genome, a RAPD type of fragment profile will be obtained. This method gives high variability and compares well with other marker systems such as RFLP and RAPDs. It has the same advantages of the classic RAPD technique but is more robust and reproducible than RAPDs (Fang & Roose, 1997; Nagaoka & Ogihara, 1997).

Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) is a genotyping technique based on DNA sequencing of housekeeping genes which are under stable selective pressure. Although the technique has originally been proposed and used for genotyping of bacteria (Maiden *et al*., 1998), it is also used for the genotyping of fungi (reviewed by Taylor & Fisher, 2003). The technique has several advantages: (i) it is based on sequence data and can therefore easily be shared between laboratories (Maiden *et al*., 1998), (ii) sequences are highly reproducible between different laboratories, and (iii) data can be placed in public databases such as NCBI's GenBank (http://www.ncbi.nih.gov/) that can be accessed and searched via the Internet. Unfortunately, MLST also has a few disadvantages: (i) multiple loci are needed to achieve optimal resolution (Maiden *et al*., 1998), (ii) more time and expenses are required with each additional locus included, (iii) not all loci are easily amplified, and (iv) not all loci are equally suitable, even between species of the same genus (Crous & Groenewald, 2005). Examples where MLST was used in fungi include the use of the mating type locus and seven nuclear genes (namely histone H3, EF1-α, reductase, phosphate permease, *Tri101*, ammonia ligase and beta-tubulin) to recognized nine phylogenetically distinct species within *Fusarium graminearum* (O'Donnell *et al*., 2004); using actin, calmodulin and histone H3 sequence data in *Mycosphaerella* to resolve *M. thailandica* and *M. colombiensis* (Crous *et al*., 2004b) and using the ITS of the rDNA operon, partial EF1-α and beta-tubulin sequences to resolve phylogenetic species within the *M. nubilosa* / *M. juvenis* species complex (Crous *et al*., 2004a). Litvintseva *et al*. (2006) applied MLST to investigate the population structure and mode of reproduction of *Cryptococcus neoformans var. grubii* (serotype A). Twelve unlinked polymorphic loci were used and their comparison of MLST analyses with conventional AFLP analysis showed a good correlation between the MLST and AFLP results.

OUTLINE OF THESIS

The *C. apii* complex is an important species complex causing leaf spots on a range of host plants. However, this complex has a long history of taxonomic confusion. The experiments and results described in this thesis provide insight into the cultural and molecular variation that exist among species that belong to the *C. apii* complex, with specific reference to *C. apii* and *C. beticola*. The host plants from which these two pathogens were isolated from is discussed and a new *Cercospora* species, *C. apiicola* that causes Cercospora leaf spot on celery, is described. Little is known about the genetic diversity that exists within *C. beticola.* Different molecular markers were therefore developed and used during this study to determine the level of genetic diversity that exist within and among six *C. beticola* populations.

The teleomorphs of most Cercospora leaf spot pathogens are unknown and one of the main objectives of this study was to understand the reproduction cycle in these apparently asexual species. Degenerate primers were developed from homologous regions within the mating type gene sequences described for three *Mycosphaerella* species, *M. graminicola*, *M. fijiensis* and *Septoria passerinii*. These primers were used to isolate and characterize the mating type genes from several *Cercospora* species as well as two related *Dothistroma* species, namely *D. pini* and *D. septosporum.* A wide range of techniques was used during this study to obtain more insight in the variation that exists within and between species that are part of the *C. apii* complex and their relatives.

Chapter 1 gives an introduction to the genus *Cercospora* with specific reference to species that belong to the *C. apii* complex. It includes an introduction to mating type genes and the possible reproduction strategies of *M. graminicola, S. passerinii* and closely related species. A brief overview of different molecular markers that are commonly used in fungal genetic diversity and population studies is provided.

Chapter 2 describes how MLST data, AFLP analyses and cultural characteristics are used to characterize morphologically similar *Cercospora* species that occur on celery and sugar beet. The development of a PCR-based diagnostic protocol to distinguish between the *Cercospora* species from sugar beet and celery is described.

In **Chapter 3** the host range of *C. apii* and *C. beticola* is examined and a description of *C. apiicola*, a novel species of *Cercospora* from celery is provided. Specific attention is given to the fact that some species are highly host-specific, while other appears to have wider host ranges.

Chapter 4 illustrates the development of mating type-specific degenerate primers and how these are used to isolate and characterize the mating type genes of *C. apii, C. apiicola*, *C. beticola*, *C. zeae-maydis* and *C. zeina* using PCR-based techniques. The distribution of the mating type genes *MAT1-1-1* and *MAT1-2* in field populations is determined.

AFLP analyses together with mating type data are used in **Chapter 5** to study the level of genetic variation within *C. beticola* populations from four European countries, Iran and New Zealand.

In **Chapter 6** the development of additional polymorphic markers that can be used in future population studies is illustrated. These markers include microsatellites, PCR-restriction

fragment length polymorphisms (PCR-RFLPs) and single nucleotide polymorphisms (SNPs).

In **Chapter 7** the isolation and characterization of the *MAT1-1-1* and *MAT1-2* genes of two relatives of *C. beticola,* namely the pine needle blight pathogens (red band needle disease) *Dothistroma septosporum* and *D. pini,* are described. Species-specific primer sets that can distinguish between the two species and simultaneously determine the mating type are designed.

Chapter 8 discusses the important findings of this dissertation. It demonstrates how the data from the present study significantly improves our understanding regarding the (i) taxonomic relationship between *C. beticola* and closely related species; (ii) the genetic variation that exists within and between geographically distinct *C. beticola* populations and (iii) the possible reproduction strategies that are present within populations of *C. beticola* and close relatives.

Appendix I is a review of data obtained during this PhD that will appear as a chapter in a book. It provides an overview on mating type genes in *Cercospora* species and close relatives and represents an expanded version of Chapter 4 of this thesis.

REFERENCES

Agrios, G.N. 1997. Plant Pathology, 4th ed. Academic Press, San Francisco, California.

Ahmed, A.O.A., Mukhtar, M.M., Kools-Sijmons, M., Fahal, A.H., de Hoog, S., Gerrits van den Ende, B., Zijlstra, E.E., Verbrugh, H., Abugroun, EL SIR A.M., Elhassan, A.M., and van Belkum, A. 1999. Development of a species-specific PCR-restriction fragment length polymorphism analysis procedure for identification of *Madurella mycetomatis*. J. Clin. Microbiol. 37:3175–3178.

Arie, T., Christiansen, S.K., Yoder, O.C., and Turgeon, B.G. 1997. Efficient cloning of ascomycete mating type genes by PCR amplification of the conserved MAT-HMG box. Fungal Genet. Biol. 21:118–130.

Arie, T., Kaneko, I., Yoshida, T., Noguchi, M., Nomura, Y., and Yamaguchi, I. 2000. Mating-type genes from asexual phytopathogenic ascomycetes *Fusarium oxysporum* and *Alternaria alternata.* Mol. Plant Microbe In. 13:1330–1339.

Astell, C.R., Ahlstrom-Jonasson, L., Smith, M., Tatchell, K., Nasmyth, K.A., and Hall, B.D. 1981. The sequence of the DNAs coding for the mating-type loci of *Saccharomyces cerevisiae*. Cell 27:15–23.

Avise, J.C. 1994. Molecular markers, natural history and evolution. New York, Chapman and Hall.

Assante, G., Locci, R., Camarda, L., Merlini, L., and Nasini, G. 1977. Screening of the genus *Cercospora* for secondary metabolites. Phytochemistry 16:243–247.

Beckman, J.S., and Soller, M. 1983. Restriction fragment length polymorphisms in genetic improvement: Methodologies, mapping and costs. Theor. Appl. Genet. 67:35–43.

Bennett, R.S., Milgroom, M.G., and Bergstrom, G.C. 2005. Population structure of seedborne *Phaeosphaeria nodorum* on New York Wheat. Phytopathology 95:300–305.

Berger, R.D., and Hanson, E.W. 1963. Pathogenicity, host-parasite relationship, and morphology of some forage legume Cercosporae, and factors related to disease development. Phytopathology 53:500–508.

Blakeslee, A.F. 1904. Sexual reproduction in the Mucorinae. P. Natl. Acad. Sci. U.S.A. 40:205–319.

Botstein, D., White, R.L., Skolnik, M., and Davis, R.W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphims. Am. J. Hum. Genet. 32:314–331.

Brayford, D. 1990. Vegetative incompatibility in *Phomopsis* from elm. Mycol. Res. 94:745–752.

Burgess, T., Wingfield, M.J., and Wingfield, B.D. 2001. Simple sequence repeat marker distinguish among morphotypes of *Sphaeropsis sapinea.* Appl. Envirion. Microbiol. 67:354–362.

Burgess, T., Wingfield, M.J., and Wingfield, B.D. 2004. Global distribution of *Diplodia pinea* genotypes revealed using simple sequence repeat (SSR) markers. Australas. Plant Pathol. 33:513–219.

Callen, D.F., Thompson, A.D., Shen, Y., Phillips, H.A., Richards, R.I., Mulley, J.C., and Sutherland, G.R. 1993. Incidence and origin of 'null' alleles in the $(AC)_n$ microsatellite markers. Am. J. Hum. Genet. 52:922–927.

Chen, W., Shi, X., and Chen, Y.-C. 2002. Microsatellite markers and clonal genetic structure of the fungal pathogen *Phialophora gregata.* Mycol. Res. 106:194–202.

Chupp, C. 1954. A monograph of the fungus genus *Cercospora*. Ithaca, New York. (dated 1953, published 1954).

Crous, P.W., and Braun, U. 2003. *Mycosphaerella* and its anamorphs: 1. Names published in *Cercospora* and *Passalora.* Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

Crous, P.W., and Groenewald, J.Z. 2005. Hosts, species and genotypes: opinions versus data. Australas. Plant Pathol. 34:463–470.

Crous, P.W., Aptroot, A., Kang, J.-C., Braun, U., and Wingfield, M.J. 2000. The genus *Mycosphaerella* and its anamorphs. Stud. Mycol. 45:107–121.

Crous, P.W, Kang, J.C., and Braun, U. 2001. A phylogenetic redefinition of anamorph genera in *Mycosphaerella* based on ITS rDNA sequences and morphology. Mycologia 88:441–458.

Crous, P.W., Groenewald, J.Z., Mansilla, J.P., Hunter, G.C., and Wingfield, M.J. 2004a. Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. Stud. Mycol. 50:195–214.

Crous, P.W., Groenewald, J.Z., Pongpanich, K., Himaman, W., Arzanlou, M., and Wingfield, M.J. 2004b. Cryptic speciation and host specificity among *Mycosphaerella* spp. occurring on Australian *Acacia* species grown as exotics in the tropics. Stud. Mycol. 50:457–469.

Deng, G. 1988. A sensitive non-radioactive PCR-RFLP analysis for detecting point mutations at 12th codon of oncogene c-Ha-*ras* in DNAs of gastric cancer. Nucleic Acids Res. 16:6231.

Ellis, M.B. 1971. Dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, UK.

Ellis, M.B. 1976. More Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, UK.

Fajola, A.O. 1978. Cercosporin, a phytotoxin from *Cercospora* spp. Physiol. Plant Pathol. 13:157–164.

Fang, D.Q., and Roose, M.L. 1997. Identification of closely related citrus cultivars with inter-simple sequence repeat markers. Theor. Appl. Genet. 95:408–417.

Field, D., and Wills, C. 1996. Long, polymorphic microsatellites in simple organisms. P. R. Soc. Lond. 263:209–215.

Fresenius, G. 1863. Beiträge zur Mykologie 3. Heinrich Ludwig Brömmer Verlag, Frankfurt, Germany.

Fuckel, K.W.G.L. 1863. Fungi Rhemani exsiccate, Fasc. I-IV. Hedwigia 2:132–136.

Fuentes, J.-J., Banchs, M.I., Volpini, V., and Estivill, X. 1991. Multiple PCR of microsatellites applied to forensic medicine. Cytogenet. Cell Genet. 58:2141.

Glass, N.L., Vollmer, S.J., Staben, C., Grotelueschen, J., Metzenberg, R.L., and Yanofsky, C. 1988. DNAs of the two mating-type alleles of *Neurospora crassa* are highly dissimilar. Science 241:570–573.

Goodwin, S.B, Dunkle, D.L., and Zismann, V.L. 2001. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. Phytopathology 91:648–658.

Goodwin, S.B., Waalwijk, C., Kema, G.H.J., Cavaletto, J.R., and Zhang, G. 2003. Cloning and analysis of the mating-type idiomorphs from the barley pathogen *Septoria passerinii*. Mol. Gen. Genomics 269:1–12.

Groenewald, S., Van den Berg, N., Marasas, W.F., and Viljoen, A. 2006. The application of high-throughput AFLP's in assessing genetic diversity in *Fusarium oxysporum* f. sp. *cubense*. Mycol. Res. 110:297–305.

Gronberg, H., Paulin, L., and Sen, R. 2003. ITS probe development for specific detection of *Rhizoctonia* spp. and *Suillus bovinus* based on Southern blot and liquid hybridization-fragment length polymorphism. Mycol. Res. 107:428–438.

Gruis, N.A., Abeln, E.C.A., Bardoel, A.F.J., Devilee, P., Frants, R.R., and Cornelisse, C.J. 1993. PCR-based microsatellite polymorphisms in the detection of loss of heterozygosity in fresh and archival tumour tissue. Brit. J. Cancer 68:308–313.

Halliday, C.L., Bui, T., Krockenberger, M., Malik, R., Ellis, D.H., and Carter, D.A. 1999. Presence of α and a mating types in environmental and clinical collections of *Cryptococcus neoformans* var. *gattii* strains from Australia. J. Clin. Microbiol. 37:2920–2926.

Hancock, J.M. 1995. The contribution of slippage-like processes to genome evolution. J. Mol. Evol. 41:1038–1047.

Hayden, H.L., Carlier, J., and Aitken, E.A.B. 2003a. Population differentiation in the banana leaf spot pathogen *Mycosphaerella musicola*, examined at a global scale. Plant Pathol. 52:713–719.

Hayden, H.L., Carlier, J., and Aitken, E.A.B. 2003b. Genetic structure of *Mycosphaerella fijiensis* populations from Australia, Papua New Guinea and the Pacific Islands. Plant Pathol. 52:703–712.

Haymer, D.S., McInnis, D.O., and Arcangeli, L. 1992. Genetic variation between strains of the Mediterranean fruit fly, *Ceratitis capitata*, detected by DNA fingerprinting. Genome 35:528–533.

Holtschulte, B. 2000. *Cercospora beticola* - world-wide distribution and incidence. Pages 5–16 in: *Cercospora beticola* Sacc. Biology, agronomic influences and control measures in sugar beet IIRB. M.J.C. Asher, B. Holtschulte, M.M. Richard, F. Rosso, G. Steinrucken and R. Beckers, eds. International Institute for Beet Research, Belgium.

Inglis, P.W., Teixeira, E.A., Ribeiro, D.M., Valadares-Inglis, M.C., Tigano, M.S., and Mello, S.C.M. 2001. Molecular markers for the characterization of Brazilian *Cercospora caricis* isolates. Curr. Microbiol. 42:194–198.

Jackson, C.J., Barton, R.C., and Evans, E.G. 1999. Species identification and strain differentiation of dermatophyte fungi by analysis of ribosomal-DNA intergenic spacer regions. J. Clin. Microbiol. 37:931–936.

Jenns, A.E., Daub, M.E., and Upchurch, R.G. 1989. Regulation of cercosporin accumulation in culture by medium and temperature manipulation. Phytopathology 79:213–219.

Johnson, E.M., and Valleau, W.D. 1949. Synonomy in some common species of *Cercospora*. Phytopathology 39:763–770.

Jones, C.J., Edwards, K.J., Castaglione, S., Winfield, M.O., Sala, F., van de Wiel, C., Bredemeijer, G., Vosman, B., Matthes, M., Daly, A., Brettschneider, R., Bettini, P., Buiatti, M., Maestri, E., Malcevschi, A., Marmiroli, N., Aert, R., Volckaert, G., Rueda, J., Linacero, R., Vazquez, A., and Karp, A. 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Mol. Breeding 3:381–390.

Kaiser, W.J., and Lukezic, F.L. 1965. Brown spot disease of banana caused by *Cercospora hayi*. Phytopathology 55:977–980.

Kauserud, H., and Schumacher, T. 2003. Regional and local population structure of the pioneer wood-decay fungus *Trichaptum abietinum*. Mycologia 95:416–425.

Ko, K.S., Hong, S.K., Lee, K.H., Lee, H.K., Park, M.Y., Miyamoto, H., and Kook, Y.H. 2003. Detection and identification of *Legionella pneumophila* by PCR-restriction fragment length polymorphism analysis of the RNA polymerase gene (*rpoB*). J. Microbiol. Meth. 54:325–337.

Kohli, Y., and Kohn, L.M. 1998. Detection of random association among loci in clonal populations of *Sclerotinia sclerotiorum*. Fungal Genet. Biol. 23:139–149.

Koike, S.T., Gilbertson, R.L., and Davis, R.M. 2004. University of California IPM Pest Management Guidelines: Celery. Online publication UC ANR 3439.

Kretzer, A.M., Molina, R., and Spatafora, J.W. 2000. Microsatellite markers for the ectomycorhizal basidiomycete *Rhizopogon vinicolor.* Mol. Ecol. 9:1171–1193.

Kucharek, T. 2004. University of Florida Institute of Food and Agricultural Sciences. Florida Plant Disease Management Guide: Carrot. 2004 Florida Plant Disease Management Guide. Online publication PDMG-V3-35.

Levy, M., Romao, J., Marchetti, M.A., and Hamer, J.E. 1991. DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in the rice blast fungus. Plant Cell 3:95–102.

Linde, C.C., Zhan, J., and McDonald, B.A. 2002. Population structure of *Mycosphaerella graminicola* from lesions to continents. Phytopathology 92:946–955.

Linde, C.C., Zala, M., Ceccarelli, S., and McDonald, B.A. 2003. Further evidence for sexual reproduction in *Rhynchosporium secalis* based on distribution and frequency of mating-type alleles. Fungal Genet. Biol. 40:115–125.

Litt, M., and Luty, J.A. 1989. A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am. J. Hum. Genet. 44:397–401.

Litvintseva, A.P., Thakur, R., Vilgalys, R., and Mitchell, T.G. 2006. Multilocus sequence typing reveals three genetic subpopulations of *Cryptococcus neoformans* var. *grubii* (serotype A), including a unique population in Botswana. Genetics 172:2223–2238.

Maheswaran, M., Subudhi, P.K., Nandi, S., Xu, J.C., Parco, A., Yang, D.C., and Huang, N. 1997. Polymorphism, distribution, and segregation of AFLP markers in a doubled haploid rice population. Theor. Appl. Genet. 94:39–45.

Maiden, M.C., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M., and Spratt, B.G. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. P. Natl. Acad. Sci. U.S.A. 95:3140–3145.

McDonald, B.A., Miles, J., Nelson, L.R., and Pettway, R.E. 1994. Genetic variability in nuclear DNA in field populations of *Stagonospora nodorum.* Phytopathology 84:250–255.

McDonald, B.A., Zhan, J., and Burdon, J.J. 1999. Genetic structure of *Rhynchosporium secalis* in Australia. Phytopathology 89:639–645.

Metzenberg, R.L., and Glass, N.L. 1990. Mating type and mating strategies in *Neurospora*. BioEssays 12:53–59.

Milgroom, M.G. 1996. Recombination and the multilocus structure of fungal populations. Annu. Rev. Phytopathol. 34:457–477.

Milgroom, M.G. 2001. The synthesis of genetics and epidemiology: contributions of population biology in plant pathology. J. Plant Pathol. 83:57–62.

Milgroom, M.G., Lipari, S.E., and Powel, W.A. 1992. DNA fingerprinting and analysis of population structure in the chestnut blight fungus, *Cryphonectria parasitica.* Genetics 131:297–307.

Mueller, U.G., and Wolfenbarger, L.L. 1999. AFLP genotyping and fingerprinting. Trends Ecol. Evol. 14:389–394.

Mueller, U.G., Lipari, S.E., and Milgroom, M.G. 1996. Amplified fragment length polymorphism (AFLP) fingerprinting of symbiotic fungi cultured by the fungus-growing ant *Cyphomyrmex minutus.* Mol. Ecol. 5:119–122.

Müller, R., Pasberg-Gauhl, C., Gauhl, F., Ramser, J., and Kahl, G. 1997. Oligonucleotide fingerprinting detects genetic variability at different levels in Nigerian *Mycosphaerella fijiensis*. J. Phytopathol. 145:25–30.

Mwenje, E., Wingfield, B.D., Coetzee, M.P.A., and Wingfield, M.J. 2003. Molecular characterisation of *Armillaria* species from Zimbabwe. Mycol. Res. 107:291–296.

Nagaoka, T., and Ogihara, Y. 1997. Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theor. Appl. Genet. 94:597–602.

Nair, S., Bentur, J.S., Prasada Rao, U., and Mohan, M. 1995. DNA markers tightly linked to a gall midge resistance gene (*Gm2*) are potentially useful for marker-aided selection in rice breeding. Theor. Appl. Genet. 91:68–73.

O'Donnell, K., Ward, T.J., Geiser, D.M., Kistler, H.C., and Aoki, T. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genet. Biol. 41:600–623.

Okori, P., Rubaihayo, P.R., Ekwamu, A., Fahleson, J., and Dixelius, C. 2004. Genetic characterization of *Cercospora sorghi* from cultivated and wild sorghum and its relationship to other *Cercospora* fungi. Phytopathology 94:743–750.

Owen, P.G., Pei, M., Karp, A., Royle, D.J., and Edwards, K.J. 1998. Isolation and characterization of microsatellite loci in the wheat pathogen *Mycosphaerella graminicola.* Mol. Ecol. 7:1611–1612.

Penzes, A. 1927. Cercospora Hungarica. Folia crypt. Zeitschr. Ergorsh. Ungar. Kryptfl. 1:288–336.

Pérez, T., Albornoz, J., and Domínguez, A. 1998. An evaluation of RAPD fragment reproducibility and nature. Mol. Ecol. 7:347–358.

Pöggeler, S. 2001. Mating-type genes for classical strain improvements of ascomycestes. Appl. Microbiol. Biotechnol. 56:589–601.

Pollack, F.G. 1987. An annotated compilation of *Cercospora* names. Mycol. Mem. 12:1–212.

Pretorius, M.C., Crous, P.W., Groenewald, J.Z., and Braun, U. 2003. Phylogeny of some cercosporoid fungi from *Citrus*. Sydowia 55:286–305.

Rivas, G.-G., Zapater, M.-F., Abadie, C., and Carlier, F. 2004. Founder effects and stochastic dispersal at the continental scale of the fungal pathogen of bananas *Mycosphaerella fijiensis*. Mol. Ecol. 13:471–482.

Rosehart, K., Richards, M.H., and Bidochka, M.J. 2002. Micorsatellite analysis of environmental and clinical isolates of the opportunist fungal pathogen *Aspergillus fumigatus.* J. Med. Microbiol. 51:1128–1134.

Rossi, V., Battilani, P., Chiusa, G., Giosue, S., Languasco, L., and Racca, P. 2000. Components of rate-reducing resistance to Cercospora leaf spot in sugarbeet: conidiation length, spore yield. J. Plant Pathol. 82:125–131.

Saccardo, P.A. 1876. Fugi Veneti novi vel critici. Series V. Nuovo Giorn. Bot. Ital. 8:162–211.

Salamati, S., Zhan, J., Burdon, J.J., and McDonald, B.A. 2000. The genetic structure of field populations of *Rhynchosporium secalis* from three continents suggests moderate gene flow and regular recombination. Phytopathology 90:901–908.

Shane, W.W., and Teng, P.S. 1992. Impact of Cercospora leaf spot on root weight, sugar yield, and purity of *Beta vulgaris.* Plant Dis. 76:812–820.

Sharon, A., Yamaguchi, K., Christiansen, S., Horwitz, B.A., Yoder, O.C., and Turgeon, B.G. 1996. An asexual fungus has the potential for sexual development. Mol. Gen. Genet. 251:60–68.

Singh, G., and Ashby, A.M. 1998. Cloning of the mating-type loci from *Pyrenopezziza brassicae* reveals the presence of a novel mating type gene within a discomycete *MAT1-2* locus encoding a putative metallothionin-like protein. Mol. Microbiol. 30:799–806.

Singh, G., and Ashby, A.M. 1999. Errratum to Shingh and Ashby 1998. Cloning of the mating-type loci from *Pyrenopezziza brassicae* reveals the presence of a novel mating type gene within a discomycete *MAT1-2* locus encoding a putative metallothionin-like protein. Mol. Microbiol. 32:1115.

Solheim, W.G. 1930. Morphological studies of the genus *Cercospora*. Illinois Biological Monographs 12(1): (dated 1929, published 1930).

Sosinski, B., and Douches, D.S. 1996. Using polymerase chain reaction-based DNA amplification to fingerprint North American potato cultivars. HortScience 31:130–133.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.

Staben, C., and Yanofsky, C. 1990. *Neurospora crassa* a mating-type region. P. Natl. Acad. Sci. U.S.A. 87:4917–4921.

Stewart, E.L., Liu, Z., Crous, P.W., and Szabo, L.J. 1999. Phylogenetic relationships among some cercosporoid anamorphs of *Mycosphaerella* based on rDNA sequence analysis. Mycol. Res. 103:1491–1499.

Taylor, J.W., and Fisher, M.C. 2003. Fungal multilocus sequence typing - it's not just for bacteria. Curr. Opin. Microbiol. 6:351–356.

Tessmann, D.J., Charudattan, R., Kistler, H.C., and Rosskopf, E.N. 2001. A molecular characterization of *Cercospora* species pathogenic to water hyacinth and emendation of *C. piaropi*. Mycologia 93:323–334.

Turgeon, B.G. 1998. Application of mating type gene technology to problems in fungal biology. Annu. Rev. Phytopathol. 36:115–137.

Turgeon, B.G., and Yoder, O.C. 2000. Proposed nomenclature for mating type genes of filamentous ascomycetes. Fungal Genet. Biol. 31:1–5.

Turgeon, B.G., Bohlmann, H., Ciuffetti, L.M., Christiansen, S.K., Yang, G., Schafer, W., and Yoder, O.C. 1993. Cloning and analysis of the mating type genes from *Cochliobolus heterostrophus*. Mol. Gen. Genet. 238:270–284.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407–4414.

Waalwijk, C., Mendes, O., Verstappen, E.C., de Waard, M.A.D., and Kema, G.H.J. 2002. Isolation and characterization of the mating-type idiomorphs from the wheat septoria leaf blotch fungus *Mycosphaerella graminicola*. Fungal Genet. Biol. 35:277–286.

Wang, X.-R., Ennos, R.A., Szmidt, A.E., and Hansson, P. 1997. Genetic variability in the canker pathogen fungus, *Gremmeniella abietina*. 2. Fine-scale investigation of the population genetic structure. Can. J. Bot. 75:1460–1469.

Wang, J., Levy, M., and Dunkle, L.D. 1998. Sibling species of *Cercospora* associated with gray leaf spot of maize. Phytopathology 88:1269–1275.

Ware, S.B. 2006. Aspects of sexual reproduction in *Mycosphaerella* species on wheat and barley: genetic studies on specificity, mapping, and fungicide resistance. PhD thesis, Wageningen University, Netherlands.

Weber, J.L., and May, P.E. 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am. J. Hum. Genet. 44:388–396.

Weiland, J., and Koch, G. 2004. Pathogen profile. Sugarbeet leaf spot disease (*Cercospora beticola* Sacc.). Mol. Plant Pathol. 5:157–166.

Welles, C.G. 1933. Taxonomic studies on the genus *Cercospora* in the Philippine Islands. Am. J. Bot. 12:195–218.

Welsh, J., and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18:7213–7218.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531–6535.

Wu, K., Jones, R., Danneberger, L., and Scolnik, P.A. 1994. Detection of microsatellite polymorphisms without cloning. Nucleic Acids Res. 22:3257–3258.

Yun, S.H., Arie, T., Kaneko, I., Yoder, O.C., and Turgeon, B.G. 2000. Molecular organization of mating type loci in heterothallic, homothallic, and asexual *Gibberella*/*Fusarium* species. Fungal Genet. Biol. 31:7–20.

Zhan, J., and McDonald, B.A. 2004. The interaction among evolutionary forces in the pathogenic fungus *Mycosphaerella graminicola*. Fungal Genet. Biol. 41:590–599.

Zhan, J., Mundt, C.C., and McDonald, B.A. 2001. Using restriction fragment length polymorphisms to assess temporal variation and estimate the number of ascospores that initiate epidemics in field populations of *Mycosphaerella graminicola*. Phytopathology 91:1011–1017.

Zhan, J., Kema, G.H.J., Waalwijk, C., and McDonald, B.A. 2002. Distribution of mating type alleles in the wheat pathogen *Mycosphaerella graminicola* over spatial scales from lesions to continents. Fungal Genet. Biol. 36:128–136.

Zhan, J., Pettway, R.E., and McDonald, B.A. 2003. The global genetic structure of the wheat pathogen *Mycosphaerella graminicola* is characterized by high nuclear diversity, low mitochondrial diversity, regular recombination, and gene flow. Fungal Genet. Biol. 38:286–297.

Zietkiewicz, E., Rafalski, A., and Labuda, D. 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20:176–183.

CHAPTER 2

Distinct species exist within the *Cercospora apii* morphotype

Marizeth Groenewald[1,2], Johannes Z. Groenewald[1], and Pedro W. Crous[1,2]

[1] *Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, Netherlands*

[2] *Laboratory of Phytopathology, Wageningen University, Binnenhaven 5, 6709 PD Wageningen, Netherlands*

Published in: Phytopathology (2005) 95: 951–959.

Key words: celery, sugar beet, multilocus sequence typing, AFLP, systematics

Abstract: The genus *Cercospora* is one of the largest genera of hyphomycetes. *Cercospora apii sensu lato* is the oldest name for a large complex of morphologically indistinguishable *Cercospora* species occurring on a wide host range. There are currently 659 recognized *Cercospora* species, and names of another 281 morphologically identical species are included in the synonymy of *C. apii sensu lato*. Two of the species that belong to the *C. apii* complex, *C. apii* and *C. beticola*, cause Cercospora leaf spot on *Apium graveolens* (celery) and *Beta vulgaris* (sugar beet), respectively. In the present study, multilocus sequence data, amplified fragment length polymorphism analysis, and cultural characteristics were used as additional features to characterize morphologically similar *Cercospora* strains occurring on celery and sugar beet. From the data obtained, it is shown that *C. apii* and *C. beticola*, although morphologically similar and able to cross-infect each others' hosts, are distinct functional species that should be retained as separate entities. Furthermore, a third, as yet undescribed species of *Cercospora* was detected in celery fields in Korea and Venezuela, suggesting that additional undescribed species also may be found to cause Cercospora leaf spot on celery. A polymerase chain reaction-based diagnostic protocol distinguishes all three *Cercospora* species.

INTRODUCTION

In his monograph of the genus *Cercospora* Fresen., Chupp (1954) accepted 1,419 species. In total, more than 3,000 species of *Cercospora* have been described, of which 659 presently are recognized (Crous & Braun, 2003). Generally, species of *Cercospora* are considered to be host specific (Chupp, 1954) at the level of the plant genus or family; this concept has led to the description of a large number of species. Several *Cercospora* species, which are morphologically indistinguishable from *Cercospora apii* Fresen., were placed in the *C. apii* complex (Ellis, 1971). Cross-inoculation studies revealed that isolates in the *C. apii* complex can infect an extremely wide host range, including *Apium graveolens* (celery) and *Beta vulgaris* (sugar beet) (Vestal, 1933; Welles, 1933; Johnston & Valleau, 1949; Berger & Hanson, 1963; Kaiser & Lukezic, 1965; Anderson & Delhey, 1997). In their revision of the genus *Cercospora*, Crous & Braun (2003) referred 281 morphologically indistinguishable species to the *C. apii sensu lato* complex. Recent genetic analyses of *Cercospora* species have relied mainly on DNA sequences of the internal transcribed spacers (ITSs) and the 5.8S ribosomal (r)RNA gene. These studies have revealed that most species of *Cercospora*, in particular the members of the *C. apii* complex, are identical or very closely related (Stewart *et al*., 1999; Goodwin *et al*., 2001; Tessmann *et al*., 2001; Pretorius *et al*., 2003). Judging from their morphological similarity as well as their proven cross-infectiveness, it is probable that the species in the *C. apii* complex should be considered synonymous.

Species seen as representative of *C. apii sensu lato* lack a known teleomorph. Although the genus *Cercospora* is a well-established anamorph of the genus *Mycosphaerella* (Crous *et al*., 2001; Goodwin *et al*., 2001), only a few teleomorphs have been elicited via cultural studies (Crous & Braun, 2003; Crous *et al*., 2004a). Phylogenetic analyses of all *Cercospora* isolates to date have placed them as a well-defined clade in the genus *Mycosphaerella.* Therefore, if a

teleomorph were to be found for *C. apii*, it should be a species of *Mycosphaerella* (Stewart *et al*., 1999; Crous *et al*., 2001; Goodwin *et al*., 2001; Pretorius *et al*., 2003).

Cercospora beticola, causal agent of Cercospora leaf spot on *B. vulgaris*, originally was described by Saccardo (1876), and is assumed to have originated in central Europe and the Mediterranean area. *Cercospora apii*, which causes Cercospora leaf spot on *A. graveolens*, was described from the region between the Netherlands and Germany (Fresenius, 1863), and is assumed to have originated in Western Europe. *Cercospora beticola* is seen as part of the *C. apii* complex (Ellis, 1971; Crous & Braun, 2003). Several studies so far have suggested that *C. beticola* on sugar beet should be treated as a synonym of *C. apii* (Vestal, 1933; Welles, 1933; Johnston & Valleau, 1949; Berger & Hanson, 1963; Ellis, 1971).

Cercospora leaf spot on sugar beet is a serious problem wherever this crop is grown. It is one of the most common and destructive sugar beet diseases, affecting more than a third of all fields worldwide (Shane & Teng, 1992; Holtschulte, 2000). A whole sugar beet field can be destroyed by an outbreak of *C. beticola*, resulting in complete loss of the crop (Duffus & Ruppel, 1993; Rossi *et al*., 2000; Weiland & Koch, 2004).

The similarity in disease symptoms and pathogen morphology seen in celery and sugar beet Cercospora leaf spot diseases led Crous & Braun (2003) to conclude that *C. beticola* should be treated as a synonym of *C. apii sensu lato.* Although Cercospora leaf spot is no longer considered the most destructive disease on celery (Koike *et al*., 2004), in some parts of the world (e.g., Florida), *C. apii* is still seen as a serious pathogen of this crop (Kucharek, 2004).

The main objective of the present study was to confirm or reject the synonymy of *C. apii* and *C. beticola*. It was felt that the same study would provide some indication as to the status of a large number of the purported synonyms of *C. apii*. To address these matters, 38 *Cercospora* isolates were collected from sugar beet and celery; representing a total of 13 countries. Isolates were subjected to multigene sequence analysis and amplified fragment length polymorphism (AFLP) analysis, as well as cultural and morphological comparisons. Here, we show that both celery and sugar beet are hosts to two species of *Cercospora*, with one of these species infecting both hosts. Although *C. apii* and *C. beticola* are able to cross-infect each other's hosts and are morphologically similar to one another, they still appear to operate as functional species on their respective primary namesake hosts in nature.

MATERIALS AND METHODS

Fungal isolates

Single-spore isolations were obtained from symptomatic celery and sugar beet leaves, and cultures were established on 2 % malt extract agar (MEA) (Gams *et al*., 1998) (Table 1). The *Cercospora* isolates were examined morphologically to confirm their identity as *C. apii sensu stricto* as described by Crous & Braun (2003). Some reference isolates were obtained from the Centraalbureau voor Schimmelcultures (CBS) culture collection in Utrecht, Netherlands.

Morphological and cultural characterization

Cercospora reference strains were selected from celery and sugar beet for morphological and cultural characterization (Table 1). Strains were plated onto 2 % MEA and oatmeal agar (OA)

Table 1. *Cercospora* isolates included in the study.

[a] Strains and Accession numbers	Host	Origin	Collector	[b] GenBank number (ITS, EF, ACT, CAL, HIS)
C. achyranthis				
[c] CPC 10091	*Achyranthes japonica*	Korea	H. D. Shin	—, —, —, —, —
C. apii				
CBS 119.25; CPC 5086	*Apium graveolens*	—	L. J. Klotz	AY840512, AY840479, AY840443, AY840410, AY840377
CBS 121.31; CPC 5073	*Beta vulgaris*	Austria	—	AY840513, AY840480, AY840444, AY840411, AY840378
CBS 127.31; CPC 5119	*B. vulgaris*	Hungary	—	AY840514, AY840481, AY840445, AY840412, AY840379
CBS 152.52; CPC 5063	*B. vulgaris*	Netherlands	G. van den Ende	AY840515, AY840482, AY840446, AY840413, AY840380
CBS 536.71; CPC 5087	*A. graveolens*	Romania	O. Constantinescu	AY752133, AY752166, AY752194, AY752225, AY752256
CBS 114416; CPC 10925	*Apium* sp.	Austria	—	AY840516, AY840483, AY840447, AY840414, AY840381
CBS 114418; CPC 10924	*A. graveolens*	Italy	Meutri	AY840517, AY840484, AY840448, AY840415, AY840382
CBS 114485; CPC 10923	*A. graveolens*	Italy	Meutri	AY840518, AY840485, AY840449, AY840416, AY840383
[d] CBS 116455; CPC 11556	*A. graveolens*	Germany	K. Schrameyer	AY840519, AY840486, AY840450, AY840417, AY840384
CBS 116504; CPC 11579	*A. graveolens*	Germany	K. Schrameyer	AY840520, AY840487, AY840451, AY840418, AY840385
CBS 116507; CPC 11582	*A. graveolens*	Germany	K. Schrameyer	AY840521, AY840488, AY840452, AY840419, AY840386
C. beticola				
CBS 116.47; CPC 5074	*B. vulgaris*	Netherlands	G. E. Bunschoten	AY752135, AY752168, AY752196, AY752227, AY752258
CBS 122.31; CPC 5072	*B. vulgaris*	Germany	—	AY752136, AY752169, AY752197, AY752228, AY752259
CBS 123.31; CPC 5071	*B. vulgaris*	Spain	—	AY840522, AY840489, AY840453, AY840420, AY840387
CBS 124.31; CPC 5070	*B. vulgaris*	Romania	—	AY840523, AY840490, AY840454, AY840421, AY840388
CBS 125.31; CPC 5069	*B. vulgaris*	Japan	—	AY840524, AY840491, AY840455, AY840422, AY840389

Table 1. Continued

[a] Strains and Accession numbers	Host	Origin	Collector	[b] GenBank number (ITS, EF, ACT, CAL, HIS)
CBS 126.31; CPC 5064	*B. vulgaris*	Germany	—	AY840525, AY840492, AY840456, AY840423, AY840390
CBS 116454; CPC 11558	*B. vulgaris*	Germany	S. Mittler	AY840526, AY840493, AY840457, AY840424, AY840391
[d] CBS 116456; CPC 11557	*B. vulgaris*	Italy	V. Rossi	AY840527, AY840494, AY840458, AY840425, AY840392
CBS 116501; CPC 11576	*B. vulgaris*	Iran	A. A. Ravanlou	AY840528, AY840495, AY840459, AY840426, AY840393
CBS 116502; CPC 11577	*B. vulgaris*	Germany	S. Mittler	AY840529, AY840496, AY840460, AY840427, AY840394
CBS 116503; CPC 11578	*B. vulgaris*	Italy	—	AY840530, AY840497, AY840461, AY840428, AY840395
CBS 116505; CPC 11580	*B. vulgaris*	France	S. Garressus	AY840531, AY840498, AY840462, AY840429, AY840396
CBS 116506; CPC 11581	*B. vulgaris*	Netherlands	—	AY840532, AY840499, AY840463, AY840430, AY840397
CPC 5125	*B. vulgaris*	New Zealand	C. F. Hill	AY752137, AY752170, AY752198, AY752229, AY752260
CPC 5128	*B. vulgaris*	New Zealand	C. F. Hill	AY752138, AY752171, AY752199, AY752230, AY752261
CPC 10168	*B. vulgaris*	New Zealand	C. F. Hill	AY840533, AY840500, AY840464, AY840431, AY840398
CPC 10171	*B. vulgaris*	New Zealand	C. F. Hill	AY840534, AY840501, AY840465, AY840432, AY840399
CPC 10197	*B. vulgaris*	New Zealand	C. F. Hill	AY840535, AY840502, AY840466, AY840433, AY840400
C. bizzozeriana				
[c] CBS 258.67; CPC 5061	*Cardaria draba*	Romania	O. Constantinescu	—, —, —, —, —
C. canescens				
[c] CPC 1138	*Vigna* sp.	South Africa	S. van Wyk	—, —, —, —, —
C. flagellaris				
[c] CPC 10124	*Phytolacca americana*	Korea	H. D. Shin	—, —, —, —, —

Table 1. Continued.

[a] Strains and Accession numbers	Host	Origin	Collector	[b] GenBank number (ITS, EF, ACT, CAL, HIS)
C. kikuchii				
[c] CBS 135.28; CPC 5067	*Glycine soja*	Japan	H. W. Wollenweber	—, —, —, —, —
C. malvacearum				
[c] CBS 126.26; CPC 5066	*Malva* sp.	—	—	—, —, —, —, —
C. penzigii				
[c] CPC 3950	*Citrus* sp.	South Africa	—	—, —, —, —, —
C. piaropi				
[c] CBS 113127	*Eichhornia crassipes*	USA	R. Charudattan	—, —, —, —, —
C. polygonacea				
[c] CPC 10117	*Persicaria* sp.	Korea	H. D. Shin	—, —, —, —, —
C. rautensis				
[c] CBS 555.71; CPC 5082	*Coronilla varia*	Romania	O. Constantinescu	—, —, —, —, —
C. ricinella				
[c] CPC 10104	*Ricinus communis*	Korea	H.D. Shin	—, —, —, —, —
C. rodmanii				
[c] CBS 113130	*Eichhornia crassipes*	USA	R. Charudattan	—, —, —, —, —
Cercospora sp.				
[d] CBS 116457; CPC 10267	*Apium* sp.	Venezuela	N. Pons	AY840536, AY840503, AY840467, AY840434, AY840401
CBS 116458; CPC 10657	*Apium* sp.	Korea	H. D. Shin	AY840537, AY840504, AY840468, AY840435, AY840402
CPC 10220	*Apium* sp.	Venezuela	N. Pons	AY840538, AY840505, AY840469, AY840436, AY840403

Table 1. Continued.

[a] Strains and Accession numbers	Host	Origin	Collector	[b] GenBank number (ITS, EF, ACT, CAL, HIS)
CPC 10248	*Apium* sp.	Venezuela	N. Pons	AY840539, AY840506, AY840470, AY840437, AY840404
CPC 10265	*Apium* sp.	Venezuela	N. Pons	AY840540, AY840507, AY840471, AY840438, AY840405
CPC 10266	*Apium* sp.	Venezuela	N. Pons	AY840541, AY840508, AY840472, AY840439, AY840406
CPC 10279	*Apium* sp.	Venezuela	N. Pons	AY840542, AY840509, AY840473, AY840440, AY840407
CPC 10666	*Apium* sp.	Korea	H. D. Shin	AY840543, AY840510, AY840474, AY840441, AY840408
CPC 10759	*A. graveolens*	Korea	H. D. Shin	AY840544, AY840511, AY840475, AY840442, AY840409
C. violae				
[c] CPC 10725	*Viola mondshivica*	Korea	H. D. Shin	—, —, —, —, —

[a] Origin of strain numbers: CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands and CPC = Collection of Pedro Crous, Netherlands.
[b] ITS = internal transcribed spacer, EF = elongation factor 1-α, ACT = actin, CAL = calmodulin, HIS = histone H3.
[c] Additional *Cercospora* species tested with the species-specific primers.
[d] *Cercospora apii, C. beticola* and *Cercospora* sp. isolates used for colony characteristics as well as growth rate measurements.

(Gams *et al*., 1998) and incubated at 24 °C in the dark for 8 days. Colony characteristics were determined and colors rated on the different growth media using a color chart (Rayner, 1970). Cardinal growth temperatures were determined on MEA (Crous *et al*., 1996). These plates were incubated in the dark for 8 days at temperatures beginning at 6 °C and progressing to 36 °C in 3 °C intervals; in addition, growth at 40 °C was studied. Several isolates taken from each of the three different groups were used (Table 1). The experiments featured three simultaneous replicates for each isolate; the whole trial was repeated once.

DNA extraction and sequencing

DNA analysis was done on all isolates listed in Table 1. The FastDNA kit (BIO 101, Carlsbad, California) was used according to the manufacturer's instructions to isolate genomic (g)DNA of 200–400 mg of fungal mycelia grown on MEA plates for 8 days at 24 °C. A sterile blade was used to scrape the mycelia from the surface of the plate. The primers ITS1 and ITS4 (White *et al*., 1990) were used to amplify the ITS areas as well as the 5.8S rRNA gene (ITS). Part of the actin gene (ACT) was amplified using the ACT512F and ACT783R primers (Carbone & Kohn, 1999) and part of the translation elongation factor 1-α gene (EF) using the primers EF728F and EF986R (Carbone & Kohn, 1999). The CAL228F and CAL737R primers (Carbone & Kohn, 1999) were used to amplify part of the calmodulin gene (CAL), and the primers CylH3F and CylH3R (Crous *et al*., 2004b) to amplify part of the histone H3 gene (HIS). The polymerase chain reaction (PCR) conditions were the same for all regions, except for the $MgCl_2$ concentration, which was 2 mM for the CAL region and 1.5 mM for the remaining areas. The reaction mixture had a total volume of 12.5 µl and contained 1 µl of diluted gDNA, 1× PCR buffer, 48 µM of each of the dNTPs, 2.5 pmol of each primer, and 0.7 units *Taq* polymerase (Bioline GmbH, Luckenwalde, Germany). The amplification reactions were done on a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, Connecticut). The initial denaturation step was done at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C (30 s), annealing 52 °C (30 s), and elongation at 72 °C (30 s). A final elongation step at 72 °C (7 min) was included in the run. The PCR products were separated by electrophoresis at 80 V for 40 min on a 0.8 % (w/v) agarose gel containing 0.1 µg/ml ethidium bromide in 1× TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualized under UV light.

The amplicons were sequenced in both directions using the PCR primers and a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Roosendaal, Netherlands) according to the manufacturer's recommendations. The products were analyzed on an ABI Prism 3700 DNA Sequencer (Perkin-Elmer, Foster City, California). A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package (DNASTAR, Madison, Wisconsin).

Phylogenetic analysis

The sequences were assembled and added to the outgroups using Sequence Alignment Editor v2.0a11 (Department of Zoology, University of Oxford, Oxford, UK), and manual adjustments for improvement were made by eye where necessary. The phylogenetic analyses of sequence data were done in Phylogenetic Analysis Using Parsimony (PAUP) v4.0b10 (Sinauer Associates, Sunderland, Massachusetts) and consisted of neighbor-joining analysis with the uncorrected

("p"), Jukes-Cantor and Kimura 2-parameter substitution models. Alignment gaps were treated as missing data and all characters were unordered and of equal weight. Any ties were broken randomly when encountered. For parsimony analysis, alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option with 100 random taxa additions and tree bisection and reconstruction as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1,000 bootstrap replications (Hillis & Bull, 1993). Other measures calculated included tree length, consistency index, retention index, and rescaled consistency index (TL, CI, RI and RC, respectively). The resulting trees were printed with TreeView v1.6.6 (Page, 1996). A partition homogeneity test was done in PAUP to test whether the different loci can be used in a combined analysis (Farris *et al*., 1994). Sequences were deposited in GenBank (accession numbers listed in Table 1) and the alignments in TreeBASE (accession no. M2242).

AFLP analysis

Restriction enzyme digestion and adaptor ligation were done using 30 ng of gDNA, 1× T4 DNA ligase buffer, 50 mM NaCl, 2 units of *Mse*I, 2 units of *EcoR*I, 40 units of T4 DNA ligase, 10 µg of bovine serum albumin, 50 pmol of *Mse*I adaptor, 5 pmol of *Eco*RI adaptor made up to a final volume of 11 µl (adapted from Vos *et al.,* 1995). All enzymes were obtained from New England BioLabs (Beverly, Massachusetts). This reaction was carried out at 37 °C for 12 h. A 1:1 dilution was made with dH_2O and 4 µl was used in the preselective PCR. The preselective PCR was performed in a 20 µl volume containing 25 pmol of primer *Eco*RI-0 (Vos *et al*., 1995), 25 pmol of primer *Mse*I-0 (Vos *et al*., 1995), 1.5 mM $MgCl_2$, 1× Bioline *Taq* reaction buffer, 0.1 mM each dNTP, and 0.75 units of Bioline *Taq* polymerase. An initial 72 °C step was done for 2 min, followed by 20 cycles of denaturation at 94 °C (20 s), annealing at 56 °C (40 s), and elongation at 72 °C (1 min). The preselective amplification was confirmed by electrophoresis on a 0.8 % (w/v) agarose gel as described above. The preamplified DNA was diluted 1:1 with dH_2O and used as template for selective amplification. Primers used in the selective amplification were *Eco*RI-A [FAM] / *Mse*I-CT, *Eco*RI-AT [JOE] / *Mse*I-C, and *Eco*RI-AG [NED] / *Mse*I-C (Applied Biosystems, Nieuwerkerk aan de IJssel, Netherlands). The reactions contained 1.5 mM $MgCl_2$, 0.5 units of Bioline *Taq* polymerase, 1× Bioline *Taq* polymerase buffer, 0.1 mM each dNTP, 0.5 µl of *Eco*RI primer, and 0.5 µl of *Mse*I primer made up to a final volume of 10 µl. Selective PCR products (2 µl), amplified with the different primer combinations for each of the isolates, were mixed together with 0.5 µl of GeneScan 500 (labeled with 6-carboxy-X-rhodamine) (Applied Biosystems) and made up to a final volume of 25 µl with formamide. The products were denatured at 100 °C for 5 min, followed by 30 min runs on an ABI 310 genetic analyzer. The AFLP data were analyzed using Bionumerics software v2.5 (Applied Maths, Kortrijk, Belgium).

Development of a species-specific diagnostic test

The CAL gene was found to be very effective for separating the three species described in the present study; therefore, this area was targeted for the development of a species-specific

diagnostic test. Primers CercoCal-F and CercoCal-R (Table 2) were designed from regions of the CAL gene that are conserved for the *Cercospora* species in our database. They act as outer primers and their amplification functions as a positive control. Three internal primers (CercoCal-beta, CercoCal-apii and CercoCal-sp), each specific for one of the three *Cercospora* species described in this study, were designed. The species-specific primers were used in separate PCR's together with the outer control primers. Strains of *C. beticola*, *C. apii*, the undescribed *Cercospora* sp., and 13 other species of *Cercospora* (Table 1) were screened with these primers. The sequences and specific nucleotide binding sites of the primers are listed in Table 2. The same PCR conditions were used for the detection of all three species. The reaction mixture had a total volume of 12.5 µl and contained 1 µl of diluted gDNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 48 µM each of the dNTPs, 1 pmol of CercoCal-F, 3 pmol of each of CercoCal-R and the specific internal primer, and 0.7 units *Taq* polymerase (Bioline). The amplification reactions were done on a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, Connecticut). The initial denaturation step was done at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C (30 s), annealing at 58 °C (30 s), and elongation at 72 °C (30 s). A final elongation step at 72 °C (7 min) was included to ensure that full-length products were obtained. The PCR products were separated on a 1.5 % agarose gel and visualized as described above.

Table 2. Primers designed from calmodulin sequences for the species identification amplifications.

Primer	Sequence (5' – 3')	[a] Nucleotide position	Description
CercoCal-F	CGCGAGGCAGAGCTAACGA	61 - 79	Positive control forward primer
CercoCal-beta	GCCCACCCTCTGCGAATGTA	117 - 137	*C. beticola*-specific primer
CercoCal-apii	GACCACCCTCTGCAACTGCG	117 - 137	*C. apii*-specific primer
CercoCal-sp	GCCCACTTTCTGTGACTGCA	117 - 137	*Cercospora* sp.-specific primer
CercoCal-R	GTGAGGAATTCGGGGAAATC	275 - 294	Reverse primer

[a] The calmodulin sequence of *Cercospora apii* strain CBS 116455 (GenBank accession no. AY840417) was used to derive the nucleotide positions of the primers.

RESULTS

Morphological and cultural characterization

The morphological characteristics of the conidia and conidiophores for all isolates obtained from celery and sugar beet (Table 1) were the same as described for *C. apii sensu lato* by Crous & Brown (2003). Isolates from celery obtained from Venezuela and Korea were distinct, however, in that conidiophores were relatively short, 25–70 × 4–6 µm, and conidia were obclavate-cylindrical, not acicular. They measured (50–)80–120(–150) × (3–)4–5 µm and were 1–6-septate.

To facilitate the standardization of further genotypic studies on the *C. apii* complex, we herewith designate new epitype (a specimen selected to serve as an interpretative type in support of other type material, to facilitate the precise application of the published name) materials with cultures for *C. apii* and *C. beticola*. For *C. apii*, the original herbarium material used for the type ("holotype") has been lost, but some of the original material might have been distributed

and a lectotype, therefore, can be designated from these duplicates. Isolectotypes are duplicate specimens of the same lectotype. All of the material originally associated with the publication of the name *C. beticola* has been lost, therefore, a specimen has to be designated to serve as if it were the holotype of the species ("neotype"). Isoneotypes are duplicate specimens of the neotype and ex-epitype cultures (to facilitate molecular studies) are derived from the epitype material.

Cercospora apii Fresen., Beitr. Mykol. 3:91. 1863.
Lectotype (proposed here): on *Apium graveolens*, Germany, Oestrich, garden, Fuckel, Fungi rhen. 117, in HAL. Fresenius (1863) cited material of *C. apii* obtained from Fuckel. This is an indirect reference to the material distributed by Fuckel as Fungi rhen. 117. Original material in the herbarium of Fresenius could not be traced, and probably is not preserved; therefore, we prefer to select one of the duplicates distributed by Fuckel to serve as lectotype. Isolectotypes: Fuckel, Fungi rhen. 117. Epitype (proposed here): on *Apium graveolens*, Germany, Landwirtschaftsamt Heilbronn, 10.08.2004, K. Schrameyer, culture ex-epitype CBS 116455.

Cercospora beticola Sacc., Nuovo Giorn. Bot. Ital. 8:189. 1876.
Neotype (proposed here): on *Beta vulgaris*, Italy, Vittorio (Treviso), Sept. 1897, Sacc., Fungi ital. 197 (PAD). Isoneotypes: Sacc., Fungi ital. 197. Epitype (proposed here): on *Beta vulgaris*, Italy, Ravenna, 10.7.2003, Rossi V., culture ex-epitype CBS 116456.

Colonies of *C. beticola* and *C. apii* are smooth, erumpent, and regular, with smooth, even margins, and sparse to moderate aerial mycelium. *Cercospora beticola* colonies on MEA are greenish gray on the surface and dark mouse-gray beneath. On OA, colonies are white to green-olivaceous. *Cercospora apii* colonies on MEA are pale greenish-gray on the surface and dark mouse-gray beneath. The surfaces of the colonies are white to green-olivaceous on OA. Morphologically divergent isolates from Venezuela and Korea are smooth to folded, erumpent with smooth, even to uneven margins, and sparse to moderate aerial mycelium. On MEA, colonies are white to smoke-gray on the surface, and olivaceous-gray to iron-gray beneath. On OA, colonies are white to olivaceous-gray on the surface.

The temperature ranges and colony diameters of three reference isolates (CBS 116455, CBS 116456 and CBS 116457), representing each of the three different species, are given in Fig. 1. The Venezuela and Korea isolates can grow at lower temperatures (6 °C) than *C. beticola* and *C. apii* (12 °C), whereas *C. beticola* and *C. apii* have a higher maximum temperature tolerance (33 °C) than the *Cercospora* sp. (30 °C). The optimal temperature for growth of the *Cercospora* sp. was observed to be 24 °C, whereas the optimal growth temperature for *C. apii* and *C. beticola* is 27 °C. The *Cercospora* sp. grows much slower than the other two species, growing only 1.72 mm/day at its optimum temperature, whereas *C. beticola* and *C. apii* grew 3.5 and 2.7 mm/day at their respective optimal temperatures. Differences in growth rate between *C. apii* and *C. beticola* were observed for most of the temperatures tested. *Cercospora beticola* grew faster than *C. apii* (Fig. 1). *Cercospora beticola* was more tolerant to temperatures higher than 30 °C (1.46 mm/day vs. 0.26 mm/day at 33 °C).

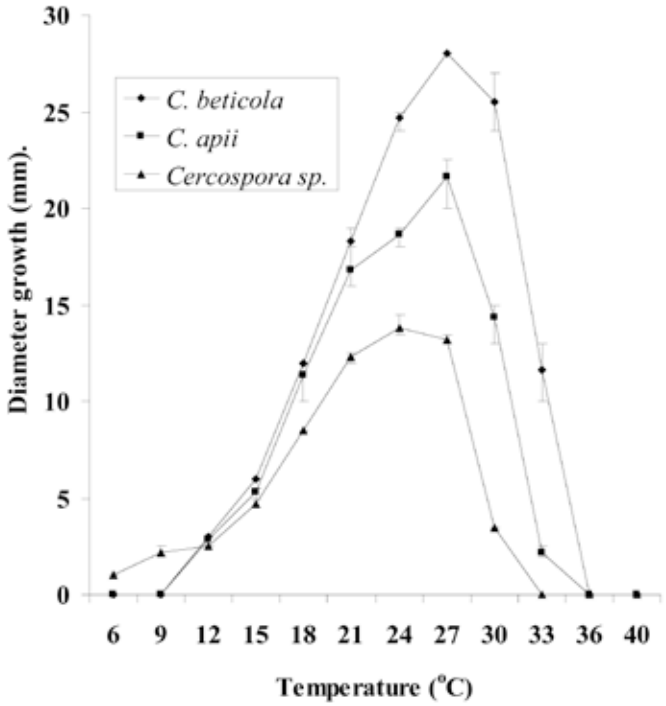


Fig. 1. Colony diameters at different temperatures ranging from 6 °C to 40 °C for 8 days on 2 % MEA were calculated for *Cercospora apii* (CBS 116455), *C. beticola* (CBS 116456) and *Cercospora* sp. from Venezuela (CBS 116457).

Phylogenetic analysis

A partition homogeneity test showed that the five data sets were combinable (P = 0.834); therefore, the sequence data were analyzed as one combined set. The combined alignment of ITS, ACT, EF, CAL and HIS contained 41 strains including the three outgroups, and had a total length of 1,611 characters, of which 1,183 were constant, three were parsimony-uninformative, and 425 were parsimony-informative. The topology of the neighbor-joining trees obtained using the different substitution models was the same. A similar topology was found for the most parsimonious trees. Parsimony analysis of the combined data resulted in 12 parsimonious trees, one of which is shown in Fig. 2 (TL = 465 steps, CI = 0.989, RI = 0.997, RC = 0.986). From the phylogenetic analysis (Fig. 2), three distinct and well-supported clades were obtained. The first clade contained isolates of the new *Cercospora* sp. from *Apium* spp. (100 % bootstrap support), the second clade contained only *Cercospora* isolates from *B. vulgaris* (91 % bootstrap support) and the third clade contained *Cercospora* isolates from both *B. vulgaris* and *Apium* spp. (100 % bootstrap support). All the isolates from the third clade were isolated in Europe. The ITS and ACT data sets showed no variation among the isolates from the second and the third clade and no significant variation could be observed between the isolates of these two clades with the EF and HIST data sets. The amount of variation observed within the CAL region of the *C. beticola* and *C. apii* isolates (96 % similarity) was significant and placed these species into two distinct phylogenetic clades, each with a high bootstrap support in the combined analysis.

AFLP analysis

Genetic differences between isolates of the different clades also were confirmed using AFLP analysis. Banding patterns obtained with the *Eco*RI-A [FAM] / *Mse*I-CT and *Eco*RI-AT [JOE] /

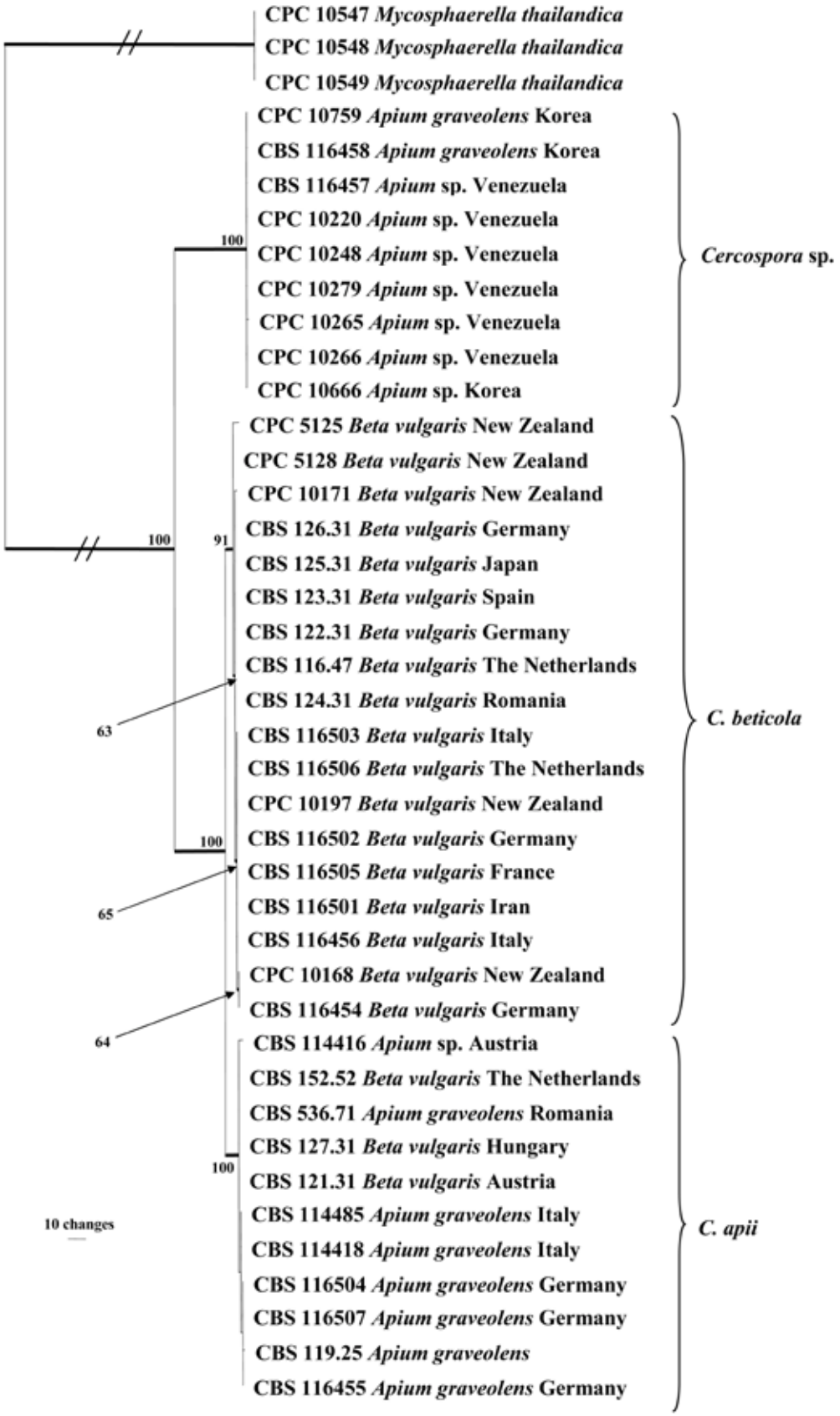


Fig. 2. One of the 12 most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the combined ITS, EF, ACT, CAL and HIS sequences alignment. The scale bar shows 10 changes and bootstrap support values from 1,000 replicates are shown in percentages at the nodes. Thickened lines indicate the strict consensus branches. The tree was rooted with three *Mycosphaerella thailandica* isolates.

*Mse*I-C primer combinations are shown in Fig. 3. The number and sizes of the polymorphic bands obtained for isolates of the *Cercospora* sp., using the *Eco*RI-A [FAM] / *Mse*I-CT primer combination, show major differences with the profiles obtained for the other two species (Fig. 3A). Although isolates from the *C. apii* and *C. beticola* clades are more similar to each other than to the *Cercospora* sp., several bands are specific to each of the species, as seen using the *Eco*RI-A [FAM] / *Mse*I-CT and *Eco*RI-AT [JOE] / *Mse*I-C primer combinations (Fig. 3). The primer combination *Eco*RI-AG [NED] / *Mse*I-C also was tested on isolates from the three *Cercospora* species and the banding patterns obtained show results similar to those obtained with the other two primer combinations (data not shown).

Species identification

Easy and rapid identification of *C. beticola*, *C. apii* and the new *Cercospora* sp. was possible using three multiplex PCR amplifications, each specific for one of the species. A 234-bp fragment, which serves as the positive control, was present for all three species, whereas a 176-bp fragment was only observed for the *Cercospora* species elucidated by the specific internal primer (Fig. 4). Only the 234-bp fragment was present for all other *Cercospora* species tested in our database representing 13 *Cercospora* species (data not shown). Therefore, primers CercoCal-beta, CercoCal-apii, and CercoCal-sp are therefore specific for *C. beticola, C. apii* and the *Cercospora* sp., respectively, and can be used for their identification and detection.

DISCUSSION

Although morphological characteristics are frequently used to identify newly isolated fungi, it is not possible to distinguish *C. apii* (celery) from *C. beticola* (sugar beet) based solely on morphology. At the onset of this study, these species were considered to be synonymous as part of the *C. apii sensu lato* complex. Our data, however, refute the hypothesis that all morphologically indistinguishable *Cercospora* forms represent one species (Ellis, 1971; Crous & Braun, 2003). *Cercospora apii sensu stricto*, which typifies the *C. apii sensu lato* complex, including *C. beticola*, which is a morphologically similar fungus originally described from sugar beet, are shown to differ genetically and with some cultural characteristics from one another to an extent confirming species-level separation. It is now possible to identify the studied species using these characteristics.

Among the sequence types studied, only CAL strongly supports the split of *C. apii* and *C. beticola* into two distinct phylogenetic groups. This grouping, however, is confirmed in the growth studies as well as in AFLP analysis. This study shows that the choice and number of loci sequenced can be crucial in elucidating phylogenetic relationships of very closely related species and that using the wrong or an insufficient number of sequence loci could result in erroneous synonymies being proposed. It also shows that phenotypic characteristics, such as growth rates and temperature thresholds, can be very important parameters in the identification of species that are morphologically identical.

From the phylogenetic data obtained, it is clear that *C. apii* occurs mainly on celery, whereas *C. beticola* occurs on sugar beet, and that cross-infection of each other's hosts is rare. We did, however, study three isolates, revealed molecularly as *C. apii sensu stricto*, that were

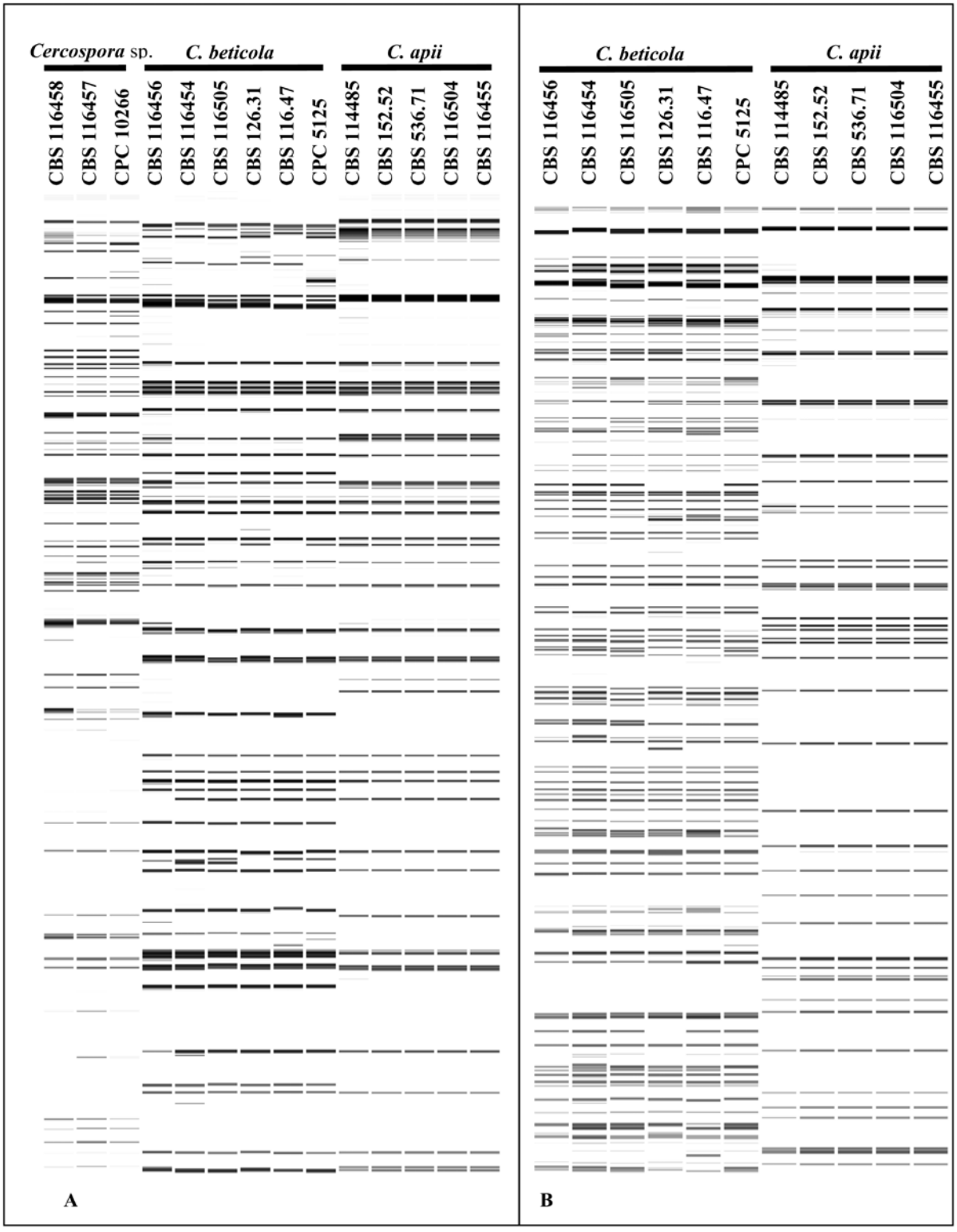


Fig. 3. Visualization of the AFLP band patterns were done using Bionumerics software. **A,** AFLP fingerprints of different isolates of the *Cercospora* sp., *C. beticola*, and *C. apii* using primer combination *Eco*RI-A [FAM] / *Mse*I-CT. **B,** AFLP fingerprints of *C. beticola* and *C. apii* isolates using primer combination *Eco*RI-AT [JOE] / *Mse*I-C.

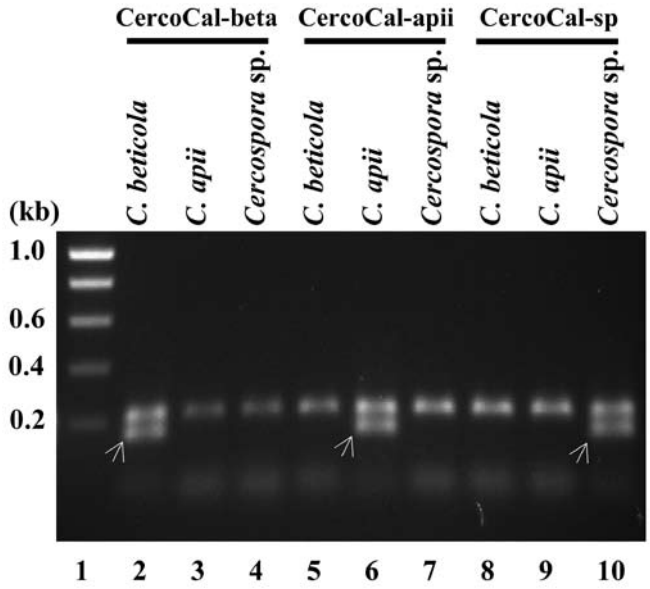


Fig. 4. Identification of *Cercospora beticola*, *C. apii* and the new *Cercospora* sp. using the different species-specific (CercoCal) primers. Lane 1 contains the DNA marker. The 234-bp fragment, the positive control, is present for all the PCR amplifications done (lane 2 to 10). The species-specific fragment (176-bp, indicated with an arrow) can be observed only when the amplification reaction contains *C. beticola* (CBS 116456) DNA with primer CercoCal-beta (lane 2), *C. apii* (CBS 116455) with primer CercoCal-apii (lane 6) or *Cercospora* sp. (CBS 116457) with primer CercoCal-sp (lane 10).

obtained from sugar beet in Europe (CBS 121.31 and CBS 127.31, deposited in 1931, and CBS 152.52 in 1952). The origin of *C. apii* is suspected to be Western Europe, and certainly the species was first described from celery collected in Germany. Because all of the *C. apii* isolates available in this study were from European countries, we do not know whether *C. apii* has been introduced on *Apium* spp. in non-European countries. It has been reported that *C. apii sensu lato* isolates can infect hosts other than the ones they were isolated from (Vestal, 1933; Welles, 1933; Johnston & Valleau, 1949; Crous & Braun, 2003). Therefore, it is quite possible that *C. apii*, which grows much more slowly than *C. beticola* at high temperatures (Fig. 1), originally was able to infect sugar beet and compete with *C. beticola* in the early 1900s, when Europe was considerably colder than is currently the case (Luterbacher *et al*., 2004). Without doubt, *Cercospora beticola* has been introduced from Europe to many other parts of the world, and this species now can be found on almost every continent (Crous & Braun, 2003; current study). The absence of *C. apii* on fresh diseased leaf material of *B. vulgaris* obtained for the purposes of this study can be ascribed to the unique growth properties of *C. beticola.* It is very probable that the faster growth rate and its ability to easily grow at higher temperatures allow *C. beticola* to outcompete *C. apii* for infection sites on *B. vulgaris*. It is clear that environmental factors, such as temperature and availability of specific plant species, play an important role in the survival and infection ability of the fungus. Thus, it seems that genotype-environment interactions (Kang & Gauch, 1996) may play a role in the fitness of species in the *C. apii* complex.

We illustrated an easy PCR-based method which can be used in laboratories that use basic PCR techniques as a diagnostic tool. Although three PCRs are necessary to distinguish between the three *Cercospora* species affecting celery and sugar beet, it is possible to limit the number of reactions according to the crop from which the pathogen was isolated. Thus far, the

new *Cercospora* sp. has never been isolated from *B. vulgaris*; however, because both *C. beticola* and *C. apii* have been isolated from sugar beet, it is important to test isolates from that source as possible representatives of both these species. None of the *C. beticola* isolates confirmed as such with molecular data have been isolated from *Apium* spp.; therefore, it remains possible that *C. beticola* might not infect celery under field conditions.

Because of the major loss in sugar beet production due to Cercospora leaf spot, naturally derived fungicides and synthetic fungicides with broad chemistries are currently being used to control *Cercospora* species infections in this crop (Ioannidis & Karaoglanidis, 2000). Several studies have indicated that *C. beticola* has become resistant to fungicides in the benzimidazole class (Georgopoulos & Dovas, 1973; Ruppel & Scott, 1974; Weiland & Halloin, 2001) and has developed increased tolerance to fungicides in the organotin and triazole classes (Cerato & Grassi, 1983; Bugbee, 1995; Karaoglanidis *et al*., 2000; Weiland & Koch, 2004). In order to reduce fungicide tolerance of *Cercospora* species and to control the severity of Cercospora leaf spot disease of sugar beet, the frequent rotation of fungicide chemistries as well as the development of crops resistant to *Cercospora* infections have been implemented (Ioannidis & Karaoglanidis, 2000; Weiland & Koch, 2004). Although *C. beticola* seems to be the main agent of Cercospora leaf spot on sugar beet, this study shows that *C. apii* can also be isolated from Cercospora leaf spot lesions on sugar beet. Fungicide trials must be done on these two species to determine their respective resistance levels against different fungicides. If there is a significant difference in their resistance levels, it might provide an explanation for the buildup of fungicide resistance of Cercospora leaf spot in sugar beet. This also can have major implications for the use of fungicides in other crops to which *Cercospora* species are pathogenic.

The relationships of all the other species that have been ascribed to the *C. apii* complex need to be studied in detail. Knowledge of whether species names previously synonymized with *C. apii* are correctly considered superfluous will enable us to better understand the diversity and host specificity of species in this complex, and will enable us to delineate the functional species units that operate in nature. The three species described in this study can be separated from one another not only on the genetic level but also by the ecological niche of each of the species. The genotypic differences observed for the three *Cercospora* species can be linked most of the time to the ecological differences between them; for example, cardinal temperature ranges and host identity.

From our data, it is clear that Chupp (1954) was not totally incorrect when he proposed that *Cercospora* species were restricted to specific host genera or families. If this concept could be used for all the *Cercospora* species-host combinations, it would be easy to identify *Cercospora* species based on their hosts. Unfortunately, the present study confirms that this concept is not applicable to the genus as whole. For instance, the *Cercospora* sp. present on typical Cercospora leaf spot symptoms of celery in Venezuela and Korea is a distinct species that matches with none of the 200 *Cercospora* sequences in our database. This species grows much more slowly than *C. apii*, and is unable to grow at 33 °C or above, but can grow at much lower temperatures than *C. apii*; for example, at 6 to 10 °C. Based on phylogenetic and AFLP analyses, this species is different from *C. apii* as well as *C. beticola*. A population representing more than 50 celery plants was collected of this species in Venezuela, indicating

that it obviously is well established on this host. The fact that this species also occurs on celery in Korea suggests that, rather than representing a pathogen that normally grows on another host but occasionally occurs on celery by chance alone, it is instead an established pathogen of celery. It probably has been overlooked in the past due to its morphological similarity to *C. apii* and similar host symptomatology. This discovery of such a widespread cryptic species on a well-studied host like celery, however, does stimulate one to question whether similar cryptic species could exist within additional "common" pathogens that we currently accept as having wide host ranges. The present study illustrates how important it is to the plant pathology community to lodge reference strains of the pathogens they are working with in long-term storage in publicly accessible collections. Had it not been for the plant pathologists who lodged their *C. apii* strains in the early 1900s, it would not have been possible to prove the presence of different *Cercospora* species on celery, or the natural occurrence of *C. apii* on sugar beet. This riddle, in spite of the advanced techniques employed here, remains unresolved to this day.

ACKNOWLEDGEMENTS

This research was financially supported by the CBS-Odo van Vloten Stichting, and the Royal Netherlands Academy of Arts and Sciences. We would like to thank D. Shin and N. Pons for supplying us with the infected *Apium* leaf materials from Korea and Venezuela, respectively, which were crucial for the identification of a new *Cercospora* species on celery; U. Braun for his assistance regarding the tracing of type materials of *C. apii* and *C. beticola*; and R.C. Summerbell for his comments on an earlier version of the manuscript.

LITERATURE CITED

Anderson, F., and Delhey, R. 1997. Mancha foliar de *Modiolastrum australe* (Malvaceae), causada por *Cercospora gossypina* (Hyphomycetes). Bol. Soc. Argent. Bot. 33:47–52.

Berger, R.D., and Hanson, E.W. 1963. Pathogenicity, host-parasite relationship, and morphology of some forage legume Cercosporae, and factors related to disease development. Phytopathology 53:500–508.

Bugbee, W.M. 1995. *Cercospora beticola* tolerant to triphenyltin hydroxide. J. Sugar Beet Res. 30:167–174.

Carbone, I., and Kohn, L.M. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91:553–556.

Cerato, C., and Grassi, G. 1983. Tolerance of organo-tin compounds among *Cercospora beticola* isolates. Informat. Fitopatol. 33:67–69.

Chupp, C. 1954. A Monograph of the fungus genus *Cercospora*. Published by the author. Ithaca, NY.

Crous, P.W., and Braun, U. 2003. *Mycosphaerella* and its anamorphs: 1. Names published in *Cercospora* and *Passalora.* CBS Biodivers. Series 1:1–571.

Crous, P.W., Gams, W., Wingfield, M.J., and van Wyk, P.S. 1996. *Phaeoacremonium* gen. nov. associated with wilt and decline diseases of woody hosts and human infections. Mycologia 88:786–796.

Crous, P.W., Kang, J.-C., and Braun, U. 2001. A phylogenetic redefinition of anamorph genera in *Mycosphaerella* based on ITS rDNA sequences and morphology. Mycologia 93:1081–1101.

Crous, P.W., Groenewald, J.Z., Pongpanich, K., Himaman, W., Arzanlou, M., and Wingfield, M.J. 2004a. Cryptic speciation and host specificity among *Mycosphaerella* spp. occurring on Australian *Acacia* species grown as exotics in the tropics. Stud. Mycol. 50:457–469.

Crous, P.W., Groenewald, J.Z., Risede, J.-M., and Hywel-Jones, N.L. 2004b. *Calonectria* species and their *Cylindrocladium* anamorphs: species with sphaeropedunculate vesicles. Stud. Mycol. 50:415–430.

Duffus, J.E., and Ruppel, E.G. 1993. Diseases. Pages 346–427 in: The sugar beet crop. D. A. Cooke and R. K. Scott, eds. Chapman and Hall, London, UK.

Ellis, M.B. 1971. Dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, UK.

Farris, J.S., Källersjö, M., Kluge, A.G., and Bult, C. 1994. Testing significance of incongruence. Cladistics 10:315–320.

Fresenius, G. 1863. Beiträge zur Mykologie 3. Heinrich Ludwig Brömmer Verlag, Frankfurt, Germany.

Gams, W., Hoekstra, E.S., and Aptroot, A. 1998. CBS Course of Mycology, 4th ed. Centraalbureau voor Schimmelcultures, Baarn, Delft, Netherlands.

Georgopoulos, S.G., and Dovas, C. 1973. Occurrence of *Cercospora beticola* strains resistant to benzimidazole fungicides in northern Greece. Plant Dis. Rep. 62:321–324.

Goodwin, S.B., Dunkley, L.D., and Zismann, V.L. 2001. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. Phytopathology 91:648–658.

Hillis, D.M., and Bull, J.J. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst. Biol. 42:182–192.

Holtschulte, B. 2000. *Cercospora beticola* - world-wide distribution and incidence. Pages 5–16 in: *Cercospora beticola* Sacc. Biology, agronomic influences and control measures in sugar beet IIRB. M.J.C. Asher, B. Holtschulte, M.M. Richard, F. Rosso, G. Steinrucken and R. Beckers, eds. International Institute for Beet Research, Belgium.

Ioannidis, P.M., and Karaoglanidis, G.S. 2000. Resistance of *Cercospora beticola* Sacc. to fungicides. Adv. Sugar Beet Res. IIRB 2:123–147.

Johnston, E.M., and Valleau, W.D. 1949. Synonymy in some common species of *Cercospora*. Phytopathology 39:763–770.

Kaiser, W.J., and Lukezic, F.L. 1965. Brown spot disease of banana caused by *Cercospora hayi*. Phytopathology 55:977–980.

Kang, M.S., and Gauch, H.G. 1996. Genotype-by-environment interaction. CRC Press, Boca Raton, FL.

Karaoglanidis, G.S., Ioannidis, P.M., and Thanassoulopoulos, C.C. 2000. Reduced sensitivity of *Cercospora beticola* to sterol-demethylation-inhibiting fungicides. Plant Pathol. 49:567–572.

Koike, S.T., Gilbertson, R.L., and Davis, R.M. 2004. University of California IPM Pest Management Guidelines: Celery. Online publication UC ANR 3439.

Kucharek, T. 2004. University of Florida Institute of Food and Agricultural Sciences. Florida Plant Disease Management Guide: Carrot. 2004 Florida Plant Disease Management Guide. Online publication PDMG-V3-35.

Luterbacher, J., Dietrich, D., Xoplaki, E., Grosjean, M., and Wanner, H. 2004. European seasonal and annual temperature variability, trends, and extremes since 1500. Science 303:1499–1503.

Page, R.D.M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12:357–358.

Pretorius, M.C., Crous, P.W., Groenewald, J.Z., and Braun, U. 2003. Phylogeny of some cercosporoid fungi from *Citrus*. Sydowia 55:286–305.

Rayner, R.W. 1970. A mycological colour chart. Commonwealth Mycological Institute and British Mycological Society, Kew, Surrey, UK.

Rossi, V., Battilani, P., Chiusa, G., Giosue, S., Languasco, L., and Racca, P. 2000. Components of rate-reducing resistance to Cercospora leaf spot in sugarbeet: conidiation length, spore yield. J. Plant Pathol. 82:125–131.

Ruppel, E.G., and Scott, P.R. 1974. Strains of *Cercospora beticola* resistant to benomyl in the USA. Plant Dis. Rep. 58:434–436.

Saccardo, P.A. 1876. Fungi Veneti novi vel critici. Series V. Nuovo Giorn. Bot. Ital. 8:162–211.

Shane, W.W., and Teng, P.S. 1992. Impact of Cercospora leaf spot on root weight, sugar yield, and purity of *Beta vulgaris*. Plant Dis. 76:812–820.

Stewart, E.L., Liu, Z., Crous, P.W., and Szabo, L.J. 1999. Phylogenetic relationships among some cercosporoid anamorphs of *Mycosphaerella* based on rDNA sequence analysis. Mycol. Res. 103:1491–1499.

Tessmann, D.J., Charudattan, R., Kistler, H.C., and Rosskopf, E.N. 2001. A molecular characterization of *Cercospora* species pathogenic to water hyacinth and emendation of *C. piaropi*. Mycologia 93:323–334.

Vestal, E.F. 1933. Pathogenicity, host response and control of Cercospora leaf spot of sugarbeet. Iowa Ahees. Res. Bull. 168:43–72.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van der Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407–4414.

Weiland, J.J., and Halloin, J.M. 2001. Benzimidazole resistance in *Cercospora beticola* sampled from sugarbeet fields in Michigan, USA. Can. J. Plant Pathol. 23:78–82.

Weiland, J., and Koch, G. 2004. Pathogen profile. Sugarbeet leaf spot disease (*Cercospora beticola* Sacc.). Mol. Plant Pathol. 5:157–166.

Welles, C.G. 1933. Taxonomic studies on the genus *Cercospora* in the Philippine Islands. Am. J. Bot. 12:195–218.

White, T.J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 282–287 in: PCR Protocols. A guide to methods and applications. M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White, eds. Academic Press, San Diego, CA.

CHAPTER 3

Host range of *Cercospora apii* and *C. beticola* and description of *C. apiicola*, a novel species from celery

Marizeth Groenewald[1,2], Johannes Z. Groenewald[1], Uwe Braun[3] and Pedro W. Crous[1,2]

[1] *Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, Netherlands*

[2] *Laboratory of Phytopathology, Wageningen University, Binnenhaven 5, 6709 PD Wageningen, Netherlands*

[3] *Martin-Luther-Universität, FB. Biologie, Institut für Geobotanik und Botanischer Garten, Neuwerk 21, D-06099 Halle (Saale), Germany*

Published in: Mycologia (2006) 98: 275–285.

Key words: Ascomycetes, *Cercospora apii* complex, Cercospora leaf spot, molecular phylogeny, species boundaries, taxonomy

Abstract: The genus *Cercospora* is one of the largest and most heterogeneous genera of hyphomycetes. *Cercospora* species are distributed worldwide and cause Cercospora leaf spot on most of the major plant families. Numerous species described from diverse hosts and locations are morphologically indistinguishable from *C. apii*, and are subsequently referred to as *C. apii sensu lato*. The importance and ecological role that different hosts play in taxon delimitation and recognition within this complex remains unclear. It has been shown that Cercospora leaf spot on celery and sugar beet are caused respectively by *C. apii* and *C. beticola*, both of which are part of the *C. apii* complex. During this study we characterized a new *Cercospora* species, *C. apiicola*, that was isolated from celery in Venezuela, Korea and Greece. The phylogenetic relationship between *C. apiicola* and other closely related *Cercospora* species was studied with five different gene areas. These analyses revealed that the *C. apiicola* isolates cluster together in a well defined clade. Both *C. apii* and *C. beticola sensu stricto* form well defined clades and are shown to have wider host ranges and to represent distinct species.

INTRODUCTION

The genus *Cercospora* Fresen. first was described in 1863 by Fresenius (Fuckel, 1863) and currently is one of the largest and most heterogeneous genera of hyphomycetes (Crous & Braun, 2003). Species belonging to this plant pathogenic genus are distributed worldwide and cause Cercospora leaf spot on most of the major plant families (Crous & Braun, 2003). Since the description of the genus, the taxonomy of its species has become difficult because *Cercospora* for many years has been a dumping ground for all dematiaceous hyphomycetes with filiform conidia (Pons & Sutton, 1988). Johnson & Valleau (1949) stated that most of the morphologically uniform *Cercospora* isolates belong to a single *Cercospora* species that occurs on a wide host range and morphologically is indistinguishable from *C. apii* Fresen. *Cercospora apii* is the oldest available name for this large complex of morphologically indistinguishable *Cercospora* taxa. This approach was questioned by Chupp (1954), who stated in his monograph that species of *Cercospora* are generally host specific. Chupp subsequently formulated the concept of "one host species, genus or family equals one *Cercospora* species". Chupp's concept led to the description of a large number of species based on host substrate, with more than 3,000 names being listed by Pollack (1987). Crous & Braun (2003) revised these species and redisposed many of them. A total of 659 *Cercospora* species were recognized, with a further 281 being referred to synonymy under *C. apii sensu lato*. This decision was substantiated by the various inoculation experiments that have been conducted on the *C. apii* complex (Vestal, 1933; Johnston & Valleau, 1949; Fajola, 1978) and that raised doubts whether host specificity existed within this complex.

To date only a few species belonging to *C. apii sensu lato* have been cultured, and molecular data addressing host specificity within this complex is still lacking (Crous *et al.,* 2004). Three scenarios are possible when examining the host-species association of taxa belonging to the *C. apii* complex. The first scenario is that a single species of *Cercospora* occurs on a wide host range; the second that several species exist with overlapping host ranges; and the third is

that some *Cercospora* species are host-specific, whereas others are not.

The first evidence that distinct species exist within the *C. apii* morphotype was published by Groenewald *et al*. (2005). The latter study focused on *Cercospora* species isolated from sugar beet (*Beta vulgaris*) and celery (*Apium graveolens*). Characteristics examined for these isolates included morphology, cultural characteristics and cardinal temperature requirements for growth. These data were supplemented with amplified fragment length polymorphism analyses and phylogenetic analyses with five different genes. Groenewald *et al*. (2005) showed that three distinct *Cercospora* species exist on sugar beet and/or celery, namely *C. beticola* on sugar beet, *C. apii* on both celery and sugar beet, and a third *Cercospora* species that was isolated from celery in Venezuela and Korea.

The ability to infect different hosts during artificial inoculation is of questionable value as a character in species delimitation. For instance, a recent study revealed that *C. beticola* could infect safflower during artificial inoculation experiments (Lartey *et al*., 2005). However, *C. beticola* has yet to be isolated from this host in the field. Only a few taxa that belong to the *C. apii* complex have been studied in the past in an attempt to elucidate the relationship between fungal species and host. The first objective of this study, therefore, was to name the new *Cercospora* species from celery. The second objective was to use DNA sequence data to examine the host range of this species, including *C. apii sensu stricto* and *C. beticola sensu stricto* as defined by Groenewald *et al*. (2005).

MATERIALS AND METHODS

Isolates

Isolates used in this study were obtained from the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, Netherlands, as well as the working collection of Pedro Crous (CPC) that is housed at CBS (Table 1). Single conidial isolates also were obtained from symptomatic material as explained in Crous (1998). Isolates were plated onto 2 % malt-extract agar (MEA) and oatmeal agar (OA) (Gams *et al*., 1998) and incubated at 24 °C for 8 days.

DNA isolation, amplification and sequencing

The FastDNA kit (BIO 101, Carlsbad, California) was used according to the manufacturer's instructions to isolate genomic DNA of 200–400 mg fungal mycelia grown on MEA plates. A sterile blade was used to scrape the mycelia from the surface of the plate. For the phylogenetic analyses, parts of these gene areas were used: the internal transcribed spacers and 5.8S rRNA gene (ITS), the actin gene (ACT), the translation elongation factor 1-α gene (EF), the calmodulin gene (CAL) and the histone H3 gene (HIS). PCR primers and amplification conditions followed the protocols outlined by Groenewald *et al*. (2005). PCR products were separated by electrophoresis at 80 V for 40 min on a 0.8 % (w/v) agarose gel containing 0.1 µg/ml ethidium bromide in 1× TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualized under UV light.

Amplicons were sequenced in both directions with the PCR primers and a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Roosendal, Netherlands) according to the manufacturer's recommendations. The products were analyzed on an ABI Prism 3700

Table 1. *Cercospora* isolates included in the study.

[a] Strains and Accession numbers	Host	Origin	Collector	[b] GenBank number (ITS, EF, ACT, CAL, HIS)
C. apii Fresen.				
CBS 119.25; CPC 5086	*Apium graveolens*	—	L. J. Klotz	AY840512, AY840479, AY840443, AY840410, AY840377
CBS 121.31; CPC 5073	*Beta vulgaris*	Austria	—	AY840513, AY840480, AY840444, AY840411, AY840378
CBS 127.31; CPC 5119	*B. vulgaris*	Hungary	—	AY840514, AY840481, AY840445, AY840412, AY840379
CBS 152.52; CPC 5063	*B. vulgaris*	Netherlands	G. van den Ende	AY840515, AY840482, AY840446, AY840413, AY840380
CBS 252.67; CPC 5084	*Plantago lanceolata*	Rumania	O. Constantinescu	DQ233318, DQ233342, DQ233368, DQ233394, DQ233420
CBS 257.67; CPC 5057	*Helianthemum sp.*	Romania	O. Constantinescu	DQ233319, DQ233343, DQ233369, DQ233395, DQ233421
CBS 536.71; CPC 5087	*A. graveolens*	Romania	O. Constantinescu	AY752133, AY752166, AY752194, AY752225, AY752256
CBS 553.71; CPC 5083	*Plumbago europaea*	Romania	O. Constantinescu	DQ233320, DQ233344, DQ233370, DQ233396, DQ233422
CBS 110813; CPC 5110	*Moluccella laevis*	USA	S. T. Koike	AY156918, DQ233345, DQ233371, DQ233397, DQ233423
CBS 110816; CPC 5111	*M. laevis*	USA	S. T. Koike	AY156919, DQ233346, DQ233372, DQ233398, DQ233424
CBS 114416; CPC 10925	*Apium* sp.	Austria	—	AY840516, AY840483, AY840447, AY840414, AY840381
CBS 114418; CPC 10924	*A. graveolens*	Italy	Meutri	AY840517, AY840484, AY840448, AY840415, AY840382
CBS 114485; CPC 10923	*A. graveolens*	Italy	Meutri	AY840518, AY840485, AY840449, AY840416, AY840383
[c] CBS 116455; CPC 11556	*A. graveolens*	Germany	K. Schrameyer	AY840519, AY840486, AY840450, AY840417, AY840384
CBS 116504; CPC 11579	*A. graveolens*	Germany	K. Schrameyer	AY840520, AY840487, AY840451, AY840418, AY840385
CBS 116507; CPC 11582	*A. graveolens*	Germany	K. Schrameyer	AY840521, AY840488, AY840452, AY840419, AY840386
CPC 5112	*M. laevis*	New Zealand	C. F. Hill	DQ233321, DQ233347, DQ233373, DQ233399, DQ233425
C. beticola Sacc.				
CBS 116.47; CPC 5074	*B. vulgaris*	Netherlands	G. E. Bunschoten	AY752135, AY752168, AY752196, AY752227, AY752258
CBS 117.47	*B. vulgaris*	Czechia	G. E. Bunschoten	DQ233322, DQ233348, DQ233374, DQ233400, DQ233426
CBS 122.31; CPC 5072	*B. vulgaris*	Germany	—	AY752136, AY752169, AY752197, AY752228, AY752259
CBS 123.31; CPC 5071	*B. vulgaris*	Spain	—	AY840522, AY840489, AY840453, AY840420, AY840387
CBS 124.31; CPC 5070	*B. vulgaris*	Romania	—	AY840523, AY840490, AY840454, AY840421, AY840388
CBS 125.31; CPC 5069	*B. vulgaris*	Japan	—	AY840524, AY840491, AY840455, AY840422, AY840389

Table 1. Continued.

[a] Strains and Accession numbers	Host	Origin	Collector	[b] GenBank number (ITS, EF, ACT, CAL, HIS)
CBS 126.31; CPC 5064	*B. vulgaris*	Germany	—	AY840525, AY840492, AY840456, AY840423, AY840390
CBS 539.71; CPC 5062	*B. vulgaris*	Romania	O. Constantinescu	DQ233323, DQ233349, DQ233375, DQ233401, DQ233427
CBS 548.71; CPC 5065	*Malva pusilla*	Romania	O. Constantinescu	DQ233324, DQ233350, DQ233376, DQ233402, DQ233428
CBS 113069; CPC 5369	*Spinacia* sp.	Botswana	L. Lebogang	DQ233325, DQ233351, DQ233377, DQ233403, DQ233429
CBS 116454; CPC 11558	*B. vulgaris*	Germany	S. Mittler	AY840526, AY840493, AY840457, AY840424, AY840391
[c] CBS 116456; CPC 11557	*B. vulgaris*	Italy	V. Rossi	AY840527, AY840494, AY840458, AY840425, AY840392
CBS 116501; CPC 11576	*B. vulgaris*	Iran	A. A. Ravanlou	AY840528, AY840495, AY840459, AY840426, AY840393
CBS 116502; CPC 11577	*B. vulgaris*	Germany	S. Mittler	AY840529, AY840496, AY840460, AY840427, AY840394
CBS 116503; CPC 11578	*B. vulgaris*	Italy	—	AY840530, AY840497, AY840461, AY840428, AY840395
CBS 116505; CPC 11580	*B. vulgaris*	France	S. Garressus	AY840531, AY840498, AY840462, AY840429, AY840396
CBS 116506; CPC 11581	*B. vulgaris*	Netherlands	—	AY840532, AY840499, AY840463, AY840430, AY840397
CPC 5113	*Limonium sinuatum*	New Zealand	C. F. Hill	DQ233326, DQ233352, DQ233378, DQ233404, DQ233430
CPC 5123	*A. graveolens*	New Zealand	C. F. Hill	DQ233327, DQ233353, DQ233379, DQ233405, DQ233431
CPC 5125	*B. vulgaris*	New Zealand	C. F. Hill	AY752137, AY752170, AY752198, AY752229, AY752260
CPC 5128	*B. vulgaris*	New Zealand	C. F. Hill	AY752138, AY752171, AY752199, AY752230, AY752261
CPC 5370	*Spinacia* sp.	Botswana	L. Lebogang	DQ233328, DQ233354, DQ233380, DQ233406, DQ233432
CPC 10166	*B. vulgaris*	New Zealand	C. F. Hill	DQ233329, DQ233355, DQ233381, DQ233407, DQ026471
CPC 10168	*B. vulgaris*	New Zealand	C. F. Hill	AY840533, AY840500, AY840464, AY840431, AY840398
CPC 10171	*B. vulgaris*	New Zealand	C. F. Hill	AY840534, AY840501, AY840465, AY840432, AY840399
CPC 10195	*B. vulgaris*	New Zealand	C. F. Hill	DQ233330, DQ233356, DQ233382, DQ233408, DQ026472
CPC 10197	*B. vulgaris*	New Zealand	C. F. Hill	AY840535, AY840502, AY840466, AY840433, AY840400
CPC 10204	*B. vulgaris*	New Zealand	C. F. Hill	DQ233331, DQ233357, DQ233383, DQ233409, DQ233433
CPC 11341	*Chrysanthemum coronarium*	Korea	H. D. Shin	DQ233332, DQ233358, DQ233384, DQ233410, DQ233434
CPC 11344	*Chrysanthemum coronarium*	Korea	H. D. Shin	DQ233333, DQ233359, DQ233385, DQ233411, DQ233435
CPC 12022	*B. vulgaris*	Germany	S. Mittler	DQ233334, DQ233360, DQ233386, DQ233412, DQ233436
CPC 12027	*B. vulgaris*	Germany	S. Mittler	DQ233335, DQ233361, DQ233387, DQ233413, DQ026468

Table 1. Continued.

[a] Strains and Accession numbers	Host	Origin	Collector	[b] GenBank number (ITS, EF, ACT, CAL, HIS)
CPC 12028	*B. vulgaris*	Egypt	M. Hasem	DQ233336, DQ233362, DQ233388, DQ233414, DQ233437
CPC 12029	*B. vulgaris*	Egypt	M. Hasem	DQ233337, DQ233363, DQ233389, DQ233415, DQ233438
CPC 12030	*B. vulgaris*	Egypt	M. Hasem	DQ233338, DQ233364, DQ233390, DQ233416, DQ233439
CPC 12031	*B. vulgaris*	Germany	S. Mittler	DQ233339, DQ233365, DQ233391, DQ233417, DQ026470
C. apiicola				
[c] CBS 116457; CPC 10267	*Apium* sp.	Venezuela	N. Pons	AY840536, AY840503, AY840467, AY840434, AY840401
CBS 116458; CPC 10657	*Apium* sp.	Korea	H. D. Shin	AY840537, AY840504, AY840468, AY840435, AY840402
CPC 10220	*Apium* sp.	Venezuela	N. Pons	AY840538, AY840505, AY840469, AY840436, AY840403
CPC 10248	*Apium* sp.	Venezuela	N. Pons	AY840539, AY840506, AY840470, AY840437, AY840404
CPC 10265	*Apium* sp.	Venezuela	N. Pons	AY840540, AY840507, AY840471, AY840438, AY840405
CPC 10266	*Apium* sp.	Venezuela	N. Pons	AY840541, AY840508, AY840472, AY840439, AY840406
CPC 10279	*Apium* sp.	Venezuela	N. Pons	AY840542, AY840509, AY840473, AY840440, AY840407
CPC 10666	*Apium* sp.	Korea	H. D. Shin	AY840543, AY840510, AY840474, AY840441, AY840408
CPC 10759	*A. graveolens*	Korea	H. D. Shin	AY840544, AY840511, AY840475, AY840442, AY840409
CPC 11641	*A. graveolens*	Greece	A. N. Jama	DQ233340, DQ233366, DQ233392, DQ233418, DQ233440
CPC 11642	*A. graveolens*	Greece	A. N. Jama	DQ233341, DQ233367, DQ233393, DQ233419, DQ233441

[a] CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands and CPC = Collection of Pedro Crous, housed at CBS, Netherlands.
[b] ITS = internal transcribed spacer, EF = elongation factor 1-α, ACT = actin, CAL = calmodulin, HIS = histone H3.
[c] Type strains of the different *Cercospora* species.

DNA Sequencer (Perkin-Elmer, Foster City, California). A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package (DNASTAR, Madison, Wisconsin).

Data analysis

The consensus sequences were assembled and added to the alignment (TreeBASE matrix number M2242) of Groenewald *et al*. (2005) using Sequence Alignment Editor v2.0a11 (Rambaut, 2002), and manual adjustments for improvement were made by eye where necessary. The phylogenetic analyses of sequence data were done in PAUP (Phylogenetic Analysis Using Parsimony) v4.0b10 (Swofford, 2003) and consisted of neighbor-joining analysis with the uncorrected ("p"), the Jukes-Cantor and the HKY85 substitution models. Alignment gaps were treated as missing data and all characters were unordered and of equal weight. Any ties were broken randomly when encountered. For parsimony analysis, alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets with the heuristic search option with 100 random taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees was evaluated by 1,000 bootstrap replications (Hillis & Bull, 1993). Other measures calculated included tree length, consistency index, retention index and rescaled consistency index (TL, CI, RI and RC). The resulting trees were printed with TreeView v1.6.6 (Page, 1996). A partition homogeneity test was done in PAUP to test whether the different loci can be used in a combined analysis (Farris *et al*., 1994). Sequences were deposited in GenBank (Accession numbers listed in Table 1) and the alignment and trees in TreeBASE (Accession number M2872).

Morphology

Fungal structures were mounted in lactic acid and examined under a light microscope (× 1000). The extremes of spore measurements (30 observations) are given in parentheses. Colony colors were rated after 8 d on MEA and OA at 24 °C in the dark, using the color charts of Rayner (1970).

RESULTS

Sequence data analyses

A partition homogeneity test showed that all five data sets were not combinable ($P = 0.001$), but that four of the data sets (ITS, EF, ACT and CAL) could be combined ($P = 1.000$) and these therefore were analyzed as one combined set. The combined alignment contained 67 strains, including the three outgroups, and had a total length of 1,262 characters, of which 935 were constant, six were parsimony uninformative, and 321 were parsimony informative. The topology of the neighbor-joining trees obtained with the different substitution models was the same. A similar topology was found for the most parsimonious trees. Parsimony analysis of the combined data resulted in a single most parsimonious tree (Fig. 1) (TL = 350 steps, CI = 0.997, RI = 0.999, RC = 0.996). From the phylogenetic analysis (Fig. 1), three distinct

and well supported clades were obtained. The first clade (99 % bootstrap support) contains *Cercospora* isolates belonging to the *C. beticola sensu stricto* clade. Twenty-nine of these isolates were obtained from *Beta* species, but several isolates in this group also were obtained from five additional hosts (two from *Chrysanthemum*, one from *Apium*, one from *Limonium*, one from *Malva* and two from *Spinacia*). The isolates were obtained from Europe, Africa, New Zealand and Asia. The second clade (100 % bootstrap support) contains *C. apii sensu stricto* isolates. These isolates were obtained from a diverse range of hosts (three from *Beta*, three from *Moluccella*, one from *Plantago*, one from *Plumbago* and one from *Helianthemum*), but the primary host infected by isolates in this group appears to be *Apium* (eight isolates). Isolates from the second clade were from Europe, America and New Zealand. The third clade (100 % bootstrap support) contains isolates of *C. apiicola* that thus far have only been isolated from *Apium* species in Venezuela, Korea and Greece.

As the HIS data set was not combinable with the other sequence data, it was analyzed separately. The HIS alignment contained 67 strains including the three outgroups, and had a total length of 380 characters, of which 319 were constant, one was parsimony uninformative, and 60 were parsimony informative. The topology of the neighbor-joining trees obtained with the different substitution models was the same and was identical to the topology of the most parsimonious tree. Parsimony analysis of the HIS data resulted in a single most parsimonious tree (Fig. 2) (TL = 73 steps, CI = 0.986, RI = 0.998, RC = 0.984). From the phylogenetic analysis (Fig. 2), three well supported clades with 100 % bootstrap values were obtained. The first clade contained eight isolates (seven from *Beta* species from different countries and one from *Helianthemum* in Rumania) that were present in the *C. beticola sensu stricto* clade obtained from the first analysis, except for the *Helianthemum* isolate which grouped in the *C. apii sensu stricto* clade (Fig. 1). The second clade contained the remaining *C. beticola sensu stricto* and *C. apii sensu stricto* isolates. The third clade consisted only of the *C. apiicola* isolates, which is consistent with the first analysis using the other four loci.

Taxonomy

Cercospora apii and *C. beticola sensu stricto* were circumscribed by Groenewald *et al*. (2005). During the present study several *Cercospora* isolates were obtained from celery exhibiting Cercospora leaf spot. A population of 47 plants collected in Venezuela by N. Pons, as well as individual diseased plants collected in Greece and Korea, were found to be associated with a novel species of *Cercospora*. The latter species is morphologically distinct from the *C. apii sensu lato* complex. Its conidiophores are relatively short, 25–70 × 4–6 µm, and the conidia are obclavate–cylindrical, not acicular, measuring (50–)80–120(–150) × (3–)4–5 µm, and being 1–6-septate (Figs. 3, 4). This species therefore is described as new:

Cercospora apiicola M. Groenewald, Crous & U. Braun, sp. nov.

Differt a C. apii (s.str. et s.lat.) conidiophoris relative brevibus, 25–70 × 4–6 µm, conidiis obclavatis-cylindraceis, non-acicularibus, tantum 1–6-septatis.

Specimen examined. VENEZUELA. La Guanota, Caripe, Edo. Monagas, 1050 m.s.n.m., *Apium* sp., 23 Jul. 2002, *N. Pons*, HOLOTYPE herb. CBS 18473, culture ex-type CBS 116457; MycoBank MB500768.

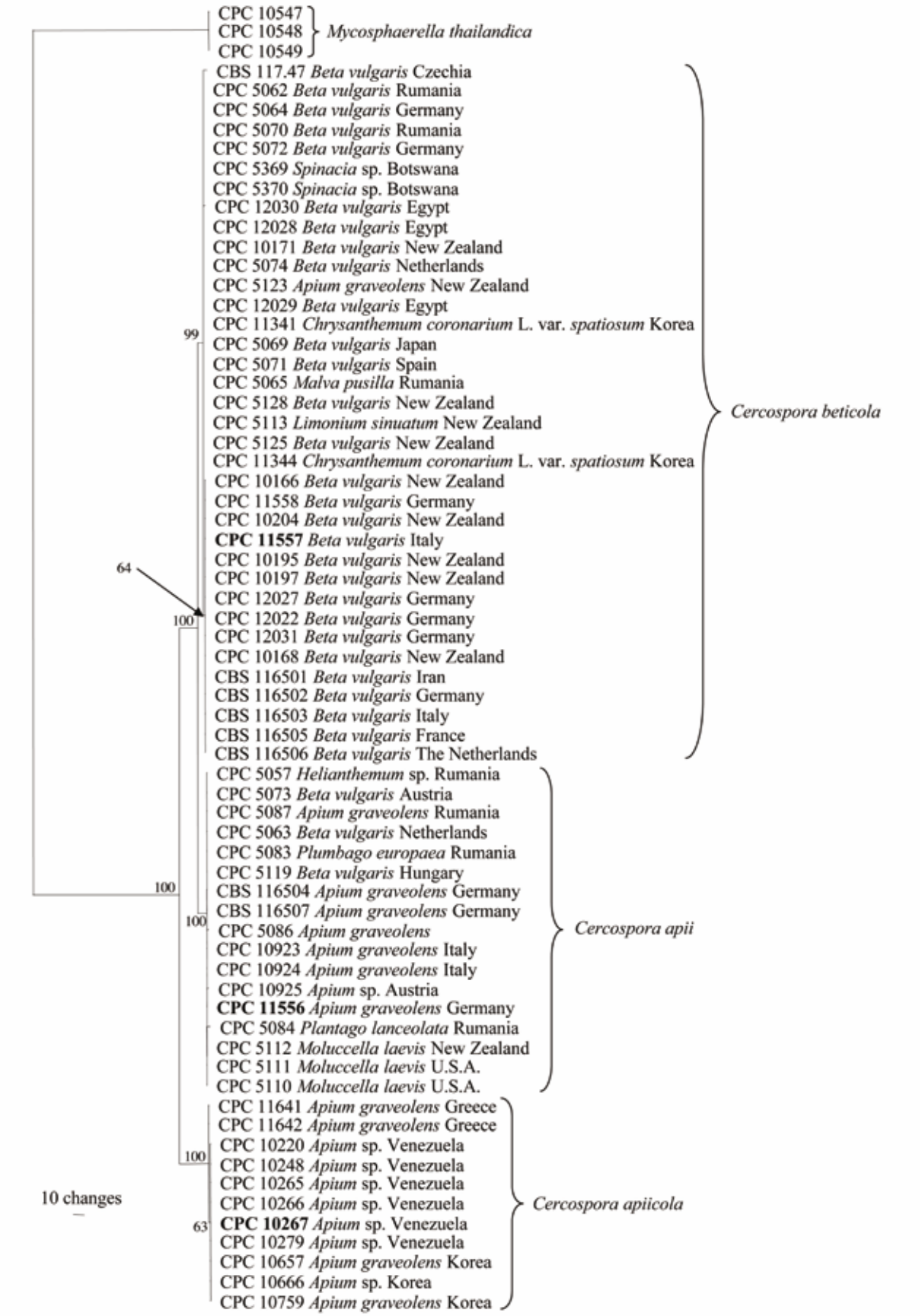


Fig. 1. Single most parsimonious tree obtained from a heuristic search with 100 random taxon additions of the combined ITS, EF, ACT and CAL sequence alignment. The scale bar shows ten changes and bootstrap support values from 1,000 replicates are shown at the nodes. Type strains are shown in boldface. The tree was rooted to three *Mycosphaerella thailandica* strains.

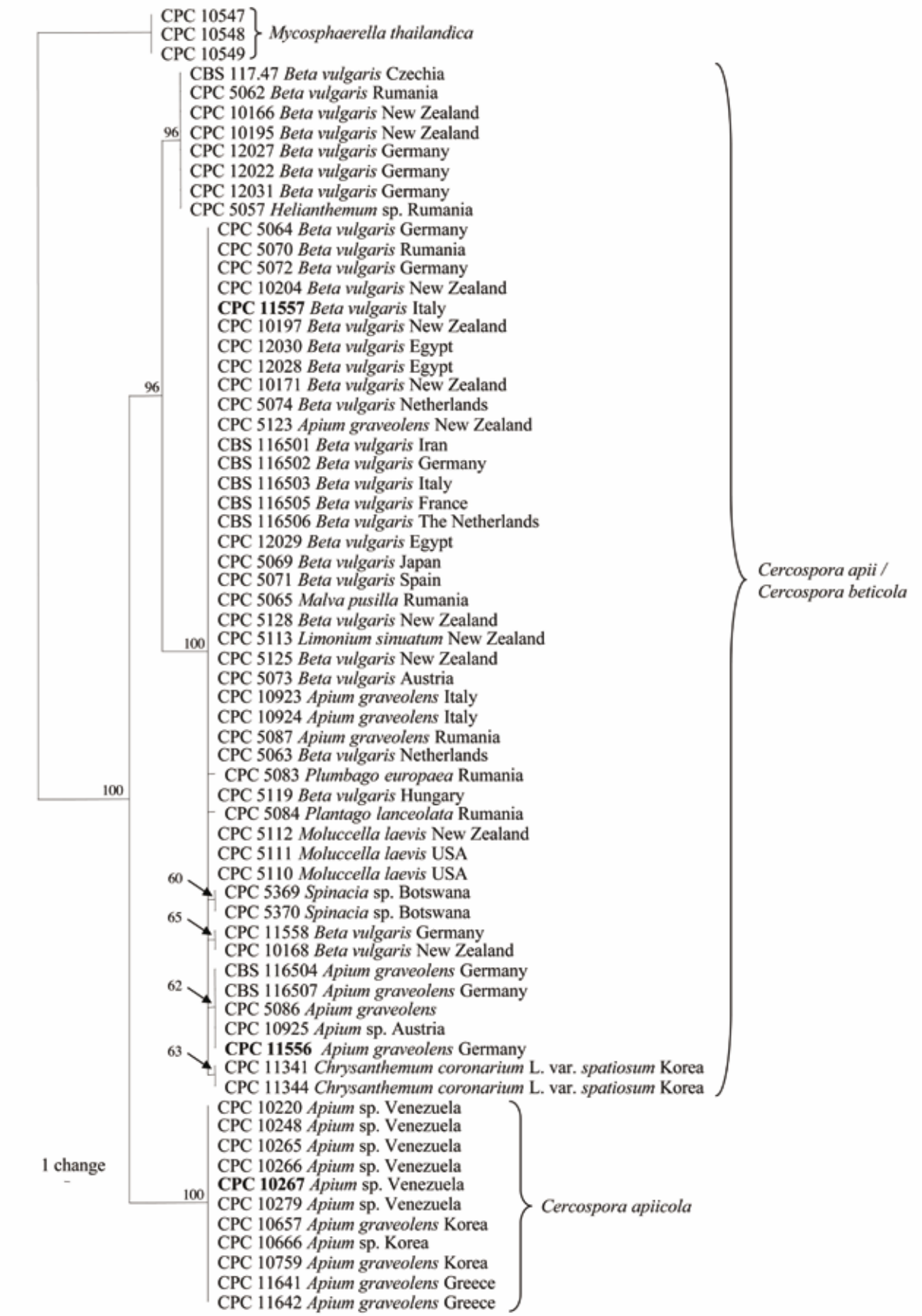


Fig. 2. The single most parsimonious tree obtained from a heuristic search with 100 random taxon additions of the histone H3 sequence alignment. The scale bar shows a single change and bootstrap support values from 1,000 replicates are shown at the nodes. Type strains are shown in boldface. The tree was rooted to three *Mycosphaerella thailandica* strains.

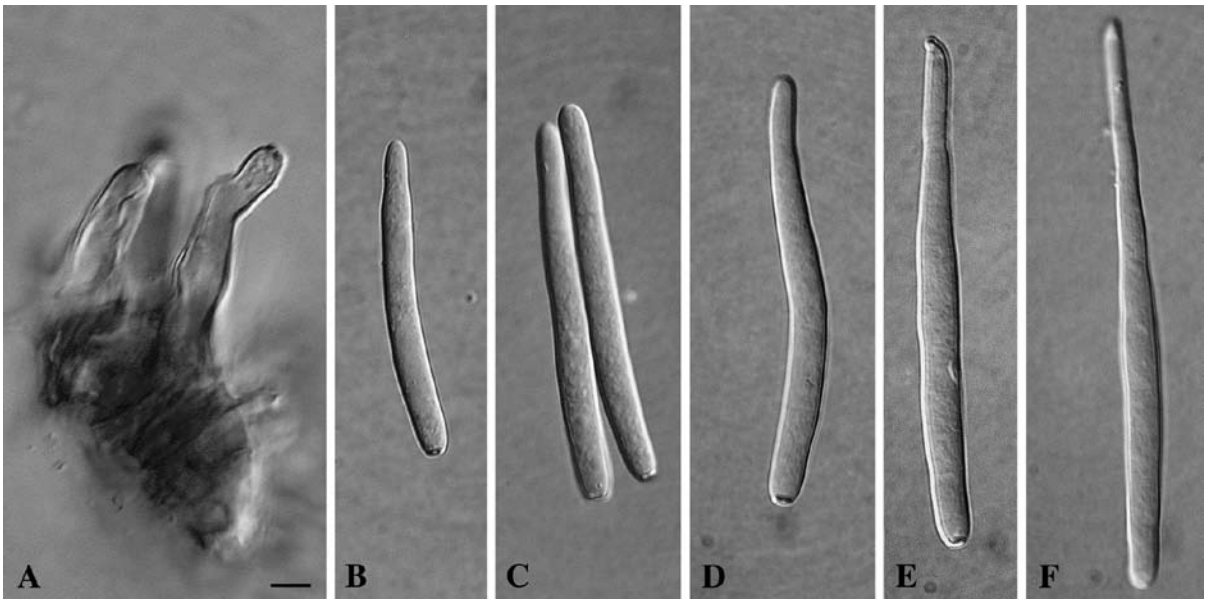


Fig. 3. *Cercospora apiicola* (holotype). **A**, Conidiophores. **B**–**F**, Conidia. Bar = 5 µm.

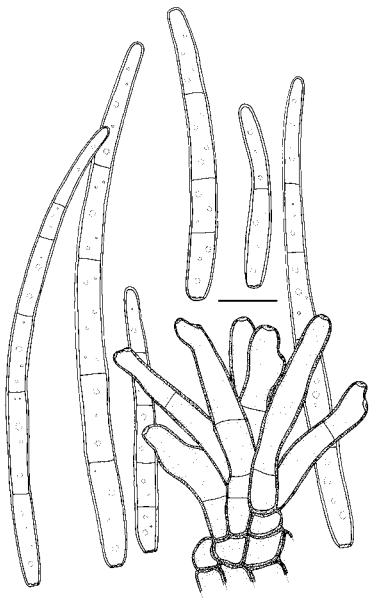


Fig. 4. Line drawing of conidiophores and conidia of the *Cercospora apiicola* holotype (CBS 116457). Bar = 10 µm.

Leaf spots amphigenous, subcircular to irregular, 3–10 mm diam, medium brown, with a raised or inconspicuous, indefinite margin, not surrounded by a border of different color. *Caespituli* amphigenous, but primarily hypophyllous. *Stromata* lacking to well-developed, 30–60 µm diam, medium brown. *Conidiophores* arising in fascicles of 4–10, moderately dense, arising from stromata, emerging through stomata or erumpent through the cuticle, subcylindrical, upper part geniculate–sinuous, unbranched, 1–3-septate, 25–70 × 4–6 µm, medium brown, becoming pale brown towards the apex, smooth, wall somewhat thickened. *Conidiogenous cells* integrated, terminal, 15–30 × 4–5 µm, occasionally unilocal, usually multilocal, sympodial; loci subcircular, planate, thickened, darkened, refractive, 2.5–3 µm wide. *Conidia* solitary, cylindrical when small, obclavate–cylindrical when mature, not acicular, (50–)80–120(–150) ×

(3–)4–5 µm, 1–6-septate; apex subobtuse, base obconically subtruncate; hila 2–2.5 µm wide, thickened, darkened, refractive.

Cultural characteristics. Colonies are smooth to folded, erumpent with smooth, even to uneven margins and sparse to moderate aerial mycelium; white to smoke-gray on MEA (surface), and olivaceous-gray to iron-gray beneath; on OA colonies are white to olivaceous-gray on the surface. Cardinal temperature requirements for growth, min 6 °C, opt 24 °C, max 30 °C.

Host range and distribution. *Apium graveolens*, *Apium* sp., Greece, Korea, Venezuela.

DISCUSSION

During a recent study in which we circumscribed *C. apii* and *C. beticola sensu stricto*, we collected isolates of several *Cercospora* spp. that are part of the *C. apii sensu lat*o species complex. A whole population of "*C. apii*" collected on celery from Venezuela was revealed to be a distinct species. Several months later we isolated the same species on celery collected from Korea. At that time it was thought that this species had not yet invaded European celery fields because it was absent from European *Cercospora* isolates from this crop (Groenewald *et al*., 2005). However, in the present study, we report the presence of this species on celery from Greece and describe it as *C. apiicola* sp. nov. Cultural and morphological examination of the *C. apiicola* strains support the observation made by Groenewald *et al*. (2005) that this new *Cercospora* species is distinct from the two closely related species, *C. beticola* and *C. apii,* that previously have been isolated from celery. The isolation of this new *Cercospora* species on a well-known crop such as celery is an indication that there may still be many other undescribed cercosporoid species on well known crops and ornamental plants awaiting description.

Chupp (1954) associated Cercospora leaf spot on sugar beet with infections of *C. beticola,* and that of celery with *C. apii*. Ellis (1971) discussed the *C. apii sensu lato* isolates in detail and described a wide host range for this species, but five years later he changed his opinion, and narrowed the host range of *C. apii* to celery and *C. beticola* to sugar beet (Ellis, 1976). Crous & Braun (2003) linked 83 host genera to *C. apii*, and nine host genera to *C. beticola* infections. Groenewald *et al*. (2005) again cast doubt on the purported wide host ranges of these species. In the present study, a survey of *Cercospora* isolates from ten host genera identified several additional hosts for both *C. apii sensu stricto* and *C. beticola sensu stricto*. From these data we can confirm four additional host genera for *C. apii* (*Helianthemum*, *Moluccella*, *Plantago*, *Plumbago*) and five additional host genera for *C. beticola* (*Apium*, *Chrysanthemum*, *Limonium*, *Malva*, *Spinacia*). According to Crous & Braun (2003) several *Cercospora* species (listed in parentheses) are associated with these hosts: *Apium* (*C. apii*), *Beta* (*C. beticola*), *Helianthemum* (*C. cistinearum*, *C. helianthemi*), *Moluccella* (*C. molucellae*), *Plantago* (*C. pantoleuca*, *C. plantaginis*), *Plumbago* (*C. apii*, *C. plumbaginea*), *Limonium* (*C. apii*, *C. insulana*, *C. statices*), *Malva* (*C. althaeina*, *C. beticola*, *C. hyalospora*, *C. malvae*, *C. malvarum*) and *Spinacia* (*C. bertrandii***,** *C. beticola*, *C. spinaciicola*). In the treatment of Crous & Braun (2003) neither *Apium*, *Chrysanthemum* or *Limonium* are listed as hosts of *C. beticola*, nor *Beta*, *Helianthemum*, *Moluccella* and *Plantago* as hosts of *C. apii*. This study provides the first molecular evidence that these two species have wider host ranges than had been accepted by

Chupp (1954) and Ellis (1976). However from the present study it appears that both species have narrower host ranges than that proposed by Crous & Braun (2003), but this has to be investigated further by conducting pathogenicity studies on all the hosts previously listed for these species.

The host range data obtained in the present study illustrate that *C. beticola sensu stricto* and *C. apii sensu stricto* are not entirely host specific and that it is not possible to identify these two species solely based on host. Despite of the additional host genera that were found for *C. apii* and *C. beticola*, it is clear that *C. apii sensu stricto* is mainly isolated from celery, whereas *C. beticola* is mainly isolated from sugar beet, even though both of these species have been isolated from the other's primary host in the past.

Crous & Groenewald (2005) introduced the pogo stick hypothesis to explain the colonization of nectrotic *Mycosphaerella* lesions by other species of *Mycosphaerella* that jump hosts in the process of reaching their real hosts. The possibility that this process of substrate colonization and host jumps also occurs in asexual *Mycosphaerella* species could explain the isolation of specific *Cercospora* species from "atypical" hosts and needs to be investigated further. It would be especially interesting to determine whether *Cercospora* species occurring on "atypical" hosts are able to cause disease on these hosts or not.

As illustrated in this study, morphology, host specificity and geographic location are not suitable characters for the identification of species of the *C. apii* complex. Groenewald *et al*. (2005) used sequence data in combination with other features such as growth rate to establish species boundaries for *C. apii*, *C. apiicola* (as *Cercospora* sp.) and *C. beticola*. From these established species boundaries, species-specific primers were designed in polymorphic areas of the calmodulin gene for the three species. This combined approach probably represents the most reliable way to characterize and identify species within this complex.

Five loci were used in this study for phylogenetic analyses, although all five loci sequenced were not congruent and therefore could not be used in a combined phylogenetic analysis. Two separate analyses were thus performed, the first combining the ITS, EF, ACT and CAL sequences and the second using only HIS sequences. The first analysis separated the *C. apii sensu stricto*, *C. beticola sensu stricto* and *C. apiicola* isolates. Although the second analysis also was able to separate the *C. apiicola* isolates from the *C. apii sensu stricto* / *C. beticola sensu stricto* isolates, it was not able to distinguish between *C. apii sensu stricto* and *C. beticola sensu stricto* isolates. Using HIS data a small cluster representing seven *C. beticola sensu stricto* and one *C. apii sensu stricto* isolate grouped separately from the other *C. apii sensu stricto* / *C. beticola sensu stricto* isolates. The unique polymorphisms (ten in total) observed in the histone H3 sequences of these isolates were identical and were not present in the other isolates or in our *Cercospora* sequence database. A possible explanation might be host jumping by the *Helianthemum* isolate, followed by recombination with the *Beta* isolates. However more *Helianthemum* isolates need to be studied to confirm whether this allele is unique to *Helianthemum* before one can address this issue. Caution should therefore be taken when using histone H3 sequence data for *Cercospora* phylogeny because variation in the histone H3 sequence may not indicate species differences.

It can be concluded from this study that strains belonging to the *C. apii sensu stricto* and *C. beticola sensu stricto* clades can be isolated from other hosts and, although these species are

mainly isolated from celery and sugar beet, they are not host specific. It seems that the new species from celery described in this paper (*viz*. *C. apiicola*) is host specific because no other *Cercospora* strain isolated from other hosts and available in our sequence database has similar sequences. The reasons why host jumping by *C. apii* and *C. beticola* is so common remains unknown. However it is not unlikely that under stress - a shortage of host tissue or suitable weather conditions - the new species might also be able to jump from celery onto other hosts.

ACKNOWLEDGEMENTS

The authors hank H.D. Shin, N. Pons and A.N. Jama for respectively supplying the infected *Apium* leaf materials from Korea, Venezuela and Greece. This research was financially supported by the CBS-Odo van Vloten Stichting, and the Royal Netherlands Academy of Arts and Sciences.

LITERATURE CITED

Chupp, C. 1954. A monograph of the fungus genus *Cercospora*. Published by the author. Ithaca, New York.

Crous, P.W. 1998. *Mycosphaerella* spp. and their anamorphs associated with leaf spot disease of Eucalyptus. Mycol. Mem. 21:1–171.

Crous, P.W., and Braun, U. 2003. *Mycosphaerella* and its anamorphs: 1. Names published in *Cercospora* and *Passalora*. CBS Biodivers. Series 1:1–571.

Crous, P.W., and Groenewald, J.Z. 2005. Hosts, species and genotypes: opinions versus data. Australas. Plant Pathol. 34:463–470.

Crous, P.W., Groenewald, J.Z., Pongpanich, K., Himaman, W., Arzanlou, M., and Wingfield, M.J. 2004. Cryptic speciation and host specificity among *Mycosphaerella* spp. occurring on Australian *Acacia* species grown as exotics in the tropics. Stud. Mycol. 50:457–469.

Ellis, M.B. 1971. Dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, UK.

Ellis, M.B. 1976. More dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, UK.

Fajola, A.O. 1978. The effect of some environmental factors on the reproductive structures of some species of *Cercospora*. Nova Hedwigia 29:922–934.

Farris, J.S., Källersjö, M., Kluge, A.G., and Bult, C. 1994. Testing significance of incongruence. Cladistics 10:315–320.

Fuckel, K.W.G.L. 1863. Fungi Rhenani exsiccati, Fasc. I-IV. Hedwigia 2:132–136.

Gams, W., Hoekstra, E.S., and Aptroot, A. 1998. CBS Course of Mycology, 4th ed. Centraalbureau voor Schimmelcultures, Baarn, Delft, Netherlands.

Groenewald, M., Groenewald, J.Z., and Crous, P.W. 2005. Distinct species exist within the *Cercospora apii* morphotype. Phytopathology 95:951–959.

Hillis, D.M., and Bull, J.J. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst. Biol. 42:182–192.

Johnson, E.M., and Valleau, W.D. 1949. Synonomy in some common species of *Cercospora*. Phytopathology 39:763–770.

Lartey, R.T., Caesar-Ton That, T.C., Caesar, A.J., Shelver, W.L., Sol, N.I., and Bergman, J.W. 2005. Safflower: A new host of *Cercospora beticola*. Plant Dis. 89:797–801.

Page, R.D.M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12:357–358.

Pollack, F.G. 1987. An annotated compilation of *Cercospora* names. Mycol. Mem. 12:1–212.
Pons, N., Sutton, B.C. 1988. *Cercospora* and similar fungi on yams (*Dioscorea* spp.). Mycol. Pap. 160:1–78.
Rambaut, A. 2002. Sequence Alignment Editor. Version 2.0. Department of Zoology, University of Oxford, Oxford.
Rayner, R.W. 1970. A mycological colour chart. CMI and British Mycological Society, Kew, Surrey, England.
Swofford, D.L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.
Vestal, E.F. 1933. Pathogenicity, host response and control of *Cercospora* leaf spot of sugar beet. Iowa Agric. Exp. Sta. Res. Bull. 168:43–72.

CHAPTER 4

Mating type gene analysis in apparently asexual *Cercospora* species is suggestive of cryptic sex

Marizeth Groenewald[1,2], Johannes Z. Groenewald[1], Thomas C. Harrington[3], Edwin C.A. Abeln[1], and Pedro W. Crous[1,2]

[1] *Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, Netherlands*

[2] *Laboratory of Phytopathology, Wageningen University, Binnenhaven 5, 6709 PD Wageningen, Netherlands*

[3] *Department of Plant Pathology, Iowa State University, 351 Bessey Hall, Ames, Iowa, 50011, USA*

Published in: Fungal Genetics and Biology (2006) 43:813–825.

Key words: Allele frequency, Cercospora leaf spot, geographic distribution, mating types, *Mycosphaerella*, PCR amplification

Abstract: The genus *Cercospora* consists of numerous important, apparently asexual plant pathogens. We designed degenerate primers from homologous sequences in related species to amplify part of the *C. apii*, *C. apiicola*, *C. beticola*, *C. zeae-maydis* and *C. zeina* mating type genes. Chromosome walking was used to determine the full-length mating type genes of these species. Primers were developed to amplify and sequence homologous portions of the mating type genes of additional species. Phylogenetic analyses of these sequences revealed little variation among members of the *C. apii* complex, whereas *C. zeae-maydis* and *C. zeina* were found to be dissimilar. The presence of both mating types in approximately even proportions in *C. beticola*, *C. zeae-maydis* and *C. zeina* populations, in contrast to single mating types in *C. apii* (MAT1) and *C. apiicola* (MAT2), suggests that a sexual cycle may be active in some of these species.

INTRODUCTION

The genus *Cercospora* was described by Fresenius (Fuckel, 1863) and is one of the largest genera of hyphomycetes. More than 3,000 names were listed by Pollack (1987), but Crous & Braun (2003) revised the genus and reduced many species to synonymy, leaving a total of 659 *Cercospora* species. There are 281 morphologically indistinguishable *Cercospora* species, infecting a wide range of plant genera and families, listed as synonyms under *C. apii sensu lato* (Crous & Braun, 2003).

Cercospora apii is the main causal agent of Cercospora leaf spot on celery, although it has also been confirmed to occur on additional host genera such as *Beta*, *Helianthemum*, *Moluccella*, *Plantago* and *Plumbago* (Crous & Braun, 2003; Groenewald *et al*., 2005, 2006). A second *Cercospora* species, *C. apiicola*, has also been found to cause Cercospora leaf spot on celery (Groenewald *et al*., 2005, 2006). A multigene phylogeny revealed *C. apiicola* to be distinct from *C. apii* (Groenewald *et al*., 2005, 2006). This species is morphologically similar, but not identical, to *C. apii,* and has thus far only been isolated from celery in Venezuela, Korea and Greece.

Cercospora beticola, which causes Cercospora leaf spot on sugar beet (Saccardo, 1876; Groenewald *et al*., 2005), is morphologically identical to *C. apii*. Although these two species were considered to be synonymous in the past (Crous & Braun, 2003), a multigene phylogenic comparison and cultural characteristics revealed them to be distinct species (Groenewald *et al*., 2005). *Cercospora beticola* has also been confirmed from additional host genera such as *Apium*, *Chrysanthemum*, *Limonium*, *Malva*, and *Spinacia* (Crous & Braun, 2003; Groenewald *et al*., 2006).

Three *Cercospora* species have been linked to grey leaf spot on maize, namely *C. zeae-maydis*, *C. zeina*, and an unnamed *Cercospora* sp. (Crous *et al*., 2006), though it appears that other *Cercospora* species may also occur on this host (Wang *et al*., 1998). The unnamed *Cercospora* sp. reported by Crous *et al*. (2006) appeared to be morphologically and phylogenetically more similar to isolates in the *C. apii* complex than to *C. zeae-maydis* and

C. zeina. The description of *C. zeina* (Crous *et al*., 2006) has resolved some of the taxonomic uncertainty surrounding groups in *C. zeae-maydis.* The previously described *C. zeae-maydis* group II is now *C. zeina*, whereas group I is *C. zeae-maydis sensu stricto* (Dunkle & Levy, 2000; Goodwin *et al*., 2001; Crous *et al*., 2006).

No teleomorphs are known for the *Cercospora* species causing leaf spot on celery, sugar beet or maize, although there was an unconfirmed report of a teleomorph for *C. zeae-maydis* (Latterell & Rossi, 1977). Wang *et al*. (1998) were unable to find evidence of the *MAT-2* idiomorph in isolates of *C. zeae-maydis*, and *in vitro* pairing studies with isolates of *C. zeae-maydis* and *C. zeina* have thus far proven unsuccessful in producing a teleomorph (Crous *et al*., 2006). Wang *et al*. (1998) reported that there is little genotypic variation in populations of Group I and Group II (*C. zeae-maydis* and *C. zeina*, respectively), which might be expected for asexual species. In contrast, high levels of genetic variation have been reported within and among *C. beticola* field populations, as well as among isolates from the same leaf lesion (Große-Herrenthey, 2001; Moretti *et al*., 2004). Phylogenetic analyses using the ITS sequences of a variety of *Cercospora* species have resolved *Cercospora* as a well-defined monophyletic clade within the teleomorph genus *Mycosphaerella* (Stewart *et al*., 1999; Crous *et al*., 2000, 2001, 2004; Goodwin *et al*., 2001; Pretorius *et al*., 2003). Based on these data, it is clear that if sexual states do exist for these species, they would reside in *Mycosphaerella*.

In the absence of a known sexual stage, several approaches can be used to test for evidence of sexual reproduction. Populations that regularly undergo sexual reproduction should have many more genotypes that result in higher levels of genotypic diversity compared to those with only asexual reproduction (Milgroom, 1996). This type of genetic structure is seen in most populations of *M. graminicola* (Linde *et al*., 2002; Zhan *et al*., 2003; Zhan & McDonald, 2004). Another method to test for the possibility of sexual reproduction is to establish at the occurrence and frequency of the mating type genes. Both mating types have been characterized for filamentous ascomycetes such as *Alternaria alternata* and *Fusarium oxysporum*, for which only asexual reproduction have been observed (Arie *et al*., 1997, 2000). Therefore, the presence of the mating type idiomorphs in a given species alone is insufficient to prove that a sexual stage exists. However, it is probable that sexual recombination does take place if the two mating types occur in approximately equal frequencies within a given population (Milgroom, 1996; Halliday *et al*., 1999; Waalwijk *et al*., 2002; Linde *et al*., 2003).

The fact that different mating types are necessary for sexual reproduction was first recognized for the genus *Rhizopus* by Blakeslee (1904); and the first molecular characterization of the mating type idiomorphs was achieved for the yeast *Saccharomyces cerevisiae* (Astell *et al*., 1981). *Neurospora crassa* was the first filamentous ascomycete for which the mating type genes (*MAT1-1-1* and *MAT1-2*) were cloned and sequenced (Glass *et al*., 1988). The direct target genes of the mating type proteins have not yet been described, although there is evidence for the control of some genes such as pheromone genes (Bobrowicz *et al*., 2002). The DNA and amino acid sequences of mating type genes show no obvious similarities, although the mating type locus is surrounded by common flanking regions (Turgeon *et al*., 1993). Except for the high mobility group (HMG)- and the alpha domains, the similarity of homologous mating type genes is usually very low between different species (Turgeon, 1998). Regions with similarities

of up to 90 % can be found in the HMG domain, and these homologous regions have been used to design degenerative primers for amplification and cloning of the *MAT1-2* gene (Arie *et al*., 1997).

Four *MAT1-1* genes have been observed in ascomycetes (Pöggeler, 2001). Three of these genes can be distinguished from one another by the specific domain they contain. The *MAT1-1-1* gene contains an alpha domain, the *MAT1-1-2* gene has a MAT A-2 domain, and the *MAT1-1-3* gene has a HMG domain, whereas the *MAT1-1-4* encodes for a metallothionein protein (Kronstad & Staben, 1997; Turgeon, 1998). Only a single gene, *MAT1-2*, is known to confer the MAT2 phenotype. The formal mating type gene nomenclature proposed by Turgeon & Yoder (2000) will be used to define the mating type locus and genes from the *Cercospora* species.

The *MAT1-2* nucleotide sequences show high variability among species but low variability within species (Du *et al*., 2005; Paoletti *et al*., 2005). Sequences of the HMG domain of the *MAT1-2* gene have been used to investigate the phylogenetic relationships among closely related species in the *Gibberella fujikuroi* complex (Steenkamp *et al*., 2000), the *Ceratocystis coerulescens* complex (Witthuhn *et al*., 2000), *Fusarium graminearum* (O'Donnell *et al*., 2004), the *Ophiostoma ulmi* complex (Paoletti *et al*., 2005), and *Colletotrichum* species (Du *et al*., 2005). Most of these studies concluded that sequences of the HMG domain gave the same and sometimes even greater resolution and stronger support for most branches in a phylogenetic tree than the sequences of the more frequently used internal transcribed spacer regions of nuclear ribosomal DNA.

Sexual reproduction frequently results in genetic recombination and this has a major impact on the dynamics and fitness of a species. The teleomorphs of the Cercospora leaf spot pathogens are unknown, and have thus far not been successfully induced by crosses in the laboratory. As a first step to understanding the reproduction cycle in the apparently asexual species of the genus *Cercospora*, our objectives are to identify which mating type(s) are present in *Cercospora* species and to characterize the mating type gene(s). To achieve this objective, we (i) sequence and characterize the full-length mating type genes of *C. apii, C. apiicola, C. beticola*, *C. zeae-maydis,* and *C. zeina* using PCR-based techniques, (ii) amplify and sequence portions of the *MAT1-1-1* and *MAT1-2* genes of other *Cercospora* species for comparison, and (iii) develop a multiplex PCR method for rapid identification of the *MAT1-1-1* and *MAT1-2* genes to determine the frequencies of the mating types in different *Cercospora* populations.

MATERIALS AND METHODS

Fungal isolation and DNA extraction

Single conidial cultures were established from Cercospora leaf spots associated with celery leaves collected in Venezuela (*C. apiicola*) on 23 June 2002 and in Germany (*C. apii*) on 10 August 2004. Isolations were also made from symptomatic sugar beet leaves obtained from the Netherlands, Germany, Italy, France and New Zealand in 2003 and from Iran in 2004. Symptomatic maize leaves were collected from fields in South Africa (*C. zeina*) in the beginning of 2005 and from Pioneer 3394, a gray leaf spot susceptible hybrid of *Zea mays*, in

the USA (*C. zeae-maydis*) on 2 August 2005. Sampling was done in an X figure across each field to ensure consistency. For each population, 50 symptomatic leaves were collected: ten of each leg and 10 from the center plant. Isolates collected were used to screen for mating type distribution. Additional isolates used during this study were obtained from the Centraalbureau voor Schimmelcultures (CBS) culture collection in Utrecht, the Netherlands. DNA analyses were done on all isolates listed in Table 1. The FastDNA kit (BIO 101, Carlsbad, California) was used according to the manufacturer's instructions to isolate genomic DNA from 200–400 mg fungal mycelia grown on MEA plates for 8 days at 24 °C.

Degenerate primer development and screening of *Cercospora* isolates

The primer pairs, MAT1-1F/R, and MAT1-2F/R, described by Waalwijk *et al*. (2002) for the screening of the *MAT1-1-1* and *MAT1-2* genes, respectively, of *M. graminicola*, as well as the degenerate *MAT1-2* primers, ChHMG1 and ChHMG2 described by Arie *et al*. (1997), were used in an attempt to amplify part of the mating type genes of *C. beticola*. The amplifications were done according to the authors' instructions, and additional annealing temperatures (47 and 50 °C) were tested.

The *MAT1-1-1* sequences of *M. graminicola* (GenBank accession no. AF440399), *S. passerinii* (GenBank accession no. AF483193) and *M. fijiensis* (E.C.A. Abeln, *unpublished data*) and the *MAT1-2* sequences of *M. graminicola* (GenBank accession no. AF440398), *S. passerinii* (GenBank accession no. AF483194) and *M. fijiensis* (E.C.A. Abeln, *unpublished data*) were aligned using MegAlign from the Lasergene package (DNASTAR, Madison, Wisconsin). Two sets of degenerate primers were designed from this alignment, one set in a conserved region of the *MAT1-1-1* (MgMfSpMat1-1f1: 5'-CATTNGCNCATCCCTTTG-3' and MgMfSpmat1-1r2: 5'-GGCTTNGANACCATGGTGAG-3') and the other in a conserved region of the *MAT1-2* (MgMfSpmat1-2f2: 5'-CAAAGAANGCNTTCNTGATCT-3' and MgMfSpmat1-2r1: 5'-TTCTTCTCNGATGGCTTGC-3') gene. Initially, five randomly selected *C. beticola* isolates from the German population were screened with these two primer sets in order to amplify a partial region of the *MAT1-1-1* or *MAT1-2* genes.

The same PCR conditions were used for the amplification of both partial mating type genes. The reaction mixtures had a total volume of 12.5 µl and contained 0.7 µl of diluted gDNA, 1× PCR buffer (Bioline, London, UK), 48 µM of each of the dNTPs, 8 pmol of each degenerate primer, 1.5 mM $MgCl_2$ and 0.7 units *Taq* polymerase (Bioline). The amplification reactions were done on a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, California). The initial denaturation step was done at 94 °C for 5 min, followed by 15 cycles of 94 °C (20 s), 52 °C (20 s) and 72 °C (50 s), followed by 25 cycles of 94 °C (20 s), 50 °C (20 s) and 72 °C (50 s). A final elongation step at 72 °C (5 min) was included in the run. The PCR products obtained were separated by electrophoresis at 80 V for 1 h on a 1 % (w/v) agarose gel containing 0.1 µg/ml ethidium bromide in 1× TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualized under UV light. Amplicons were sequenced in both directions using the PCR primers and a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Roosendaal, Netherlands) according to the manufacturer's recommendations. The products were analyzed on an ABI Prism 3700 DNA Sequencer (Applied Biosystems).

Table 1. *Cercospora* isolates included in this study.

[a] Strains and Accession numbers	Host genus	Origin	Collector	GenBank number (*MAT1-1-1*; *MAT1-2*)
C. acaciae-mangii				
CPC 10527	*Acacia*	Thailand	K. Pongpanich	— ;DQ264749
C. achyranthis				
CPC 10091	*Achyranthes*	Korea	H.D. Shin	DQ264733; —
C. apii				
CPC 5057; CBS 257.67	*Helianthemum*	Romania	O. Constantinescu	DQ264734; —
CPC 5086; CBS 119.25	*Apium*	—	G.H. Coons	DQ264735; —
[b] CPC 11556; CBS 116455	*Apium*	Germany	K. Schrameyer	DQ264736; —
C. "apii"				
CPC 5329; CBS 115536	*Cajanus*	South Africa	L. van Jaarsveld	— ;DQ264750
CPC 5365; CBS 114817	*Fuchsia*	New Zealand	C.F. Hill	DQ264737; —
CPC 5366; CBS 115060	*Gaura*	New Zealand	C.F. Hill	— ;DQ264751
C. apiicola				
CPC 10266	*Apium*	Venezuela	N. Pons	— ;DQ264753
[b] CPC 10267; CBS 116457	*Apium*	Venezuela	N. Pons	— ;DQ264752
C. berteroae				
CPC 5090; CBS 538.71	*Berteroa*	Romania	O. Constantinescu	— ;DQ264754
C. beticola				
CPC 5065; CBS 548.71	*Malva*	Romania	O. Constantinescu	— ;DQ264755
CPC 5069; CBS 125.31	*Beta*	Japan	—	— ;DQ264756
CPC 5128	*Beta*	New Zealand	C.F. Hill	— ;DQ264757
CPC 5125	*Beta*	New Zealand	C.F. Hill	DQ264738; —
[b] CPC 12190	*Beta*	Germany	S. Mittler	— ;DQ192582
[b] CPC 12191	*Beta*	Germany	S. Mittler	DQ192581; —
C. canescens				
CPC 1138; CBS 111134	*Vigna*	South Africa	S. van Wyk	DQ264739; —
C. erysimi				
CPC 5361; CBS 115059	*Erysimum*	New Zealand	C.F. Hill	DQ264740; —
C. ipomoeae-pedis-caprae				
CPC 10094	*Ipomoea*	Korea	H.D. Shin	— ;DQ264758
C. kikuchii				
CPC 5067; CBS 135.28	*Glycine*	Japan	H.W. Wollenweber	DQ264741; —
C. lactucae-sativae				
CPC 10082	*Ixeris*	Korea	H.D. Shin	— ;DQ264759
C. malvacearum				
CPC 5066; CBS 126.26	*Malva*	—	C. Killian	DQ264742; —
C. modiolae				
CPC 5115	*Modiola*	New Zealand	C.F. Hill	— ;DQ264760

Table 1. Continued.

[a] Strains and Accession numbers	Host genus	Origin	Collector	GenBank number (*MAT1-1-1*; *MAT1-2*)
C. penzigii				
CPC 4001	*Citrus*	Swaziland	M.C. Pretorius	DQ264743; —
CPC 4410; CBS 115482	*Citrus*	South Africa	M.C. Pretorius	DQ264744; —
C. polygonaceae				
CPC 10117	*Persicaria*	Korea	H.D. Shin	DQ264745; —
C. violae				
CPC 5079; CBS 251.67	*Viola*	Romania	O. Constantinescu	DQ264746; —
C. zeae-maydis				
[b] CBS 117758	*Zea*	Iowa, U.S.A.	B. Fleener	DQ264747; —
[b] CBS 117760	*Zea*	Pennsylvania, U.S.A.	B. Fleener	— ;DQ264761
C. zeina				
[b] CPC 11995	*Zea*	South Africa	P. Caldwell	— ;DQ264762
[b] CPC 11998	*Zea*	South Africa	P. Caldwell	DQ264748; —
Cercospora sp.				
CPC 5126	*Oenothera*	New Zealand	C.F. Hill	— ;DQ264763
CPC 10627	*Delairea*	South Africa	C.L. Lennox	— ;DQ264764
CPC 12062	*Zea*	South Africa	—	— ;DQ264765

[a] CBS: Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CPC: Collection of Pedro Crous, housed at CBS.
[b] Strains used for characterization of full-length *MAT1-1-1* and *MAT1-2* sequences.

A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package.

The degenerate primers and the amplification and sequencing conditions described above were also used to screen *C. apii*, *C. apiicola*, *C. zeae-maydis* and *C. zeina* isolates to obtain portions of their mating type genes.

Isolation and characterization of *Cercospora MAT1-1-1* and *MAT1-2* genes

Internal primers were designed in the partially sequenced *MAT1-1-1* and *MAT1-2* genes for each of the species. These internal primers were used together with the appropriate primers from the DNA walking speedup kit (Seegene Inc., Rockville, USA) to determine additional sequences upstream and downstream of the partial sequences in order to obtain the full-length genes. In total, 57 primers were designed and used for the chromosome walking. Blastx (Altschul *et al*., 1997) was used to compare the sequences obtained from the five *Cercospora* species with protein sequences of other fungi present in the NCBI non-redundant protein database. The geneid v1.2 web server (http://www1.imim.es/geneid.html - Research Unit on Biomedical Informatics of IMIM, Barcelona, Spain) was used to predict the gene and intron / exon boundaries using the genetic code of *Neurospora crassa*. The conversion of DNA sequences to putative amino acid sequences was done using the translate tool of ExPASy (Gasteiger *et al*., 2003). The percentage identities between the predicted *MAT1-1-1* and *MAT1-2* gene sequences for the

different *Cercospora* species were calculated using the alignment tool of ALIGN (Pearson *et al*., 1997).

Obtaining partial MAT sequences of additional *Cercospora* isolates

Cercospora-specific primers for the mating type genes were designed from the aligned sequences of *C. apii*, *C. apiicola*, *C. beticola*, *C zeae-maydis*, and *C. zeina*. The aligned *MAT1-1-1* sequences included *C. beticola*, *C. apii*, *C. zeae-maydis* and *C. zeina* (GenBank accession nos. DQ192581, DQ264736, DQ264747 and DQ264748, respectively). The aligned *MAT1-2* sequences included those of *C. beticola*, *C. apiicola*, *C. zeae-maydis* and *C. zeina* (GenBank accession nos. DQ192582, DQ264752, DQ264761 and DQ264762, respectively). The sequences of each gene were aligned using MegAlign from the Lasergene package (DNASTAR). To robustly amplify partial *Cercospora* mating type genes, the primers CercosporaMat1f (5'-CTTGCAGTGAGGACATGG-3') and CercosporaMat1r (5'-GAGGCCATGGTGAGTGAG-3') were designed from the conserved regions of the *MAT1-1-1* gene, and primers CercosporaMat2f (5'-GATNTACCNTCTCGACCTC-3') and CercosporaMat2r (5'-CTGTGGAGCAGTGGTCTC-3') were designed from the conserved regions of the *MAT1-2* gene. Twenty-six additional *Cercospora* isolates representing species that belong to the *C. apii* complex (Table 1) were screened with the CercosporaMat1 and CercosporaMat2 primer sets in two separate amplification reactions.

For amplification of the *MAT1-1-1* and *MAT1-2* gene regions, primer concentrations were halved and the other reagent concentrations were as described above. The initial denaturation was done at 94 °C for 5 min, followed by 20 cycles of 94 °C (20 s), 58 °C (20 s) and 72 °C (50 s), followed by 20 cycles of 94 °C (20 s), 55 °C (20 s) and 72 °C (50 s). A final elongation step at 72 °C (5 min) was included. The obtained PCR products were visualized and sequenced as described above.

Phylogenetic analyses and protein alignment

The partial *MAT1-1-1* and *MAT1-2* sequences of the *Cercospora* isolates were analyzed using the mating type gene sequences of *M. graminicola* (GenBank accession nos. AF440399 and AF440398, respectively) and *S. passerinii* (GenBank accession nos. AF483193 and AF483194, respectively) as outgroup taxa. All phylogenetic analyses were done in PAUP (Phylogenetic Analysis Using Parsimony) v4.0b10 (Swofford, 2003). Maximum parsimony and neighbor-joining analyses were conducted as described by Groenewald *et al*. (2005). All sequences generated were deposited in GenBank (Table 1), and the alignments and trees were deposited in TreeBASE (TreeBASE accession no. M2871).

Amino acid sequences of the alpha domain (MAT1) and/or HMG domain (MAT2) of *M. graminicola* and *S. passerinii* were downloaded from NCBI's GenBank database. The downloaded amino acid sequences of both of the mating type proteins were aligned to that of the five *Cercospora* species using Sequence Alignment Editor v2.0a11 (Rambaut, 2002).

Mating type distribution in *Cercospora* populations

The two primer pair sets, CercosporaMat1 and CercosporaMat2, were used in a multiplex PCR to screen for the presence of the two mating type genes in the *C. apii*, *C. apiicola*, *C. beticola*,

C. zeae-maydis and *C. zeina* populations. Reagent concentrations were as described above and all four primers were present at equal concentrations. The initial denaturation step was done at 94 °C for 5 min, followed by 40 cycles of 94 °C (20 s), 60 °C (30 s) and 72 °C (50 s); a final elongation step at 72 °C (5 min) was included. The products were separated on a 1 % agarose gel and visualized as described above. The mating type frequency and the *MAT1-1-1/MAT1-2* ratios were calculated for each population.

RESULTS

MAT1-1-1 isolation and characterization in *Cercospora* species

The MAT1-1F and MAT1-1R primers that were designed to amplify part of the *MAT1-1-1* of *M.graminicola* (Waalwijk *et al*., 2002) were not successful in amplifying the mating type 1 region of *C. beticola*. The degenerate primers, MgMfSpMAT1-1f1 and MgMfSpMAT1-1r2, designed from the *M. graminicola*, *S. passerinii* and *M. fijiensis* sequences, amplified a fragment of 922 bp for three of the five *C. beticola* isolates tested (Fig. 1). The fragment obtained from strain CPC 12191 was sequenced, and the translated sequence showed 77 % identity to a 57 amino acid region of the *S. passerinii* MAT1 protein and 54 % identity to a 57 amino acid region as well as 34 % identity to a 82 amino acid region of the *M. graminicola* MAT1 protein using Blastx on the GenBank database. This confirmed that the 922 bp fragment is part of the *MAT1-1-1* gene of *C. beticola*. A homologous fragment was also obtained from *C. apii*, *C. zeae-maydis* and *C. zeina* isolates during the first round of amplification using the MgMfSpMAT1-1f1 and MgMfSpMAT1-1r2 degenerate primers. The *C. apiicola* population of 47 isolates, as well as 11 additional *C. apiicola* isolates, that were obtained from Greece, Korea and Venezuela and used in previous studies by Groenewald *et al*. (2005, 2006), were screened for the presence of the mating type genes, but all isolates were found to only contain the *MAT1-2* gene.

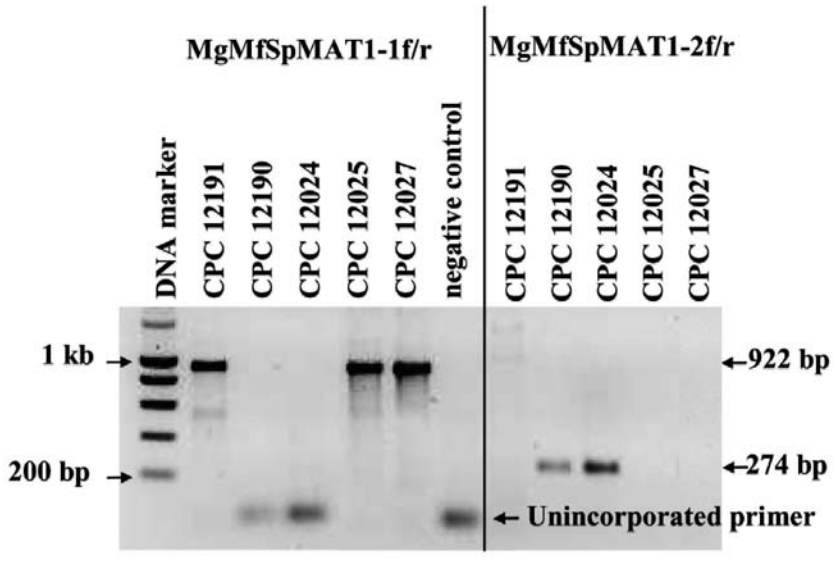


Fig. 1. Amplification products obtained from *Cercospora beticola* isolates containing the *MAT1-1-1* (922 bp) and *MAT1-2* (274 bp) genes using the degenerate primer pairs MgMfSpMAT1-1 and MgMfSpMAT1-2 respectively.

The full-length *MAT1-1-1* gene sequences for all four *Cercospora* species were obtained by chromosome walking. The geneid software predicted that the *MAT1-1-1* sequences of all four species contain four exons (Fig. 2). Although the number of amino acids was the same for all three species (335 aa), several differences were observed between the *MAT1-1-1* of the two maize pathogens and that of *C. apii* and *C. beticola*. The predicted length of the genes, as well as exon and intron positions are illustrated in Fig. 2 and the percentage sequence similarities between the different *Cercospora* species are listed in Table 2. Perfect lariat sequences (RCTRAC) (Bruchez *et al*., 1993) were present in the introns of all four *Cercospora* species, except in the first intron of *C. beticola* and *C. apii*, that contained a GCTGAT sequence starting at 16 nt upstream from the likely 3' splice site. The number of predicted introns (two) in the conserved alpha domain region of the *Cercospora* species studied correlates with the number predicted for the same region in *M. graminicola* (Waalwijk *et al*., 2002) and *S. passerinii* (Goodwin *et al*., 2003).

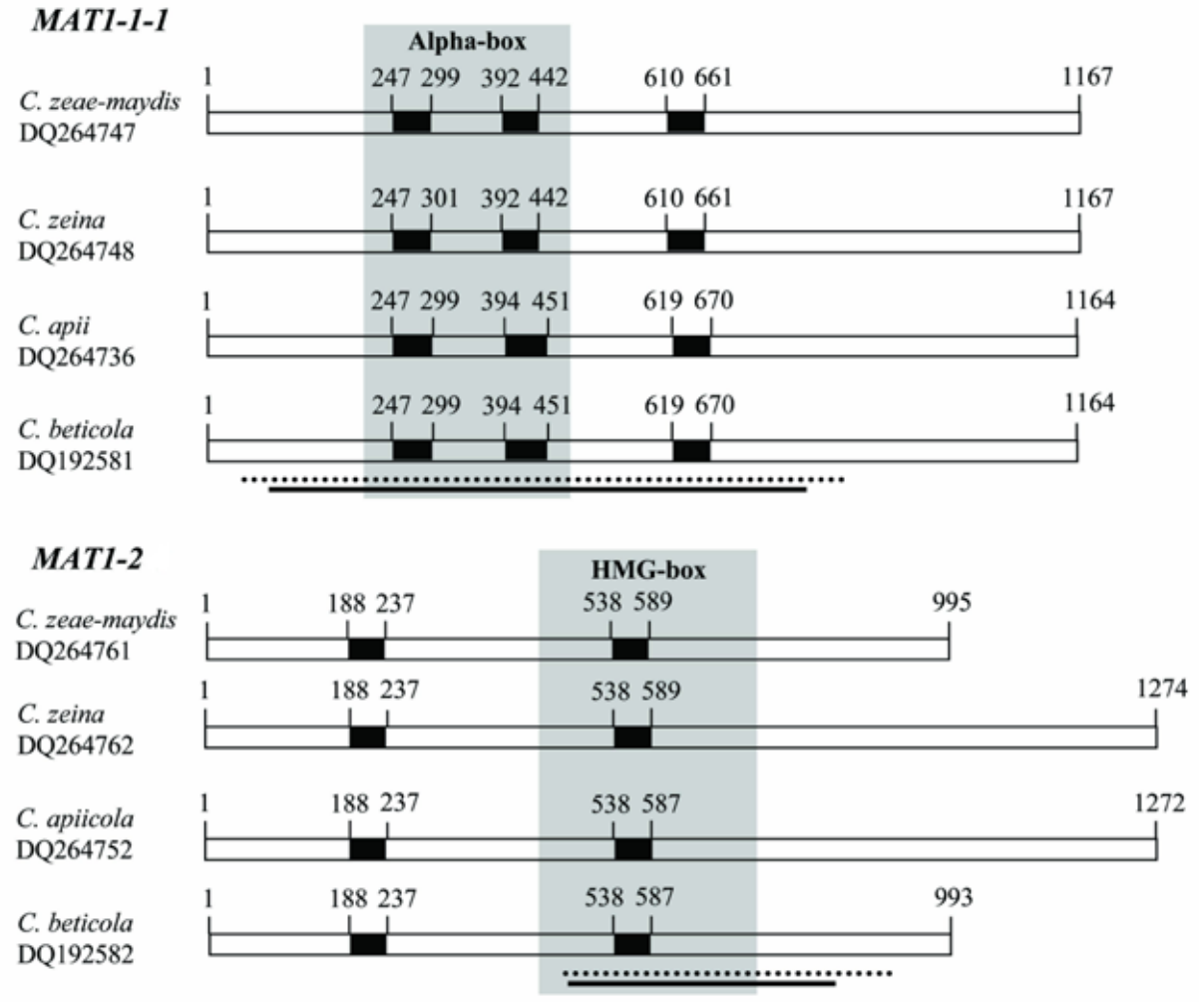


Fig. 2. Diagrammatic representation of the full-length mating type genes of *Cercospora zeae-maydis, C. zeina, C. apiicola, C. apii* and *C. beticola*. The predicted sites of exons (white bars), and introns (black bars) are shown, and their locations (nucleotide position) are indicated. The lines at the bottom of each diagram indicate the area amplified by the CercosporaMat1 and CercosporaMat2 primer sets (dotted line) and the area used for the phylogenetic analyses (solid black line).

Table 2. Percentage nucleotide identity across the whole *MAT1-1-1* (upper right triangle) and *MAT1-2* (lower left triangle) genes between the *Cercospora* species studied.

	C. zeae-maydis	*C. zeina*	*C. apiicola*	*C. apii*	*C. beticola*
C. zeae-maydis	—	92.6	NA	87.4	87.3
C. zeina	74.5	—	NA	87.3	87.2
C. apiicola	70.3	90.8	—	NA	NA
C. apii	NA	NA	NA	—	99.9
C. beticola	90.2	70.6	76.4	NA	—

NA = Not available due to the absence of the specific gene in the isolates tested.

MAT1-2 isolation and characterization in *Cercospora* species

The *MAT1-2* region in the *C. beticola* genome could not be amplified using the MAT1-2F and MAT1-2R primers of *M. graminicola* (Wang *et al*., 1998) nor using the degenerate ChHMG1 and ChHMG2 primers of Arie *et al*. (1997). The degenerate primers (MgMfSpMAT1-2f2 and MgMfSpMAT1-2r1) designed in this study resulted in a 274 bp PCR product in those *C. beticola* isolates of the test panel which did not amplify with the MgMfSpMAT1-1f1 and MgMfSpMAT1-1r2 primers (Fig. 1). The fragment obtained from CPC 12190 was sequenced and the translated sequence showed 59 % identity to a 76 amino acid region of the *S. passerinii* MAT2 protein and 61 % identity to a 76 amino acid region of the *M. graminicola* MAT2 protein using Blastx. This confirmed that a part of the *MAT1-2* gene of *C. beticola* had been amplified using the newly developed degenerate primers.

A 274 bp fragment was also amplified in three of the additional four *Cercospora* species (*C. apiicola*, *C. zeae-maydis* and *C. zeina*) using the degenerate primers. A *C. apii* population of 32 isolates as well as 17 additional *C. apii* isolates, that were obtained from different countries and used in previous studies by Groenewald *et al*. (2005, 2006), were screened for the presence of the mating type genes, but only the *MAT1-1-1* gene was found. The sequence of these products corresponded with the *MAT1-2* sequence found for *C. beticola*. Chromosome walking enabled us to obtain the full-length *MAT1-2* genes of *C. apiicola, C. beticola*, *C. zeae-maydis* and *C. zeina*. The predicted length of the genes, as well as exon and intron positions are illustrated in Fig. 2. Both introns in all four *MAT1-2* genes contain a perfect lariat sequence (RCTRAC). The predicted presence of a single intron in the conserved HMG domain region of the *Cercospora* species corresponded with the predicted intron for the same region in *M. graminicola* (Waalwijk *et al*., 2002) and *S. passerinii* (Goodwin *et al*., 2003).

The percentage sequence identities between the different *Cercospora* species are listed in Table 2. Because the putative *MAT1-2* gene of *C. beticola* and *C. zeae-maydis* is much shorter than that of the other species, the similarities among the *MAT1-2* sequences vary greatly. The high similarity (90.2 %) between *C. zeae-maydis* and *C. beticola* is largely due to their similarity in number of nucleotides. The number of amino acids predicted for the MAT2 protein of *C. beticola* and *C. zeae-maydis* was 299, whereas for *C. zeina* and *C. apiicola* it was 392 amino acids.

Partial *MAT1-1-1* and *MAT1-2* sequences from additional *Cercospora* species

The *Cercospora*-specific mating type primer sets CercosporaMat1 and CercosporaMat2 were successful in amplifying a portion (location indicated with a dashed black line in Fig. 2) of the *MAT1-1-1* or the *MAT1-2* genes, respectively, of 26 additional *Cercospora* isolates representing 17 putative species. The primer pair CercosporaMat1f and CercosporaMat1r amplified a fragment of approximately 805 bp in half of the isolates tested, and the CercosporaMat2f and CercosporaMat2r primer set a 442 bp fragment in the rest of the isolates (Fig. 3). These sequences, which included the alpha and the HMG domain respectively, were aligned with the corresponding *MAT* regions of the *Cercospora* species characterized in this study. The sequences were of relatively high similarity, even in the variable regions flanking the conserved domains (alignments available in TreeBASE accession number M2871).

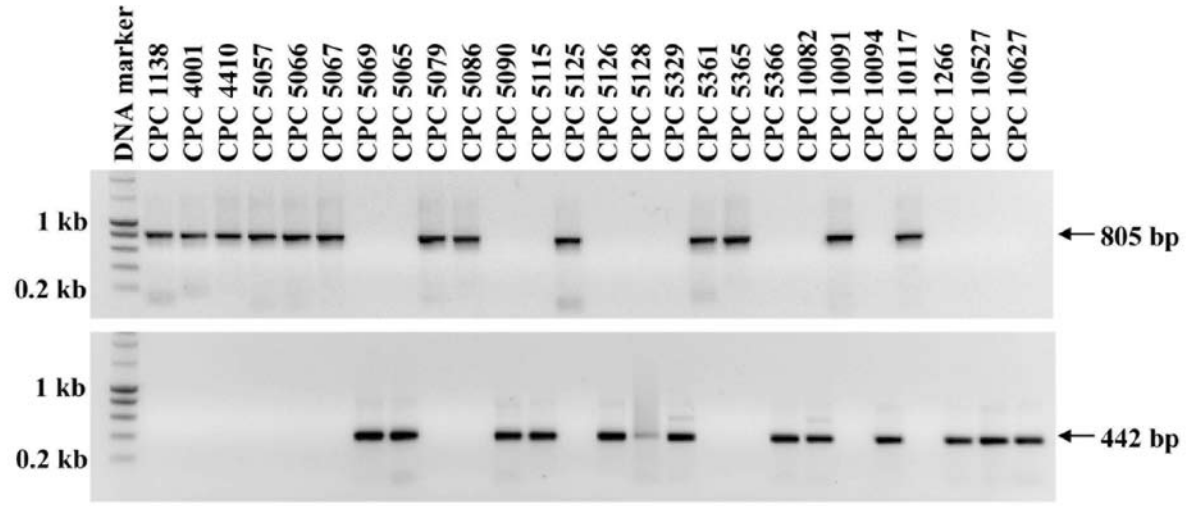


Fig. 3. Different *Cercospora* species screened using the CercosporaMat1 primer set (805 bp fragment; top part of photo) and the same *Cercospora* isolates screened with the CercosporaMat2 primer set (442 bp fragment; lower part of photo).

Phylogenetic analyses of nucleic acid sequences

The *MAT1-1-1* alignment (TreeBASE accession number M2871) contained 19 taxa, including the two outgroups, and 702 characters, including alignment gaps. Of these characters, 290 were constant, 139 were variable and parsimony-uninformative, and 273 characters were parsimony-informative. The *MAT1-2* alignment (TreeBASE accession number M2871) contained 20 taxa, including the two outgroups, and 362 characters, including alignment gaps. Of these characters, 181 were constant, 68 were variable and parsimony-uninformative, and 113 characters were parsimony-informative.

Similar trees were obtained irrespective of whether neighbor-joining or parsimony was used. Five most parsimonious trees were obtained from the *MAT1-1-1* sequences, and three most parsimonious trees were obtained from the *MAT1-2* sequences. The most parsimonious trees differed somewhat in the arrangement of the taxa within the clade containing the *C. apii* complex (Fig. 4). Limited variation was observed among the isolates belonging to the *C. apii* complex, and these isolates clustered together with bootstrap support values of 100 % (*MAT1-1-1*) and

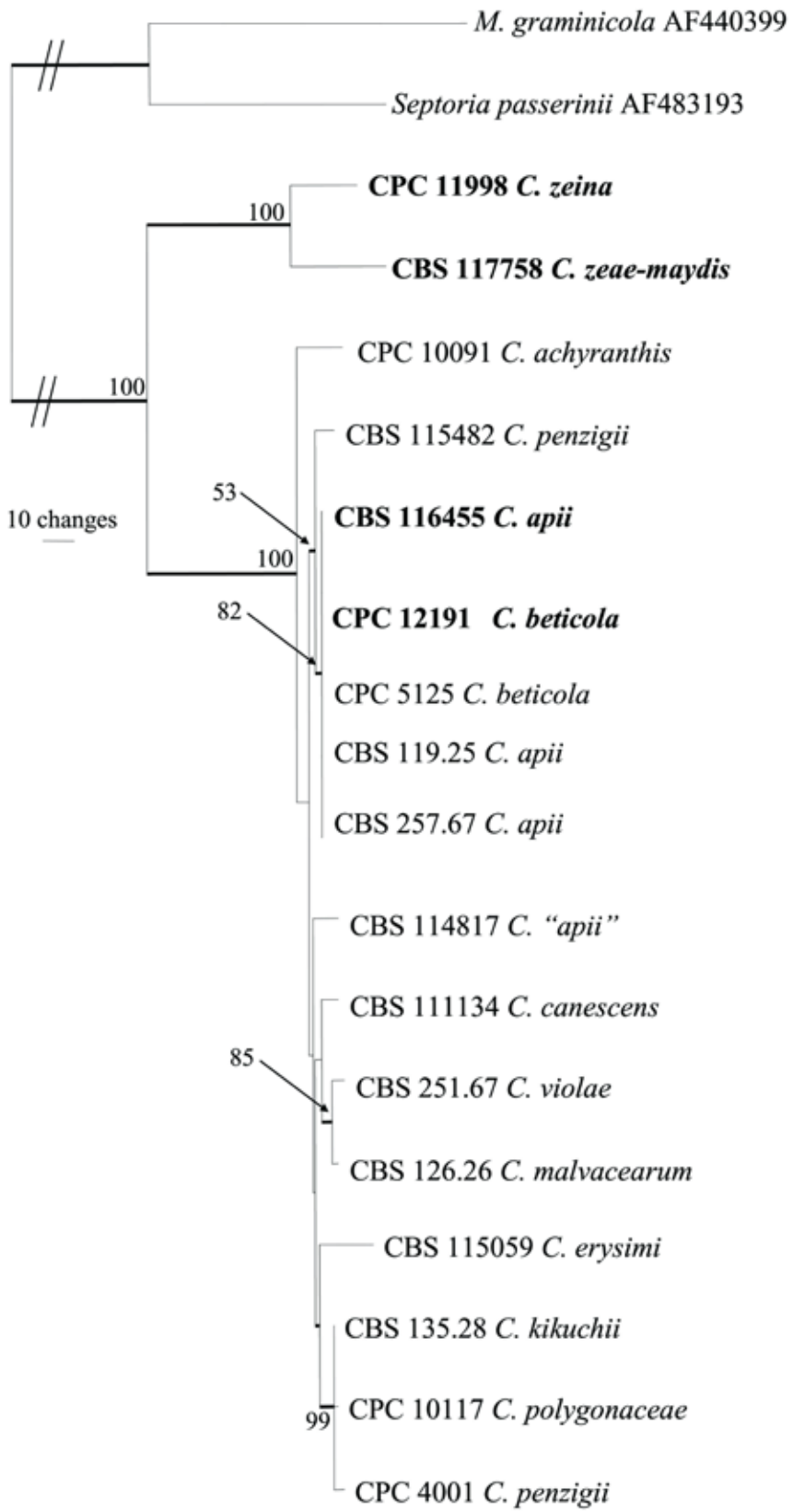


Fig. 4. A, one of five most parsimonious trees obtained from the *MAT1-1-1* sequence alignment. Bootstrap support values from 1,000 replicates are shown at the nodes. The tree was rooted to *Mycosphaerella graminicola* (AF440399) and *Septoria passerinii* (AF483193). (Tree length = 622 steps, CI = 0.904, RI = 0.857, RC = 0.774). Thickened lines indicate the strict consensus branches. Labels in boldface represent species for which full-length genes were sequenced.

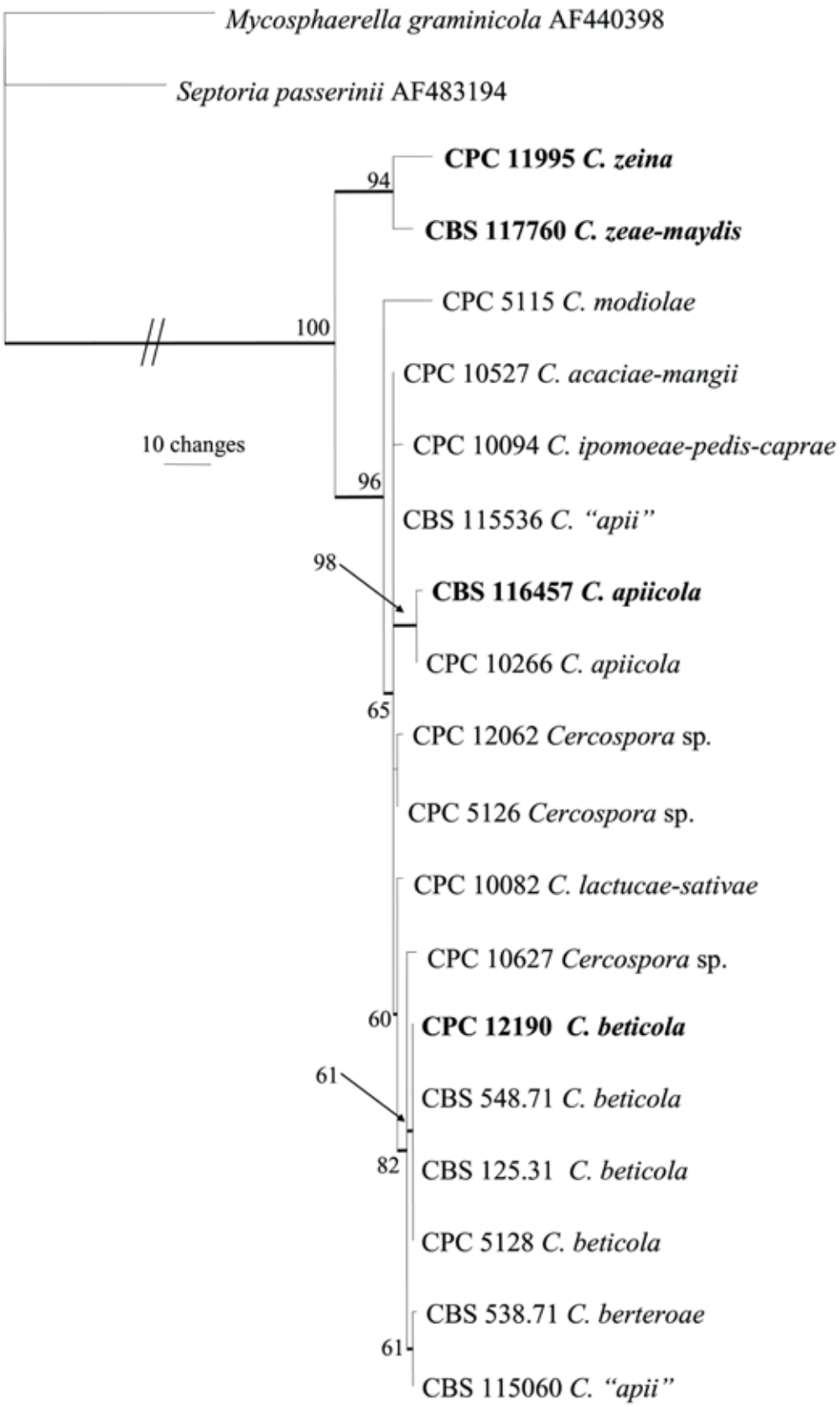


Fig. 4. B, one of three most parsimonious trees obtained from the *MAT1-2* sequence alignment. Bootstrap support values from 1,000 replicates are shown at the nodes. The tree was rooted to *Mycosphaerella graminicola* (AF440398) and *Septoria passerinii* (AF483194). (Tree length = 247 steps, CI = 0.943, RI = 0.917, RC = 0.865). Thickened lines indicate the strict consensus branches. Labels in boldface represent species for which full-length genes were sequenced.

96 % (*MAT1-2*). The trees obtained for both the *MAT1-1-1* and *MAT1-2* datasets showed that the two isolates that do not belong to the *C. apii* complex, namely *C. zeae-maydis* and *C. zeina*, group together with a 100 % bootstrap support for *MAT1-1-1* and 94 % bootstrap support for *MAT1-2*. The phylogenetic trees obtained from these sequences are congruent with the main groupings of the housekeeping gene trees published for the *Cercospora* species (Crous *et al*., 2006; Groenewald *et al*., 2005, 2006).

The *MAT1-1-1* phylogeny showed that all the isolates from *C. apii* (CBS 116455, CBS 119.25 and CBS 257.67) and *C. beticola* (CPC 5125 and CPC 12191) group together with a bootstrap support value of 82 % (Fig. 4A). The unnamed *Cercospora* sp. from maize (CPC 12062) did not group with the other maize isolates in the *MAT1-2* analysis, but it did group with the rest of the *Cercospora* isolates with a bootstrap support value of 96 % (Fig. 4A). The analyses of the *MAT1-1-1* sequences showed that the isolate from *Helianthemum* (CBS 257.67) identified as *C. apii* in an earlier study (Groenewald *et al*., 2006) grouped together with the other *C. apii* isolates obtained from celery (CBS 116455 and CBS 119.25) (Fig. 4B). The analysis using the *MAT1-2* dataset showed that the isolate from *Malva* (CBS 548.71) and identified as *C. beticola* using sequence data (Groenewald *et al*., 2006) grouped with the *C. beticola* isolates (CBS 125.31, CPC 5128, CPC 12190) from sugar beet (Fig. 4B).

Comparison of predicted amino acid sequences

The predicted amino acid sequences in the alpha (MAT1) and HMG (MAT2) domain showed very high similarity among the four *Cercospora* species (Fig. 5A). For the alpha domain only three amino acid changes were detected between *C. beticola* and *C. zeina*, and only two between *C. beticola* and *C. zeae-maydis.* The amino acid compositions of the alpha domain of *C. beticola* and *C. apii* were identical. For the HMG domain, two amino acid changes were predicted between *C. beticola* and each of *C. zeae-maydis*, *C. apiicola* and *C. zeina* (Fig. 5B). The *C. beticola* predicted amino acid sequences showed moderate identitiy (Fig. 5) to the alpha domain (MAT1) and HMG domain (MAT2) regions of *S. passerinii* (53.6 % and 67.5 %, respectively) and *M. graminicola* (57.1 % and 67.5 %, respectively).

Distribution of *MAT1-1-1* and *MAT1-2* in *Cercospora* populations

A total of 255 *C. beticola* isolates (46 from France, 41 from Germany, 33 from Italy, 48 from the Netherlands, 50 from Iran and 37 from New Zealand) were screened with a multiplex PCR assay using primer pairs CercosporaMAT1-1F/R (805 bp fragment) and CercosporaMAT1-2F/R (442 bp fragment). Each tested isolate showed either the 442-bp fragment or the 805-bp fragment of the respective *MAT1-1-1* or *MAT1-2* genes, and no isolate showed both fragments. The *MAT1-1-1* and *MAT1-2* genes were equally distributed in most of the *C. beticola* populations. The ratios were in most cases near to 1.00 (0.85 – 1.19), except for the Italian population, in which a ratio of 0.50 was found (Table 3). There was no significant deviation ($P < 0.05$) from a 1:1 ratio for the *MAT1-1-1*:*MAT1-2* ratio calculated for each of the populations tested.

A total of 43 *C. zeae-maydis*, 49 *C. zeina*, 32 *C. apii* and 47 *C. apiicola* isolates were screened for the presence of the mating type genes, and no isolate showed both fragments. The *MAT1-1-1* and *MAT1-2* genes were distributed in the *C. zeae-maydis* and *C. zeina* populations at observed *MAT1-1-1*:*MAT1-2* ratios of 0.95 and 1.58, respectively, which did not differ

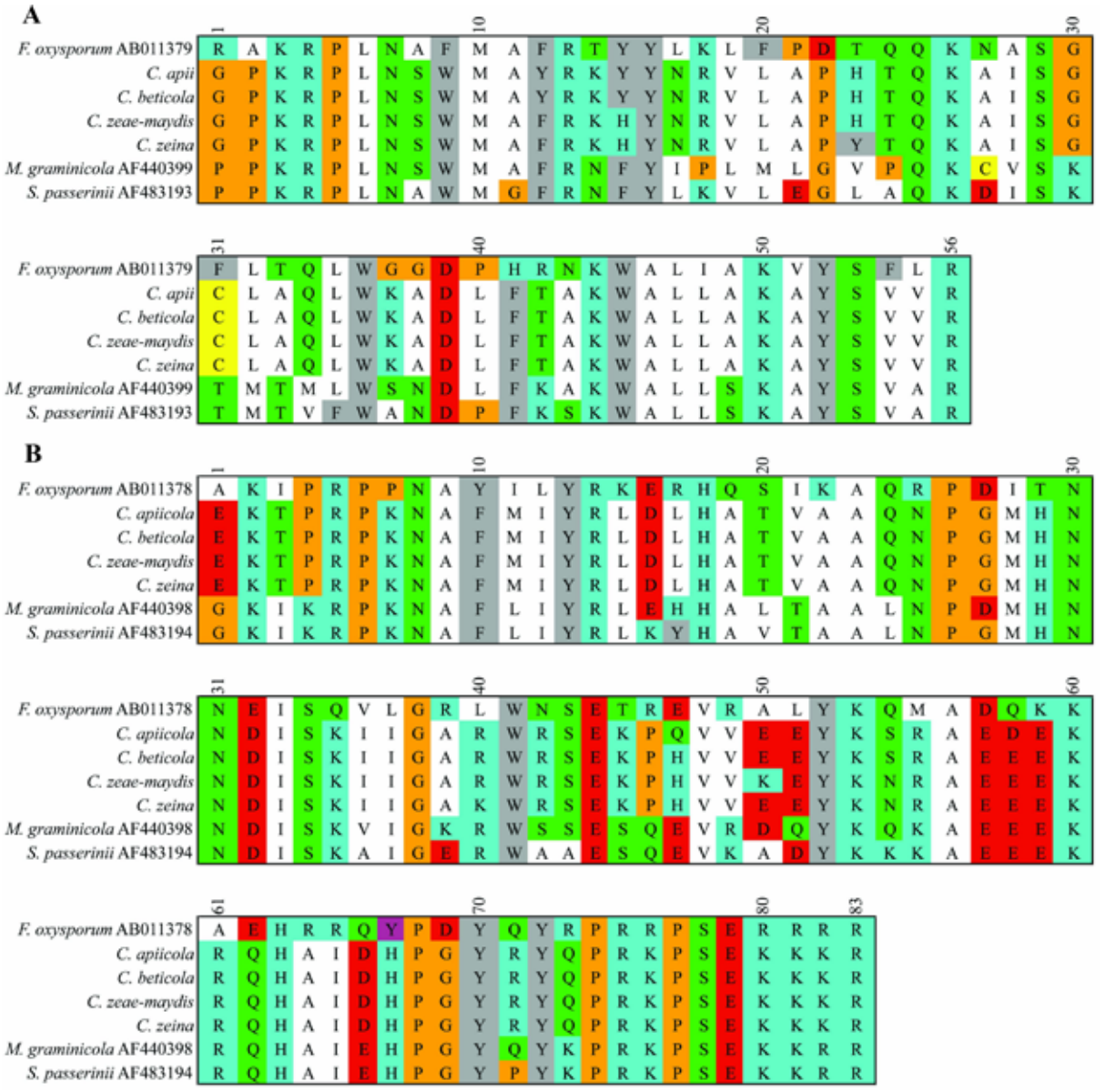


Fig. 5. Protein sequence alignments of the conserved **A**, alpha domain and **B**, HMG domain of the mating type genes of *Cercospora* species and closely related fungi.

($P < 0.05$) from the expected 1:1 ratio based on Chi-square analyses (Table 3). All of the *C. apiicola* isolates obtained from Venezuela were found to be *MAT1-2*, whereas all the *C. apii* isolates obtained from Germany were found to be *MAT1-1-1*.

DISCUSSION

Very little is known about the occurrence or importance of sex in apparently asexual species of *Cercospora*. During this study the mating type genes of a sugar beet pathogen, *C. beticola*, two celery pathogens, *C. apii* and *C. apiicola*, and two maize pathogens, *C. zeae-maydis* and *C. zeina*, were sequenced and characterized. The degenerate primer sets MgMfSpMAT1-1 and MgMfSpMAT1-2 successfully amplified a portion of the mating type genes, and these sequences led to the characterization of the full-length *MAT1-1-1* and/or *MAT1-2* sequences

Table 3. Occurrence and frequency of the *MAT1-1-1* and *MAT1-2* genes in *Cercospora* populations. The numbers in brackets represent the frequency of the gene.

Populations (country; region)	N[a]	*MAT1-1-1*	*MAT1-2*	Ratio[b]	χ^{2c}	*P*[d]
C. beticola (France; Longvic)	46	25 (0.54)	21 (0.46)	1.19	0.35	0.55
C. beticola (Germany; Niedersachsen)	41	22 (0.54)	19 (0.46)	1.16	0.22	0.64
C. beticola (Italy; Ravenna)	33	11 (0.33)	22 (0.67)	0.5	3.77	0.05
C. beticola (Netherlands; Bergen op Zoom)	48	22 (0.46)	26 (0.54)	0.85	0.33	0.57
C. beticola (Iran; Pakajik)	50	26 (0.52)	24 (0.48)	1.08	0.08	0.78
C. beticola (New Zealand; Unknown)	37	19 (0.51)	18 (0.49)	1.06	0.03	0.86
C. zeae-maydis (USA; Iowa)	43	21 (0.49)	22 (0.51)	0.95	0.02	0.89
C. zeina (South Africa; KwaZulu-Natal)	49	30 (0.61)	19 (0.39)	1.58	2.5	0.11
C. apiicola (Venezuela; Caripe)	47	0 (0)	47 (1)	[e]	62.67	< 0.001
C. apii (Germany; Baden Württemberg)	32	32 (1)	0 (0)	[f]	58.33	< 0.001

[a]Number of isolates analyzed.
[b]*MAT1-1-1*:*MAT1-2* ratio.
[c]χ^2 value for the deviation from the expected 1:1 ratio.
[d]Probability of a greater χ^2 value under the null hypothesis of 1:1 ratio (1 degree of freedom).
[e]*MAT1-1-1* was not detected in *C. apiicola*.
[f]*MAT1-2* was not detected in *C. apii*.

of *Cercospora* species. Preliminary data reveal that these degenerate primer sets can also amplify the corresponding areas within the mating type genes of other species belonging to the *Mycosphaerellaceae* and allied *Davidiellaceae*. These species include some important pathogens of pines (*Dothistroma pini*, *D. septosporum*), tomatoes (*Passalora fulva*), bananas (*M. musicola, M. musae*), eucalypts (*M. marksii*, *M. thailandica*), or are important as agents in human health or food spoilage (*Cladosporium herbarum*), and will be treated elsewhere in future studies.

The *MAT1-1-1* gene characterized during this study contains an area that corresponds to a putative alpha domain of *MAT1-1-1*, and DNA sequences in the *MAT1-2* gene correspond to the HMG domain described from other ascomycetes. As illustrated in this and other studies, these two domains are also found in the mating type genes of a wide range of ascomycetes. The putative introns in these domains of the *Cercospora* mating type genes are also found in *M. graminicola* and *S. passerinii* (Waalwijk *et al*., 2002; Goodwin *et al*., 2003). However, additional introns are predicted in the areas flanking the conserved boxes of each of the respective genes for *Cercospora*. The number of putative introns also varies for the *MAT1-1-1* and *MAT1-2* genes of other ascomycetes. Species containing only one putative intron in both of these genes include *Alternaria alternata* (Arie *et al*., 2000), *Ascochyta rabiei* (Barve *et al*., 2003), *Cochliobolus heterostrophus* (Turgeon *et al*., 1993) and *Pyrenopeziza brassicae* (Singh & Ashby, 1998, 1999). *Fusarium oxysporum* (Arie *et al*., 2000), *Giberella fujikuroi* and *G. zeae* (Yun *et al*., 2000) have two introns in the *MAT1-2* region, whereas *Ophiostoma novo-ulmi* has one intron in the *MAT1-2* gene (Paoletti *et al*., 2005). The putative intron splicing sites and gene predictions of only a few filamentous ascomycetes, e.g., *A. alternata* (*MAT1-1-1* and *MAT1-2*), *F. oxysporum* (*MAT1-1-1* and *MAT1-2*) and *O. novo-ulmi* (*MAT1-2*), have been confirmed by mRNA studies. Further studies at the mRNA and protein level are necessary to confirm the

exact length of the coding regions as well as the intron and exon boundaries for the mating type genes of the *Cercospora* species.

The predicted length of the encoded proteins among different *MAT1-1-1* and *MAT1-2* genes of ascomycetes varies greatly (Pöggeler, 2001; Goodwin *et al*., 2003). Usually the MAT1 protein is much larger than the MAT2 protein of the same species. However, this is not the case for *M. graminicola*, where the predicted MAT1 protein (296 amino acids) is smaller than the predicted MAT2 protein (394 amino acids) (Waalwijk *et al*., 2002), and for *C. zeina* (predicted MAT1 = 339 amino acids and MAT2 protein = 392 amino acids).

Most protein coding genes used in previous taxonomic studies of *Cercospora* lack resolution to distinguish closely related *Cercospora* species (Groenewald *et al*., 2005, 2006). This study is the first to conduct phylogenetic analyses of partial mating type genes to determine whether they have sufficient discriminatory resolution between closely related *Cercospora* species, particularly those included in the *C. apii* complex. The *Cercospora* mating type-specific primer sets (CercosporaMat1 and CercosporaMat2) amplifies the three introns of *MAT1-1-1* and the intron that is present in the HMG domain of the *MAT1-2*. One of the biggest problems encountered when using *MAT* genes in phylogenetic analyses is that sometimes only one mating type is known in the species, or only one isolate of a species is available, and this isolate carries only one of the two mating type genes. This was the case for most of the *Cercospora* species tested, and these taxa could only be compared to taxa with sequences of the same mating type. Another problem is that the *MAT* gene sequences differ a great deal among different genera and even among species of the same genus. This may restrict analyses to related species and to only a small portion of the gene, specifically, to the more conserved regions (alpha or HMG domains) of these genes. The conserved regions may lack the resolution to distinguish among closely related species, as was the case within the group of isolates belonging to the *C. apii* complex and it is clear that the *MAT1-1-1* sequences cannot separate *C. apii* and *C. beticola.* Mating type genes therefore do not appear to represent promising loci for phylogenetic studies aimed at distinguishing cryptic species belonging to the *C. apii* complex.

Both mating type genes have been isolated from strains of *C. beticola*, *C. zeae-maydis* and *C. zeina*. The *Cercospora* mating type-specific primer sets (CercosporaMat1 and CercosporaMat2) can be used in a multiplex PCR assay for amplification of these two genes in *Cercospora* populations. The two mating types are approximately evenly distributed within the six sampled populations of *C. beticola* as well as in the *C. zeae-maydis* population in the USA and in the *C. zeina* population in South Africa, suggesting that the genes may be functional in these populations. If *C. beticola*, *C. zeae-maydis* and *C. zeina* were strictly asexual, we would expect that with time there would be a skewed distribution of the mating types, or perhaps only a single mating type would be found. Also, if these populations arose from a human introduction of a single genotype, we might expect only one mating type to be present, as was found for the *C. apii* and *C. apiicola* populations. The presence of both mating type genes in the USA population of *C. zeae-maydis* and the South African population of *C. zeina* further strengthens the hypothesis (Dunkle & Levy, 2000; Crous *et al*., 2006) that these species are native to North America and Africa, respectively. Though the teleomorph has not been confirmed for these three *Cercospora* species, we would expect their teleomorphs to be in the

genus *Mycosphaerella*. Detailed analyses have been done on the distribution of the mating types of *M. graminicola* and an equal distribution of the mating types were found in different populations of this sexually reproducing fungus (Waalwijk *et al*., 2002; Zhan *et al*., 2002). It is therefore probable that these *Cercospora* species that contain both mating types, are also able to reproduce sexually, but that the teleomorph is not readily observed in nature nor induced under laboratory conditions. However, Halliday & Carter (2003) found segregation of the mating types in natural populations of *Cryptococcus gattii* but, on studying the population structure using AFLP fingerprinting, did not find any evidence supporting genetic exchange between members of the population. These results indicated a clonal population structure even though both mating types were present. All attempts to obtain successful matings between these isolates failed, and the authors concluded that heterogeneity in genome composition resulted in mating incompatibility which gave rise to the clonal population structure (Halliday & Carter, 2003). Contrary to Halliday *et al*. (1999), who found severely skewed distributions of up to 30:1 for the mating types of some *Cryptococcus gattii* populations, all the *Cercospora* populations we sampled containing both mating types favored a 1:1 ratio, being more consistent with the distribution pattern observed for the sexually reproducing *M. graminicola*. A detailed study on the genetic population structure and the genome composition (for example chromosome number and genome size) of the *Cercospora* species characterized in this study is needed to further evaluate the effect of mating type distribution in these species.

Only the *MAT1-2* gene was present in the *C. apiicola* isolates tested, including isolates from Korea and Greece that were used in previous studies (Groenewald *et al*., 2005, 2006), as well as a field population of 47 isolates from Venezuela. Although it is possible that a *MAT1-1-1* gene may exist for this species, these data suggest that it would rarely occur, if it were to be present. Without sexual recombination, a species may not be able to rapidly evolve, and it is subsequently more difficult for these species to easily adapt to different environmental conditions. Alternatively, *C. apiicola* may be native to another part of the world, and the sampled populations may be introductions of a single mating type. The tested isolates of *C. apii sensu stricto* contained only the *MAT1-1-1* gene. Based on our current sampling, we predict that *C. apii* is asexual. However, more populations need to be studied, but due to the cultivation of celery under controlled greenhouse conditions we were unsuccessful in obtaining more populations. Unlike *C. apiicola, C. apii* has an extremely wide host range (Crous & Braun, 2003; Groenewald *et al*., 2006). The geographic origin of *C. apii* is Western Europe, whereas *C. apiicola* was originally described from Korea and Venezuela (Groenewald *et al*., 2005). Recently, Groenewald *et al*. (2006) showed that *C. apiicola* also occurs in Europe (i.e. Greece). As only one mating type has until now been found for *C. apii* (MAT1) and *C. apiicola* (MAT2), it is possible that these two species lack the ability to reproduce sexually due to the absence of the opposite mating type. If these species are homothallic, they will still be able to reproduce sexually. Our attempts to induce mating between isolates of *C. apii* have failed. In the sexually reproducing basidiomycetous yeast *Cryptococcus neoformans*, laboratory matings produce offspring with an equal distribution of the mating types (Kwon-Chung, 1976). However, in environmental and clinical isolates the majority of isolates belong to one mating type; yet they still retain their sexual reproductive potential by means of fruiting, a process of diploidization

followed by reduction to haploid basidiospores which results in a high rate of recombination (Lin *et al*., 2005). Similar methods of sexual recombination have not yet been observed or reported for the *Cercospora* species characterized here, and strictly asexual reproduction can not be ruled out.

Mating type genes play an important part in the biology and evolution of fungal species. Knowledge of these genes can provide insight in the potential prevalence of sex in species of *Cercospora*, the majority of which are currently thought to be asexual. The primers that were developed during this study allowed us to determine and characterize the mating type genes of several agronomically important *Cercospora* species. The even distribution of the mating types for most species studied here do not favor asexual reproduction; however, further studies are needed to determine whether recombination is taking place. The primers designed here will allow the identification and characterization of mating type genes, or portions thereof, of other important *Cercospora* species and other members of the Mycosphaerellaceae.

ACKNOWLEDGEMENTS

This research was financially supported by the CBS-Odo van Vloten Stichting, and the Royal Netherlands Academy of Arts and Sciences. The authors thank Dr. Gary Munkvold of Pioneer Hi-Bred International, Inc. who kindly supplied some of the isolates of *C. zeae-maydis*.

REFERENCES

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.

Astell, C.R., Ahlstrom-Jonasson, L., Smith, M., Tatchell, K., Nasmyth, K.A., and Hall, B.D. 1981. The sequence of the DNAs coding for the mating-type loci of *Saccharomyces cerevisiae*. Cell 27:15–23.

Arie, T., Kaneko, I., Yoshida, T., Noguchi, M., Nomura, Y., and Yamaguchi, I. 2000. Mating-type genes from asexual phytopathogenic ascomycetes *Fusarium oxysporum* and *Alternaria alternata.* Mol. Plant Microbe In. 13:1330–1339.

Arie, T., Christiansen, S.K., Yoder, O.C., and Turgeon, B.G. 1997. Efficient cloning of ascomycete mating type genes by PCR amplification of the conserved MAT-HMG box. Fungal Genet. Biol. 21:118–130.

Barve, M.P., Arie, T., Salimath, S.S., Muehlbauer, F.J., and Peever, T.L. 2003. Cloning and characterization of the mating type (*MAT*) locus from *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) and a *MAT* phylogeny of legume-associated *Ascochyta* spp. Fungal Genet. Biol. 39:151–167.

Blakeslee, A.F. 1904. Sexual reproduction in the Mucorinae. Proc. Am. Acad. Sci. 40:205–319.

Bobrowicz, P., Pawlak, R., Correa, A., Bel-Pedersen, D., and Ebbole, D.J. 2002. The *Neurospora crassa* pheromone precursor genes are regulated by the mating type locus and the circadian clock. Mol. Microbiol. 45:795–804.

Bruchez, J.J.P., Eberle, J., and Russo, V.E.A. 1993. Regulatory sequences in the transcription of *Neurospora crassa* genes: CAAT box, TATA box, introns, poly(A) tail formation sequences. Fungal Genet. Newslett. 40:89–96.

Crous, P.W., and Braun, U. 2003. *Mycosphaerella* and its anamorphs: 1. Names published in *Cercospora* and *Passalora*. CBS Biodivers. Series 1:1–571.

Crous, P.W., Aptroot, A., Kang, J.-C., Braun, U., and Wingfield, M.J. 2000. The genus *Mycosphaerella* and its anamorphs. Stud. Mycol. 45:107–121.

Crous, P.W., Kang, J.-C., and Braun, U. 2001. A phylogenetic redefinition of anamorph genera in *Mycosphaerella* based on ITS rDNA sequences and morphology. Mycologia 93:1081–1101.

Crous, P.W., Groenewald, J.Z., Pongpanich, K., Himaman, W., Arzanlou, M., and Wingfield, M.J. 2004. Cryptic speciation and host specificity among *Mycosphaerella* spp. occurring on Australian *Acacia* species grown as exotics in the tropics. Stud. Mycol. 50:457–469.

Crous, P.W., Groenewald, J.Z., Groenewald, M., Caldwell, P., Braun, U., and Harrington, T.C. 2006. Species of *Cercospora* associated with grey leaf spot of maize. Stud. Mycol. 55:189–197.

Du, M., Schardl, C.L., Nuckles, E.M., and Vaillancourt, L.J. 2005. Using mating-type gene sequences for improved phylogenetic resolution of *Colletotrichum* species complexes. Mycologia 97:641–658.

Dunkle, L.D., and Levy, M. 2000. Genetic relatedness of African and United States populations of *Cercospora zeae-maydis*. Phytopathology 90:486–490.

Fuckel, K.W.G.L. 1863. Fungi Rhenani exsiccati, Fasc. I-IV. Hedwigia 2:132–136.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R.D., and Bairoch, A. 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis, Nucleic Acids Res. 31:3784–3788.

Glass, N.L., Vollmer, S.J., Staben, C., Grotelueschen, J., Metzenberg, R.L., and Yanofsky, C. 1988. DNAs of the two mating-type alleles of *Neurospora crassa* are highly dissimilar. Science 241:570–573.

Goodwin, S.B., Dunkle, D.L., and Zismann, V.L. 2001. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. Phytopathology 91:648–658.

Goodwin, S.B., Waalwijk, C., Kema, G.H.J., Cavaletto, J.R., and Zhang, G. 2003. Cloning and analysis of the mating-type idiomorphs from the barley pathogen *Septoria passerinii*. Mol. Gen. Genomics 269:1–12.

Groenewald, M., Groenewald, J.Z., Braun, U., and Crous, P.W. 2006. Host range of *Cercospora apii* and *C. beticola*, and description of *C. apiicola*, a novel species from celery. Mycologia 98:275–285.

Groenewald, M., Groenewald, J.Z., and Crous, P.W. 2005. Distinct species exist within the *Cercospora apii* morphotype. Phytopathology 95:951–959.

Große-Herrenthey, U. 2001. Molekulare analyse der genetischen variabilität von *Cercospora beticola* Sacc., dem Erreger der cercospora-blattfleckenkrankheiten bei zuckerrüben, und untersuchungen zur resistenz von *Beta vulgaris* ssp. maritima gegen *C. beticola* unter anwendung unterschiedlicher resistenztestsysteme. PhD thesis, Christian-Albrechts university Kiel, Germany.

Halliday, C.L., and Carter, D.A. 2003. Clonal reproduction and limited dispersal in an environmental population of *Cryptococcus neoformans* var. *gatii* isolates from Australia. J. Clin. Microbiol. 41:703–711.

Halliday, C.L., Bui, T., Krockenberger, M., Malik, R., Ellis, D.H., and Carter, D.A. 1999. Presence of α and **a** mating types in environmental and clinical collections of *Cryptococcus neoformans* var. *gattii* strains from Australia. J. Clin. Microbiol. 37:2920–2926.

Kronstad, J.W., and Staben, C. 1997. Mating type in filamentous fungi. Annu. Rev. Genet. 31:245–276.

Kwon-Chung, K.J. 1976. Morphogenesis of *Filobasidiella neoformans,* the sexual state of *Cryptococcus neoformans.* Mycologia 68:821–833.

Latterell, F.M., and Rossi, A.E. 1977. Further evidence for the genetic relationship between *Cercospora zeae-maydis* and a species of *Mycosphaerella*. In: Proceedings of the 2nd International Mycological Congress, p. 374.

Lin, X., Hull, C.M., and Heitman, J. 2005. Sexual reproduction between partners of the same mating type in *Cryptococcus neoformans.* Nature 434:1017–1021.

Linde, C.C., Zhan, J., and McDonald, B.A. 2002. Population structure of *Mycosphaerella graminicola* from lesions to continents. Phytopathology 92:946–955.

Linde, C.C., Zala, M., Ceccarelli, S., and McDonald, B.A. 2003. Further evidence for sexual reproduction in *Rhynchosporium secalis* based on distribution and frequency of mating-type alleles. Fungal Genet. Biol. 40:115–125.

Milgroom, M.G. 1996. Recombination and the multilocus structure of fungal populations. Annu. Rev. Phytopathol. 34:457–477.

Moretti, M., Saracchi, M., and Farina, G. 2004. Morphological, physiological and genetic diversity within a small population of *Cercospora beticola* Sacc. Ann. Microbiol. 54:129–150.

O'Donnell, K., Ward, T.J., Geiser, D.M., Kistler, H.C., and Aoki, T. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genet. Biol. 41:600–623.

Paoletti, M., Buck, K.W., and Brasier, C.M. 2005. Cloning and sequence analysis of the *MAT-B* (*MAT-2*) genes from the three Dutch elm disease pathogens, *Ophiostoma ulmi*, *O. novo-ulmi*, and *O. himal-ulmi.* Mycol. Res. 109:983–991.

Pearson, W.R., Wood, T., Zhang, Z., and Miller, W. 1997. Comparision of DNA sequences with protein sequences. Genomics 46:24–36.

Pöggeler, S. 2001. Mating-type genes for classical strain improvements of ascomycestes. Appl. Microbiol. Biotechnol. 56:589–601.

Pollack, F.G. 1987. An annotated compilation of *Cercospora* names. Mycol. Mem. 12:1–212.

Pretorius, M.C., Crous, P.W., Groenewald, J.Z., and Braun, U. 2003. Phylogeny of some cercosporoid fungi from *Citrus*. Sydowia 55:286–305.

Rambaut, A. 2002. Sequence Alignment Editor. Version 2.0. Department of Zoology, University of Oxford, Oxford.

Saccardo, P.A. 1876. Fungi veneti novi vel critici. Series V. Nuovo Giorn. Bot. Ital. 8:162–211.

Singh, G., and Ashby, A.M. 1998. Cloning of the mating-type loci from *Pyrenopezziza brassicae* reveals the presence of a novel mating type gene within a discomycete *MAT1-2* locus encoding a putative metallothionin-like protein. Mol. Microbiol. 30:799–806.

Singh, G., and Ashby, A.M. 1999. Errratum to Shingh and Ashby 1998. Cloning of the mating-type loci from *Pyrenopezziza brassicae* reveals the presence of a novel mating type gene within a discomycete MAT1-2 locus encoding a putative metallothionin-like protein. Mol. Microbiol. 32:1115.

Steenkamp, E.T., Wingfield, B.D., Coutinho, T.A., Zeller, K.A., Wingfield, M.J., Marasas, W.F.O., and Leslie, J.F. 2000. PCR-based identification of MAT-1 and MAT-2 in the *Gibberella fujikuroi* species complex. Appl. Environ. Microbiol. 66:4378–4382.

Stewart, E.L., Liu, Z., Crous, P.W., and Szabo, L.J. 1999. Phylogenetic relationships among some cercosporoid anamorphs of *Mycosphaerella* based on rDNA sequence analysis. Mycol. Res. 103:1491–1499.

Swofford, D.L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Turgeon, B.G. 1998. Application of mating type gene technology to problems in fungal biology. Annu. Rev. Phytopathol. 36:115–137.

Turgeon, B.G., Bohlmann, H., Ciuffetti, L.M., Christiansen, S.K., Yang, G., Schafer, W., and Yoder, O.C. 1993. Cloning and analysis of the mating type genes from *Cochliobolus heterostrophus*. Mol. Gen. Genetics 238:270–284.

Turgeon, B.G., and Yoder, O.C. 2000. Proposed nomenclature for mating type genes of filamentous ascomycetes. Fungal Genet. Biol. 31:1–5.

Waalwijk, C., Mendes, O., Verstappen, E.C., de Waard, M.A.D., and Kema, G.H.J. 2002. Isolation and characterization of the mating-type idiomorphs from the wheat septoria leaf blotch fungus *Mycosphaerella graminicola*. Fungal Genet. Biol. 35:277–286.

Wang, J., Levy, M., and Dunkle, L.D. 1998. Sibling species of *Cercospora* associated with gray leaf spot of maize. Phytopathology 88:1269–1275.

Witthuhn, R.C., Harrington, T.C., Steimel, J.P., Wingfield, B.D., and Wingfield, M.J. 2000. Comparison of isozymes, rDNA spacer regions and *MAT-2* DNA sequences as phylogenetic characters in the analysis of the *Ceratocystis coerulescens* complex. Mycologia 92:447–452.

Yun, S.H., Arie, T., Kaneko, I., Yoder, O.C., and Turgeon, B.G. 2000. Molecular organization of mating type loci in heterothallic, homothallic, and asexual *Gibberella*/*Fusarium* species. Fungal Genet. Biol. 31:7–20.

Zhan, J., Kema, G.H.J., Waalwijk, C., and McDonald, B.A. 2002. Distribution of mating type alleles in the wheat pathogen *Mycosphaerella graminicola* over spatial scales from lesions to continents. Fungal Genet. Biol. 36:128–136.

Zhan, J., and McDonald, B.A. 2004. The interaction among evolutionary forces in the pathogenic fungus *Mycosphaerella graminicola*. Fungal Genet. Biol. 41:590–599.

Zhan, J., Pettway, R.E., and McDonald, B.A. 2003. The global genetic structure of the wheat pathogen *Mycosphaerella graminicola* is characterized by high nuclear diversity, low mitochondrial diversity, regular recombination, and gene flow. Fungal Genet. Biol. 38:286–297.

CHAPTER 5

Indirect evidence for sexual reproduction in *Cercospora beticola* populations from sugar beet

Marizeth Groenewald[1,2], Celeste C. Linde[3], Johannes Z. Groenewald[1], and Pedro W. Crous[1,2]

[1] *Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, Netherlands*
[2] *Laboratory of Phytopathology, Wageningen University, Binnenhaven 5, 6709 PD Wageningen, Netherlands*
[3] *School of Botany and Zoology, Daley Rd, The Australian National University, Canberra 0200, ACT, Australia*

Submitted for publication.

Key words: AFLP, gene flow, genetic diversity, mating type genes, population structure

Abstract *Cercospora beticola* is the main causal agent of Cercospora leaf spot on sugar beet and has a large negative impact on the yield and quality of sugar beet production worldwide. Previous studies have shown that both mating type genes of *C. beticola* are present in field populations, suggesting that *C. beticola* is heterothallic and may be reproducing sexually. *Cercospora beticola* isolates are diverse in the morphology of their conidia, onset of disease symptoms and fungicide resistance. In an attempt to find the source of this diversity and to determine whether the level of genotypic differentiation among isolates supports the possibility that sexual recombination occurs in this fungus, we collected *C. beticola* populations from Western Europe, Iran and New Zealand. The mating types of these isolates were determined and amplified fragment length polymorphism (AFLP) analyses were used to study the genetic diversity in these populations. The mating type ratios did not deviate significantly from a 1:1 ratio in most of the populations and AFLP analyses showed high levels of genetic variation within and between the populations with 86.4 % of the isolates having unique genotypes. From these results we conclude that there is a high possibility that sexual recombination does occur. Gene flow among especially the European populations unites populations into one panmictic *C. beticola* population. This, however, was not found for the New Zealand isolates that are clearly genetically distant. This study confirms *C. beticola* to be a genetically highly diverse species, supporting the assumption that it could also reproduce sexually.

INTRODUCTION

More than 3,000 species have been named in the genus *Cercospora* (Pollack, 1987), which is currently regarded as one of the largest genera of hyphomycetes. Following the recent revision by Crous & Braun (2003), this number was significantly reduced to 659 species, with a further 281 species that are treated as morphologically indistinguishable from *C. apii sensu lato. Cercospora beticola* belongs to the *C. apii* complex and is the main causal agent of Cercospora leaf spot of sugar beet (Saccardo, 1876; Groenewald *et al*., 2005, 2006a). Some confusion existed in the past about whether or not *C. beticola* and *C. apii*, the main leaf spot causing agent of *Apium* species, are synonymous. Groenewald *et al*. (2005) conducted a detailed study of the cultural characteristics, cardinal temperature requirements for growth and molecular analyses to show that these two *Cercospora* species are indeed distinct.

Cercospora beticola is considered to be one of the most destructive foliar pathogens of sugar beet, causing yield losses of up to 40 % (Shane & Teng, 1992; Holtschulte, 2000). For most *Cercospora* species, including *C. beticola*, no sexual stage is known. The genus *Cercospora* is a well-established anamorph of *Mycosphaerella* (Crous & Braun, 2003), and phylogenetic analyses on a variety of *Cercospora* species have placed them as a well-defined clade within *Mycosphaerella* (Crous *et al*., 2001, 2006a, 2006b; Goodwin *et al*., 2001). Therefore, if a sexual stage does exist for *C. beticola*, it would be a species of *Mycosphaerella*.

In contrast to what is expected in a strictly asexually reproducing fungus, a surprisingly wide array of phenotypic diversity has been described for *C. beticola.* This includes variation

in e.g. spore morphology and production, cultural characteristics, pathogenicity and fungicide resistance (Ruppel, 1971; Rossi, 1995; Moretti *et al.*, 2004). In fungi, gene diversity is not necessarily affected by the mating structure (McDonald, 1997) but sexually reproducing fungi usually have high levels of genotypic diversity and alleles among loci are randomly associated (Milgroom, 1996). Even though phenotypic markers indicate high levels of variation, little is known on the population genetic structure of *C. beticola* populations we aim to investigate the population genetic structure of *C. beticola*.

Limited studies have been done on the genetic structure of *Cercospora* populations. A substantial amount of genetic variation was found within *Cercospora* strains isolated from sugar beet fields in Italy (Moretti *et al*., 2006), whereas genetic variation was also observed in *C. beticola* isolates isolated from lesions of the same plant (Moretti *et al.*, 2004). This is in contrast to the data available for other *Cercospora* species which have low genetic diversity e.g. *C. sorghii* (Okori *et al.,* 2004). Similarly, the genetic variation among isolates of *C. zeae-maydis* from Africa (Okori *et al.*, 2003) and the United States (Wang *et al.*, 1998; Crous *et al*., 2006a) is low with little genetic differentiation within a population.

The population dynamics of three *Mycosphaerella* species (*M. graminicola*, *M. musicola* and *M. fijiensis*) have been extensively studied in the past. In *M. graminicola* it has been demonstrated that the sexual stage has a major impact on its genetic structure (Chen & McDonald, 1996). Several studies have shown a high level of genetic variation, not only among different *M. graminicola* populations, but also among isolates obtained from the same lesion (McDonald & Martinez, 1990; Linde *et al.,* 2002; Zhan *et al*., 2003; Zhan & McDonald, 2004). High levels of genetic diversity and random mating in *M. fijiensis* were illustrated by Carlier *et al*. (1996). Hayden *et al*. (2003a, 2003b) found high levels of genetic diversity in populations of *M. musicola,* especially in the South-east Asian region, the possible origin of this pathogen. Genetic variability was also found among isolates of *M. fijiensis* that were obtained from the same lesion on banana leaves (Müller *et al.*, 1997; Rivas *et al*., 2004). In all these studies a high number of unique genotypes were present in their populations, illustrating the scale in which genetic variation, which is predominantly due to sexual reproduction, occurs in these pathogens.

Mating type genes play an important role in the biology and evolution of fungal species and are under frequency dependent selection in randomly mating populations. Mating type genes (*MAT1-1-1* and *MAT1-2*) of *C. beticola* were isolated and characterized, and showed that the fungus has a bipolar mating system (Groenewald *et al*., 2006b). Ascomycetes that are heterothallic have a single locus, two allele mating system which requires two nuclei of opposite mating-type to fuse in order for sexual reproduction to occur (Kronstad & Staben, 1997). *Cercospora* mating type-specific primers were developed for use in a multiplex PCR to determine the frequencies of these genes in field populations (Groenewald *et al*., 2006b). They found that mating types occurred in similar frequencies in *C. beticola* field populations, a phenomenon that is commonly accepted as indicative of random mating, such as in e.g. *M. graminicola* (Waalwijk *et al*., 2002; Zhan *et al*., 2002). Groenewald *et al*. (2006b) therefore suggested that some *Cercospora* species cannot be strictly asexual and that another method of reproduction has to occur to account for the high levels of genetic variation observed within field populations.

Although previous studies showed that high levels of genotypic variation could be found in populations of *C. beticola* (Moretti *et al*., 2004, 2006), these studies were based on small sample sizes (N ≤ 13 per population). As knowledge of the distribution of the mating types together with the amount of genotypic variation observed within a specific fungal population can provide a strong indication whether or not sexual reproduction is likely to occur, our main objectives were to (i) determine the population genetic structure of *C. beticola* populations including genotypic diversity and gametic disequilibrium, and (ii) to determine whether there is frequency dependent selection on mating types. This knowledge will provide indirect evidence for the possible presence of a sexual cycle occurring in this fungus. In order to achieve our objectives we analysed populations from Netherlands, Germany, France, Italy, Iran and New Zealand with AFLPs, using four selective primer combinations, and mating type-specific primers.

MATERIALS AND METHODS

Fungal isolation and DNA extraction

Beta vulgaris leaves were sampled from single sugar beet fields in four European countries (Netherlands, Germany, France and Italy) as well as in Iran and New Zealand (Table 1). The sampling was done in an X figure across each field. For each population, ten symptomatic leaves were collected from ten different plants in each leg of the cross. Ten symptomatic leaves were collected from a single plant in the middle of the field. Single-spore isolations were made from the symptomatic leaf material and cultures were established on 2 % malt extract agar (MEA). The isolates were examined morphologically to confirm their identity as *C. apii sensu lato* as described by Crous & Braun (2003). All isolates were also screened with *C. beticola*-specific primers to confirm that they are truly *C. beticola* before being included in the analyses (Groenewald *et al*., 2005). Isolates were cultured on MEA plates for 8 days at 24 °C and 200–400 mg mycelium were used in the DNA extraction using the FastDNA kit (BIO101, Carlsbad, CA, USA) according to the manufacturer's instructions.

Table 1. *Cercospora beticola* populations included in this study.

Country of origin	Sample size	Location	Collector
France (Fr)	46	Longvic	S. Garressus
Germany (Ger)	39	Niedersachsen	S. Mittler
Italy (It)	32	Ravenna	V. Rossi
Netherlands (Neth)	48	Bergen op Zoom	Unknown
New Zealand (NZ)	35	Unknown	C.F. Hill
Iran (Ir)	50	Pakajik	A.A. Ravanlou

Screening of markers

Degenerate mating type gene primers designed by Groenewald *et al*. (2006b) were used to screen all the isolates from the six *C. beticola* populations as described previously. AFLP analyses were performed according to Vos *et al*. (1995), with minor modifications as described by Groenewald *et al*. (2005). Genomic DNA (100 µg) from 250 isolates was digested with the restriction enzymes *Eco*RI and *Mse*I and ligated to the corresponding adaptors. Four selective primer combinations were used, namely *Eco*RI-A-[FAM] / *Mse*I-CT, *Eco*RI-AT-[JOE] / *Mse*I-C, *Eco*RI-AG-[NED] / *Mse*I-C and *Eco*RI-G-[JOE] / *Mse*I-CG (Applied Biosystems, Nieuwerkerk aan de IJssel, Netherlands) for the final amplification step. To test the reproducibility of the AFLP profiles, separate DNA extractions, PCR amplifications and AFLP analyses were performed in duplicate on 10 isolates (using the four primer combinations). An error rate of 1 % (1 to 2 bands difference per population among 206 loci) was observed. Only polymorphic loci (78) were included in the analyses.

Data analyses

The presence and absence of the bands obtained from the AFLP analyses were scored as 1 and 0, respectively, and these results were combined for the statistical analyses. Isolates were considered members of the same clone or clonal lineage if they had 99 % similar bands. Clones identified with AFLPs which had different mating type alleles were considered different haplotypes. To quantify genotypic variation within populations, the genotype diversity was measured with a Shannon-Weaver index (Shannon & Weaver, 1949) and normalised for sample size (Elstrand & Levin, 1982).

To evaluate the associations among loci in each sample, we used the index of association (I_A) and an unbiased estimate of multilocus linkage disequilibrium, $\bar{\mathbf{r}}_\mathbf{d}$. I_A and $\bar{\mathbf{r}}_\mathbf{d}$ values were calculated by using Multilocus 1.3 software, and 1,000 artificially recombined data sets were used to determine the statistical values of the test (Agapow & Burt, 2001). Significant departures from an expected 1:1 ratio in mating type frequencies were tested with a χ^2 test.

Three programs, namely TFPGA (Miller, 1997), POPGENE v1.32 (Yeh *et al.*, 1997) and TREECON (Van de Peer, 1994), were used to analyse the 0/1 matrix. The population genetic analyses program TFPGA was used to calculate the gene diversity (Nei, 1978), percentage of polymorphic loci, F-statistics, genetic distances and the exact tests. The percentage polymorphic loci were based on 99 % criteria. The F-statistic (F_{ST}) was calculated using the method of Weir & Cockerham (1984), jackknife over loci was done with 10,000 iterations using a confidence level (C.I.) of 95 %. Genetic distances between the populations were calculated using Wright's (1978) modification of Rogers' (1972) distance. For this study, a value of < 0.1 indicates small genetic distances, 0.10 – 0.15 indicates moderate genetic distances, 0.15 – 0.2 indicates high genetic distances and > 0.2 indicates very large genetic distances. A graphical representation of the genetic distance data was done using the UPGMA algorithm. Bootstrap support values were calculated over all the loci using 1,000 repetitions. The exact test was used to determine if significant differences in allele frequencies exist between populations (Sokal & Rohlf, 1995). The Markov Chain Monte Carlo approach that was used to calculate the exact test values gives an approximation of the exact probability of the observed differences in allele frequencies (Raymond & Rousset, 1995).

POPGENE was used to calculate the gene flow (*Nm*) between any two populations, between the four Western European populations, between the five Eurasian populations and between all six populations. The grouping of populations into major geographic areas; Asia (Iran), Europe (Netherlands, France, Italy and Germany) and New Zealand allowed the analysis of variation (analysis of molecular variance, or AMOVA) at three levels; (i) within individual populations, (ii) between populations within geographic regions, and (iii) between geographic regions. All calculations, including random-permutation procedures to assess statistical significance, were performed by use of the GenALEx 6 package (Peakall & Smouse, 2005). TREECON was used for distance estimations between the different *C. beticola* isolates.

RESULTS

AFLP markers

Moderate levels of polymorphism were obtained from the four AFLP primer combinations used in this study (Table 2). In total, 206 bands could be scored unambiguously. The number of polymorphic bands obtained from all six populations varied from 15 to 22 (Table 2) and the band sizes ranged from 50 to 500 base pairs. The AFLP primer sets *Eco*RI-AG / *Mse*I-C amplified the largest number of polymorphic bands (22) whereas AFLP primer pair *Eco*RI-G / *Mse*I-CG amplified the lowest number of polymorphic bands (15) (Table 2). The percentage polymorphic loci ranged from 20.9 % in the New Zealand population to 30.6 % in the German population (Table 2).

Table 2. Results obtained with four AFLP primer combinations on 250 *Cercospora beticola* isolates from the six populations listed in Table 1.

		Number of polymorphic bands						
Primer pair	**Number of bands**	**NZ**	**Fr**	**Ger**	**Ir**	**It**	**Neth**	**All[a]**
*Eco*RI-A / *Mse*I-CT	54	14	14	16	14	16	16	21
*Eco*RI-AG / *Mse*I-C	52	11	16	17	17	16	15	22
*Eco*RI-G / *Mse*I-CG	52	8	11	13	12	12	12	15
*Eco*RI-AT / *Mse*I-C	48	10	11	17	9	14	16	21
Total	206	43	52	63	52	58	59	79
% Polymorphic loci		20.9	25.2	30.6	25.2	28.6	28.6	38.3

[a]All: total of all six populations.

Population genetic analyses

Genotypic diversity (*H'*) ranged from 0.85 (Netherlands) to 1.0 (France and Italy, Table 3). Gene diversity (H) is the lowest in the New Zealand population (0.19), and the highest in the German and Italian populations (0.27) (Table 3). The F_{ST} value shows high genetic differentiation (0.17) across the six populations, and moderate genetic differentiation across the four European populations (0.07) and five Eurasian populations (0.07) (Table 4). The pairwise comparisons of population differentiation between the New Zealand population and other populations was high (F_{ST} = 0.33 – 0.41). The pairwise comparison of the F_{ST} values between the remaining populations vary between 0.02 (Dutch / Italian) and 0.13 (French / German).

Table 3. The number of haplotypes, genotypic diversity and gene diversity obtained from six populations of *Cercospora beticola* analysed with AFLPs.

	NZ	Fr	Ger	Ir	It	Neth
Sample size	35	46	39	50	32	48
Number of haplotypes	27	46	32	43	32	37
H'^{a}	0.91	1.00	0.92	0.94	1.00	0.85
H^{b}	0.19	0.23	0.27	0.24	0.27	0.25

[a]Shannon-Weaver index (Shannon & Weaver, 1949) normalised for sample size.
[b]Gene diversity (Nei, 1987).

The high *Nm* values of 6.75 and 5.80 across the four European populations and five Eurasian populations respectively indicate high genetic exchange between these populations, but gene flow was low when the New Zealand population was included in the calculation (*Nm* = 2.18) (Table 4). Low *Nm* values (1.30 – 1.83) were observed between the New Zealand population and every other population analysed. The highest *Nm* values were obtained in pairwise comparisons between Italy and the Netherlands (*Nm* = 19.08), followed by Netherlands / France (*Nm* = 11.42) (Table 4).

The high genetic distance values (0.22 – 0.25) for the New Zealand population with pairwise comparisons to the other populations, show that this population is genetically distinct from the rest (Table 5). The genetic distance values between the remaining populations are lower and vary between 0.07 and 0.13. The Exact test (Table 5) shows significant differences between the New Zealand populations and the rest ($P < 0.001$) as well as for the pair-wise comparison between populations of France / Germany ($P = 0.02$).

Table 4. The gene flow values (Nm) (lower left part of table) and the F-statistics (upper right part of table) for the pair-wise comparisons between the six populations, over all the populations and all the populations obtained from Europe or Eurasia as calculated with POPGENE and TFPGA respectively.

	NZ	Fr	Ger	Ir	It	Neth
NZ	*****	0.41	0.33	0.40	0.36	0.36
Fr	1.30	*****	0.13	0.08	0.06	0.06
Ger	1.83	5.70	*****	0.10	0.05	0.06
Ir	1.35	8.52	6.62	*****	0.06	0.09
It	1.59	11.06	10.85	11.30	*****	0.02
Neth	1.59	11.42	10.36	8.04	19.08	*****
Nm (all populations)	2.18					
Nm (European)	6.75					
Nm (Eurasian)	5.80					
F_{ST} (all populations)	0.17					
F_{ST} (European)	0.07					
F_{ST} (Eurasian)	0.07					

Table 5. The exact test (lower left part of table) and the genetic distance (upper right part of table) for the pair-wise comparison between the six populations as calculated with TFPGA.

	NZ	Fr	Ger	Ir	It	Neth
NZ	*****	0.25	0.22	0.24	0.23	0.23
Fr	0.00	*****	0.13	0.10	0.09	0.09
Ger	0.00	0.02	*****	0.12	0.10	0.10
Ir	0.00	0.93	0.64	*****	0.09	0.11
It	0.00	1.00	1.00	1.00	*****	0.07
Neth	0.00	1.00	1.00	0.98	1.00	*****

Cluster analysis

Figure 1 is a graphical representation of the genetic distance data obtained between populations using the TFPGA program with UPGMA clustering. The nodes for the New Zealand and Eurasian populations are very well supported by high bootstrap values (100 % and 99 %, respectively). The New Zealand population is genetically distant from the Eurasian population and there is not sufficient support for any population structure within the Eurasian populations with bootstrap values ranging from 36 % – 59 %.

The tree obtained with TREECON (data not shown) shows that 216 unique genotypes were obtained for the 250 isolates; 86.4 % of the isolates studied have a unique genotype. Unique genotypes refer to isolates with dissimilar AFLP profiles, but also to isolates with identical AFLP profiles but different mating types.

Mating type ratios did not deviate significantly from a 1:1 ratio, except in the population from Italy where MAT2 isolates were more predominant (Table 6), suggesting frequency dependent selection. In 14 cases, isolates with the same multilocus AFLP haplotype had different mating type alleles (data not shown). The I_A and $\bar{r}_d$ values were significantly higher than zero ($P < 0.01$) for all the populations, suggesting that recombination is rare or absent (Table 6).

AMOVA analyses revealed that the percentage of genetic variation among individuals within populations was 75 %. Only 4 % of the variation is due to differences among populations within a region (European populations) and 21 % to differences among geographic regions.

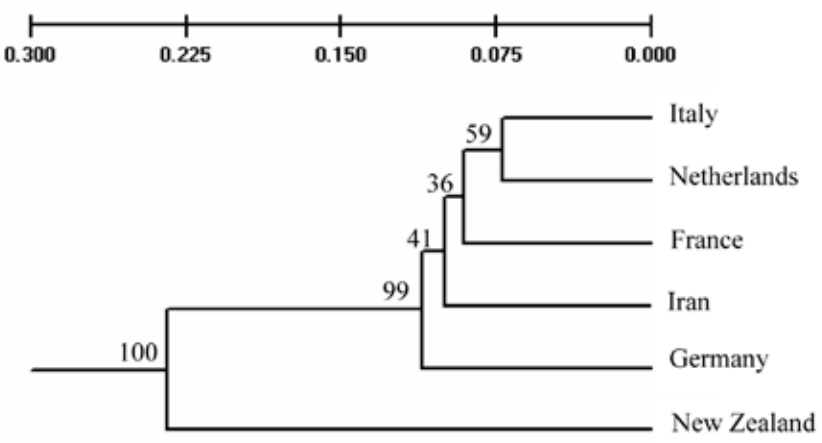


Fig. 1. Graphical representation of the genetic distance data generated by UPGMA clustering in the software TFPGA. The scale bar shows the genetic distance and bootstrap support values (in percentage) from 1,000 replicates are shown at the nodes.

Table 6. Mating type frequencies and tests of multilocus association in six *Cercospora beticola* clone-corrected populations.

	Number of isolates		Mating type frequency					
Population	**Mat1-1-1**	**Mat1-2**	**Mat1-1-1**	**Mat1-2**	**N**	χ^{2a}	$I_A{}^b$	$\bar{r}_d{}^b$
NZ	14	13	0.52	0.48	27	0.037	1.530*	0.037*
Fr	25	21	0.54	0.46	46	0.347	0.729*	0.015*
Ger	19	13	0.59	0.41	32	1.125	1.934*	0.032*
Ir	21	22	0.49	0.51	43	0.023	1.025*	0.021*
It	10	22	0.31	0.69	32	4.500*	0.377*	0.006*
Neth	21	16	0.57	0.43	37	0.675	0.214*	0.004*
Total	110	107	0.51	0.49	217	6.668	1.135*	0.016*

[a]χ^2 value based on 1:1 ratio and 1 degree of freedom for clone-corrected populations and 5 degrees of freedom for the contingency χ^2 analyses of the total data set. * Indicates mating type frequencies which are significantly different at $P < 0.05$.

[b]* Indicate I_A and $\bar{r}_d$ values significantly higher then zero ($P < 0.01$).

DISCUSSION

This study is the first to report on the genotypic structure, diversity and mating type distribution of *C. beticola* populations from different geographic localities, and adds to our understanding of the population structure of *C. beticola*. The results obtained from the F_{ST}, gene flow and genetic distance analyses suggest that the populations from Europe and Iran are genetically similar, whereas the New Zealand population is significantly different. High levels of genetic variation were found among the *C. beticola* isolates tested. This variation, illustrated by the high number of distinct haplotypes obtained with the AFLP analyses, compares well with earlier studies that also reported high levels of genetic variation among isolates obtained from the same lesion on a sugar beet plant in Italy (Moretti *et al*., 2004), and between isolates from Italy (Moretti *et al*., 2006). Most of the isolates that were obtained from one plant during our study also had a distinct multilocus AFLP haplotype, except for clonal isolates from two plants in the Netherlands. Our sampling allowed partitioning of genetic variation and showed that most variation could be found within populations (75 %), whereas only 4 % of the variation is due to differences among populations within a region (European populations) and 21 % to differences among geographic regions.

To date no teleomorph has been found for *C. beticola* (Groenewald *et al*., 2006b) and the mating structure of this pathogen has been considered clonal. However, we found high levels of genotypic diversity in all six populations analysed. It is known that populations that regularly undergo sexual reproduction should have many more genotypes that result in higher levels of genotypic diversity compared to those that reproduce only asexually (Milgroom, 1996). This type of genetic structure is seen in most populations of e.g. *M. graminicola* (Linde *et al*., 2002; Zhan *et al*., 2003; Zhan & McDonald, 2004). Thus, the genotypic diversity observed for *C. beticola* is exceptionally high for a presumed asexually reproducing organism.

Milgroom (1996) and Zhan *et al*. (2002) found that a combination of high levels of genetic diversity and the equal distribution of the mating types in a given population are very

strong indications that sexual recombination does occur. Therefore, to rule out that only asexual recombination is the cause of the observed genetic variation, we screened for the presence and frequency of the mating type genes in our populations. The equal distribution of mating types in most populations (except Italy) suggests frequency dependent selection and thus random mating. Both mating types could also be found on the same plant, providing opportunity for genetic exchange. Thus the high levels of genotypic diversity that we have encountered in our *C. beticola* populations, together with equal mating type ratios, indicate that sexual recombination could play an important role in the life cycle of this fungus. If *C. beticola* was strictly asexual, we would expect that, over time, there would be a skewed distribution of the mating types, or that only one mating type would be present as was found for other *Cercospora* species such as *C. apii* and *C. apiicola* (Groenewald *et al*., 2006b). Although the MAT2 was more prevalent in the Italian population of *C. beticola*, this could be an artefact of the sampling and, although the majority of the isolates were of MAT2, the presence of MAT1 isolates make sexual recombination possible within the population. *Cercospora beticola* has also been observed to form spermatogonia on leaf residue collected during this study, which is also indicative of a possible sexual cycle. Although no sexual stage has yet been found for *C. beticola,* it is not unlikely that it does exist, but that the teleomorph is not readily observed in nature nor induced under laboratory conditions.

Tests for multilocus associations (I_A and $\bar{r}_d$) showed that all six populations were significantly different from zero ($P < 0.01$) and thus in gametic disequilibrium. This suggests that asexual production is predominant and that random mating occurs only rarely, if at all. However, although significant, the values of I_A and $\bar{r}_d$ were only moderately low. Furthermore, $\bar{r}_d$ was similar or even lower in *C. beticola* (0.004 – 0.037) than that estimated for *Pyrenophora teres* f. sp. *teres* (0.037 – 0.039), which is known to undergo regular sexual recombination (Rau *et al*., 2003). Frequent population expansions during an epidemic can result in populations dominated by closely related individuals (Maynard-Smith *et al*., 2000). Thus multilocus association tests indicate that asexual reproduction is an important mode of reproduction for *C. beticola*, as it is for *P. teres*, but recombination cannot be discarded.

The high levels of genetic variation in *C. beticola* can also be explained by other factors. It has been shown that the genome of *C. beticola* can undergo major chromosome changes, especially after sub-culturing (Weiland & Koch, 2004), and this can have a major influence on the results obtained using marker systems, such as AFLP, that sample the whole genome. In order to limit these chromosomal rearrangements in our isolates, sub-culturing during this study has been kept to the minimum and the DNA was extracted from the cultures directly after the original isolation. We can therefore conclude that the genetic variation observed in the populations screened during this study occurred during the life cycle of the fungus in its natural field environment. Unfortunately, these chromosome changes can potentially make it problematic to study the relationships between cultures that are preserved in culture collections by continuous sub-culturing of older cultures.

Genetic diversity within a species can also be caused by asexual events that include hyphal anastomosis (Molnar *et al.*, 1990), normal mutations (Koenig *et al*., 1997; Bentley *et al*., 1998; O'Donnell *et al*., 1999) and events occurring during parasexual cycles (Kuhn *et al*., 1995,

Taylor *et al*., 1999). It is known that *C. beticola* is able to persist on infected sugar beet leaf residue as stromata on the soil surface (Wolf & Verreet, 2005). It is therefore possible that, during this over-wintering period, asexual reproduction can readily take place, which might explain these high levels of genetic variation that exist in the studied populations. No geographic boundaries can be enforced to any extent on the European populations based on the country of isolation. This can be based on the diverse genotypes that are present, and intermingled between the different European countries. There is a significant amount of gene flow between these populations, with low levels of genetic distance and differentiation. Therefore it can be concluded that these populations can be seen as one big diverse European population rather than distinct populations. Sharing of haplotypes among geographic populations could be explained by man-mediated dispersal. The import and export of host material between countries in the European Union readily occurs because of the open borders, and could provide a mechanism for man-mediated dispersal. The high gene flow and low genetic distance and differentiation values observed between European populations and Iran indicate that genotype transfer also readily takes place between these countries. The Iranian genotypes are also intermingled with those of the European isolates, but this was not found for the New Zealand isolates.

According to Dingley (1969), *C. beticola* was common in New Zealand as a leaf spot on red beet, silver beet and mangels, with only minor economic importance. Pennycook (1989) recorded *C. beticola* on sugar beet in New Zealand, and during the last few years it has been isolated from different localities in New Zealand (New Zealand Fungi Database, 2002). New Zealand is not connected to a mainland and the migration of isolates in and out of the country therefore cannot take place on a regular basis as within Europe. The geographic isolation of the country is probably why the *C. beticola* population from New Zealand is genetically distant from the European isolates. It is known that founder effects can cause genetic differentiation among populations (Boileau *et al*., 1992), as was also observed for *M. fijiensis* (Hayden *et al*., 2003b). A possible explanation for the high genetic differentiation that exists between the New Zealand and Eurasian populations is that a founder effect shaped the population structure that exists in New Zealand. The specific origin of *C. beticola* in New Zealand is unclear and requires further investigation; more populations from New Zealand and other parts of the world have to be screened. As the *C. beticola* centre of origin is central Europe and the Mediterranean area (Saccardo, 1876), it is most likely that earlier sugar beet trade introduced *C. beticola* to New Zealand, and that a distinct population structure was shaped on this isolated island without any outside influences, such as gene flow.

Several studies have reported high levels of variation during the onset and progression of Cercospora leaf spot on sugar beet (Wolf *et al*., 2000; Wolf & Verreet, 2002, 2005), and that *C. beticola* has become resistant or has developed an increased tolerance to fungicides (Ruppel & Scott, 1974; Karaoglanidis *et al.*, 2000; Weiland & Koch, 2004). Variation in fungicide resistance and variability in disease symptoms on resistant sugar beet plants make effective disease management difficult. It is most likely that the high level of genetic variation that exists within *C. beticola* plays an important role in the pathogen's ability to develop new virulent alleles and fungicide resistance.

Previous studies showed that some genetic variation exists within *C. beticola,* but it was not known whether this variation was due to chromosomal rearrangements, asexual or sexual recombination. Our results indicate that the genetic variation observed in the isolates studied was most likely caused by recombination events occurring in nature. We conclude that a sexual stage is probably part of this fungus's life cycle, and that it is unlikely that strictly asexual reproduction occurs in *C. beticola*. The high levels of genotypic variation, low gametic disequilibrium and the equal distribution of the mating types within populations suggest that sexual recombination events most likely play an important role in the reproductive cycle of this apparently asexual species. These findings significantly improve our knowledge regarding the population structure and reproduction strategies of this important plant pathogen.

ACKNOWLEDGEMENTS

This research was financially supported by the CBS-Odo van Vloten Stichting and the Royal Netherlands Academy of Arts and Sciences.

REFERENCES

Agapow, P.-M., and Burt, A. 2001. Indices of multilocus linkage disequilibrium. Mol. Ecol. Notes 1:101–102.

Barraclough, T.G., Birky, C.W., and Burt, A. 2003. Diversification in sexual and asexual organisms. Evolution 57:2166–2172.

Boileau, M.G., Hebert, P.D.N., and Schwartz, S.S. 1992. Non-equilibrium gene frequency divergence: persistent founder effects in natural populations. J. Evolution. Biol. 5:25–39.

Bentley, S., Pegg, K.G., Moore, N.Y., Davis, R.D., and Buddenhagen, I.W. 1998. Genetic variation among vegetative compatibility groups of *Fusarium oxysporum* f. sp. *cubense* analyzed by DNA fingerprinting. Phytopathology 88:1283–1293.

Carlier, J., Lebrun, M.H., Zapater, M.F., Dubois, C., and Mourichon, X. 1996. Genetic structure of the global population of banana black leaf streak fungus, *Mycosphaerella fijiensis*. Mol. Ecol. 5:499–510.

Chen, R.S., and McDonald, B.A. 1996. Sexual reproduction plays a major role in the genetic structure of populations of the fungus *Mycosphaerella graminicola*. Genetics 142:1119–1127.

Crous, P.W., and Braun, U. 2003. *Mycosphaerella* and its anamorphs: 1. Names published in *Cercospora* and *Passalora*. CBS Biodivers. Series 1:1–571.

Crous, P.W., Kang, J.-C., and Braun, U. 2001. A phylogenetic redefinition of anamorph genera in *Mycosphaerella* based on ITS rDNA sequences and morphology. Mycologia 93:1081–1101.

Crous, P.W., Groenewald, J.Z., Groenewald, M., Caldwell, P., Braun, U., and Harrington, T.C. 2006a. Species of *Cercospora* associated with grey leaf spot of maize. Stud. Mycol. 55:189–197.

Crous, P.W., Wingfield, M.J., Mansilla, J.P., Alfenas, A.C., and Groenewald, J.Z. 2006b. Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. II. Stud. Mycol. 55:99–131.

Dingley, J.M. 1969. Records of plant diseases in New Zealand. New Zeal. DSIR B. 192:1–297.

Elstrand, N.C., and Levin, D.A. 1982. Genotypic diversity in *Oenothera laciniata* (Onagraceae), a permanent translocation heterozygote. Evolution 36:63–69.

Goodwin, S.B., Dunkle, D.L., and Zismann, V.L. 2001. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. Phytopathology 91:648–658.

Groenewald, M., Groenewald, J.Z., and Crous, P.W. 2005. Distinct species exist within the *Cercospora apii* morphotype. Phytopathology 95:951–959.

Groenewald, M., Groenewald, J.Z., Braun, U., and Crous, P.W. 2006a. Host range of *Cercospora apii* and *C. beticola*, and description of *C. apiicola*, a novel species from celery. Mycologia 98:275–285.

Groenewald, M., Groenewald, J.Z., Harrington, T.C., Abeln, E.C.A., and Crous, P.W. 2006b. Mating type gene analysis in apparently asexual *Cercospora* species is suggestive of cryptic sex. Fungal Genet. Biol. 43:813–825.

Hayden, H.L., Carlier, J., and Aitken, E.A.B. 2003a. Population differentiation in the banana leaf spot pathogen *Mycosphaerella musicola*, examined at a global scale. Plant Pathol. 52:713–719.

Hayden, H.L., Carlier, J., and Aitken, E.A.B. 2003b. Genetic structure of *Mycosphaerella fijiensis* populations from Australia, Papua New Guinea and the Pacific Islands. Plant Pathol. 52:703–712.

Holtschulte, B. 2000. *Cercospora beticola* - world-wide distribution and incidence. Pages 5–16 in: *Cercospora beticola* Sacc. Biology, agronomic influences and control measures in sugar beet IIRB. M.J.C. Asher, B. Holtschulte, M.M. Richard, F. Rosso, G. Steinrucken and R. Beckers, eds. International Institute for Beet Research, Belgium.

Karaoglanidis, G.S., Ioannidis, P.M., and Thanassoulopoulos, C.C. 2000. Reduced sensitivity of *Cercospora beticola* to sterol-demethylation-inhibiting fungicides. Plant Pathol. 49:567–572.

Koenig, R.L., Ploetz, R.C., and Kistler, H.C. 1997. *Fusarium oxysporum* f. sp. *cubense* consists of a small number of divergent and globally distributed clonal lineages. Phytopathology 87:915–923.

Kronstad, J.W., and Staben, C. 1997. Mating type in filamentous fungi. Annu. Rev. Genet. 31:245–276.

Kuhn, D.N., Cortes, B.R., Pinto, T., and Weaver, J. 1995. Parasexuality and heterokaryosis in *Fusarium oxysporum* f. sp. *cubense*. Phytopathology 85:119.

Linde, C.C., Zhan, J., and McDonald, B.A. 2002. Population structure of *Mycosphaerella graminicola* from lesions to continents. Phytopathology 92:946–955.

Maynard-Smith, J., Feil, E.J., and Smith, N.H. 2000. Population structure and evolutionary dynamics of pathogenic bacteria. Bioessays 22:1115–1122.

McDonald, B.A., and Martinez, J.P. 1990. DNA restriction fragment length polymorphisms among *Mycosphaerella graminicola* (anamorph *Septoria tritici*) isolates collected from a single wheat field. Phytopathology 80:1368–1373.

McDonald, B.A. 1997. The population genetics of fungi: Tools and techniques. Phytopathology 87:448–453.

Milgroom, M.G. 1996. Recombination and the multilocus structure of fungal populations. Annu. Rev. Phytopathol. 34:457–477.

Miller, M.P. 1997. Tools for population genetics analyses (TFPGA): A Windows program for the analysis of allozyme and molecular population genetic data. On-line: http://herb.bio.nau.edul~miller/tfpga.htm.

Molnar, A., Sulyok, L., and Hornok, L. 1990. Parasexual recombination between vegetatively incompatible strains in *Fusarium oxysporum*. Mycol. Res. 94:393–398.

Moretti, M., Saracchi, M., and Farina, G. 2004. Morphological, physiological and genetic diversity within a small population of *Cercospora beticola* Sacc. Ann. Microbiol. 54:129–150.

Moretti, M., Karaoglanidis, G., Saracchi, M., Fontana, A., and Farina, G. 2006. Analysis of genotypic diversity in *Cercospora beticola* Sacc. field isolates. Ann. Microbiol. 56:215–221.

Müller, R., Pasberg-Gauhl, C., Gauhl, F., Ramser, J., and Kahl, G. 1997. Oligonucleotide fingerprinting detects genetic variability at different levels in Nigerian *Mycosphaerella fijiensis*. J. Phytopathol. 145:25–30.

Nei, M. 1978. Molecular Evolutionary Genetics. Columbia University Press, New York.

New Zealand Fungi Database. 2002. http://nzfungi.landcareresearch.co.nz/html/mycology.asp.

O'Donnell, K., Gherbawy, Y., Schweigkofler, W., Adler, A., and Prillinger, H. 1999. Phylogenetic analyses of DNA sequence and RAPD data compared in *Fusarium oxysporum* and related species from maize. J. Phytopathol. 147:445–452.

Okori, P., Rubaihayo, P.R., Fahleson, J., Adipala, E., and Dixelius, C. 2003. Assessment of genetic variation among East African *Cercospora zeae-maydis* populations. Afr. Crop Sci. J. 11:75–85.

Okori, P., Rubaihayo, P.R., Ekwamu, A., Fahleson, J., and Dixelius, C. 2004. Genetic characterization of *Cercospora sorghi* from cultivated and wild sorghum and its relationship to other *Cercospora* fungi. Phytopathology 94:743–750.

Peakall, R., and Smouse, P. 2005. GenALEx 6: Genetic Analysis in Excel. Population genetic software for teaching and research. Australian National University, Canberra, Australia. http://www.anu.edu.au/BoZo/GenALEx/.

Pennycook, S.R. 1989. 'Plant diseases recorded in New Zealand. Vol. 2. Fungal plant diseases recorded from New Zealand.' Plant Diseases Division, DSIR, Auckland.

Pollack, F.G. 1987. An annotated compilation of *Cercospora* names. Mycol. Mem. 12:1–212.

Rao, D., Brown, A.H.D., Brubaker, C.L., Attene, G., Balmas, V., Saba, E., and Papa, R. 2003. Population genetic structure of *Pyrenophora teres* Drechs., the causal agent of net blotch of barley (*Hordeum vulgare* L.). Theor. Appl. Genet. 106:947–959.

Raymond, M., and Rousset, F. 1995. An exact test for population differentiation. Evolution 49:1280–1283.

Rivas, G.-G., Zapater, M.-F., Abadie, C., and Carlier, F. 2004. Founder effects and stochastic dispersal at the continental scale of the fungal pathogen of bananas *Mycosphaerella fijiensis*. Mol. Ecol. 13:471–482.

Rogers, J.S. 1972. Measures of genetic similarity and genetic distance. University of Texas Publication 7213:145–153.

Rossi, V., and Battilani, P. 1991. CERCOPRI: A Forecasting model for primary infections of Cercospora leaf spot of sugar beet. EPPO Bulletin 21:527–531.

Rossi, V. 1995. Effect of host resistance in decreasing infection rate of Cercospora leaf spot epidemics on sugarbeet. Phytopathol. Mediterr. 34:149–156.

Ruppel, E.G. 1971. Variation among isolates of *Cercospora beticola* from sugar beet. Phytopathology 62:134–136.

Ruppel, E.G., and Scott, P.R. 1974. Strains of *Cercospora beticola* resistant to benomyl in the USA. Plant Dis. Rep. 58:434–436.

Saccardo, P.A. 1876. Fungi veneti novi vel critici. Series V. Nuovo Giorn. Bot. Ital. 8:162–211.

Shane, W.W., and Teng, P.S. 1992. Impact of Cercospora leaf spot on root weight, sugar yield, and purity of *Beta vulgaris*. Plant Dis. 76:812–820.

Shannon, C.E., and Weaver, W. 1949. The mathematical theory of communication. University of Illinois Press, Urbana.

Sokal, R.R., and Rohlf, F.J. 1995. Biometry. W.H. Freeman and Company, New York, New York, USA.

Taylor, J.W., Jacobson, D.J., and Fisher, M.C. 1999. The evolution of asexual fungi: reproduction, speciation, and classification. Annu. Rev. Phytopathol. 37:197–246.

Van de Peer, Y., and de Wachter, Y. 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Appl. Biosci. 10:569–570.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van der Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407–4414.

Waalwijk, C., Mendes, O., Verstappen, E.C., de Waard, M.A.D., and Kema, G.H.J. 2002. Isolation and characterization of the mating-type idiomorphs from the wheat septoria leaf blotch fungus *Mycosphaerella graminicola*. Fungal Genet. Biol. 35:277–286.

Wang, J., Levy, M., and Dunkle, L.D. 1998. Sibling species of *Cercospora* associated with gray leaf spot of maize. Phytopathology 88:1269–1275.

Weiland, J.J., and Koch, G. 2004. Sugar-Beet Leaf Spot Disease (*Cercospora beticola* SACC.). Mol. Plant Pathol. 5:157–166.

Weir, B.S., and Cockerham, C.C. 1984. Estimating F-statistics for the analysis of population structure. Evolution 38:1358–1370.

Wolf, P.F.J., and Verreet, J.A. 2002. Development and implementation of an integrated pest management system in Germany for the control of fungal leaf diseases in sugar beet: The IPM sugar beet model. Plant Dis. 86:336–344.

Wolf, P.F.J., and Verreet, J.A. 2005. Factors affecting the onset of Cercospora leaf spot epidemics in sugar beet and establishment of disease-monitoring thresholds. Phytopathology 95:269–274.

Wolf, P.F.J., Verreet, J.A., Maier, H., and Köhler, R. 2000. Pages 103–121 in: An Integrated Pest Management Model (IPM Sugar Beet Model) for Threshold-Oriented Control of *Cercospora beticola* on Sugar Beet, Developed Under Conditions in Southern Germany. International Institute for Beet Research, Brussels.

Wright, S. 1978. Evolution and the genetics of populations, Vol. 4: Variability within and among natural populations. University of Chicago Press, Chicago.

Yeh, F.C., Yang, R.-C., Boyle, T.B.J., Ze, Z.H., and Mao, J.X. 1997. Popgene: Microsoft Windows®-Based Freeeware for Population Genetic Analysis, Version 1.32. University of Alberta and Centre for International Forestry Research, Canada. Free program distributed by the authors over the internet from: http://www.ualberta.ca/~fyeh.

Zhan, J., Kema, G.H.J., Waalwijk, C., and McDonald, B.A. 2002. Distribution of mating type alleles in the wheat pathogen *Mycosphaerella graminicola* over spatial scales from lesions to continents. Fungal Genet. Biol. 36:128–136.

Zhan, J., Pettway, R.E., and McDonald, B.A. 2003. The global genetic structure of the wheat pathogen *Mycosphaerella graminicola* is characterized by high nuclear diversity, low mitochondrial diversity, regular recombination, and gene flow. Fungal Genet. Biol. 38:286–297.

Zhan, J., and McDonald, B.A. 2004. The interaction among evolutionary forces in the pathogenic fungus *Mycosphaerella graminicola*. Fungal Genet. Biol. 41:590–599.

CHAPTER 6

Development of polymorphic microsatellite and single nucleotide polymorphism markers for *Cercospora beticola*

Marizeth Groenewald[1,2], Johannes Z. Groenewald[1], and Pedro W. Crous[1,2]

[1] *Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, Netherlands*
[2] *Laboratory of Phytopathology, Wageningen University, Binnenhaven 5, 6709 PD Wageningen, Netherlands*

Submitted for publication.

Key words: mating types, *Mycosphaerella*, RFLP, SNP, sugar beet

Abstract: *Cercospora beticola* causes the most important foliage disease of sugar beet. Previous studies have detected genetic diversity within this pathogen by means of amplified fragment length polymorphism (AFLP) or randomly amplified polymorphic DNA (RAPD) analyses. The aim of this study was to develop additional, highly reproducible polymorphic markers for analysis of genetic diversity within natural populations of *C. beticola*. Five microsatellite and four single nucleotide polymorphism (SNP) markers were developed that allowed rapid screening of *C. beticola* populations.

Cercospora beticola is the main causal agent of Cercospora leaf spot on sugar beet (Saccardo, 1876). Few studies have been conducted on the genetic structure of *Cercospora* populations. A substantial amount of genetic variation was found within *Cercospora* populations isolated from sugar beet fields in Germany and Italy (Moretti *et al*., 2006) using a RAPD approach. Genetic variation was also observed between *C. beticola* isolates from the same plant lesion (M. Groenewald, *unpublished data*) using AFLP analyses. Groenewald *et al.* (2006) isolated and characterized the mating type genes (*MAT1-1-1* and *MAT1-2*) of *C. beticola* and showed that the two mating types are present in equal proportions in field populations. The aim of this study was thus to develop higly reproducible and easy to use polymorphic markers for *C. beticola* that could be used in future population studies.

The method described by Cortinas *et al*. (2006) was used to isolate microsatellite regions from the *C. beticola* genome (strain CBS 116456). Different temperatures (23 °C, 40 °C, 45 °C, 50 °C, 55 °C and 60 °C) were used for the annealing of probes. A total of 550 clones were sequenced, analyzed and screened for microsatellites. Four clones were found that contain eight or more perfect repeats and an additional five were selected that contain non perfect repeats (Table 1). Nine primer sets were developed (Table 1) and preliminary tests were performed on nine isolates. The PCR conditions were the same for all markers, except for marker SSRCb3 for which the annealing temperature was 55 °C. The reaction mixture contained 10 ng of genomic DNA, 1× PCR buffer, 48 µM of each of the dNTPs, 2.5 pmol of each of the specific forward and reverse SSRCb primers, 1.5 mM $MgCl_2$, and 0.7 units of Bioline *Taq* polymerase (Bioline, London, UK). The initial denaturation step was performed at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C or 58 °C for 20 s and elongation at 72 °C for 30 s with a final elongation step at 72 °C for 7 min.

The amplification products were sequenced using the PCR primers and a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Roosendal, Netherlands). The products were analyzed on an ABI Prism 3730 DNA Sequencer (Perkin-Elmer, Norwalk, Foster City, California). A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package (DNASTAR, Madison, Wisconsin). The microsatellites were evaluated for sequence polymorphism among the nine isolates. It was found that five primer pairs amplified microsatellite regions that were polymorphic and four were monomorphic (Table 1). The four monomorphic microstellite markers showed

Table 1. Characteristics of the nine polymorphic molecular markers developed for *Cercospora beticola*.

Primer	Fluorescent Label	Primer Sequence (5' – 3')	Tm (°C)	[a] Marker Type	Product Size (bp)	Core SSR Sequence	GenBank no.
SSRCb1F	NED	TGCGATCTGGGCATAAATATC	58	Microsatellite	223-225	(TC)	DQ902564
SSRCb1R		AGATTTGCATTTGCCCACAC					
SSRCb2F	6-FAM	TTGATGGCTGCGTGAGATAG	58	Microsatellite	195-197	(TC)	DQ902565
SSRCb2R		AGTGTGCGACTGACAGATGG					
SSRCb3F		CATCTCGTGCTCAACGGGTAC	55	Microsatellite	244-372	(AC)	DQ902566
SSRCb3R	6-FAM	ATAGAGTCAAACCAAGCCAAG					
SSRCb4F	NED	CCCGTTATAGCGCCCTTAG	58	Microsatellite	156-188	(AAAAGAAGAGAGA)	DQ902567
SSRCb4R		CAGAGGTAGAGCCGACGAC					
SSRCb6F	VIC	TTCGCGCCGATGCTGACTCG	58	Microsatellite	223-241; null allele	(CT)	DQ902568
SSRCb6R		TACAACTCGCACGCTTGATG					
SSRCb10F	none	TGTGATTCTTTCTTCCCTGT	58	SNP	115/247	—	DQ902569
SSRCb10R		CATCTGCAGCACGAATAA					
SSRCb10F$_2$		GCCAGAGTGTGCATAGATTG					
SSRCb11F	none	CGCGTGAGATAGTAACGATT	58	SNP	192/284	—	DQ902570
SSRCb11R		CTCCAAGGTTGCCAATGT					
SSRCb11F$_2$		TTCTCTGGAGCAGCAGTGG					
SSRCb12F	none	CGATATCATCGCGAAAGG	58	SNP	238/291	—	DQ902571
SSRCb12R		CCAGACATGATTAACAGAAGTAC					
SSRCb12F$_2$		CAAGGAACTTTGGAAGTCACTG					
SSRCb16F	none	GGGTGATAAGGGAGTCTTCA	58	PCR-RFLP	73+212/285	—	DQ902572
SSRCb16R		GCTATGATTGAAGAGATTGC					

[a] SNP = single nucleotide polymorphism marker; PCR-RFLP = PCR-based restriction fragment length polymorphism marker.

single nucleotide differences in the regions flanking the microsatellite repeats and these were converted to a RFLP and three SNP markers (Table 1). For the RFLP screening each reaction mixture was incubated at 37 °C for 10 hours and contains 200 ng of PCR product amplified with the SSRCb16 primers, 1× *Eco*RV buffer and 5 units *Eco*RV (Promega Benelux B.V., Leiden, Netherlands). The PCR products were visualized on a 1 % agarose gel stained with ethidium bromide and viewed under ultra violet light. For this marker the 285-bp PCR fragment is digested with *Eco*RV into a 212-bp and a 73-bp fragment.

For the SNP screening internal primers were designed (SSRCb10F_2, SSRCb11F_2 and SSRCb12F_2) within each of the polymorphic areas (Table 1) and used in a multiplex PCR together with the SSRCb10, SSRCb11 and SSRCb12 primer sets, respectively. The PCR conditions were the same for all markers. The mixture contained 10 ng gDNA, 1× buffer, 1.5 mM $MgCl_2$, 48 µM dNTPs, 2 pmol forward primer, 3 pmol each for the reverse as well as internal primer, and 0.7 units *Taq* polymerase. The same program was used as described above with an annealing temperature of 60 °C and the amplified fragments were separated on a 2 % agarose gel and visualized under UV light. For the SSRCb10F/F_2/R reaction a 247-bp fragment is present in all reaction whereas a 115-bp fragment is observed only when the specific polymorphism is present. For the SSRCb11F/F_2/R multiplex PCR, a 284-bp fragment is present in all reactions but only those containing the polymorphism, recognized by the internal primer, have the 192-bp fragment. A 238-bp fragment is amplified by the SSRCb12F/F_2/R primer combination only when the polymorphism is present, but due to competition between the two forward primers, a 291-bp fragment, which serves as the positive control, is only present in the absence of the smaller band.

For microsatellite analyses all forward primers were labelled with fluorescent dyes, except for primer pair SSRCb3 for which the reverse primer was fluorescently labelled (Table 1). Genomic DNA from 244 isolates of *C. beticola* was amplified with the fluorescently labelled primers. Allele sizes of amplified PCR products were determined by electrophoresis on an ABI PRISM™ 3730 Automated DNA sequencer (Perkin-Elmer) and data analysis was conducted with GENEMAPPER v4 (Applied Biosystems, Nieuwerkerk aan de IJssel, Netherlands).

Across the four SNP and the five microsatellite markers a total of 61 alleles was obtained for the 244 isolates (Table 2). The most polymorphic locus was SSRCb3 with 38 alleles (Table 2). A null allele was found with SSRCb6 for isolates from France, Germany and Italy. These isolates were screened three times with different conditions to verify the absence of the band. Our results show that polymorphic markers for *C. beticola* developed in this study can be applied to populations of this species to allow a better understanding of the population genetic structure of this pathogen.

ACKNOWLEDGEMENTS

This research was financially supported by the CBS-Odo van Vloten Stichting and the Royal Netherlands Academy of Arts and Sciences.

Table 2. Allele size and prevalence (number of isolates in round parentheses) for isolates of *Cercospora beticola* from various countries. The total number of isolates per population is given in square parentheses.. H: Gene Diversity (Weir, 1990).

Marker	France [44]	Germany [40]	Italy [30]	Netherlands [46]	Iran [46]	New Zealand [38]	Number of alleles	H
SSRCb1	223 (35); 225 (9)	223(40); 225 (0)	223(27); 225(3)	223(37); 225(9)	223(36); 225(10)	223 (29); 225 (9)	2	0.27
SSRCb2	195 (39); 197 (5)	195 (35); 197 (5)	195 (28); 197 (2)	195 (43); 197 (3)	195 (46); 197 (0)	195 (7); 197 (31)	2	0.31
SSRCb3	244 (3); 246 (5); 272 (2); 274 (17); 276 (3); 282 (2); 298 (1); 300 (1); 304 (1); 306 (1); 308 (1); 330 (1); 334 (1); 336 (1); 358 (3); 360 (1)	244 (3); 246 (1); 248 (1); 250 (6); 258 (3); 276 (3); 282 (3); 296 (1); 308 (1); 312 (3); 314 (6); 316 (6); 318 (1); 324 (1); 372 (1)	244 (7); 246 (4); 248 (1); 250 (3); 252 (1); 278 (1); 304 (1); 306 (2); 308 (1); 314 (1); 318 (1); 326 (3); 330 (1); 334 (2); 338 (1)	244 (13); 246 (5); 248 (1); 272 (4); 282 (4); 294 (1); 298 (1); 304 (3); 306 (3); 308 (4); 314 (3); 322 (1); 324 (1); 326 (2)	264 (1); 282 (2); 288 (8); 290 (1); 300 (6); 304 (14); 306 (4); 308 (1); 312 (2); 314 (3); 320 (1); 330 (2); 340 (1)	264 (2); 274 (6); 276 (11); 278 (12); 280 (1); 294 (1); 318 (1); 320 (3); 322 (1)	38	0.95
SSRCb4	188 (8); 156 (36)	188 (10); 156 (30)	188 (8); 156 (22)	188 (22); 156 (24)	188 (40); 156 (6)	188 (37); 156 (1)	2	0.5
SSRCb6	null (19); 227 (1); 229 (13); 231 (4); 233 (5); 237 (2)	null (6); 223 (1); 225(1); 227 (9); 229 (5); 231 (1); 233 (17)	null (4); 225(2); 229 (8); 231 (5); 233 (10); 237 (1)	227 (3); 229 (19); 231 (14); 233 (10)	229 (2); 231 (39); 233 (5)	227 (2); 231 (34); 233 (1); 241 (1)	9	0.75
SSRCb10	115 (44)	115 (40)	115 (30)	115 (46)	115 (41); 247 (5)	115 (34); 247 (4)	2	0.07
SSRCb11	192 (35); 284 (9)	192 (34); 284 (6)	192 (30)	192 (44); 284 (2)	192 (36); 284 (10)	192 (18); 284 (20)	2	0.30
SSRCb12	238 (37); 291 (7)	238 (22); 291 (18)	238 (17); 291 (13)	238 (25); 291 (21)	238 (36); 291 (10)	238 (15); 291 (23)	2	0.47
SSRCb16	212 (7); 285 (37)	212 (8); 285 (32)	212 (12); 285 (18)	212 (9); 285 (37)	212 (27); 285 (19)	285 (38)	2	0.38

REFERENCES

Cortinas, M.N., Barnes, I., Wingfield, B.D., and Wingfield, M.J. 2006. Polymorphic microsatellite markers for the *Eucalyptus* fungal pathogen *Colletogloeopsis zuluensis*. Mol. Ecol. Notes 6:780.

Groenewald, M., Groenewald, J.Z., Harrington, T.C., Abeln, E.C.A., and Crous, P.W. 2006. Mating type gene analysis in apparently asexual *Cercospora* species is suggestive of cryptic sex. Fungal Genet. Biol. 43:813–825.

Moretti, M., Karaoglanidis, G., Saracchi, M., Fontana, A., and Farina, G. 2006. Analysis of genotypic diversity in *Cercospora beticola* Sacc. field isolates. Ann. Microbiol. 56:215–221.

Saccardo, P.A. 1876. Fungi veneti novi vel critici. Series V. Nuovo Giorn. Bot. Ital. 8:162–211.

Weir, B.S. 1990. Genetic data analysis methods for discrete genetic data. Sinauer Association, Sunderland, Massachusetts.

CHAPTER 7

Characterization and distribution of mating type genes in the Dothistroma needle blight pathogens

Marizeth Groenewald[1], Irene Barnes[2], Rosie E. Bradshaw[3], Anna V. Brown[4], Angie Dale[5], Johannes Z. Groenewald[1], Kathy J. Lewis[5], Brenda D. Wingfield[2], Michael J. Wingfield[2], and Pedro W. Crous[1]

[1] *Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, Netherlands*

[2] *Forestry and Agricultural Biotechnology Institute (FABI), Department of Genetics, University of Pretoria, Pretoria 0002, South Africa*

[3] *Institute of Molecular BioSciences, Massey University, Palmerston North, New Zealand*

[4] *Forest Research, Alice Holt Lodge, Wrecclesham, Farnham, Surrey, GU10 4LH, United Kingdom*

[5] *University of Northern British Columbia, 3333 University Way, Prince George, BC V2N 4Z9, Canada*

Submitted for publication.

Key words: allele frequency, ascomycete, *Dothistroma pini*, *Dothistroma septosporum*, fungi, geographic distribution, heterothallic, mating types, *Mycosphaerella*, PCR amplification, sexual reproduction

Abstract: *Dothistroma septosporum* and *D. pini* are the two main causal agents of Dothistroma needle blight of *Pinus* spp. in natural forests and plantations. Degenerate primers were used to amplify the partial mating type genes (*MAT1-1-1* and *MAT1-2*) and chromosome walking was applied to obtain the full-length genes in both species. The mating type-specific primers designed in this study could distinguish between the morphologically similar *D. pini* and *D. septosporum* as well as between the different mating types of these species. Screening of isolates from global collections of *D. septosporum*, showed that only MAT2 isolates are present in Australian and New Zealand collections where only the asexual form of the fungus has been found. In contrast, both mating types of *D. septosporum* were present in collections from Canada where the sexual state of the fungus is known. Intriguingly, collections from South Africa, where the sexual state of the fungus is unknown, included both mating types. In *D. pini*, for which no teleomorph is known, both mating types were present in collections from the United States of America. These results provided new insights into the biology and global distribution of two of the world's most important pine pathogens and they should facilitate management of the diseases caused by these fungi.

INTRODUCTION

Dothistroma needle blight, also known as red band needle blight, is one of the most important diseases of *Pinus* spp., both in natural forest ecosystems especially of non-native pines (Ivory, 1967; Cobb & Miller, 1968; Gibson, 1972, 1974). The disease owes its international notoriety to the fact that it has been one of the most important constraints to the development of plantation forestry in many countries of Africa as well as in New Zealand, Australia, Chile and other South American countries (Ivory, 1967; Gibson, 1972, 1974). The disease is particularly severe on *Pinus radiata* D. Don. This species is highly desirable for its rapid growth and exceptional high quality "timber", and consequently one of the first non-native tree species established in intensively managed plantations in the tropics and Southern Hemisphere. Outbreaks of Dothistroma needle blight on *P. radiata* led to devastating losses and resulted in its abandonment from plantation forestry in many countries (Marks *et al*., 1989; Coops *et al*., 2003; M.J. Wingfield, *unpublished data*).

The main causal agent of Dothistroma needle blight has been a matter of considerable taxonomic confusion. In various parts of the world, the disease has thus been attributed to a single pathogen, different species of a pathogen as well as varieties of a species. This has also differed depending on whether the pathogen was considered introduced or native in areas where the disease has been studied. Recent studies based on DNA sequence comparisons have provided robust evidence to show that two fungi, namely *D. septosporum* and *D. pini*, cause Dothistroma needle blight (Barnes *et al*., 2004). This study also showed that the disease which devastated plantations of *P. radiata* in the Southern Hemisphere, is caused by *D. septosporum*. Barnes *et al*. (2004) identified two distinct phylogenetic lineages for *Dothistroma* isolates. These clearly separated *D. septosporum*, which has a world-wide distribution, and *D. pini*, until recently only found in north central USA. Recently, *D. pini*, has been found infecting *P. pallassiana* D. Don.

in the Ukraine (I. Barnes, *unpublished data*) and it clearly has a distribution much wider than was believed at the time of the study of Barnes *et al*. (2004).

Dothistroma needle blight, now known to have been caused by *D. septosporum*, resulted in huge damage to *P. radiata* plantations in the Southern Hemisphere in the 1950s and 1960s (Ivory, 1967; Cobb & Miller, 1968; Gibson, 1972, 1974). Consequently, considerable research was conducted on the disease and great efforts were made to minimize its impact (Gibson, 1972, 1974; Ray & Vanner, 1988; Carson *et al*., 1991; Stone *et al*., 2003). These included selection of alternative species, tree breeding, agricultural practices and the first examples of aerial applications of chemical fungicides in forest plantations (Gibson, 1972). Although the disease has continued to be important, it is generally considered to be under reasonable control. There has however been a recent resurgence of the disease in various Northern Hemisphere countries and this has raised concern that a new wave of losses might occur elsewhere in the world (Woods, 2003; Bradshaw, 2004).

Almost nothing is known on the genetic diversity amongst isolates of *D. septosporum* and *D. pini*. The single study by Hirst *et al*. (1999) applied random amplified polymorphic DNA (RAPD) markers to a population of *D. septosporum* (as *D. pini*) from New Zealand. Results showed no genetic variation, supporting the hypothesis that it is an introduced pathogen that has been spreading asexually ever since its introduction into that country.

The sexual state of *D. septosporum* is a species of *Mycosphaerella* known as *M. pini* E. Rostr. in Munk (Funk & Parker, 1966). In most countries of the Southern Hemisphere where *D. septosporum* has long been an important forest pathogen, only the anamorph has been reported (Evans, 1984; Barnes *et al*., 2004; Bradshaw, 2004; M.J. Wingfield, *unpublished data*). However, Barnes *et al*. (2004) linked the fungus to the teleomorph state *M. pini* using nucleotide sequence data. In contrast, no sexual state has ever been reported for *D. pini*. The absence or rarity of a sexual state for either of these fungi could be the result of selection pressure and a reduced need for sexual reproduction (Evans, 1984). Likewise, lower frequency and limited distribution of the teleomorph compared with the anamorph suggests that the primary method of dispersal of the fungus could be by asexually produced conidia. Thus conidia, rather than ascospores, would represent the inoculum of primary epidemiological importance (Cobb *et al*., 1969; Karadzic, 1989).

Mating type genes play an important part in the biology and evolution of fungal species. Thus, knowledge of these genes can provide insight into the potential prevalence of sexual reproduction in different species. Some heterothallic pyrenomycetes and discomycetes can contain up to four mating type 1 (MAT1) genes (Sing & Ashby, 1998, 1999; Yun *et al*., 2000; Pöggeler, 2001). These include the *MAT1-1-1* encoding an alpha domain protein, the *MAT1-1-2* encoding an amphipathic alpha helix protein, the *MAT1-1-3* gene encoding a HMG domain protein and the *MAT1-1-4* gene encoding a metallothionein protein. Only one mating type 2 (MAT2) gene has been characterized and it encodes a regulatory protein with a high mobility group (HMG) domain. The formal nomenclature that is proposed for mating type genes of heterothallic ascomycetes is used here for the *MAT1-1-1*, and since only a single MAT2 gene has been identified for filamentous ascomycetes, this gene is referred to as *MAT1-2* (Turgeon & Yoder, 2000).

DNA and amino acid sequences of the *MAT1-1-1* and *MAT1-2* in fungi show no obvious similarities, although the mating type locus has common flanking regions (Turgeon *et al*., 1993). Except for the HMG and alpha domains, the similarity of homologous mating type genes is usually very low between different species (Turgeon, 1998). The direct target genes of the mating type proteins have not yet been described, although there is evidence for the control of some genes such as pheromone genes (Bobrowicz *et al*., 2002). Mating type genes have been described for various sexual and presumably asexual fungi that are close relatives of *Dothistroma* (Mycosphaerellaceae). Detailed analyses have been performed on the distribution of the mating types of the sexually reproducing *Mycosphaerella graminicola* (Waalwijk *et al*., 2002; Zhan *et al*., 2002) and the presumably asexual species *Septoria passerinii* (Goodwin *et al*., 2003), *Cercospora beticola*, *C. zeae-maydis* and *C. zeina* (Groenewald *et al*., 2006). Equal distribution of the mating types was found in most of the populations from these five species sampled from different geographical scales indicating that sexual stages probably exist for the latter four apparently asexual species.

In most parts of the world where *D. septosporum* has been introduced and causes serious disease, only the asexual state of the fungus is ever seen. This raises the interesting question as to whether this could be attributed to the introduction of only one mating type into these new environments. The aims of this study were thus to characterize the mating type gene(s) of the causal agents of Dothistroma needle blight and to ascertain which mating type(s) are present in the different countries where diseases caused by these fungi occur. To achieve this objective, the full-length *MAT1-1-1* and *MAT1-2* genes of *D. septosporum* and *D. pini* were sequenced using PCR-based techniques. This made it possible to develop a multiplex PCR method for the rapid screening for the presence of the *MAT1-1-1* and *MAT1-2* genes in isolates of the pathogens. A global collection of isolates was subsequently screened to determine mating type distributions in these populations.

MATERIALS AND METHODS

Fungal isolates

A total of 227 *Dothistroma* strains obtained from various locations in 15 countries were chosen to represent a global distribution of *Dothistroma* (Table 1). Countries for which more than one isolate was screened included Austria (10), Canada (106), Chile (10), New Zealand (36), Poland (11), South Africa (11), Ukraine (4), United Kingdom (10) and USA (17). Isolates were obtained from different culture collections and standard protocols were used to isolate the genomic DNA.

The initial screening of the mating type genes was undertaken for *D. septosporum* using two isolates. These included CBS 116489 obtained from *P. radiata* in Tzaneen, South Africa and ATCC MYA-605 obtained from *P. radiata* in Rotorua, New Zealand. For *D. pini* four isolates were used; CBS 116485 obtained from *P. nigra* in Crystal Township, Michigan, USA; CBS 116487 obtained from *P. nigra* in Evergreen Township, Michigan, USA; CBS 116483 obtained from River Township, Michigan, USA and CBS 117609 obtained from *P. palassiana* in Tsyurupinsk, Ukraine. The identities of the six isolates used for the screening of the mating

Table 1. Origins of the *Dothistroma septosporum* and *D. pini* strains used during this study and the distribution of their mating types.

Country/Area/Site	Collector	Species	Number of Strains	MAT1	MAT2
Australia					
A.C.T. Canberra	K. Old	*D. septosporum*	10	0	10
Austria					
Thenneberg	T. Kirisits	*D. septosporum*	10	6	4
Brazil					
São Paulo	T. Namekata	*D. septosporum*	1	0	1
Canada					
Northwest BC					
Brown Bear Road	K. Lewis & A. Dale	*D. septosporum*	10	5	5
Bell Irving River	K. Lewis & A. Dale	*D. septosporum*	1	0	1
Bulkley Canyon	K. Lewis & A. Dale	*D. septosporum*	9	5	4
Evelyn Pasture	K. Lewis & A. Dale	*D. septosporum*	1	0	1
Jonas Creek	K. Lewis & A. Dale	*D. septosporum*	2	0	2
Kinskutch Road	K. Lewis & A. Dale	*D. septosporum*	8	7	1
Kuldo Creek	K. Lewis & A. Dale	*D. septosporum*	7	2	5
Kisgegas Canyon	K. Lewis & A. Dale	*D. septosporum*	5	2	3
Squingula River Mine	K. Lewis & A. Dale	*D. septosporum*	8	1	7
Mosque River	K. Lewis & A. Dale	*D. septosporum*	6	1	5
Mitten Road	K. Lewis & A. Dale	*D. septosporum*	7	4	3
Nangeese Road	K. Lewis & A. Dale	*D. septosporum*	8	4	4
North Kuldo Road	K. Lewis & A. Dale	*D. septosporum*	4	1	3
Sanyam river	K. Lewis & A. Dale	*D. septosporum*	1	0	1
Nash Y	K. Lewis & A. Dale	*D. septosporum*	9	7	2
Orendo	K. Lewis & A. Dale	*D. septosporum*	7	6	1
Motaze lake & Squingula River	K. Lewis & A. Dale	*D. septosporum*	8	6	2
Sunday Lake	K. Lewis & A. Dale	*D. septosporum*	4	1	3
Goldstream River BC	D. Morrison	*D. septosporum*	1	0	1
Chile					
Valdivia	M.J. Wingfield	*D. septosporum*	10	0	10
France					
Meurthe-et-Moselle	M. Morelet	*D. septosporum*	1	0	1
Germany					
Bavarian Alps	L. Pehl	*D. septosporum*	1	0	1
Guatemala					
Sierra de Chuacús	Unknown	*D. septosporum*	1	0	1
New Zealand					
Bay of Plenty	M.A. Dick	*D. septosporum*	1	0	1
Golden Downs sites 1/2/3	P. Hirst	*D. septosporum*	4	0	4
Kaingora Forest	M.J. Wingfield	*D. septosporum*	10	0	10
Kaingora sites 1/2/3	P. Hirst	*D. septosporum*	11	0	11
Kinleith	P. Hirst	*D. septosporum*	5	0	5

Table 1. Continued.

Country/Area/Site	Collector	Species	Number of Strains	MAT1	MAT2
Mt Maunganui	K. Dobbie	*D. septosporum*	1	0	1
Rotorua	M.E. Buchanan	*D. septosporum*	2	0	2
Tongariro	J.W. Gilmour	*D. septosporum*	1	0	1
West Coast South Island	B. Doherty	*D. septosporum*	1	0	1
Poland					
Miechow Forest, Cracow	T. Kowalski	*D. septosporum*	11	3	8
Slovakia	E. Foffova	*D. septosporum*	1	1	0
South Africa					
Hogsback	J. Roux	*D. septosporum*	10	3	7
Tzaneen	I. Barnes	*D. septosporum*	1	1	0
Ukraine					
Tsyurupinsk	A.C. Usichenko	*D. pini*	4	4	0
UK					
West Midlands	A. Coggin	*D. septosporum*	1	0	1
South East England	A.V. Brown	*D. septosporum*	1	0	1
Forest of Dean	R. Beasley	*D. septosporum*	1	1	0
New Forest	A.V. Brown	*D. septosporum*	7	1	6
USA					
Oregon, Bandon	S. Cooley	*D. septosporum*	1	0	1
Michigan					
Crystal Township	G. Adams	*D. pini*	10	4	6
Evergreen Township	G. Adams	*D. pini*	1	1	0
River Township	G. Adams	*D. pini*	1	0	1
Central Minnesota	T. Nicholls	*D. pini*	1	1	0
Nebraska, Lincoln	G. Peterson	*D. pini*	3	2	1
Total			230	80	150

types had previously been confirmed using comparisons of DNA sequence data for the Internal Transcribed Spacer (ITS) regions (Barnes *et al*., 2004; J.Z. Groenewald, *unpublished data*).

Isolation and characterization of *MAT1-1-1* of *Dothistroma* species

The *MAT1-1-1*-specific degenerate primers (MgMfSpMat1-1f1 and MgMfSpMat1-1r2) (Table 2), designed by Groenewald *et al*. (2006), were used to screen and amplify a partial region of the *MAT1-1-1* genes of the *Dothistroma* isolates.

The reaction mixtures had a total volume of 12.5 µl and contained 0.7 µl of diluted gDNA, 1× PCR buffer (Bioline, London, UK), 48 µM of each of the dNTPs, 8 pmol of each degenerate primer, 1.5 mM $MgCl_2$ and 0.7 units *Taq* polymerase (Bioline). The amplification reactions were performed on a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, California). The initial denaturation step was performed at 94 °C for 5 min, followed by 25 cycles of 94 °C (20 s), 52 °C (20 s) and 72 °C (30 s), followed by 25 cycles of 94 °C (20 s), 50 °C (20 s) and 72 °C (50 s). A final elongation step at 72 °C (5 min) was included in the run. The PCR

Table 2. Primers used during this study.

Primer	5' — 3'	Description
MgMfSpMat1-1f1	CATTNGCNCATCCCTTTG	*MAT1-1-1*-specific degenerate primer
MgMfSpMat1-1r2	GGCTTNGANACCATGGTGAG	*MAT1-1-1*-specific degenerate primer
MgMfSpMat1-2f2	CAAAGAANGCNTTCNTGATCT	*MAT1-2*-specific degenerate primer
MgMfSpMat1-2r1	TTCTTCTCNGATGGCTTGC	*MAT1-2*-specific degenerate primer
DseptoMat1f	CGCAGTAAGTGATGCCCTGAC	*D. septosporum MAT1-1-1*-specific primer
DpiniMat1f2	AGTAAGCGACGCGCTCCCATG	*D. pini MAT1-1-1*-specific primer
DotMat1r	TTGCCTGACCGGCTGCTGGTG	*Dothistroma MAT1-1-1*-specific primer
DseptoMat2f	GTGAGTGAACGCCGCACATGG	*D. septosporum MAT1-2*-specific primer
DpiniMat2f	GTAAGTGATCGTTGAACATGC	*D. pini MAT1-2*-specific primer
DotMat2r	CTGGTCGTGAAGTCCATCGTC	*Dothistroma MAT1-2*-specific primer

products obtained were separated by electrophoresis at 80 V for 1 h on a 1 % (w/v) agarose gel containing 0.1 µg/ml ethidium bromide in 1× TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualized under UV light. Amplicons were sequenced in both directions using the PCR primers and a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Roosendaal, Netherlands) following the manufacturer's recommendations. The products were analyzed on an ABI Prism 3730 DNA Sequencer (Applied Biosystems). A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package (DNA-STAR, Madison, Wisconsin).

Internal primers were designed in the partially sequenced *MAT1-1-1* genes for each of the species (CBS 116489 for *D. septosporum* and CBS 116487 for *D. pini*). In order to obtain the full-length genes, these internal primers were used together with the appropriate primers from the DNA walking speedup kit (Seegene Inc., Rockville, USA) to determine additional sequences upstream and downstream of the partial *MAT1-1-1* sequences. In total, 12 primers were designed and used for chromosome walking. Blastx (Altschul *et al*., 1997) was used to compare the sequences obtained from the two *Dothistroma* species with protein sequences of other fungi present in the NCBI non-redundant protein database. The geneid v1.2 web server (http://www1.imim.es/geneid.html - Research Unit on Biomedical Informatics of IMIM, Barcelona, Spain) was used to predict the gene and intron/exon boundaries using the genetic code of *Neurospora crassa*. The conversion of DNA sequences to putative amino acid sequences was performed using the translation tool of ExPASy (Gasteiger *et al*., 2003). The percentage identities between the predicted *MAT1-1-1* gene sequences for the *Dothistroma* species were calculated using the alignment tool of ALIGN (Pearson *et al*., 1997).

Isolation and characterization of *MAT1-2* of *Dothistroma* species

The *MAT1-2*-specific degenerate primers (MgMfSpMat1-2f2 and MgMfSpMat1-2fr1) (Table 1), designed by Groenewald *et al*. (2006), were used to screen isolates of *D. septosporum* and *D. pini* to obtain a partial region of the *MAT1-2* genes. The same PCR conditions described above were used to amplify the partial *MAT1-2* regions. Internal primers were designed in the partially sequenced *MAT1-2* sequences for both species (ATCC MYA-605 for *D. septosporum* and CBS 116485 for *D. pini*). These primers were used together with the primers from the

DNA walking speedup kit to determine the regions flanking the sequences. In total, 12 primers were designed and used for chromosome walking to obtain the full-length *MAT1-2* genes. The same procedure and programs described for the characterization and analyses of the *MAT1-1-1* sequences were used to characterize and analyze the *Dothistroma MAT1-2* sequences.

Development and screening of *D. pini* and *D. septosporum* mating type-specific primers

Dothistroma MAT1-1-1-specific primers (Table 2) were designed from the aligned *MAT1-1-1* sequences of *D. pini* and *D. septosporum* (GenBank accession nos. DQ915449 and DQ915450, respectively). The forward primers were designed to be specific for *D. septosporum* (DseptoMat1f) or *D. pini* (DpiniMat1f2) and are, therefore, both species- and mating type-specific. The reverse primer (DotMat1r) was designed from homologous regions within the *MAT1-1-1* genes and is, therefore, only mating type-specific.

Dothistroma MAT1-2-specific primers (Table 2) were designed from the aligned *MAT1-2* sequences of *D. pini* and *D. septosporum* (GenBank accession nos. DQ915451 and DQ915452, respectively). The two forward primers were designed in regions of the genes that were variable between the two species. DseptoMat2f was designed to be specific for *D. septosporum* and DpiniMat2f for *D. pini,* and both are, therefore, species- and mating type-specific. The reverse primer (DotMat2r) was designed from homologous regions within both the *MAT1-2* genes, and is thus, only mating type-specific.

Multiplex PCR was used to screen for the *MAT1-1-1* or the *MAT1-2* of *D. pini* and *D. septosporum* in two separate reactions. The reaction mixtures had a total volume of 12.5 µl and contained 0.7 µl of diluted gDNA, 1× PCR buffer (Bioline), 48 µM of each of the dNTPs, 4 pmol of each primer, 1 mM $MgCl_2$ and 0.7 units *Taq* polymerase (Bioline). The amplification reactions were performed on a GeneAmp PCR System 9600 (Applied Biosystems). The initial denaturation step was performed at 94 °C for 5 min, followed by 40 cycles of 94 °C (20 s), 65 °C (20 s) and 72 °C (40 s). A final elongation step at 72 °C (5 min) was included in the run. The resulting PCR products were visualized as described above. Twenty *D. pini* and 210 *D. septosporum* isolates (Table 1) were screened with the two mating type-specific primer sets to determine the mating type and to confirm the identity of each isolate.

Phylogenetic analyses

The nucleotide sequences of the alpha domain (*MAT1-1-1*) and HMG domain (*MAT1-2*) of *D. septosporum* and *D. pini* determined in this study and additional mating type sequences for other species representing different fungal orders downloaded from NCBI's GenBank database, were used for phylogenetic analyses. These sequences were analyzed using the mating type gene sequences of *Magnaporthe grisea* (GenBank accession nos. AB080672 and AB080673, respectively) as the outgroup. All phylogenetic analyses were performed in PAUP (Phylogenetic Analysis Using Parsimony) v4.0b10 (Swofford, 2003). Maximum parsimony analyses were conducted as described by Groenewald *et al*. (2005). All sequences generated were deposited in GenBank, and the alignments and trees were deposited in TreeBASE (accession number SN3047).

RESULTS

Isolation and characterization of *MAT1-1-1* in *Dothistroma* species

The degenerate primers MgMfSpMAT1-1f1 and MgMfSpMAT1-1r2 amplified a fragment of 914 bp for three of the six *Dothistroma* isolates tested (Fig. 1). The fragments obtained from strains CBS 116489, CBS 117609 and CBS 116487 were sequenced. The translated sequence of the fragment obtained from strain CBS 116489 (*D. septosporum*) showed 39 % and 46 % identity to a 229 and 63 amino acid region of the *M. graminicola* MAT1 protein and 32 % identity to a 213 amino acid region of the *S. paserinii* MAT1 protein using Blastx on the GenBank database. This confirmed that the 914-bp fragment is part of the *MAT1-1-1* gene of *D. septosporum*.

Sequences for the fragments obtained from the *D. pini* strains (CBS 117609 and CBS 116487) showed 100 % identity to each other in this region. The translated sequences showed 39 % and 37 % identity to a 226 and 78 amino acid region of the *M. graminicola* MAT1 protein and 32 % identity to a 218 amino acid region of the *S. paserinii* MAT1 protein. This confirmed that the 914-bp fragment is part of the *MAT1-1-1* gene of *D. pini*.

The full-length *MAT1-1-1* gene sequences for *D septosporum* and *D. pini* were obtained by chromosome walking. The geneid software predicted that the *MAT1-1-1* genes of both species contained four exons. The predicted length of the genes and the exon and intron positions are illustrated in Fig. 2. Although the number of amino acids was the same for both species (387 aa), an identity of 94.1 % was found between the 1,311 nucleotide residues of the *MAT1-1-1* of *D. septosporum* and *D. pini.* All introns of the *MAT1-1-1* from both species contained a perfect lariat sequence (RCTRAC), except for the second intron of the *MAT1-1-1* of *D. septosporum*. When this intron is included in the coding region, an early stop codon is introduced in the reading frame, indicating that this is a true intron. The positions of the three predicted introns in the *Dothistroma* species studied correlate with those found for *Cercospora* species (Groenewald *et al*., 2006). The number of predicted introns (two) in the conserved alpha domain of the *Dothistroma* species correlated with the number predicted for the same region in *M. graminicola* (Waalwijk *et al*., 2002) and *S. passerinii* (Goodwin *et al*., 2003).

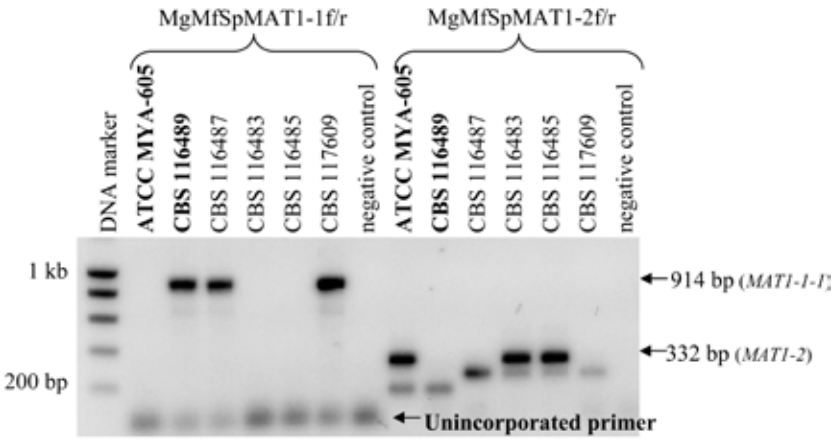


Fig. 1. Amplification products obtained from *Dothistroma septosporum* (in boldface) and *D. pini* isolates containing the partial *MAT1-1-1* (914 bp) and *MAT1-2* (332 bp) genes using the degenerate primer sets MgMfSpMAT1-1 and MgMfSpMAT1-2, respectively.

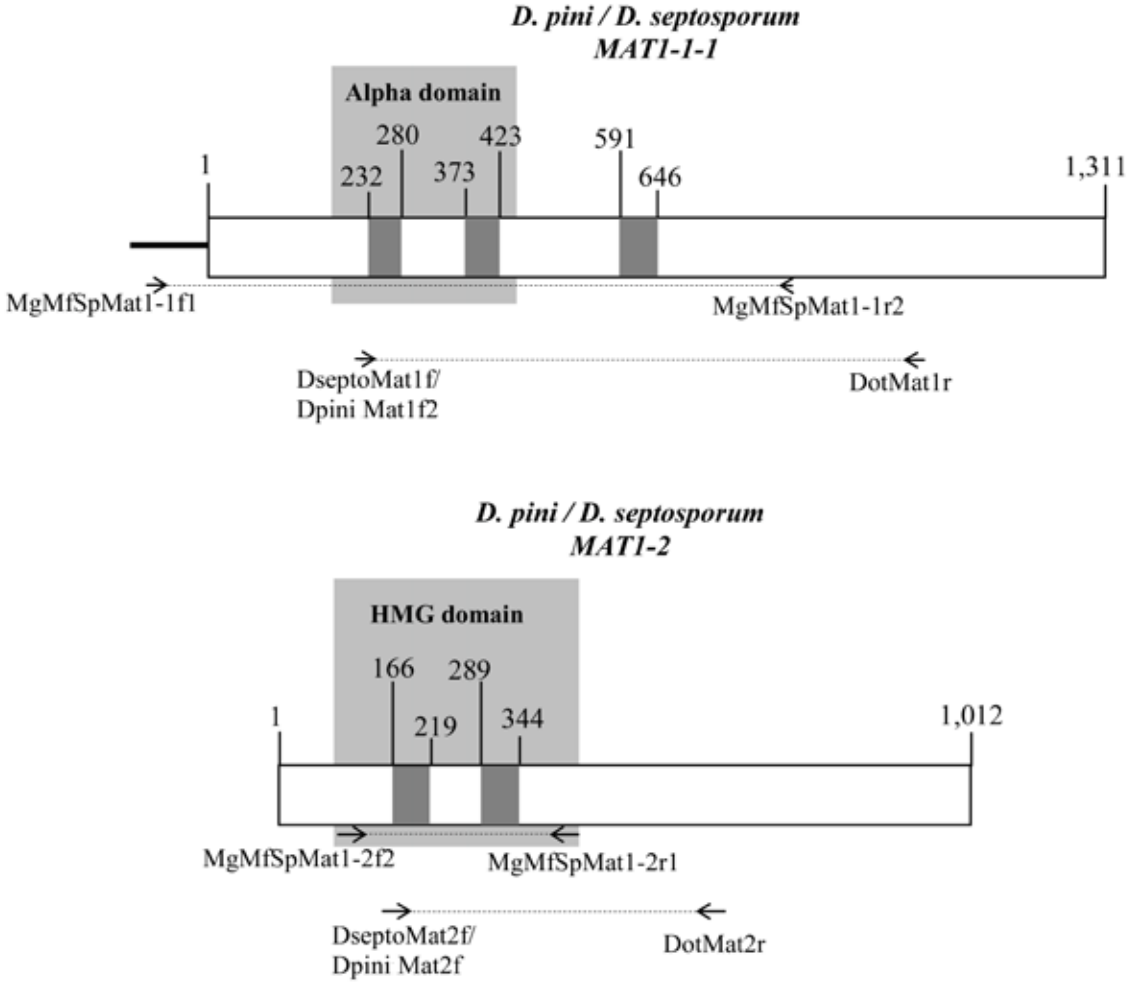


Fig. 2. Diagrammatic representation of the full-length *MAT1-1-1* and *MAT1-2* genes of *Dothistroma septosporum* and *D. pini*. The predicted sites of exons (white bars), and introns (shaded bars) are shown, and their locations (nucleotide position) are indicated. The areas amplified by the MgMfSpMAT1-1 and MgMfSpMAT1-2 primer sets as well as the mating type specific primers for each species are indicated.

Isolation and characterization of *MAT1-2* of *Dothistroma* species

The degenerate primers MgMfSpMAT1-2f2 and MgMfSpMAT1-2r1 amplified a fragment of 332 bp for the *Dothistroma* isolates that did not amplify the 914-bp fragment using the *MAT1-1-1* degenerate primers (Fig. 1). An additional 180 bp fragment was also obtained from the two *D. septosporum* strains as well as an additional 280 bp fragment from the four *D. pini* strains. The 332-bp fragment obtained from strain ATCC MYA-605 (*D. septosporum*) was sequenced, and the translated sequence showed 55 % and 70 % identity to a 65 and 27 amino acid region of the *M. graminicola* MAT2 protein and 50 % identity to a 65 amino acid region of the *S. passerinii* MAT2 protein using Blastx on the GenBank database. This confirmed that the 332-bp fragment is part of the *MAT1-2* gene of *D. septosporum*. The 332-bp translated sequences for the fragments obtained from the two *D. pini* strains (CBS 116483 and CBS 116485) showed 52 % and 68 % identity to a 65 and 29 amino acid region of the *M. graminicola* MAT2 protein as well as 47 % and 68 % identity to the same amino acid regions of the *S. passerinii* MAT2 protein. This confirmed that the 332-bp fragment is part of the *MAT1-2* gene of *D. pini*. Sequences for the 180-bp (*D. septosporum*) and 280-bp (*D. pini*) fragments showed no homology to protein sequences available in GenBank.

The full-length *MAT1-2* gene sequences for both *Dothistroma* species were obtained by chromosome walking. The geneid software predicted that the *MAT1-2* sequences of both species contain three exons. The predicted length of the genes, as well as exon and intron positions, is illustrated in Fig. 2. The number of amino acids was the same for both species (302 aa), and the identity over the 1,012 nucleotides of the *MAT1-2* of the two species was 94.4 %. All the introns found for both species contained a perfect lariat sequence. The number of predicted introns (two) of the *Dothistroma* species studied correlates with the number predicted for *Cercospora* species (Groenewald *et al*., 2006), but the specific locations of these introns within the gene differed. Only one predicted intron was found in the HMG domain of species of *Cercospora* (Groenewald *et al*., 2006), *M. graminicola* (Waalwijk *et al*., 2002) and *S. passerinii* (Goodwin *et al*., 2003), whereas two predicted introns were found in the same region of the *Dothistroma* species studied.

Screening with *D. pini* and *D. septosporum* mating type-specific primers

In the *D. pini* MAT1 isolates, DpiniMat1f2 and DotMat1r amplified an 820 bp fragment and in the *D. pini* MAT2 isolates DpiniMat2f and DotMat2r amplified a 480 bp fragment (Fig. 3). Each isolate tested showed either the 820-bp or the 480-bp fragment of the respective *MAT1-1-1* or *MAT1-2* genes. None of the isolates contained both fragments. The *D. pini* mating type-specific primers did not amplify the *MAT1-1-1* and *MAT1-2* fragments in any of the *D. septosporum* isolates (Fig. 3). The majority of the *D. pini* isolates were from areas in the USA where both mating types are known to exist. An equal distribution of the mating types (eight isolates of each mating type) was found for these *D. pini* isolates. Only one mating type (MAT1) was found for the *D. pini* isolates from Ukraine. In the *D. septosporum* MAT1 isolates, DseptoMat1f2 and DotMat1r amplified an 820 bp fragment and in the *D. septosporum* MAT2 isolates, DseptoMat2f and DotMat2r amplified a 480 bp fragment (Fig. 3). Each isolate tested showed either the 820-bp or the 480-bp fragment of the *MAT1-1-1* or *MAT1-2* genes, respectively. None of the isolates amplified both fragments.

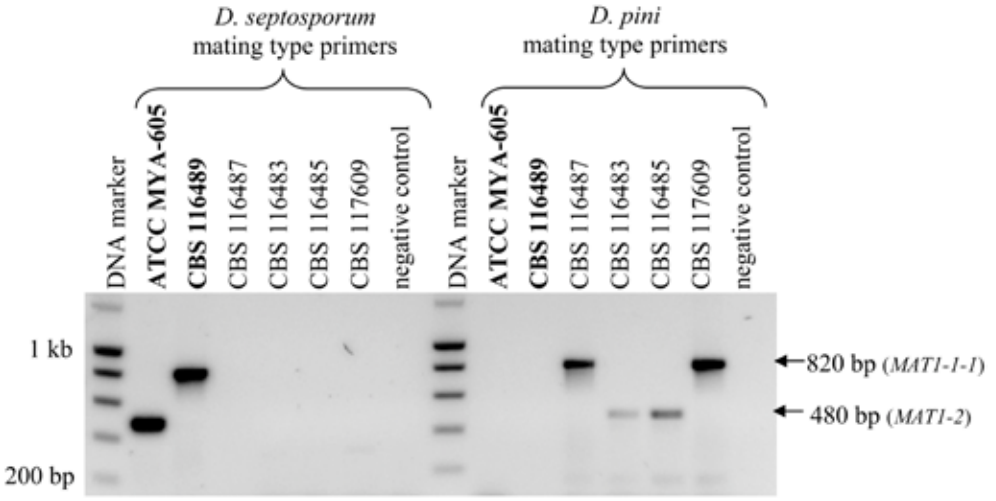


Fig. 3. *Dothistroma septosporum* (in boldface) and *D. pini* isolates screened using the Dsepto/Dpini/DotMat1 primer set (820 bp fragment) and the same *Dothistroma* isolates screened with the Dsepto/Dpini/DotMat2 primer set (480 bp fragment).

The *D. septosporum* mating type-specific primers did not amplify the *MAT1-1-1* and *MAT1-2* fragments of the *D. pini* isolates (Fig. 3). All *D. septosporum* isolates obtained from Chile, Australia and New Zealand contained only the *MAT1-2*. In contrast, isolates representing both mating types were present in Austria, Canada, Poland, South Africa and United Kingdom collections. Only one isolate was available from Germany, Brazil, France, Guatemala, Slovakia and USA, respectively. All of these isolates contained the *MAT1-2* except for the isolate from Slovakia that contained the *MAT1-1-1*.

Phylogenetic analyses

The alignment of partial *MAT1-1-1* nucleotide sequences (alpha domain) contained 21 strains including *Magnaporthe grisea* as the outgroup, and had a total length of 174 characters (TreeBASE accession number SN3047). Of the 174 characters, 23 were constant, 15 were variable and uninformative and 136 were parsimony informative. The alignment of partial *MAT1-2* nucleotide sequences (HMG domain) contained 21 strains including *Magnaporthe grisea* as outgroup, and had a total length of 249 characters (TreeBASE accession number SN3047). Of the 249 characters, 37 were constant, 13 were variable and uninformative and 199 were parsimony informative. Two equally parsimonious trees were obtained from each of the *MAT1-1-1* alignment (Fig. 4A with tree length of 638 steps; CI = 0.498, RI = 0.649, RC = 0.324) and from the *MAT1-2* alignment (Fig. 4B with a tree length of 886 steps; CI = 0.512, RI = 0.659, RC = 0.338).

The topology of the phylogenetic trees using the alpha domain (Fig. 4A) and HMG domain (Fig. 4B) sequences was similar. The Capnodiales, Hypocreales and Pleosporales clades showed high bootstrap support (92 % – 97 %) in both trees. The phylogenetic analysis using the DNA sequences in the HMG-box and alpha domain, respectively, showed that *D. pini* and *D. septosporum* are phylogenetically closely related to *Cercospora*, *M. graminicola* and *S. passerinii* as illustrated by the 92 % (*MAT1-1-1*) and 97 % (*MAT1-2*) bootstrap support values.

DISCUSSION

This study represents a first attempt to understand the distribution of mating types and the mating behavior of the important pine needle pathogens *D. septosporum* and *D. pini*. In this regard, emphasis is on *D. septosporum*, because it has been introduced into numerous Southern Hemisphere countries, where it has caused very damaging disease problems. Thus, the degenerate primer sets MgMfSpMAT1-1 and MgMfSpMAT1-2 (Groenewald *et al*., 2006) were successfully used to amplify portions of the mating type genes of *D. septosporum* and *D. pini*. This made it possible to characterize the full-length *MAT1-1-1* and/or *MAT1-2* genes of both species.

The *MAT1-1-1* and *MAT1-2* genes characterized for *D. septosporum* and *D. pini* in this study contained areas that correspond to a putative alpha domain and a HMG domain also described for the *MAT1-1-1* and *MAT1-2* of other ascomycetes. The two putative introns in the alpha domains of the *Dothistroma MAT1-1-1* have also been found in corresponding areas in *M. graminicola* (Waalwijk *et al*., 2002), *S. passerinii* (Goodwin *et al*., 2003) and several

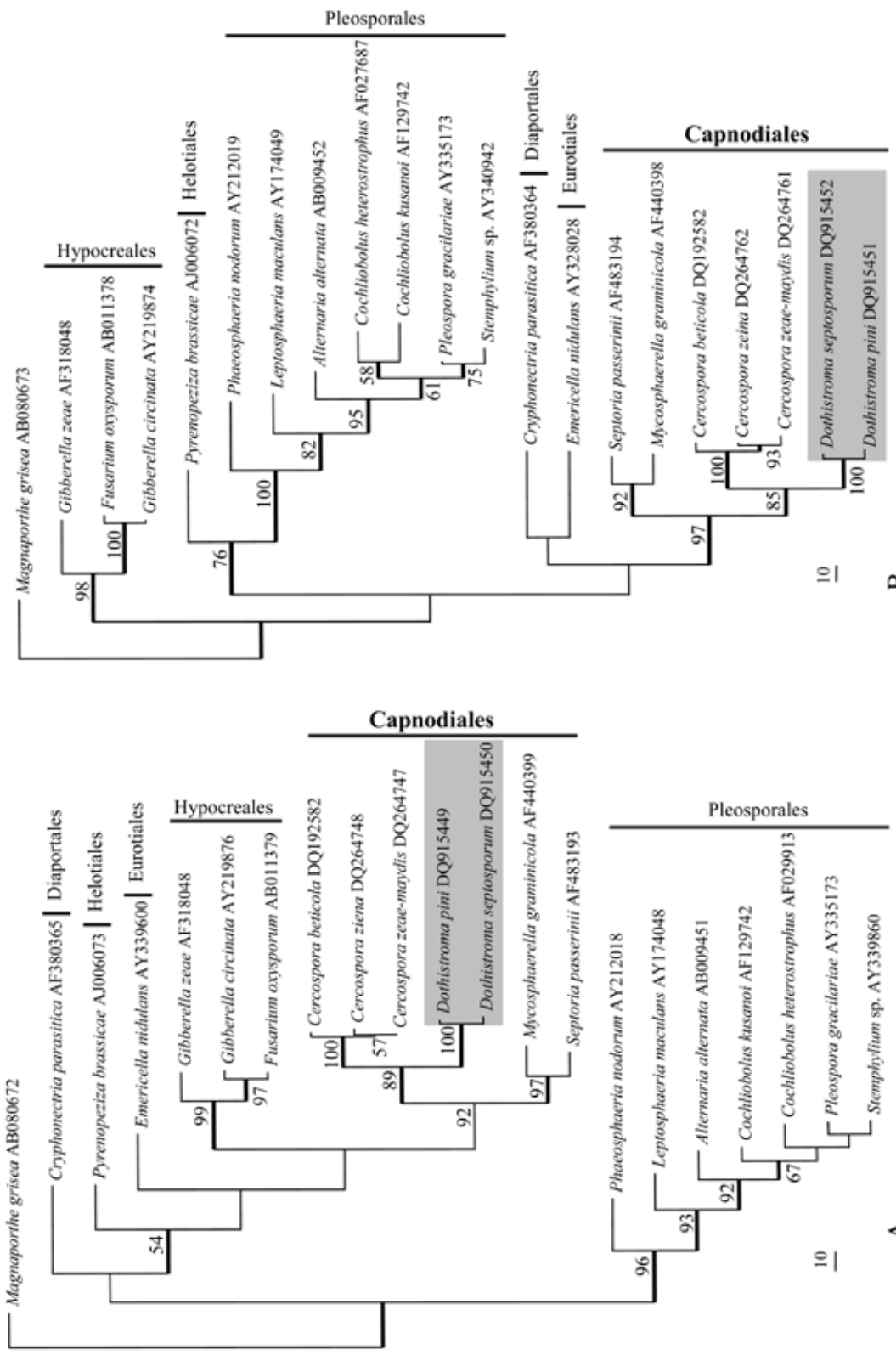


Fig. 4. One of two equally parsimonious trees obtained from each of the **A**, *MAT1-1-1* sequence alignment rooted to *Magnaporthe grisea* (AB080672) and **B**, *MAT1-2* sequence alignment rooted to *M. grisea* (AB080673). In both trees, bootstrap support values from 1,000 replicates are shown at the nodes while thickened lines indicate strict consensus branches.

Cercospora species (Groenewald *et al*., 2006). However, the third predicted intron in the downstream area flanking the alpha domain of the *MAT1-1-1* of both *Dothistroma* species is present in the *Cercospora* species, but not in *M. graminicola* or *S. passerinii.* The number of predicted introns found in the HMG domain of the *MAT1-2* in both *Dothistroma* species differed from that of closely related species. The first predicted intron is also present in *M. graminicola* (Waalwijk *et al*., 2002), *S. passerinii* (Goodwin *et al*., 2003) and *Cercospora* species (Groenewald *et al*., 2006). In contrast, the second predicted intron is only present in the *MAT1-2* of the *Dothistroma* species but not in any other members of the Mycosphaerellaceae thus far studied. In addition the *P. fulva MAT1-2* gene carries a third intron upstream of the HMG domain and is so far the only fungus with three introns in the *MAT1-2* gene. These data indicate that clear differences can be found even within the conserved regions of the corresponding genes in different *Mycosphaerella* spp.

The predicted length of the encoded proteins among different *MAT1-1-1* and *MAT1-2* genes of ascomycetes varies greatly (Pöggeler, 2001; Goodwin *et al*., 2003; Groenewald *et al.*, 2006). In most species, the MAT1 protein is much larger than the MAT2. Results of this study have shown that this is also the case for the *Dothistroma* species where 387 amino acids were found for MAT1 and 302 amino acids for MAT2. Expression studies have not been performed on the mating type genes of any of the above-mentioned members of the Mycosphaerellaceae. Additional studies at the mRNA and protein levels would be necessary to confirm the exact length of the coding regions and the intron and exon boundaries for the mating type genes of the *Dothistroma* species.

Results of this study showed substantial differences between the mating type nucleotide sequences and amino acid identities for *D. septosporum* and *D. pini*. Using nucleotide sequences for phylogenetic inference in these fungi is consistent with previous studies where conserved domains within the mating type genes have been used to study the phylogenetic relationships among different fungal species and families (Witthuhn *et al*., 2000; O'Donnell *et al*., 2004; Du *et al*., 2005; Paoletti *et al*., 2005; Groenewald *et al*., 2006). Differences in mating type sequences for *D. septosporum* and *D. pini* show that these species are distinct entities and provide strong support for the results of Barnes *et al*. (2004) who provided the first DNA-based evidence that the species are distinct. Based on morphological characteristics, Barr (1996) attempted to reclassify *M. pini* in a new genus outside of *Mycosphaerella*. However, molecular phylogenetic analyses have shown that *Mycosphaerella* is the most appropriate designation for this fungus classification (Goodwin *et al*., 2001; Barnes *et al*., 2004). Mating type gene sequences generated in this study also confirm that *Dothistroma* species are members of the Mycosphaerellaceae. The sequences of the HMG and alpha domains grouped all the species within their corresponding families, indicating that these areas provide useful tools for higher order phylogenies.

The mating type-specific primer sets developed in this study, DpiniMat1 and DpiniMat2 as well as DseptoMat1 and DseptoMat2, can be effectively used in multiplex PCR assays to amplify areas within the mating type genes for *D. pini* and *D. septosporum* populations, respectively. These primers can also be used to distinguish between the two *Dothistroma* species, making them useful tools for rapid and accurate diagnoses of two important pathogens that are

morphological similar. Prior to this study, the only diagnostic tool available to distinguish between *D. pini* and *D. septosporum*, was to amplify the ITS region with universal primers and then to digest the amplicon with the restriction endonuclease *Alu*I (Barnes *et al*., 2004). While the latter technique is useful, the ITS amplicon of *D. pini* is digested into two fragments whereas that of *D. septosporum* is not. Therefore, to prevent a false positive result for *D. septosporum*, a prior confirmation that the fungus is a *Dothistroma* species is required. The mating type-specific primer sets emerging from this study are species-specific and do not require a prior view on the identity of unknown isolates. They are, therefore, multifunctional and can be used for the rapid identification of the species as well as its mating type.

While results of this study have shown that *D. pini* is heterothallic with a single isolate containing only one of the two mating type genes, no teleomorph has yet been linked to this species. Where both mating types were observed for the isolates from the USA, the sexual state is most likely present, but has not been observed. In contrast, the *M. pini* teleomorph of *D. septosporum* has previously been observed in some parts of the USA (Cobb & Miller, 1968; Peterson & Harvey, 1976; Peterson, 1982) where *D. pini* is predominantly found. Given that the anamorphs of these fungi are morphologically similar and have been confused in the past, it is possible that teleomorph structures reported for *D. septosporum c*ould have been linked to *D. pini* and not to *D. septosporum*.

This study has shown that *D. septosporum* isolates are heterothallic and it seems that one mating type (MAT2) is more prevalent in the fungus. Although sexual reproduction has been confirmed in *D. septosporum,* asexual reproduction happens more frequently, and the absence or rarity of the opposite mating type (MAT1) in most of the populations can explain the common occurrence of the asexual stage. It is, therefore, also possible that the teleomorph is not as rare as first believed. We found that both mating types exist within *D. septosporum* populations from Europe (Poland and Austria) and Canada where the sexual stage (*M. pini*) has been reported in the past (Funk & Parker 1966; Butin & Richter, 1983; Karadzic, 1989; Fonseca, 1998; Kowalski & Jankowiak, 1998). However, the teleomorph has never been found in countries in the Southern Hemisphere such as Chile, Australia and New Zealand where these pathogens have long been a major problem (Evans, 1984; Marks *et al*., 1989). These are also the countries for which only one mating type (MAT2) has been observed and this might explain the absence or rarity of the sexual stage.

Discovery in this study of only a single mating type of *D. septosporum* in New Zealand, Australian and Chilean populations can be explained by the fact that the fungus is an introduced pathogen in those countries. For New Zealand, Hirst *et al*. (1999) also found no genetic diversity for this fungus, which is strongly supported by the results of the present study. Dothistroma needle blight was introduced in Australia in the 1970s and it was suggested that this occurred by natural means with conidia being blown across the Tasman Sea from New Zealand. This view was supported by the fact that the strict quarantine regulations in Australia would have made it unlikely that infected plant material entered the country (Edwards & Walker, 1978; Marks *et al*., 1989; Mireku & Simpson, 2002). The presence of only one mating type shown in this study and the very low genetic diversity for the pathogen in New Zealand (Hirst *et al*., 1999) supports the view that only one genotype was introduced into or became established in

Australia and New Zealand. Asexual reproduction has evidently perpetuated the spread of the fungus subsequently. We suspect that the same situation might apply for Chile.

An intriguing result of this study has been the discovery that both mating types of *D. septosporum* exist in the South African population of the fungus. This is especially interesting because the pathogen is non-native in the country and it might have been expected that the situation would have been similar to that in other countries such as New Zealand. In addition, the teleomorph of *D. septosporum* has never been observed in South Africa, despite concerted efforts to detect it (M.J. Wingfield, *unpublished data*).

It is important to recognize that the presence of both mating types of *D. septosporum* in South Africa could indicate the presence of clandestine sex in the fungus. This would indicate the potential for the pathogen to evolve more effectively in this country than would be true elsewhere in the world where only a single mating type exists. Such change in the fungus could complicate efforts to develop trees resistant to red pine needle blight infection in South Africa. In this regard, it has previously been shown that the introduction of the second mating type of a pathogen can cause rapid increase in virulence as a result of increased genetic variation, as reported for *Phytophthora infestans* (Fry & Goodwin, 1997; Smart & Fry, 2001; Lee & Kim, 2002; Ristaino, 2002) and *Ophiostoma novo-ulmi* (Paoletti *et al*., 2006). This implies that the accidental introduction of the opposite mating type of *D. septosporum* into countries such as New Zealand, Australia and Chile, could seriously exacerbate red band needle disease in those countries. Every effort must thus be made to ensure that new mating types of *D. septosporum* do not enter these countries.

There has been a dramatic increase in the impact of Dothistroma needle blight caused by *D. septosporum* in western Canada and the United States in recent years (Woods, 2003; Bradshaw, 2004). Possible reasons for this change in the disease situation in these countries are an abundance of host material or a directional climate change as suggested by Woods *et al*. (2005). It is also possible that the fungus has changed in these areas and this would be possible given the presence of both mating types as shown in this study and associated sexual reproduction. Further investigation is, therefore, necessary to determine whether the presence of both mating types, which could increases the genetic diversity, a dramatic climate change or possibly a combination of both these factors might account for the drastic increase in the severity of this disease.

Because only one mating type of *D. septosporum* is present in most countries of the Southern Hemisphere, it is important to restrict the MAT1 isolates to their present locations. This can be achieved through refining quarantine regulations based on the knowledge that only one mating type of the pathogen is present in the country. The mating type-specific PCR developed during this study could easily be implemented as a control method to test for the presence of the mating types for *Dothistroma* species in pine plantations. One of the weaknesses of quarantine regulations internationally is that they typically rely on lists of names of pathogens rather than on knowledge of their biology and population genetics. Results of this study have provided valuable new insights into the distribution of mating types of *D. septosporum* and *D. pini* that should enhance the quality of quarantine regulations in the future.

ACKNOWLEDGEMENTS

We are grateful to the CBS-Odo van Vloten Stichting, and the Royal Netherlands Academy of Arts and Sciences for financial support to MG. We also thank Dr Gerald C. Adams (USA), Dr Thomas Kirisits (Austria), Dr Tadeusz Kowalski (Poland), Mr Rodrigo Ahumada (Chile), Dr Angus Carnegie (Australia) for providing specimens of *D. pini* and *D. septosporum* and Melissa Guo are thanked for technical assistance.

REFERENCES

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.

Barnes, I., Crous, P.W., Wingfield, B.D., and Wingfield, M.J. 2004. Multigene phylogenies reveal that red band needle blight of *Pinus* is caused by two distinct species of *Dothistroma, D. septosporum* and *D. pini.* Stud. Mycol. 50:551–565.

Barr, M.E. 1996. Planistromellaceae, a new family in the Dothideales. Mycotaxon 60:433–442.

Bobrowicz, P., Pawlak, R., Correa, A., Bel-Pedersen, D., and Ebbole, D.J. 2002. The *Neurospora crassa* pheromone precursor genes are regulated by the mating type locus and the circadian clock. Mol. Microbiol. 45:795–804.

Bradshaw, R.E. 2004. Dothistroma (red-band) needle blight of pines and the dothistromin toxin: a review. Forest Pathol. 34:163–185.

Butin, H., and Richter, J. 1983. Dothistroma needle blight: a new pine disease in the Federal Republic of Germany. Nachrichtenbl. Deut. Pflanzenschutzd. 35:129–131.

Carson, S.D., Dick, A.M.P., and West, G.G. 1991. Benefits of the Dothistroma-resistant breed of radiata pine. Pages 251–262 in: New Directions in Forestry Costs and Benefits of Change. J.C. Allen, and A.G.D. Whyte, eds. Christchurch, New Zealand. Aust. & N.Z. Inst. Forestry Conf.

Cobb, F.W., and Miller, D.R. 1968. Hosts and geographic distribution of *Scirrhia pini* – the cause of red band needle blight in California. J. Forest. 66:930–933.

Cobb, F., Uhrenholdt, B., and Drohn, R. 1969. Epidemiology of *Dothistroma pini* needle blight on *Pinus radiata*. Phytopathology 59:1021–1022.

Coops, N., Stanford, M., Old, K., Dudzinski, M., Culvenor, D., and Stone, C. 2003. Assessment of Dothistroma needle blight of *Pinus radiata* using airborne hyperspectral imagery. Phytopathology 93:1524–1532.

Du, M., Schardl, C.L., Nuckles, E.M., and Vaillancourt, L.J. 2005. Using mating-type gene sequences for improved phylogenetic resolution of *Colletotrichum* species complexes. Mycologia 97:641–658.

Edwards, D.W., and Walker, J. 1978. Dothistroma needle blight in Australia. Aust. For. Res. 8:125–137.

Evans, H.C. 1984. The genus *Mycosphaerella* and its anamorphs *Cercoseptoria*, *Dothistroma* and *Lecanosticta* on pines, CMI Mycological Paper no. 153. Surrey U.K.: Commonwealth Agricultural Bureaux.

Fonseca, N. 1998. *Mycosphaerella pini* (*Scirrhia pini*), the perfect state of *Dothistroma septospora*: first observation in Portugal. Pages 69–74 in: Foliage, Shoot and Stem Diseases of Trees. Proc. Int. Union For. Res. Org., Quebec City, Canada. May, 25–31, 1997. Ed. by Laflamme, G.; Bèrubé, J. A.; Hamelin, R. C. Can. For. Serv.

Fry, W.E., and Goodwin, S.B. 1997. Re-emergence of potato and tomato late blight in the United States and Canada. Plant Dis. 81:1349–1357.

Funk, A., and Parker, A.K. 1966. *Scirrhia pini* n. sp., the perfect state of *Dothistroma pini* Hulbary. Can. J. Bot. 44:1171-1176.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R.D., and Bairoch, A. 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. 31:3784–3788.

Gibson, I.A.S. 1974. Impact and control of Dothistroma blight of pines. Eur. J. For. Pathol. 4:89–100.

Gibson, I.A.S. 1972. Dothistroma blight of *Pinus radiata*. Annu. Rev. Phytopathol. 10:51–72.

Goodwin, S.B., Dunkle, L.D., and Zismann, V.L. 2001. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. Phytopathology 91:648–658.

Goodwin, S.B., Waalwijk, C., Kema, G.H.J., Cavaletto, J.R., and Zhang, G. 2003. Cloning and analysis of the mating-type idiomorphs from the barley pathogen *Septoria passerinii*. Mol. Gen. Genomics 269:1–12.

Groenewald, M., Groenewald, J.Z., and Crous, P.W. 2005. Distinct species exist within the *Cercospora apii* morphotype. Phytopathology 95:951–959.

Groenewald, M., Groenewald, J.Z., Harrington, T.C., Abeln, E.C.A., and Crous, P.W. 2006. Mating type gene analysis in apparently asexual *Cercospora* species is suggestive of cryptic sex. Fungal Genet. Biol. 43:813-825.

Hirst, P., Richardson, T.E., Carson, S.D., and Bradshaw, R.E. 1999. *Dothistroma pini* genetic diversity is low in New Zealand. N.Z.J. For. Sci. 29:459–472.

Ivory, M.H. 1967. A new variety of *Dothistroma pini* in Kenya. Trans. Br. Mycol. Soc. 50:289–297.

Karadzic, D. 1989. *Scirrhia pini* Funk et Parker. Life cycle of the fungus in plantations of *Pinus nigra*. Arn. in Serbia. Eur. J. For. Pathol. 19:231–236.

Kowalski, T., and Jankowiak, R. 1998. First record of *Dothistroma septospora* (Dorog.) Morelet in Poland: a contribution to the symptomology and epidemiology. Phytopathol. Pol. 16:15–29.

Lee, Y.S., and Kim, K.J. 2002. Genetic DNA marker for A2 mating type in *Phytophthora infestans*. J. Microbiol. 40:254–259.

Marks, G.C., Smith, I.W., and Cook, I.O. 1989. Spread of *Dothistroma septospora* in plantations of *Pinus radiata* in Victoria between 1979 and 1988. Aust. Forestry 52:10–19.

Mireku, E., and Simpson, J.A. 2002. Fungal and nematode threats to Australian forests and amenity trees from importation of wood and wood products. Can. J. Plant Pathol. 24:117–124.

O'Donnell, K., Ward, T.J., Geiser, D.M., Kistler, H.C., Aoki, T. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genet. Biol. 41:600–623.

Paoletti, M., Buck, K.W., and Brasier, C.M. 2005. Cloning and sequence analysis of the *MAT-B* (*MAT-2*) genes from the three Dutch elm disease pathogens, *Ophiostoma ulmi*, *O. novo-ulmi*, and *O. himal-ulmi.* Mycol. Res. 109:983–991.

Paoletti, M., Buck, K.W., and Brasier, C.M. 2006. Selective acquisition of novel mating type and vegetative incompatibility genes via interspecies gene transfer in the globally invading eukaryote *Ophiostoma novo-ulmi.* Mol. Ecol. 15:249–262.

Pearson, W.R., Wood, T., Zhang, Z., and Miller, W. 1997. Comparison of DNA sequences with protein sequences. Genomics 46:24–36.

Peterson, G.W. 1982. Dothistroma Needle Blight of Pines. Forest Insect and Disease Leaflet 143. Washington, DC: US Department of Agriculture, Forest Service.

Peterson, G.W., and Harvey, G.M. 1976. Dispersal of *Scirrhia (Dothistroma) pini* conidia and disease development in a shore pine plantation in Western Oregon. Plant Dis. Reptr. 60:761–764.

Pöggeler, S. 2001. Mating-type genes for classical strain improvements of ascomycestes. Appl. Microbiol. Biotechnol. 56:589–601.

Ray, J.W., and Vanner, A.L. 1988. Improvements in the technology of *Dothistroma* control. What's new in forest research no. 169. Rotorua, New Zealand Research Institute.

Ristaino, J.B. 2002. Tracking historic migrations of the Irish potato famine pathogen, *Phytophthora infestans.* Microbes Infect. 4:1369–1377.

Singh, G., and Ashby, A.M. 1998. Cloning of the mating-type loci from *Pyrenopezziza brassicae* reveals the presence of a novel mating type gene within a discomycete *MAT1-2* locus encoding a putative metallothionin-like protein. Mol. Microbiol. 30:799-806.

Singh, G., and Ashby, A.M. 1999. Errratum to Shingh and Ashby 1998. Cloning of the mating-type loci from *Pyrenopezziza brassicae* reveals the presence of a novel mating type gene within a discomycete *MAT1-2* locus encoding a putative metallothionin-like protein. Mol. Microbiol. 32:1115.

Smart, C.D., and Fry, W.E. 2001. Invasions by the late blight pathogen: Renewed sex and enhanced fitness. Biol. Invas. 3:235–243.

Stone, C., Chisholm, L.A., and McDonald, S. 2003. Spectral reflectance characteristics of *Pinus radiata* needles affected by Dothistroma needle blight. Can. J. Bot. 81:560–569.

Swofford, D.L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Turgeon, B.G. 1998. Application of mating type gene technology to problems in fungal biology. Annu. Rev. Phytopathol. 36:115–137.

Turgeon, B.G., and Yoder, O.C. 2000. Proposed nomenclature for mating type genes of filamentous ascomycetes. Fungal Genet. Biol. 31:1–5.

Turgeon, B.G., Bohlmann, H., Ciuffetti, L.M., Christiansen, S.K., Yang, G., Schafer, W., and Yoder, O.C. 1993. Cloning and analysis of the mating type genes from *Cochliobolus heterostrophus.* Mol. Gen. Genetics 238:270–284.

Waalwijk, C., Mendes, O., Verstappen, E.C., de Waard, M.A.D., and Kema, G.H.J. 2002. Isolation and characterization of the mating-type idiomorphs from the wheat septoria leaf blotch fungus *Mycosphaerella graminicola*. Fungal Genet. Biol. 35:277–286.

Witthuhn, R.C., Harrington, T.C., Steimel, J.P., Wingfield, B.D., Wingfield, M.J. 2000. Comparison of isozymes, rDNA spacer regions and *MAT-2* DNA sequences as phylogenetic characters in the analysis of the *Ceratocystis coerulescens* complex. Mycologia 92:447–452.

Woods, A.J. 2003. Species diversity and forest health in northwest British Columbia. For. Chronobiol. 79:892–897.

Woods, A., Coates, K.D., and Hamann, A. 2005. Is an unprecedented Dothistroma needle blight epidemic related to climate change? BioScience 55:761–769.

Yun, S.H., Arie, T., Kaneko, I., Yoder, O.C., and Turgeon, B.G. 2000. Molecular organization of mating type loci in heterothallic, homothallic, and asexual *Gibberella*/*Fusarium* species. Fungal Genet. Biol. 31:7–20.

Zhan, J., Kema, G.H.J., Waalwijk, C., and McDonald, B.A. 2002. Distribution of mating type alleles in the wheat pathogen *Mycosphaerella graminicola* over spatial scales from lesions to continents. Fungal Genet. Biol. 36:128–136.

CHAPTER 8

General discussion

GENERAL DISCUSSION

The objectives of the research described in this thesis were to (i) resolve the taxonomy of *Cercospora beticola*, a species that is part of the *C. apii* complex and causes Cercospora leaf spot on sugar beet, and (ii) examine the genetic diversity and possible reproductive strategy of this species and its close relatives. This concluding chapter summarizes the results obtained in the previous chapters and discusses how our results provide a better understanding of (i) species included in the *C. apii* complex and the potential host range of these taxa; (ii) the genetic variation that exists within *C. beticola* and (iii) differences between *C. beticola* and other closely related species with respect to the presence and distribution of their mating type genes.

The *C. apii* complex contains distinct species

Morphological characteristics are routinely used to identify and name newly isolated fungi. However, based solely on morphology it is not possible to discriminate between the morphologically indistinguishable *Cercospora* species comprising the *C. apii* complex (Johnson & Valleau, 1949; Crous & Braun, 2003). The taxonomy of this species was laden with difficulty since the first description of the genus *Cercospora*. Johnson & Valleau (1949) stated that all morphologically uniform *Cercospora* strains are a single species, but Chupp (1954) questioned this approach, as he stated that numerous species were present that could be separated based on their hosts. Previous genetic analyses of *Cercospora* species have relied mainly on part of the internal transcribed spacer areas as well as the 5.8S rRNA gene (ITS) sequences and these studies revealed that members of the *C. apii* complex are identical or very closely related (Goodwin *et al*., 2001; Pretorius *et al*., 2003). Before the present study, *C. beticola* and *C. apii* were considered to be synonymous as part of the *C. apii sensu lato* complex (Ellis, 1971; Crous & Braun, 2003). Data shown in Chapter 2, however, refuted the hypothesis that all morphologically indistinguishable *Cercospora* strains represent a single species (Groenewald *et al*., 2005). We show that a combined approach using cultural characteristics (growth rates and temperature thresholds), amplified fragment length polymorphism (AFLP) analyses as well as multilocus sequence typing (MLST) analyses using part of the ITS region, actin gene (ACT), translation elongation factor 1-α gene (EF1-α), calmodulin gene (CAL) and histone H3 gene (HIS) represent the most reliable way to characterize and identify species within the *C. apii* complex (Groenewald *et al*., 2005). These results revealed that *C. beticola* and *C. apii* should be treated distinct at species level. This combined approach was also successfully employed to delineate a novel *Cercospora* species, originally identified as *C. apii*, causing Cercospora leaf spot on celery in Venezuela, Korea and Greece. This species was formally described as *C. apiicola* in Chapter 3 (Groenewald *et al*., 2006a). Although very few differences in colony morphology between these three species were observed (Chapter 2), meaningful differences were found in the growth rates, temperature thresholds (Tables 1, 2) and CAL sequences (Table 3) of *C. beticola*, *C. apii* and *C. apiicola,* illustrating that they should be considered as functional species (Groenewald *et al*., 2005, 2006a). Species-specific primers from polymorphic areas within the calmodulin gene are now available as diagnostic tools to easily distinguish between these three species (Chapter 2).

Table 1. Differences in growth rate and temperature thresholds among *Cercospora beticola, C. apii* and *C. apiicola* isolates measured after growth for eight days on malt extract agar (MEA) with diameter growth rates at the different temperature thresholds in parentheses.

	Growth temperature		
	Minimum	**Maximum**	**Optimum**
C. beticola	12 °C (2.8 mm)	33 °C (11.7 mm)	27 °C (28 mm)
C. apii	12 °C (2.8 mm)	33 °C (2.2 mm)	27 °C (21.7 mm)
C. apiicola	6 °C (1 mm)	30 °C (3.5 mm)	24 °C (13.8 mm)

Table 2. Differences in cultural characteristics among *Cercospora beticola, C. apii* and *C. apiicola* isolates observed after growth for eight days on malt extract agar (MEA) and oatmeal agar (OA).

	MEA (surface)	**MEA (beneath)**	**OA (colony)**	**OA (agar)**
C. beticola	greenish gray	dark mouse gray	white to green-olivaceous	–
C. apii	greenish gray	dark mouse gray	white to green-olivaceous	Yellow diffusing pigment
C. apiicola	white to smoke gray	olivaceous gray to iron-gray	white to olivaceous-gray	–

Table 3. Polymorphic sites within the calmodulin gene sequences of *Cercospora beticola*, *C. apii* and *C. apiicola.* The highlighted area indicates the nucleotides that are included in the species-specific primers.

	[a] **Positions of polymorphic nucleotides in calmodulin gene**																							
	22	**38**	**46**	**80**	**85**	**113**	**116**	**119**	**124**	**125**	**130**	**131**	**133**	**136**	**137**	**142**	**147**	**151**	**187**	**199**	**220**	**223**	**244**	**247**	**253**
C. beticola	T	G	A	C	A	T	G	C	C	C	C	G	A	T	A	A	C	G	T	C	G	T	C	A	C
C. apii	C	G	A	G	C	A	G	A	C	C	C	A	C	C	G	A	T	A	T	C	G	T	T	G	C
C. apiiicola	C	A	G	C	C	T	A	C	T	T	T	G	C	C	A	G	C	A	C	T	A	C	C	G	T

[a] The calmodulin sequence of *C. apii* strain CBS 116455 (GenBank accession no. AY840417) was used to derive the nucleotide positions of the polymorphisms.

Overall we have illustrated how crucial the choice and number of loci sequenced can be in elucidating phylogenetic relationships between very closely related species or within species complexes. The wrong or an insufficient number of sequenced loci could result in erroneous synonymies being proposed. Our data suggest that the CAL gene is the most appropriate locus studied to date to distinguish between closely related species within the *C. apii* complex. This gene could also be useful for distinguishing other species that belong to the *C. apii* complex. We also show that cultural characteristics in combination with sequence data can be used successfully to establish species boundaries for *C. beticola*, *C. apii* and *C. apiicola.*

Isolation of *C. beticola*, *C. apii* and *C. apiicola* from various hosts

Host specificity has often been used in the past as criteria to distinguish *Cercospora* species (Chupp, 1954; Crous & Braun, 2003). Previous cross-inoculation studies revealed that isolates in the *C. apii* complex can infect an extremely wide range of host including sugar beet (*Beta vulgaris*) and celery (*Apium graveolens*) (Johnson & Valleau, 1949) and this raised doubt whether host specificity exists within this complex. Prior to the work described in Chapters 2

and 3, all the *Cercospora* strains isolated from celery were named *C. apii* (Fresenius, 1863) and those from sugar beet *C. beticola* (Saccardo, 1876). The data presented in these chapters, however, refute the hypothesis that *C. beticola* and *C. apii* are each restricted to only one of the two host plants (Groenewald *et al*., 2005, 2006a).

Based on the data obtained from the phylogenetic analyses described in Chapter 3 using the ITS, ACT, EF1-α, CAL and HIS areas, only the newly described species, *C. apiicola*, was found to be host-specific. This proves that Chupp (1954) was partly correct when he proposed that some *Cercospora* species were restricted to specific host genera or families. Unfortunately this concept cannot be applied to the genus as a whole and can therefore not be used for all *Cercospora* / host combinations. We show that strains belonging to *C. beticola sensu stricto* and *C. apii sensu stricto* are mainly isolated from sugar beet and celery, respectively, but that they also can be isolated from each other's as well as additional hosts. Crous & Braun (2003) linked nine host genera to *C. beticola* and 83 host genera to *C. apii*. In Chapter 3, three new host records were found for *C. beticola* and three for *C. apii* (Table 4). In our study we have provided the first molecular evidence that *C. beticola* and *C. apii* are not entirely host-specific and that they have wider host ranges than have been proposed by Chupp (1954) and Ellis (1976). These two species can therefore not be identified accurately based solely on the host from which the strain was isolated and this may be true for most species belonging to the *C. apii* complex. Although *C. beticola* and *C. apii* are able to infect other hosts they still appear to operate as functional species on their respective primary namesake hosts in nature. Whether the isolates found in the lesions on the additional hosts were primary pathogens, or whether the infected host tissue acts as a substrate for a secondary infection or as a surrogate host until the pathogen reaches its actual host, still has to be investigated. This points out the complexity of host-specificity and taxonomy of species within the *C. apii* complex and caution

Table 4. Host range of *Cercospora beticola*, *C. apii* and *C. apiicola* with the primary host indicated in boldface. Names of *Cercospora* spp. linked to the host by Crous & Braun (2003) are shown and new hosts found during this study have been added.

Species	Host	*Cercospora* spp. associated with host
C. beticola	[a]*Apium*	*C. apii*
	Beta	*C. beticola*
	[a]*Chrysanthemum*	–
	[a]*Limonium*	*C. apii*, *C. insulana*, *C. statices*
	Malva	*C. althaeina*, *C. beticola*, *C. hyalospora*, *C. malvae*, *C. malvarum*
	Spinacia	*C. bertrandii*, *C. beticola*, *C. spinaciicola*
C. apii	***Apium***	*C. apii*
	[b]*Helianthemum*	*C. cistinearum*, *C. helianthemi*
	[b]*Moluccella*	*C. molucellae*
	[b]*Plantago*	*C. pantoleuca*, *C. plantaginis*
	Plumbago	*C. apii*, *C. plumbaginea*
C. apiicola	***Apium***	*C. apii*

[a] New host records found for *C. beticola.*
[b] New host records found for *C. apii.*

should therefore be taken when choosing a rotation crop in fields were *Cercospora* species are known to be present. Also, knowing the identity of the species causing Cercospora leaf spot disease symptoms may lead to the development and utility of more efficient methods of disease prevention and treatment. We showed that determination of such identities is indeed possible and we caution against the use of morphology and the pathogen's host name as sole criteria for species identification.

Mating type genes in *Cercospora* and *Dothistroma* species

Mating type genes play an important part in the biology and evolution of fungal species. Knowledge of these genes can provide insight in the potential prevalence of sex in species that are currently thought to be asexual, for example most of the *Cercospora* spp. It is thought that populations that regularly undergo sexual reproduction should have many more genotypes than its asexual relatives and that the mating types will be present in equal proportions (Milgroom, 1996). Mating type genes have been isolated previously from sexually as well as presumably asexually reproducing filamentous ascomycetes. These include the sexual reproducing species *Mycosphaerella graminicola* (Waalwijk *et al*., 2002) and *Cochliobolus heterostrophus* (Arie *et al*., 2000); and the presumably asexually reproducing species *Septoria passerinii* (Goodwin *et al*., 2003), *Passalora fulva* (Stergiopoulos *et al*., 2007) and *Alternaria alternata* (Arie *et al*., 2000). No teleomorphs have been linked to the presumably asexually reproducing *Cercospora* species causing leaf spots on celery, sugar beet and maize as well as *Dothistroma pini* causing red band needle blight of pines. A second *Dothistroma* species linked to red band needle blight of pines, namely *D. septosporum*, has *Mycosphaerella pini* as teleomorph. Previously published primer sets (Arie *et al*., 2000; Waalwijk *et al*., 2002) failed to amplify parts of the mating type genes of these species, making it impossible to characterize these genes and to determine the distribution of these genes in populations.

The degenerate primer sets MgMfSpMAT1-1 and MgMfSpMAT1-2 designed in Chapter 4 were successfully used to amplify a part of the mating type genes of the sugar beet pathogen *C. beticola*, the two celery pathogens *C. apii* and *C. apiicola*, the two maize pathogens *C. zeae-maydis* (formerly *C. zeae-maydis* Group 1) and *C. zeina* (formerly *C. zeae-maydis* Group 2), and the two pine pathogens *D. pini* and *D. septosporum*. The sequences of these parts allowed the characterization of the full-length *MAT1-1-1* and/or *MAT1-2* sequences of these species. Figures 1 and 2 provide an overview of the differences observed among the mating type genes that were characterized in this thesis as well as those of their close relatives, *P. fulva* (recently characterized as a result of collaboration between Marizeth Groenewald and the Department of Phytopathology, Wageningen University), *S. passerinii* and *M. graminicola*, with regards to the predictions made of the length of the open reading frame (ORF), intron and exon boundaries and number of introns. Expression studies have not been performed on the mating type genes of any Mycosphaerellaceae species, therefore additional studies at the mRNA and protein levels are necessary to confirm the exact length of the coding regions as well as the intron and exon boundaries of these available genes. The overall structure of the characterized mating type genes of the genus *Mycosphaerella* is not conserved among the species and more studies are needed to determine whether the structure reflects the evolutionary history of the genus. The degenerate primer sets described in Chapter 4 also amplify parts of the mating

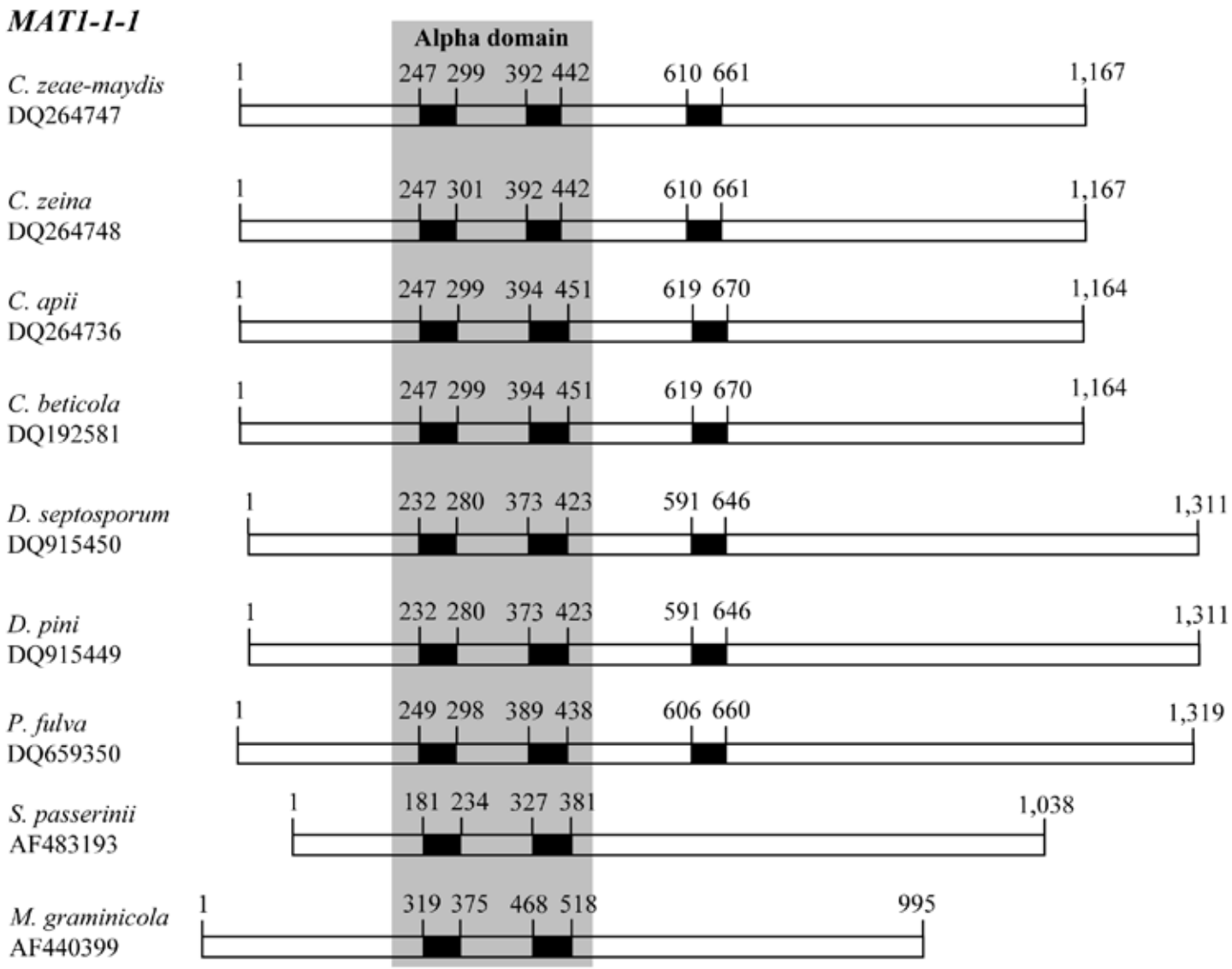


Fig .1. Diagrammatic representation of the full-length *MAT1-1-1* genes of characterized Mycosphaerellaceae. The predicted sites of exons (white bars), and introns (black bars) are shown, and their locations (nucleotide positions) are indicated.

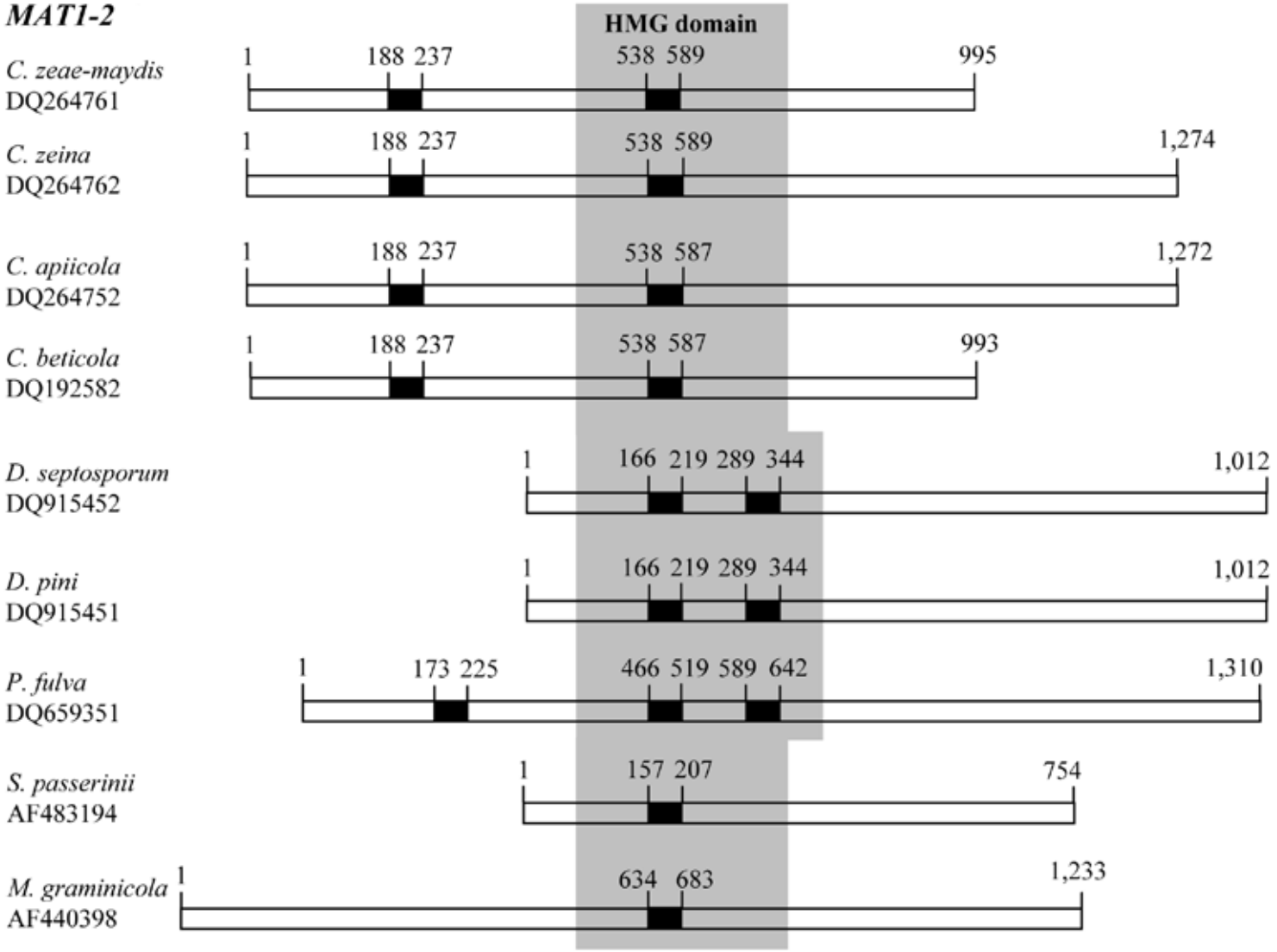


Fig. 2. Diagrammatic representation of the full-length *MAT1-2-1* genes of characterized Mycosphaerellaceae. The predicted sites of exons (white bars), and introns (black bars) are shown, and their locations (nucleotide positions) are indicated.

type genes of other species belonging to the Mycosphaerellaceae and allied Davidiellaceae (Groenewald *et al*., 2006b) for which working mating type gene primers were previously not available.

Cercospora as well as *Dothistroma* mating type-specific primer sets are now available which can be used in multiplex PCR assays to amplify portions of these genes in populations of the respective species. In addition, the *Dothistroma* mating type-specific primers can also be used to distinguish between *D. pini* and *D. septosporum*. These species-specific / mating type-specific primer sets are extremely valuable multifunctional research tools because these two species are morphologically indistinguishable. In chapters 4 and 7 we show that *C. beticola*, *C. zeina*, *C. zeae-maydis, D. septosporum* and *D. pini* are heterothallic as an isolate contains only one of the two mating type genes. The two mating types are approximately evenly distributed within sampled populations of *C. beticola*, *C. zeae-maydis* and *C. zeina*, indicating that it is quite possible that a sexual cycle is active. Unfortunately attempts to induce mating between isolates of different mating types of *C. beticola* were not successful (Groenewald *et al*., 2006b). Crous *et al*. (2006) attempted to mate isolates of *C. zeina* as well as those of *C. zeae-maydis,* but without success.

Previous phylogenetic analyses using housekeeping gene sequences resolved *Cercospora* as a monophyletic lineage within the genus *Mycosphaerella* (Crous *et al*., 2000, 2001, 2004; Goodwin *et al*., 2001) and if a sexual stage does exist for *Cercospora* species, it is expected to be a *Mycosphaerella* species. Although no teleomorph has been found so far for these three *Cercospora* species, the presence of both mating types in approximately equal proportions suggest that one could exist in nature, albeit at a low frequency. Although only the *MAT1-2* gene was present in the *C. apiicola* isolates tested and only the *MAT1-1-1* in *C. apii*, it is possible that the opposite mating types may exist for these species. However, the data suggest that, if it were to be present, it would be rare. In Chapter 7 we suggest that *D. septosporum* (teleomorph = *M. pini*) and *D. pini* isolates are heterothallic. As a *Mycosphaerella* stage has already been linked to *D. septosporum*, we can assume that the teleomorph stage of *D. pini,* if it does exist, would also be a *Mycosphaerella* species. In contrast to the three *Cercospora* species, an equal distribution of the mating types in *D. septosporum* was not found and MAT2 is more prevalent in most populations studied. Although sexual reproduction has been confirmed in *D. septosporum,* asexual reproduction happens more frequently, and the rarity of the MAT1 isolates may play a role in the uncommonness of the sexual stage. Further investigation is necessary to clarify whether the increase in the level of genetic diversity due to the presence of both mating types, the dramatic climate change or maybe a combination of both, is the source of the drastic increase in the severity of Dothistroma red band needle blight. As reported in Chapter 7, MAT1 is still absent from most countries in the Southern Hemisphere and it is therefore important to restrict the MAT1 isolates to their present locations. Stricter legislation regarding the import of plant material is necessary in these countries. It is also important to screen populations regularly to ensure that only one mating type is still present.

Nucleotide sequences of the conserved domains within the mating type genes have been used to study the phylogenetic relationships among different fungal species and families (Witthuhn *et al*., 2000; O'Donnell *et al*., 2004; Paoletti *et al*., 2005; Du *et al*., 2005). In

chapter 4 we show that these conserved regions lack the resolution to distinguish among closely related species within the group of isolates belonging to the *C. apii* complex and it is clear that the *MAT1-1-1* sequences cannot separate *C. beticola* and *C. apii.* It appears that mating type genes therefore do not represent promising loci for phylogenetic studies aimed at distinguishing cryptic species belonging to the *C. apii* complex. However, in future studies we could probably determine specific primers in other parts of the idiomorph. In contrast the *MAT1-1-1* genes allowed us to distinguish between the closely related *D. pini* and *D. septosporum* and between *M. graminicola* and *S. passerinii* (Chapters 4 and 7) and might be useful to distinguish species above the species complex level as well.

***Cercospora beticola* populations are genetically diverse**

Only a few studies have been performed on the genetic structure of *Cercospora* populations. Isolates from each of the following species, *C. zeae-maydis*, *C. sorghi*, *C. zeina* and *C. caricis*, were found to be genetically highly similar (Wang *et al*., 1998; Inglis *et al*., 2001; Okori *et al*., 2003, 2004). Previous studies showed that some genetic variation exists within *C. beticola,* but it was not known whether this variation was due to chromosomal rearrangements, asexual or sexual recombination (Große-Herrenthey, 2001; Moretti *et al*., 2004). Data presented in Chapter 5 suggest that the genetic variation observed in the isolates studied was not caused by chromosome rearrangements but by recombination events occurring in nature and that the genetic diversity observed within *C. beticola* is in agreement with the observation of approximately equal distribution of the mating type genes in these populations.

It is known that populations that regularly undergo sexual reproduction should have many more genotypes that result in higher levels of genotypic diversity compared to those that reproduce only asexually (Milgroom, 1996). Analyses using four AFLP primer combinations together with data on the mating type present were used to study the genotypic variation that exists among *C. beticola* isolates from different countries. High levels of dissimilar genotypes were found on the field as well as on the single plant level, indicating that recombination occurs frequently in *C. beticola*.

We also show that genetic exchange occurs regularly between isolates from *C. beticola* populations from different countries in Europe. This is probably due to the frequent movement of plant material between different European countriesby traiders. Although high genetic variation also exists in isolates from New Zealand, the genetic distance between New Zealand and European isolates is relatively high. As the centre of origin of *C. beticola* has been suggested to be the Mediterranean parts of Europe, it is assumed that the population of New Zealand was seeded from Europe, after which the population in New Zealand was shaped without outside influences.

Several studies have reported that variation also exists in the onset and progression of Cercospora leaf spot on sugar beet (Wolf & Verreet, 2002, 2005). It has also been shown that *C. beticola* has become resistant or has developed an increased tolerance to fungicides in recent years (Karaoglanidis *et al*., 2000; Weiland & Koch, 2004). Variation in fungicide resistance and adaptation and specialization of some isolates on previously resistant sugar beet cultivars make effective disease management very difficult. It is possible that the high level of genetic variation that exists within *C. beticola* plays an important role in the pathogen's ability

to develop new virulent alleles and fungicide resistance. The high level of genotypic variation, low gametic disequilibrium (Chapter 5) and the equal distribution of the mating types within populations (Chapter 4 and 5) are indicating that a sexual stage is probably part of *C. beticola*'s life cycle. Sexual recombination events most likely play an important role in the reproductive cycle of this genetically highly diverse and heterogeneous, apparently asexual, species.

Novel molecular markers for *C. beticola*

Although AFLP analysis was successfully used to study the genetic diversity within and between *C. beticola* populations in Chapter 5, this method requires extremely pure, high molecular weight genomic DNA, and is a very expensive and time consuming method. Several additional marker systems are available that are frequently used to study the genetic diversity and population structure of fungal species. These include microsatellites (Chen *et al*., 2002; Burgess *et al*., 2001, 2004) and restriction fragment length polymorphisms (RFLPs) (Zhan *et al*., 2003; Hayden *et al*., 2003a, 2003b; Zhan & McDonald, 2004).

In Chapter 6, we developed five microsatellite, one RFLP and three single nucleotide polymorphism (SNP) polymorphic markers were developed for *C. beticola*. Several *Cercospora* isolates were screened and it was found that these markers are highly polymorphic and that they can be used in future studies to analyze the genetic diversity of *C. beticola* isolates and populations. These marker systems have several advantages over the AFLP marker system as the screening process is relatively inexpensive, fairly fast and the results are reproducible and can therefore easily be compared between different laboratories.

Unfortunately the identification and development of these markers are expensive and time-consuming. Five hundred and fifty clones were sequenced and only a few were identified that contained long repeats that were polymorphic among the isolates tested. Nine novel molecular markers are now available that will allow rapid assessment of genetic diversity within *C. beticola* populations in future studies.

CONCLUSIONS

The main aims of this thesis were to resolve the taxonomic status of *C. beticola* with regard to the *C. apii* complex, and to determine the extent and basis of genetic diversity within *C. beticola* populations. We showed that *C. beticola* is indeed a distinct species in its own right and species-specific primers were developed for easy identification of it and other related species. It was also shown that *C. beticola* is not only restricted to sugar beet, but that it can be isolated from other hosts and that it even occurs on hosts traditionally infected by other *Cercospora* spp. The mating type genes of *C. beticola* and other species of the Mycosphaerellaceae were characterized and primers are now available for the first time for the characterization of even more species. The supposedly asexually reproducing *C. beticola* was shown to be heterothallic, a fact that could not be determined before because of the absence of the teleomorph. An approximately equal distribution of the mating type genes in field populations was linked with the high level of genetic diversity calculated within and between *C. beticola* populations and gene flow between Eurasian populations was demonstrated. Nine novel molecular markers are now available for future *C. beticola* population studies.

REFERENCES

Arie, T., Kaneko, I., Yoshida, T., Noguchi, M., Nomura, Y., and Yamaguchi, I. 2000. Mating-type genes from asexual phytopathogenic ascomycetes *Fusarium oxysporum* and *Alternaria alternata*. Mol. Plant Microbe In. 13:1330–1339.

Burgess, T., Wingfield, M.J., and Wingfield, B.D. 2001. Simple sequence repeat marker distinguish among morphotypes of *Sphaeropsis sapinea.* Appl. Envirion. Microbiol. 67:354–362.

Burgess, T., Wingfield, M.J., and Wingfield, B.D. 2004. Global distribution of *Diplodia pinea* genotypes revealed using simple sequence repeat (SSR) markers. Australas. Plant Pathol. 33:513–219.

Chen, W., Shi, X., and Chen, Y.-C. 2002. Microsatellite markers and clonal genetic structure of the fungal pathogen *Phialophora gregata.* Mycol. Res. 106:194–202.

Chupp, C. 1954. A monograph of the fungus genus *Cercospora.* Published by the author. Ithaca, New York.

Crous, P.W., and Braun, U. 2003. *Mycosphaerella* and its anamorphs: 1. Names published in *Cercospora* and *Passalora*. CBS Biodivers. Series 1:1–571.

Crous, P.W., Aptroot, A., Kang, J.-C., Braun, U., and Wingfield, M.J. 2000. The genus *Mycosphaerella* and its anamorphs. Stud. Mycol. 45:107–121.

Crous, P.W., Kang, J.-C., and Braun, U. 2001. A phylogenetic redefinition of anamorph genera in *Mycosphaerella* based on ITS rDNA sequences and morphology. Mycologia 93:1081–1101.

Crous, P.W., Groenewald, J.Z., Pongpanich, K., Himaman, W., Arzanlou, M., and Wingfield, M.J. 2004. Cryptic speciation and host specificity among *Mycosphaerella* spp. occurring on Australian *Acacia* species grown as exotics in the tropics. Stud. Mycol. 50:457–469.

Crous, P.W., Groenewald., J.Z., Groenewald., M., Caldwell, P., Braun, U., and Harrington, T.C. 2006. Species of *Cercospora* associated with grey leaf spot of maize. Stud. Mycol. 55:189–197.

Du, M., Schardl, C.L., Nuckles, E.M., and Vaillancourt, L.J. 2005. Using mating-type gene sequences for improved phylogenetic resolution of *Colletotrichum* species complexes. Mycologia 97:641–658.

Ellis, M.B. 1971. Dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, UK.

Ellis, M.B. 1976. More dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, UK.

Fresenius, G. 1863. Beiträge zur Mykologie 3. Henrich Ludwig Bröömmer Verlag, Frankfurt, Germany.

Goodwin, S.B., Dunkle, D.L., and Zismann, V.L. 2001. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. Phytopathology 91:648–658.

Goodwin, S.B., Waalwijk, C., Kema, G.H.J., Cavaletto, J.R., and Zhang, G. 2003. Cloning and analysis of the mating-type idiomorphs from the barley pathogen *Septoria passerinii*. Mol. Gen. Genomics 269:1–12.

Groenewald, M., Groenewald, J.Z., and Crous, P.W. 2005. Distinct species exist within the *Cercospora apii* morphotype. Phytopathology 95:951–959.

Groenewald, M., Groenewald, J.Z., Braun, U., and Crous, P.W. 2006a. Host range of *Cercospora apii* and *C. beticola,* and description of *C. apiicola,* a novel species from celery. Mycologia 98:275–285.

Groenewald, M., Groenewald, J.Z., Harrington, T.C., Abeln, E.C.A., and Crous, P.W. 2006b. Mating type gene analysis in apparently asexual *Cercospora* species is suggestive of cryptic sex. Fungal Genet. Biol. 43:813–825.

Große-Herrenthey, U. 2001. Molekulare analyse der genetischen variabilität von *Cercospora beticola* Sacc., dem Erreger der cercospora-blattfleckenkrankheiten bei zuckerrüben und untersuchungen zur resistenz von *Beta vulgaris* ssp. maritima gegen *C. beticola* unter anwendung unterschiedlicher resistenztestsysteme. PhD thesis, Christian-Albrechts University, Kiel, Germany.

Hayden, H.L., Carlier, J., Aitken, E.A.B. 2003a. Population differentiation in the banana leaf spot pathogen *Mycosphaerella musicola*, examined at a global scale. Plant Pathol. 52:713–719.

Hayden, H.L., Carlier, J., Aitken, E.A.B. 2003b. Genetic structure of *Mycosphaerella fijiensis* populations from Australia, Papua New Guinea and the Pacific Islands. Plant Pathol. 52:703–712.

Inglis, P.W., Teixeira, E.A., Ribeiro, D.M., Valadares-Inglis, M.C., Tigano, M.S., and Mello, S.C.M. 2001. Molecular markers for the characterization of Brazilian *Cercospora caricis* isolates. Curr. Microbiol. 42:194–198.

Johnson, E.M., and Valleau, W.D. 1949. Synonymy in some common species of *Cercospora.* Phytopathology 39:763–770.

Karaoglanidis, G.S., Ioannidis, P.M., and Thanassoulopoulos, C.C. 2000. Reduced sensitivity of *Cercospora beticola* to sterol-demethylation-inhibiting fungicides. Plant Pathol. 49:567–572.

Milgroom, M.G. 1996. Recombination and multilocus structure of fungal populations. Annu. Rev. Phytopathol. 34:457–477.

O'Donnell, K., Ward, T.J., Geiser, D.M., Kistler, H.C., and Aoki, T. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genet. Biol. 41:600–623.

Okori, P., Rubaihayo, P.R., Fahleson, J., Adipala, E., and Dixelius, C. 2003. Assessment of genetic variation among East African *Cercospora zeae-maydis* populations. Afr. Crop Sci. J. 11:75–85.

Okori, P., Rubaihayo, P.R., Ekwamu, A., Fahleson, J., and Dixelius, C. 2004. Genetic characterization of *Cercospora sorghi* from cultivated and wild sorghum and its relationship to other *Cercospora* fungi. Phytopathology 94:743–750.

Paoletti, M., Buck, K.W., and Brasier, C.M. 2005. Cloning and sequence analysis of the *MAT-B* (*MAT-2*) genes from the three Dutch elm disease pathogens, *Ophiostoma ulmi, O. novo-ulmi*, and *O. himal-ulmi*. Mycol. Res. 109:983–991.

Saccardo, P.A. 1876. Fungi Veneti novi vel critici. Series V. Nuovo Giorn. Bot. Ital. 8:162–211.

Stergiopoulos, I., Groenewald, M., Staats, M., Lindhout, P., Crous, P.W., and de Wit, P.J.G.M. 2007. Mating-type genes and the genetic structure of a world-wide collection of the tomato pathogen *Cladosporium fulvum*. Fungal Genet. Biol. dio: 10.1016/j.fgb.2006.11.004.

Waalwijk, C., Mendes, O., Verstappen, E.C., de Waard, M.A.D., and Kema, G.H.J. 2002. Isolation and characterization of the mating-type idiomorphs from the wheat septoria leaf blotch fungus *Mycosphaerella graminicola.* Fungal Genet. Biol. 35:277–286.

Wang, J., Levy, M., and Dunkle, L.D. 1998. Sibling species of *Cercospora* associated with gray leaf spot of maize. Phytopathology 88:1269–1275.

Weiland, J.J., and Koch, G. 2004. Sugar-beet leaf spot disease (*Cercospora beticola* SACC.) Mol. Plant Pathol. 5:157–166.

Witthuhn, R.C., Harrinton, T.C., Steimel, J.P., Wingfield, B.D., and Wingfield, M.J. 2000. Comparison of isozymes, rDNA spacer regions and *MAT-2* DNA sequences as phylogenetic characters in the analysis of the *Ceratocystis coerulescens* complex. Mycologia 92: 447–452.

Wolf, P.F.J., and Verreet, J.A. 2002. Development and implementation of an integrated pest management system in Germany for the control of fungal leaf diseases in sugar beet: the IPM sugar beet mode. Plant Dis. 86:336–344.

Wolf, P.F.J., and Verreet, J.A. 2005. Factors affecting the onset of Cercospora leaf spot epidemics in sugar beet and establishment of disease-monitoring thresholds. Phytopathology 95:269–274.

Zhan, J., and McDonald, B.A. 2004. The interaction among evolutionary forces in the pathogenic fungus *Mycosphaerella graminicola*. Fungal Genet. Biol. 41:590–599.

Zhan, J., Pettway, R.E., and McDonald, B.A. 2003. The global genetic structure of the wheat pathogen *Mycosphaerella graminicola* is characterized by high nuclear diversity, low mitochondrial diversity, regular recombination, and gene flow. Fungal Genet. Biol. 38:286–297.

APPENDIX I

Mating type genes in *Cercospora beticola*

Marizeth Groenewald[1,2], Johannes Z. Groenewald[1], and Pedro W. Crous[1,2]

[1] *Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, Netherlands*
[2] *Laboratory of Phytopathology, Wageningen University, Binnenhaven 5, 6709 PD Wageningen, Netherlands*

Chapter in book (1st International Symposium on Cercospora Leaf Spot of Sugar Beet).

Key words: Allele frequency, Cercospora leaf spot, MAT1, MAT2, *Mycosphaerella*

INTRODUCTION

The genus *Cercospora* was first described by Fresenius (Fuckel, 1863) and is one of the largest genera of hyphomycetes with more than 3,000 names listed by Pollack (1987). Crous & Braun (2003) revised the genus and reduced many species to synonymy, leaving a total of 659 species with a further 281 morphologically indistinguishable *Cercospora* species listed as synonyms under *C. apii sensu lato* (Crous & Braun, 2003). *Cercospora beticola*, also listed as a synonym under *C. apii sensu lato*, is the main causal agent of Cercospora leaf spot on *Beta vulgaris* (sugar beet) (Saccardo, 1876; Crous & Braun, 2003; Groenewald *et al*., 2005, 2006a). Some confusion existed in the past about whether or not *C. beticola* and *C. apii*, the causal agent of Cercospora leaf spot of celery (*Apium*), were synonymous. Groenewald *et al*. (2005) conducted a detailed molecular comparison (multigene phylogeny and AFLPs), including cultural characteristics and growth rates, and showed that these two taxa are indeed distinct species.

Phylogenetic analyses using the internal transcribed spacer (ITS) sequences of a variety of *Cercospora* species have resolved *Cercospora* as a well-defined monophyletic clade within the teleomorph genus *Mycosphaerella* (Stewart *et al*., 1999; Crous *et al*., 2000, 2001, 2004; Goodwin *et al*., 2001; Pretorius *et al*., 2003). As no teleomorph is known for *C. beticola*, neither from nature nor by artificial crosses in the laboratory, very little is known about the occurrence or importance of sexual reproduction in this apparently asexual fungal species. Groenewald *et al*. (2006b) showed that genes with high homology to mating type (*MAT*) genes of other fungi are present in *C. beticola,* and that isolates with opposite mating types are equally distributed in natural populations of *C. beticola*. As it has been shown that the two opposite mating types are equally distributed in fungal populations that regularly reproduce sexually (Milgroom, 1996), it is possible that sexual reproduction does take place in this apparently asexually reproducing fungal species.

This chapter gives a general overview on aspects regarding the molecular characteristics of mating systems in ascomycetes, with specific reference to the general structure and organization of *MAT* genes and focuses on mating type gene analyses of *C. beticola* and closely related species such as *Mycosphaerella graminicola*, *Septoria passerinii*, additional *Cercospora* species (Mycosphaerellaceae) and *Alternaria alternata* (Pleosporaceae). The main focus is on the *MAT* genes of *C. beticola*, the comparison of the genes and derived proteins to those of closely related species and the use of mating type sequences in phylogenetic analyses of the *Cercospora* species complex.

General organization of mating type genes

The existence of mating types was first recognized by Blakeslee (1904) when he discovered sexual fusion in *Rhizopus* (Mucorineae) who demonstrated that "sexes" (mating types) exist in fungi and introduced the terms homothallism for self-fertile and heterothallism for self-sterile individuals. Sexual reproduction has been studied for more than 80 years in ascomycetes, and in the early 1980s the first *MAT* loci were molecularly characterized when the complete DNA sequences of the *MAT* alleles of *Saccharomyces cerevisiae* were determined (Astell *et al*., 1981). The first filamentous ascomycete *MAT* genes that were cloned were from *Neurospora crassa* (Glass *et al*., 1988; Staben & Yanofsky, 1990), and the development of new techniques

allowed the isolation and characterization of additional *MAT* genes of economically important fungi (Turgeon *et al*., 1993; Arie *et al*., 1997, 2000; Waalwijk *et al*., 2002; Goodwin *et al*., 2003; Groenewald *et al*., 2006b).

A standardized nomenclature was proposed by Turgeon & Yoder (2000) that can be used for *MAT* genes of filamentous ascomycetes in order to simplify the communication between investigators that work with *MAT* genes from diverse genera. For most heterothallic species of ascomycetes studied thus far, only one *MAT* locus is present (*MAT1*) with two different alleles. The DNA sequences at this locus show no homology between strains of opposite mating type that belong to the same species and they have been termed idiomorphs in stead of alleles (Glass *et al*., 1988; Metzenberg & Glass, 1990). The two idiomorphs at *MAT1* are designated *MAT1-1* and *MAT1-2*, where each of these idiomorphs contains open reading frames that encode proteins with confirmed or putative DNA-binding domains. The *MAT1-2* idiomorph contains the *MAT1-2-1* gene that encodes a regulatory protein with a high mobility group (HMG) domain. The *MAT1-1* idiomorph of all the heterothallic loculoascomycetes studied thus far contains the *MAT1-1-1* gene encoding a protein with an alpha domain (Turgeon *et al*., 1993). It has been found that more than one gene can be present in the *MAT1-1* idiomorph of some heterothallic pyrenomycetes and discomycetes (Sing & Ashby, 1998, 1999; Yun *et al*., 2000; Pöggeler, 2001). These include the *MAT1-1-1,* the *MAT1-1-2* encoding an amphipathic alpha helix protein, the *MAT1-1-3* gene encoding a HMG domain protein and the *MAT1-1-4* gene encoding a metallothionein protein.

In homothallic fungi, homologs of genes from both *MAT1-1* and *MAT1-2* idiomorphs often are present within the same nucleus, and this phenomenon is much more complex than heterothallism. Complete copies of both the *MAT1-1-1* and *MAT1-2-1* genes can be found within a genome, but these genes can also be fused, resulting in truncated MAT proteins (Pöggeler *et al*., 1997; Yun *et al*., 1999). When both the *MAT1-1* and *MAT1-2* idiomorphs exist in one nucleus, regardless whether the genes are fused, such a genotype is called *MAT1/2* (Turgeon & Yoder, 2000). It has also been reported that some homothallic species contain the *MAT* genes of only one of the two idiomorphs of the heterothallic relatives (Glass *et al*., 1990; Glass & Smith, 1994; Pöggeler *et al*., 1999; Yun *et al*., 1999).

The specific function(s) of the mating type proteins have not yet been identified, but it is most likely that these proteins are transcription factors that control pathways of cell speciation and sexual morphogenesis (Turgeon, 1998). The formal nomenclature that is proposed for *MAT* genes of heterothallic ascomycetes will be used throughout this chapter for the *MAT1-1-1*, and since only a single gene has been identified in the *MAT1-2* idiomorph thus far for ascomycetes, we will refer to this gene as *MAT1-2* and not the more elaborate nomenclature *MAT1-2-1*.

Although sexual reproduction is absent in a large number of filamentous ascomycetes, mating type sequences have been isolated from several apparently asexual fungi, such as *A. alternata* (Arie *et al*., 2000), *S. passerinii* (Goodwin *et al*., 2003) and several *Cercospora* species (Groenewald *et al*., 2006b). The finding that asexual ascomycetes also carry *MAT* genes, introduced the possibility that a sexual cycle may also be active in members of some of these supposedly asexual species, but that the sexual cycle has not yet been identified. Another possibility is that asexual fungi with functional *MAT* genes may lack some other attributes that

are required for mating (Sharon *et al*., 1996; Arie *et al*., 2000; Yun *et al*., 2000). Some fungal species with no known sexual stage are closely related to species that have highly active sexual cycles from which the *MAT* genes have already been characterized. This is true for the majority of *Cercospora* species (anamorph genus) that are related to sexual species of *Mycosphaerella*, such as *M. graminicola* (Waalwijk *et al*., 2002), for which both *MAT* genes (*MAT1-1-1* and *MAT1-2*) have been characterized.

CHARACTERIZATION OF *CERCOSPORA BETICOLA* MATING TYPE GENES

Isolation of MAT sequences

Different methods have been used to isolate *MAT* genes from a wide range of fungal species. Most of these genes have been isolated by heterologous hybridization, or by using PCR techniques based on sequence homologies found within the idiomorphs or regions are flanking the idiomorphs (Turgeon *et al*., 1993; Arie *et al*., 1997, 2000; Waalwijk *et al*., 2002; Goodwin *et al*., 2003). Homologous regions within the *MAT* genes of the closely related species *M. graminicola*, *M. fijiensis* and *S. passerinii* were used to design two sets of degenerate primers to isolate these genes from *C. beticola* (Groenewald *et al*., 2006b). The first set (MgMfSpMAT1-1) was designed in homologous regions within the *MAT1-1-1* genes of these three species. The degenerate primers MgMfSpMAT1-1f1 and MgMfSpMAT1-1r2 (indicated as 1 and 2 in Fig. 1) amplified a fragment of 922 bp in *C. beticola* that showed 77 % and 54 % identity to a 57 amino acid region of the *S. passerinii* and *M. graminicola* MAT1 protein, respectively. The second set (MgMfSpMAT1-2) was designed in homologous regions within the *MAT1-2* genes of *M. graminicola*, *M. fijiensis* and *S. passerinii*. The degenerate primers MgMfSpMAT1-2f2 and MgMfSpMAT1-2r1 (indicated as 11 and 12 in Fig. 1) amplified a 274 bp fragment in *C. beticola*. This fragment showed 59 % and 61 % identity to a 76 amino acid region of the *S. passerinii* and *M. graminicola* MAT2 proteins, respectively. These partial *MAT1-1-1* and *MAT1-2* sequences were used by Groenewald *et al.* (2006b) as template in order to obtain the full-length *MAT1-1-1* and *MAT1-2* as well as additional flanking sequences (Fig. 1) by using a commercial chromosome walking kit. A 2,539 bp fragment (GenBank no. DQ192581) was obtained by amplifying the regions upstream and downstream of the partial *MAT1-1-1* sequence (Fig. 1) (Groenewald *et al.,* 2006b). The binding sites of the chromosome walking primers used are indicated in Fig. 1 with numbers 3–8. A 2,307 bp fragment (GenBank no. DQ192582) was obtained after sequencing the area flanking the partial *MAT1-2* sequence (Fig. 1). The binding sites of the chromosome walking primers used are indicated in Fig. 1 with numbers 13–24. Groenewald *et al*. (2006b) indicated that these degenerate primer sets can also amplify the corresponding areas within the *MAT* genes of other species that include *Dothistroma pini*, *D. septosporum*, *Mycosphaerella musicola, M. musae*, *M. marksii*, *M. thailandica*, *Cladosporium herbarum* and several diverse *Cercospora* species including *C. apii*, *C. apiicola*, *C. zeae-maydis* and *C. zeina*.

Gene and protein annotations

The 2,539 bp fragment (GenBank no. DQ192581) that was obtained from the *MAT1-1* idiomorph included the full-length *C. beticola MAT1-1-1* gene (1,164 bp) containing three putative introns

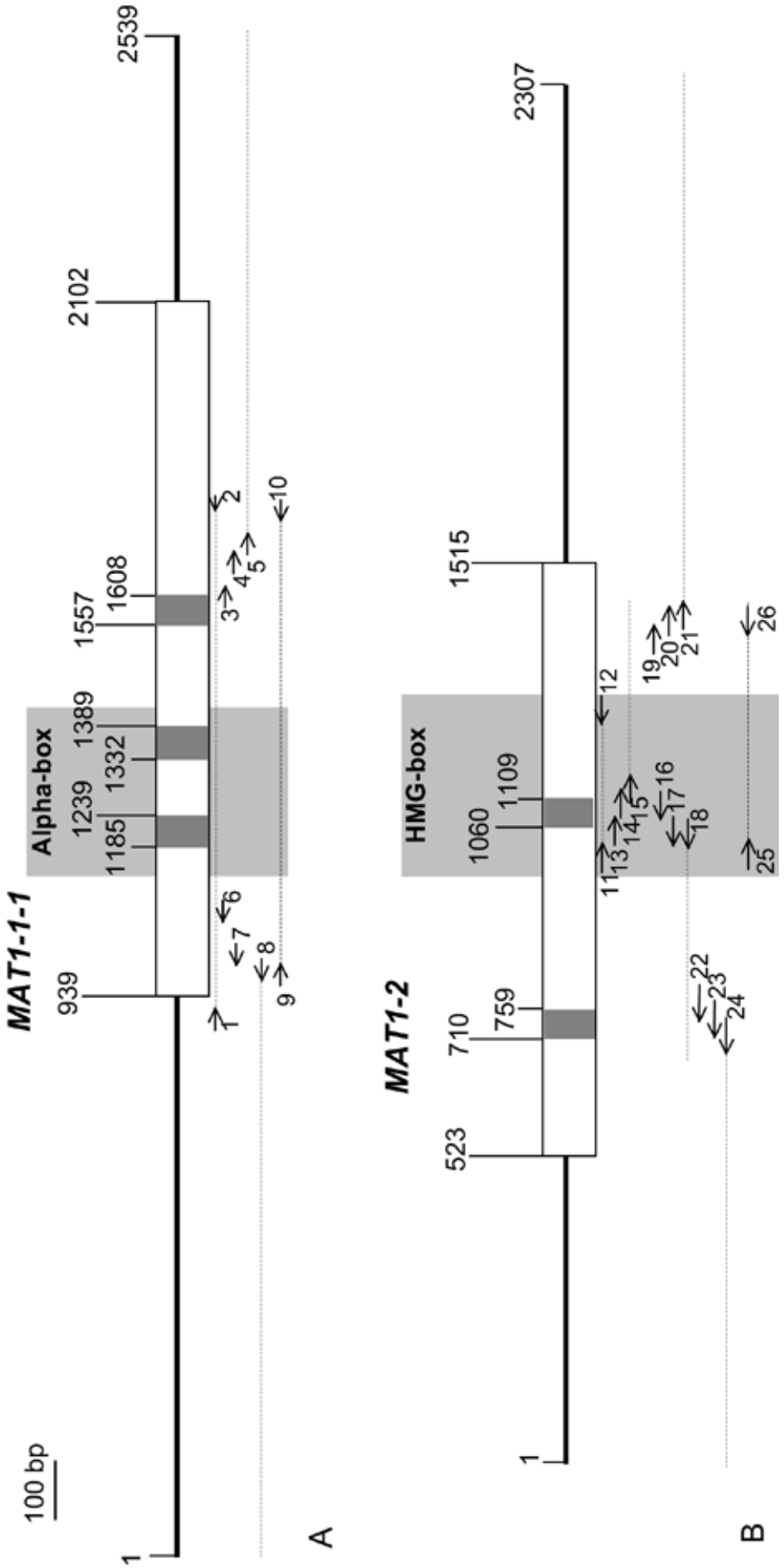


Fig. 1. Schematic representation of the **A**, *MAT1-1-1* and **B**, *MAT1-2* gene area of *Cercospora beticola*. The exon (white box) and intron (gray boxes) boundaries are shown and the sizes are indicated by a number in base pairs. The approximate binding sites of the primers are indicated with arrows and the areas sequenced using those primers are indicated with dashed lines.

(51 bp, 56 bp and 50 bp, respectively). The last two introns contained a perfect lariat sequence (RCTRAC) starting at 15 nt (second intron) and 17 nt (third intron) upstream of the likely 3' splice site (Bruchez *et al*., 1993). The first intron, however, did not contain a perfect lariat sequence and two protein predictions were performed to test whether this is a true intron, the first including it as coding nucleotides and the second excluding it from the prediction. When the intron is included in the coding domains, a frame shift causes the amino acid sequences of the resulting MAT1 protein to be highly different from other MAT1 proteins and it also results in an early stop codon. This suggests that the *MAT1-1-1* gene of *C. beticola* most likely contains three exons. The predicted 1,005 nt mRNA fragment translates into a 335 amino acid protein with a putative alpha domain. This domain of MAT1 of *C. beticola* also shows relatively high identity to the alpha domain of the MAT1 in several different fungal species (Fig. 2).

The 2,307 bp fragment (GenBank no. DQ192582) obtained from the *MAT1-2* idiomorph contained the full-length *C. beticola MAT1-2* gene of 993 bp and included two putative introns (48 bps each). Both introns contained a lariat sequence starting at 15 nt (first intron) and 16 nt (second intron) upstream from the likely 3' splice site. The predicted 897 nt mRNA translates into a MAT2 protein of 299 amino acids with a putative HMG domain that is fairly identical to those present in MAT2 proteins of several other fungal species (Fig. 3). Groenewald *et al.* (2006b) used *Cercospora*-specific primers to screen for the presence of the *MAT1-1-1* and *MAT1-2* genes in several *C. beticola* strains, and the data showed that either the *MAT1-1-1* or *MAT1-2* is present in a single strain and that this fungus is potentially heterothallic.

Comparisons of mating type sequences of related species

The *MAT* genes of a variety of *Cercospora* species were isolated using the same method that was used for isolating the corresponding genes of *C. beticola* (Groenewald *et al*., 2006b). The *MAT1-1-1* genes of three additional *Cercospora* species, *C. apii* (1,164 bp), *C. zeae-maydis* (1,167 bp) and *C. zeina* (1,167 bp), were successfully isolated (Groenewald *et al*., 2006b). The *MAT1-1-1* sequence identity between *C. beticola* and that of *C. apii* was much higher (99.9 %) than that observed between the sequence of *C. beticola* and that of *C. zeae-maydis* (87.3 %) and *C. zeina* (87.2 %). All of these *MAT1-1-1* sequences contained three putative introns and encoded a 335 amino acid MAT1 protein for all three species. The *MAT1-2* genes of *C. apiicola* (1,272 bp), *C. zeae-maydis* (995 bp) and *C. zeina* (1,274 bp) were also obtained (Groenewald *et al*., 2006b). The putative *MAT1-2* genes of *C. beticola* and *C. zeae-maydis* are shorter than those present in the additional *Cercospora* species analyzed and the identities among the overall *MAT1-2* sequences vary from 70.6 % (between *C. beticola* and *C. zeina*) to 90.2 % (between *C. beticola* and *C. zeae-maydis*). The additional *Cercospora MAT1-2* sequences also contained two putative introns that correlate with those found in *C. beticola*. The number of amino acids predicted for the MAT2 protein was 299 for *C. zeae-maydis* which is identical to the number found in *C. beticola,* and 392 for both *C. zeina* and *C. apiicola*. Comparisons between the *MAT* genes and proteins of *C. beticola* and *C. zeina* are shown in Table 1.

Both *MAT* loci of the heterothallic sexually reproducing fungal species *M. graminicola* have been isolated and characterized by Waalwijk *et al*. (2002), a species that is phylogenetically related to *C. beticola* (Mycosphaerellaceae). The *MAT1-2* was identified by screening a genomic library from a mating type 2 isolate using a probe that spans the conserved HMG domain in

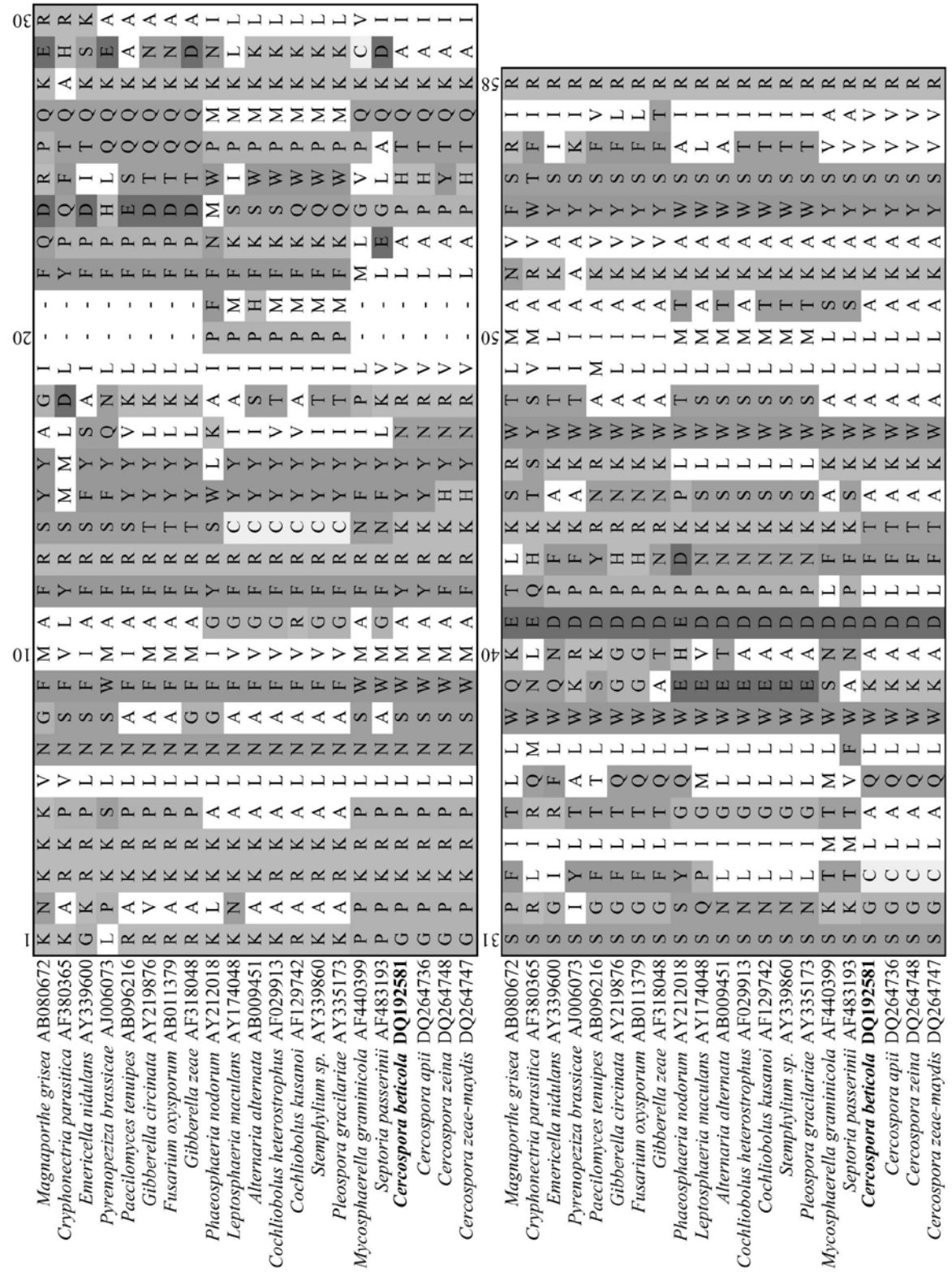


Fig. 2. Amino acid alignment of the alpha domain of the MAT1 protein of *Cercospora beticola* and other species.

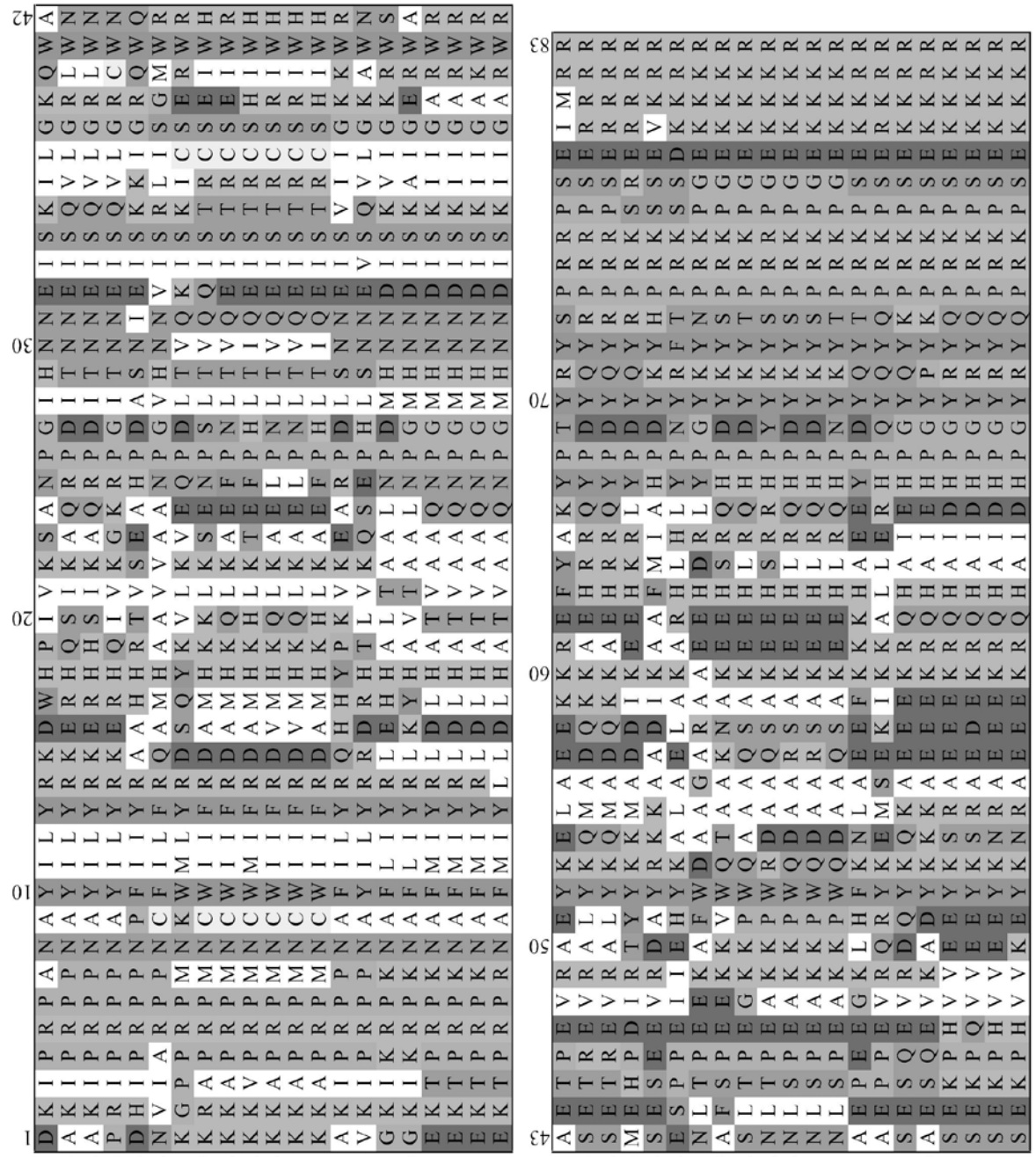


Fig. 3. Amino acid alignment of the HMG domain of the MAT2 protein of *Cercospora beticola* and other species.

the *MAT1-2-1* gene of *Oculimacula yallundae* (= *Tapesia yallundae*). The *MAT1-1* idiomorph was obtained by long-range PCR using primers that were designed within the upstream and downstream flanking regions of the *MAT1-2* idiomorph. Molecular data showed that either the *MAT1-1* or *MAT1-2* is present in a single strain and therefore confirming that *M. graminicola* is potentially heterothallic, as was already known from conventional crosses. Comparisons between the *MAT* genes and proteins of *C. beticola* and *M. graminicola* are listed in Table 1.

Both *MAT* loci of the presumably asexual species, *S. passerinii*, phylogenetically closely related to *M. graminicola* have been isolated and characterized by Goodwin *et al*. (2003). The *MAT1-2* was identified by screening a genomic library from a *S. passerinii* isolate using a probe derived from *MAT1-2* of *M. graminicola*. The clone containing the *MAT1-2* was sequenced and the full-length *MAT1-2* with its flanking regions were obtained. The primers that were used to amplify the *MAT1-1* idiomorph of *S. passerinii* were designed in homologous areas within the sequences from the flanking regions of both mating types of *M. graminicola* and that of the *MAT1-2* of *S. passerinii.* Long-range PCRs were successfully used to obtain the *MAT1-1-1* from a strain that does not contain the *MAT1-2*. As the two *MAT* genes were never found to be present in the same strain, this species was classified as potentially heterothallic. Comparisons between the *MAT* genes and proteins of *C. beticola* and *S. passerinii* are shown in Table 1.

Table 1. Comparisons of mating type genes and proteins of *Cercospora beticola* to that of *C. zeina*, *Septoria passerinii* and *Mycosphaerella graminicola*.

Region	***C. beticola***	*C. zeina*	*S. passerinii*	*M. graminicola*
MAT1-1-1				
Coding region	**1,164**	1,167	1,038	995
Exon I	**247**	247	181	319
IntronI	**51**	53	52	55
Exon II	**96**	92	94	94
Intron II	**56**	49	53	49
Exon III	**169**	169	658	478
Intron III	**50**	50	-	-
Exon IV	**495**	507	-	-
Amino acids	**335**	335	310	297
MAT1-2				
Coding region	**993**	1,274	754	1,233
Exon I	**188**	188	157	634
IntronI	**48**	48	49	48
Exon II	**302**	302	548	551
Intron II	**48**	50	-	-
Exon III	**407**	685	-	-
Amino acids	**299**	392	234	394

Both *M. graminicola* and *S. passerinii* have two putative introns in the conserved alpha domain of the *MAT1-1-1* gene and one in the conserved HMG domain of the *MAT1-2* gene (Waalwijk *et al*., 2002; Goodwin *et al*., 2003). The *MAT* genes of *C. beticola* also have these putative introns in the conserved domains, as well as an additional intron predicted in the areas

flanking the conserved domains of each of the respective genes. These additional introns found in the *MAT1-1-1* and *MAT1-2* of *C. beticola* are also present in the *MAT* genes of other *Cercospora* species (Groenewald *et al*., 2006b).

Degenerate primers, designed within the conserved HMG domains of the *MAT1-2* of *Cochliobolus* spp. (*C. heterostrophus, C. carbonum,* and *C. victoriae*) (Arie *et al*., 1997), were used for the preliminary screening of the *MAT1-2* of *A. alternata* (Arie *et al*. 2000), also known to be an asexually reproducing fungus. The complete *MAT1-2* idiomorph together with its flanking regions were obtained using PCR. Primers were designed in the upstream and downstream regions flanking the *MAT1-2* sequences that are known to be identical between the *MAT1-1-1* and *MAT1-2* strains. The complete *MAT1-1* idiomorph was obtained by long-range PCR using strains that did not give a PCR product with the *MAT1-2*-specific primers for *A. alternata*. As the two *MAT* genes were never found in the same strain, this species was also classified to be potentially heterothallic. The *MAT1-1* idiomorph (1,906 bp) of *A. alternata* contains the *MAT1-1-1* gene (1,214 bp) that encodes for a 389 amino acid protein containing an alpha domain. The *MAT1-2* idiomorph (2,220 bp) contains the *MAT1-2* gene (1,080 bp) that encodes for a 342 amino acid protein containing a HMG domain. They have found that, despite of the fact that *A. alternata* is an asexual species, the *MAT* genes are indeed expressed in this fungus. Heterologous expression studies showed that the *A. alternata MAT1-1-1* and *MAT1-2* genes are indeed functional and can be expressed in its close relative, *Cochliobolus heterostrophus*, which is a sexual reproducing species (Arie *et al*., 2000).

The number of introns in the *MAT1-1-1* and *MAT1-2* genes can vary among genera and species. Several ascomycetes contain only one putative intron in both the *MAT1-1-1* and *MAT1-2* genes. The putative intron splicing sites in the *MAT* genes and gene predictions of only a few filamentous ascomycetes, e.g., *A. alternata* (*MAT1-1-1* and *MAT1-2*), *Fusarium oxysporum* (*MAT1-1-1* and *MAT1-2*) and *Ophiostoma novo-ulmi* (*MAT1-2*), have been confirmed with mRNA studies. An example of a species that contains only one intron in both genes is *A. alternata* (Arie *et al*., 2000), whereas *F. oxysporum* (Arie *et al*., 2000) has two introns in the *MAT1-2* and one in the *MAT1-1-1*, and *O. novo-ulmi* one intron in the *MAT1-2* (Paoletti *et al*., 2005). All the *Cercospora* species studied thus far contain three introns in the *MAT1-1-1* and two in the *MAT1-2* (Groenewald *et al*., 2006b). Further studies at mRNA and protein levels are required to confirm the exact length of the coding regions as well as the intron and exon boundaries of the *MAT* genes of *C. beticola*.

The predicted amino acid sequences of the alpha domain (MAT1) and HMG domain (MAT2) region are very similar to those of other *Cercospora* species (Figs. 2, 3). For the alpha domain the percentage identity between *C. beticola* and that of *C. apii*, *C. zeina* and *C. zeae-maydis* were 100 %, 94.6 % and 96.4 %, respectively (Fig. 2), and for the HMG domain the percentage identity between *C. beticola* and that of *C. apiicola* and *C. zeina* were 97.6 % and *C. zeae-maydis* 96.4 % (Fig. 3). In addition, the *C. beticola* predicted amino acid sequences showed moderate identity to the alpha and HMG domain regions of *S. passerinii, M. graminicola* and other fungi belonging to different orders (Figs. 2, 3). Of the 56 amino acids that are included in the alpha domain of the MAT1 of *C. beticola*, 32 amino acids (57.1 %) are identical to that of *M. graminicola* and 30 (53.6 %) to that of *S. passerinii* (Fig. 2). The identities

observed between the alpha domain of the MAT1 of *C. beticola* and that of the rest of the fungal isolates ranged from 21.4 % for *Cryphonectria parasitica* to 51.8 % for *Emericella nidulans* (Fig. 2). A 67.5 % identity was observed between the HMG domain sequences (83 amino acids) of *C. beticola* and both *M. graminicola* and *S. passerinii* (Fig. 3). The identities observed for this area of *C. beticola* and that of the rest of the fungal isolates ranged from 34.94 % for *Leptosphaeria maculans* and *Cochliobolus kusanoi* to 53 % for *Magnaporthe grisea* (Fig. 3).

MATING TYPE GENES IN *CERCOSPORA* PHYLOGENIES

Several phylogenetic studies based on sequences of *MAT* genes and other housekeeping gene sequences suggest that *MAT* genes evolve rapidly (Whitfield *et al*., 1993; Goodenough, 1995; Waalwijk *et al*., 2002). Although the *MAT* genes vary substantially between distinct species, these genes appear to be highly conserved within species (Turgeon, 1998; Pöggeler, 1999). Previous studies showed that phylogenetic trees that were constructed with mating type sequences resolved the relationships between plant pathogens that remained unresolved in trees constructed with ITS sequences alone (Barve *et al*., 2003; Goodwin *et al*., 2003; O'Donnell *et al*., 2004). It has therefore been assumed that the *MAT* genes can be used as an excellent molecular tool for phylogenetic analyses of closely related species, for species identification as well as for the classification of both sexual and asexual species. If this is true for all fungal species, phylogenetic analyses using mating type sequences are not only powerful tools for the determination of relationships between pathogens, but also may prove to be an important method in identifying the relationship of economically valuable species. As most housekeeping genes sequenced thus far are not sufficient to distinguish between closely related *Cercospora* species that belong to the *C. apii* complex (Groenewald *et al*., 2006c), these species were used in phylogenetic analyses using the *MAT1-1-1* and *MAT1-2* sequences to get an indication whether the *MAT* genes can provide sufficient resolution at the species level. All maximum parsimony and neighbor-joining analyses performed by us were conducted as described by Groenewald *et al*. (2005).

Higher order phylogenies

The alignment of partial *MAT1-1-1* nucleotide sequences (alpha domain) contained 20 strains including *Magnaporthe grisea* as outgroup, and comprized of 174 characters in length of which 25 were constant, 15 were variable and uninformative, and 134 were parsimony informative. The alignment of partial *MAT1-2* nucleotide sequences (HMG domain) contained 20 strains including *Magnaporthe grisea* as outgroup, and comprized of 249 characters in lenght. Of the 249 characters, 38 were constant, 13 were variable and uninformative and 198 were parsimony informative. The three equally most parsimonious trees obtained from the *MAT1-1-1* alignment (Fig. 4) had a length of 602 steps (CI = 0.515, RI = 0.656, RC = 0.330); whereas 14 equally most parsimonious trees were obtained from the *MAT1-2* alignment (Fig. 5), and had a length of 849 steps (CI = 0.528, RI = 0.665, RC = 0.351).

The topology of the phylogenetic trees using the alpha domain (Fig. 4) and HMG domain (Fig. 5) sequences was approximately the same. All the isolates cluster within their respective orders. The Capnodiales, Hypocreales and Pleosporales clades showed high bootstrap supports

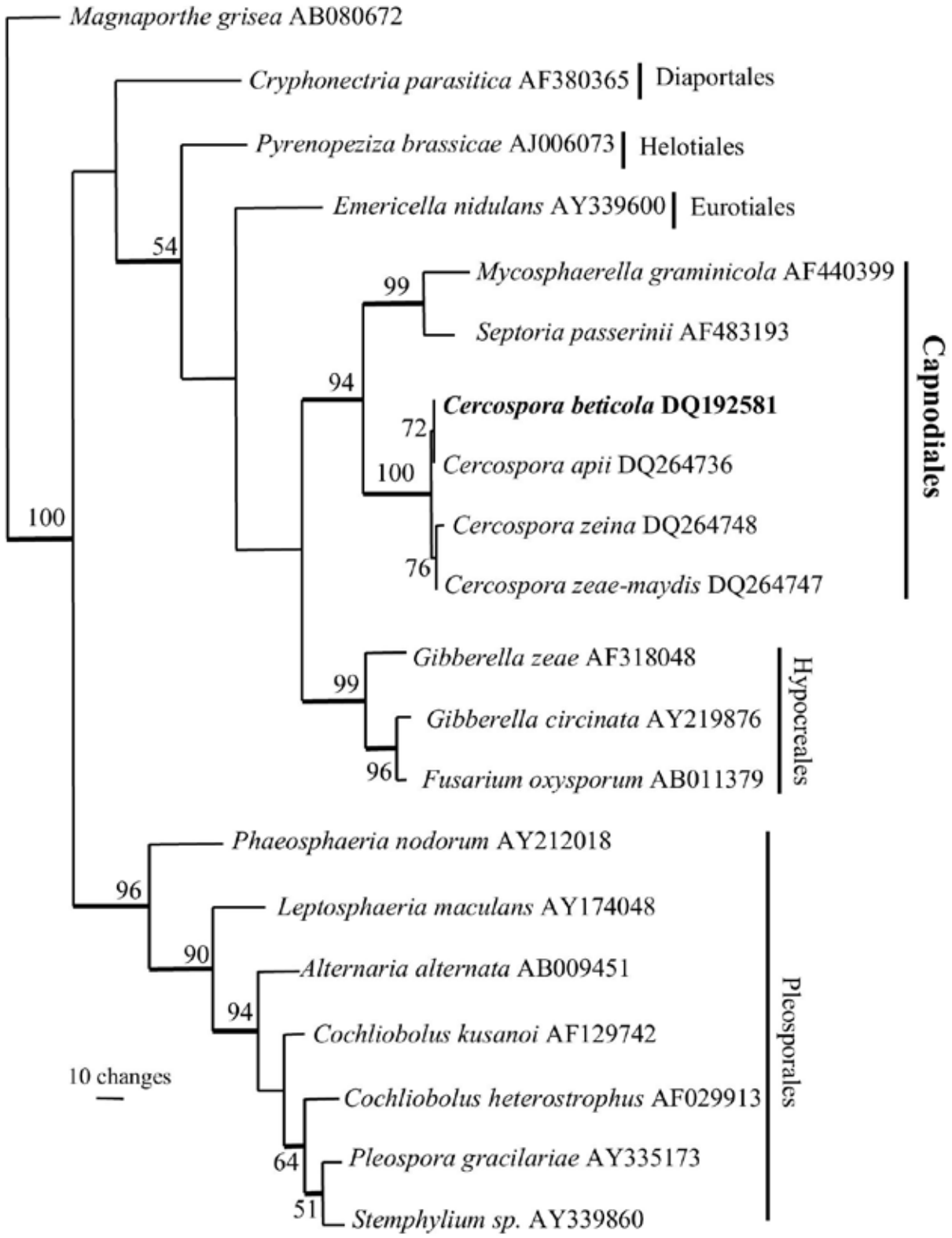


Fig. 4. Single most parsimonious tree obtained from heuristic searches of the conserved alpha domain using 100 random taxon additions. Bootstrap support values from 1,000 replicates are shown at the nodes. Fungal orders are shown to the right of the tree. The tree was rooted to *Magnaporthe grisea.*

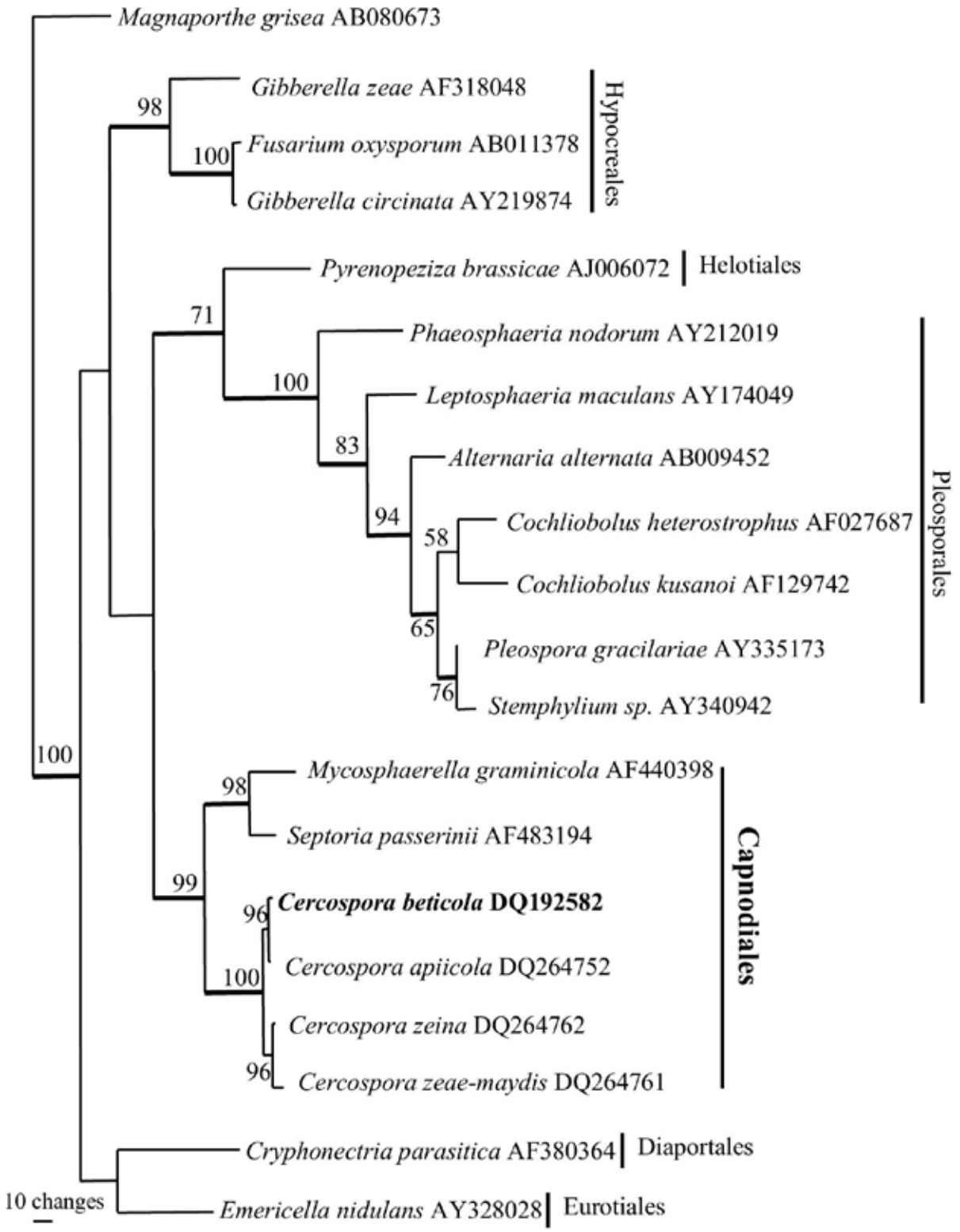


Fig. 5. One of three equally parsimonious tree obtained from heuristic searches of the conserved HMG domain using 100 random taxon additions. Bootstrap support values from 1,000 replicates are shown at the nodes. Fungal orders are shown to the right of the tree and strict consensus branches are thickened. The tree was rooted to *Magnaporthe grisea.*

(96 % – 100 %) in both trees. The phylogenetic analysis using the DNA sequences in the HMG domain and alpha domain, respectively showed that *C. beticola* is indeed genetically mostrelated to *M. graminicola* and *S. passerinii* as illustrated by the 94 % (*MAT1-1-1*) and 99 % (*MAT1-2*) bootstrap support values. These results correlate with the results obtained with other phylogenetic analyses that placed *Cercospora* within the Mycosphaerellaceae (Stewart *et al*. 1999; Crous *et al*., 2000, 2001, 2004, 2006b; Goodwin *et al*., 2001; Pretorius *et al*., 2003). The remaining *Cercospora* species cluster together with *C. beticola* with a 100 % bootstrap support value in both analyses (Figs. 4, 5). Two groups were found within the *Cercospora* cluster for the *MAT1-2* analysis, both with 96 % bootstrap support. One group contains the two isolates from maize, *C. zeina* and *C. zeae-maydis* (Crous *et al*., 2006a), and the other group contains two species that belong to the *C. apii* complex. Unfortunately, these two groups are not well supported when the alpha domain sequences were used.

These data indicate that genera belonging to the same order group together, which indicates that MAT sequences could be used for higher order phylogenetic analyses.

***Cercospora* phylogenies**

Most protein coding genes used in previous taxonomic studies of *Cercospora* lack resolution to distinguish closely related *Cercospora* species (Groenewald *et al*., 2005, 2006a, 2006c). Groenewald *et al*. (2006b) was the first to conduct phylogenetic analyses of partial *MAT* genes to determine whether they have sufficient discriminatory resolution between closely related *Cercospora* species, particularly those included in the *C. apii* complex. The *Cercospora* mating type-specific primer sets, CercosporaMat1 (indicated with numbers 9 and 10 in Fig. 1A) and CercosporaMat2 (indicated with numbers 25 and 26 in Fig. 1B), amplify the three introns of *MAT1-1-1* and the intron that is present in the HMG domain of the *MAT1-2* respectively. Sequences of introns are usually more variable than those of exons, and these two fragments could therefore be useful for phylogenetic analyses of closely related *Cercospora* species. The alignment of partial *MAT1-1-1* nucleotide sequences contained 14 strains including *M. graminicola* as outgroup, and had a total length of 731 characters (Fig. 6A). Of the 731 characters, 331 were constant, 280 were variable and uninformative and 120 were parsimony informative. The alignment of partial *MAT1-2* nucleotide sequences contained 12 strains including *M. graminicola* as outgroup, and had a total length of 436 characters (Fig. 6B). Of the 436 characters, 220 were constant, 182 were variable and uninformative and 34 were parsimony informative. A single most parsimonious tree was obtained for the *MAT1-1-1* alignment and it had a length of 516 steps (CI = 0.936, RI = 0.822, RC = 0.769). Two equally parsimonious trees were obtained for the *MAT1-2* alignment and consisted of 258 steps (CI = 0.965, RI = 0.809, RC = 0.780).

In the *MAT1-1-1* and *MAT1-2* analyses (Fig. 6), the two maize species, *C. zeina* and *C. zeae*-maydis, grouped together with bootstrap support values of 100 % and 96 %, respectively, whereas the additional *Cercospora* species, belonging to the *C. apii* complex, grouped together with a 100 % bootstrap support value for *MAT1-1-1* and 94 % for *MAT1-2*. Groenewald *et al*. (2006b) pointed out a number of potential problems when using *MAT* genes in phylogenetic analyses. Firstly, although several isolates may be available for a species, only one mating type is known, and secondly, only a single isolate of a species is available for study, and this

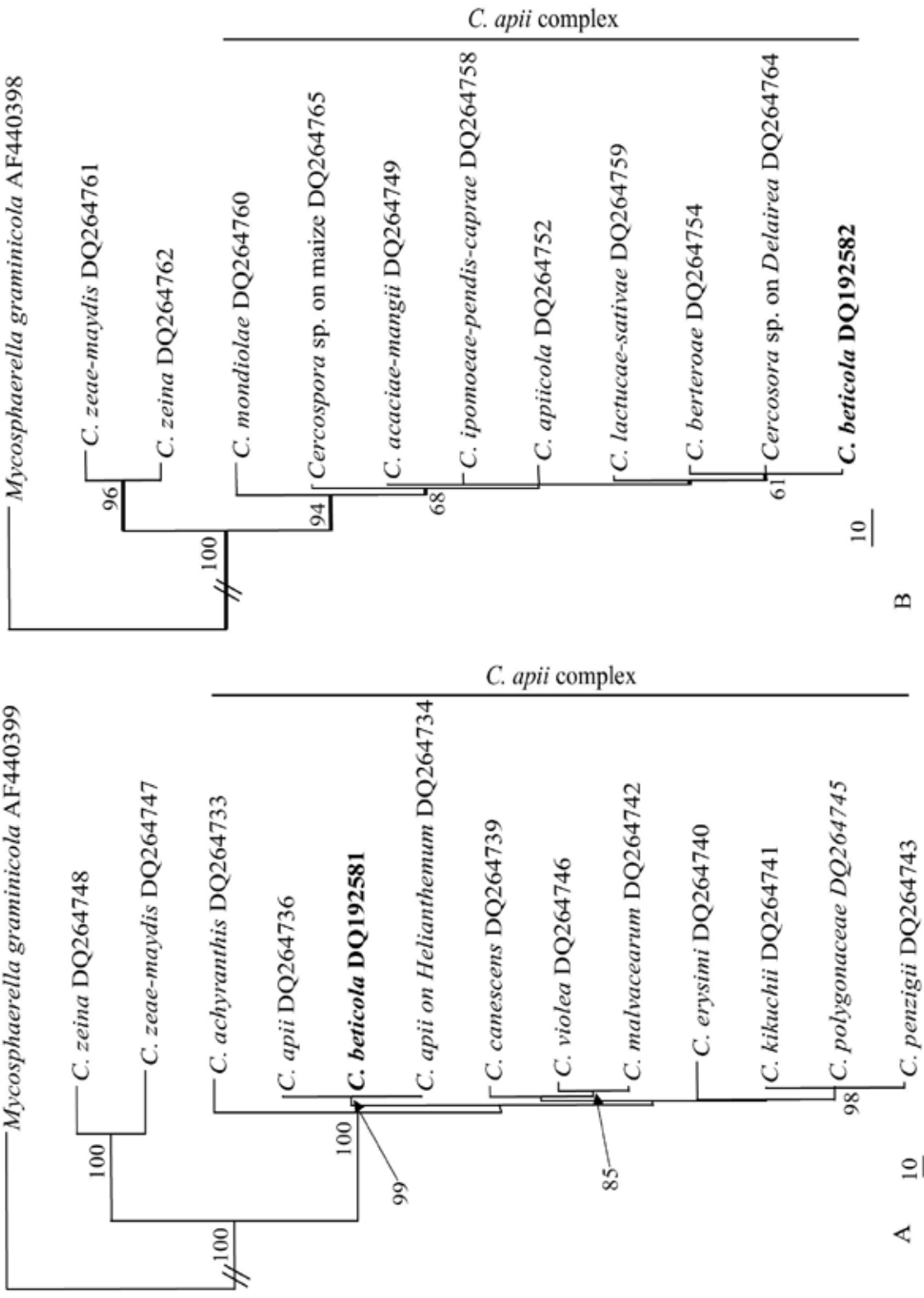


Fig. 6. A, single most parsimonious tree obtained from the *MAT1-1-1* sequence alignment, **B**, one of two equally parsimonious trees obtained from the *MAT1-2* sequence alignment. The trees were rooted to *Mycosphaerella graminicola.*

isolate carries only one of the two *MAT* genes. This was the case for most of the *Cercospora* species tested, and these taxa could only be compared to others with sequences of the same mating type. A third problem is that the *MAT* gene sequences differ a great deal among different genera and even among species of the same genus. This may restrict analyses to related species and to only a small portion of the gene; specifically, to the more conserved regions (alpha or HMG domains) of these genes. The conserved regions may lack the resolution to distinguish among closely related species, as was the case within the group of isolates belonging to the *C. apii* complex, and it is clear that the *MAT1-1-1* sequences do not resolve the two distinct species, *C. apii* and *C. beticola* (99 % support), or *C. kikuchii*, *C. polygonaceae* and *C. penzigii* (98 % support). The *MAT1-2* analysis did not provide resolution within the tree between the closely related *Cercospora* species. *MAT* genes could therefore not be successfully used in phylogenetic analyses to distinguish between closely related species that belong to the *C. apii* complex (this study; Groenewald *et al*., 2006b).

MATING TYPE DISTRIBUTION

A total of 255 *C. beticola* isolates (46 from France, 41 from Germany, 33 from Italy, 48 from the Netherlands, 50 from Iran and 37 from New Zealand) were screened by Groenewald *et al*. (2006b) and each isolate tested showed either the *MAT1-1-1* or the *MAT1-2* fragment. The *MAT1-1-1* and *MAT1-2* genes were approximately equally distributed in the *C. beticola* populations with MAT1:MAT2 ratios close to 1 (1.19 for France, 1.16 for Germany, 0.5 for Italy, 0.85 for Netherlands, 1.08 for Iran and 1.06 for New Zealand) and with no significant deviation ($P < 0.05$) from a 1:1 ratio. The approximately equal distribution of the two mating types within the six sampled populations of *C. beticola* suggests that the genes may be functional in these populations. If *C. beticola* was strictly asexual, a skewed distribution of the mating types would be expected, or perhaps only a single mating type would be found in the populations. Also, if these populations arose from a single introduction of a specific genotype, we might expect only one mating type to be present.

Conflicting results were observed for populations of other supposedly asexually reproducing *Cercospora* species. For populations of *C. zeae-maydis* and *C. zeina* the mating types were found in approximate equal distribution whereas data obtained from *C. apii* and *C. apiicola* showed that each of these two species contains only one of the two *MAT* genes (*MAT1-1-1* for *C. apii* and *MAT1-2* for *C. apiicola*) (Groenewald *et al*., 2006b). Furthermore, *C. zeae-maydis*, *C. zeina* and *C. beticola* produce spermatogonia in culture, which is indicative of a sexual cycle, but spermatogonia are absent in *C. apii* and *C. apiicola* (P.W. Crous, *unpublished data*). Equal distribution of the *MAT* genes was also found for other fungal species for which a sexual cycle has not yet been observed such as *Rhynchosporium secalis* (Linde *et al*., 2003). For *R. secalis*, isolates with opposite mating types were even found to co-exist in the same lesion (Linde *et al*., 2003). This was also found to be true for *C. beticola* (M. Groenewald, *unpublished data*). Goodwin *et al*. (2003) found both mating types to occur on the same leaf and showed that both mating types were present in approximately equal frequencies in natural populations from North Dakota and Minnesota. They concluded that the equal distribution of the mating types and the high number of unique genotypes found on the same leaf, are indications that *S. passerinii*

undergoes regular cycles of sexual reproduction in the field. Recent studies confirmed this by successfully crossing isolates of *S. passerinii* in culture, resulting in a *Mycosphaerella* state with viable ascospores (Ware, 2006). Although a teleomorph has not been found for *C. beticola*, it is expected to be a species of *Mycosphaerella*. Detailed analyses have been performed on the distribution of the mating types of the sexually reproducing *M. graminicola* (Waalwijk *et al*., 2002; Zhan *et al*., 2002). Equal distribution of the mating types were found in most of the populations sampled from different geographical scales that included different plots within the same field as well as different fields within the same region or country. It is therefore probable that *C. beticola* is also able to reproduce sexually but that the teleomorph is not readily observed in nature, and can not be easily induced under laboratory conditions.

CONCLUDING REMARKS

Very little is known about the occurrence or importance of sex and recombination strategies taking place in the apparently asexual *C. beticola*. *MAT* genes play an important role in the biology and evolution of fungal species and knowledge of these genes can provide insight in the potential prevalence of sex in *C. beticola*. The first steps were already taken in an attempt to unravel the biological aspects of the reproduction strategies within this important plant pathogen by isolating and characterizing the *MAT* genes. The data obtained from the mating type analyses of *C. beticola* showed that intact *MAT* genes are present in this fungus and that both mating types occur in equal ratios in natural populations. These findings indicate that there is a very high probability that these genes are still functional in *C. beticola* and that sexual reproduction does occur. However, the teleomorph still needs to be discovered. The possibility exist that the *MAT* genes are functional but other components required for successful sexual reproduction are absent or not functional. It is still unknown whether the *MAT* genes of *C. beticola* are expressed. But if so, analyses of mRNAs and translated proteins will enable us to confirm the exact length of the coding regions as well as the intron and exon boundaries for the *MAT* genes of this fungus. The *MAT* genes have potential for resolving the phylogeny of closely related *Cercospora* species. Unfortunately, these loci failed at distinguishing cryptic species belonging to the *C. apii* complex and other loci are needed to resolve the *C. apii* species complex. Degenerate primers are now available that can amplify portions of the *MAT* genes in other fungi belonging to the Mycosphaerellaceae and it will allow the identification and characterization of additional *MAT* genes in this family. With the improved technologies available to isolate and characterize unidentified genes, the list of characterized fungal *MAT* genes is bound to increase rapidly in the future.

LITERATURE CITED

Astell, C.R., Ahlstrom-Jonasson, L., Smith, M., Tatchell, K., Nasmyth, K.A., and Hall, B.D. 1981. The sequence of the DNAs coding for the mating-type loci of *Saccharomyces cerevisiae*. Cell 27:15–23.

Arie, T., Kaneko, I., Yoshida, T., Noguchi, M., Nomura, Y., and Yamaguchi, I. 2000. Mating-type genes from asexual phytopathogenic ascomycetes *Fusarium oxysporum* and *Alternaria alternata.* Mol. Plant Microbe In. 13:1330–1339.

Arie, T., Christiansen, S.K., Yoder, O.C., and Turgeon, B.G. 1997. Efficient cloning of ascomycete mating type genes by PCR amplification of the conserved MAT-HMG box. Fungal Genet. Biol. 21:118–130.

Barve, M.P., Arie, T., Salimath, S.S., Muehlbauer, F.J., and Peever, T.L. 2003. Cloning and characterization of the mating type (*MAT*) locus from *Ascochyta rabiei* (teleomorph: *Didymella rabiei*) and a *MAT* phylogeny of legume-associated *Ascochyta* spp. Fungal Genet. Biol. 39:151–167.

Blakeslee, A.F. 1904. Sexual reproduction in the Mucorinae. P. Natl. Acad. Sci. U.S.A. 40:205–319.

Bruchez, J.J.P., Eberle, J., and Russon, V.E.A. 1993. Regulatory sequences in the transcription of *Neurospora crassa* genes: CAAT box, TATA box, introns, poly(A) tail formation sequences. Fungal Genet. Newslett. 40:89–96.

Crous, P.W., and Braun, U. 2003. *Mycosphaerella* and its anamorphs: 1. Names published in *Cercospora* and *Passalora*. CBS Biodivers. Series 1:1–571.

Crous, P.W., Aptroot, A., Kang, J.-C., Braun, U., and Wingfield, M.J. 2000. The genus *Mycosphaerella* and its anamorphs. Stud. Mycol. 45:107–121.

Crous, P.W., Kang, J.-C., and Braun, U. 2001. A phylogenetic redefinition of anamorph genera in *Mycosphaerella* based on ITS rDNA sequences and morphology. Mycologia 93:1081–1101.

Crous, P.W., Groenewald, J.Z., Pongpanich, K., Himaman, W., Arzanlou, M., and Wingfield, M.J. 2004. Cryptic speciation and host specificity among *Mycosphaerella* spp. occurring on Australian *Acacia* species grown as exotics in the tropics. Stud. Mycol. 50:457–469.

Crous, P.W., Groenewald, J.Z., Groenewald, M., Caldwell, P., Braun, U., and Harrington, T.C. 2006a. Species of *Cercospora* associated with grey leaf spot of maize. Stud. Mycol. 55:189–197.

Crous, P.W., Wingfield, M.J., Mansilla, J.P., Alfenas, A.C., and Groenewald, J.Z. 2006b. Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. II. Stud. Mycol. 55:99–131.

Fuckel, K.W.G.L. 1863. Fungi Rhenani exsiccati, Fasc. I-IV. Hedwigia 2:132–136.

Glass, N.L., Grotelueschen, J., and Metzenberg, R.L. 1990. *Neurospora crassa* a mating-type region. P. Natl. Acad. Sci. U.S.A. 87:4912–4916.

Glass, N.L., and Smith, M.L. 1994. Structure and function of a mating-type gene from the homothallic species *Neurospora africana.* Mol. Gen. Genet. 244:401–409.

Glass, N.L., Vollmer, S.J., Staben, C., Grotelueschen, J., Metzenberg, R.L., and Yanofsky, C. 1988. DNAs of the two mating-type alleles of *Neurospora crassa* are highly dissimilar. Science 241:570–573.

Goodenough, U.W. 1995. A dramatic evolution of sex genes is associated with speciation. Mol. Biol. Cell 6:6A.

Goodwin, S.B., Dunkle, D.L., and Zismann, V.L. 2001. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. Phytopathology 91:648–658.

Goodwin, S.B., Waalwijk, C., Kema, G.H.J., Cavaletto, J.R., and Zhang, G. 2003. Cloning and analysis of the mating-type idiomorphs from the barley pathogen *Septoria passerinii*. Mol. Gen. Genomics 269:1–12.

Groenewald, M., Groenewald, J.Z., and Crous, P.W. 2005. Distinct species exist within the *Cercospora apii* morphotype. Phytopathology 95:951–959.

Groenewald, M., Groenewald, J.Z., Braun, U., and Crous, P.W. 2006a. Host range of *Cercospora apii* and *C. beticola*, and description of *C. apiicola*, a novel species from celery. Mycologia 98:275–285.

Groenewald, M., Groenewald, J.Z., Harrington, T.C., Abeln, E.C.A., and Crous, P.W. 2006b. Mating type gene analysis in apparently asexual *Cercospora* species is suggestive of cryptic sex. Fungal Genet. Biol. 43:813–825.

Groenewald, J.Z., Groenewald, M., Braun, U., and Crous, P.W. 2006c. *Cercospora* speciation and host range. Chapter in: Proceedings of the First International symposium on Cercospora leaf spot of sugar beet, 29 July – 2 August 2006, Quebec City, Canada. (in press).

Linde, C.C., Zala, M., Ceccarelli, S., McDonald, B.A., 2003. Further evidence for sexual reproduction in *Rhynchosporium secalis* based on distribution and frequency of mating-type alleles. Fungal Genet. Biol. 40:115–125.

Metzenberg, R.L., and Glass, N.L. 1990. Mating type and mating strategies in *Neurospora*. BioEssays 12:53–59.

Milgroom, M.G. 1996. Recombination and the multilocus structure of fungal populations. Annu. Rev. Phytopathol. 34:457–477.

O'Donnell, K., Ward, T.J., Geiser, D.M., Kistler, H.C., and Aoki, T. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genet. Biol. 41:600–623.

Paoletti, M., Buck, K.W., and Brasier, C.M. 2005. Cloning and sequence analysis of the *MAT-B* (*MAT-2*) genes from the three Dutch elm disease pathogens, *Ophiostoma ulmi*, *O. novo-ulmi*, and *O. himal-ulmi.* Mycol. Res. 109:983–991.

Pöggeler, S. 1999. Phylogenetic relationships between mating type gene sequences from homothallic and heterothallic ascomycetes. Curr. Genet. 36:222–231.

Pöggeler, S. 2001. Mating-type genes for classical strain improvements of ascomycestes. Appl. Microbiol. Biotechnol. 56:589–601.

Pöggeler, S., Risch, S., Kück, U., and Osiewacz, H.D. 1997. Mating-type genes from the homothallic fungus *Sordaria macrospora* are functionally expressed in a heterothallic ascomycete. Genetics 147:567–580.

Pollack, F.G. 1987. An annotated compilation of *Cercospora* names. Mycol. Mem. 12:1–212.

Pretorius, M.C., Crous, P.W., Groenewald, J.Z., and Braun, U. 2003. Phylogeny of some cercosporoid fungi from *Citrus*. Sydowia 55:286–305.

Saccardo, P.A. 1876. Fungi veneti novi vel critici. Series V. Nuovo Giorn. Bot. Ital. 8:162–211.

Sharon, A., Yamaguchi, K., Christiansen, S., Horwitz, B.A., Yoder, O.C., and Turgeon, B.G. 1996. An asexual fungus has the potential for sexual development. Mol. Gen. Genet. 251: 60–68.

Singh, G., and Ashby, A.M. 1998. Cloning of the mating-type loci from *Pyrenopezziza brassicae* reveals the presence of a novel mating type gene within a discomycete MAT1-2 locus encoding a putative metallothionin-like protein. Mol. Microbiol. 30:799–806.

Singh, G., and Ashby, A.M. 1999. Errratum to Shingh and Ashby 1998. Cloning of the mating-type loci from *Pyrenopezziza brassicae* reveals the presence of a novel mating type gene within a discomycete *MAT1-2* locus encoding a putative metallothionin-like protein. Mol. Microbiol. 32:1115.

Staben, C., and Yanofsky, C. 1990. *Neurospora crassa* a mating-type region. P. Natl. Acad. Sci. U.S.A. 87:4917–4921.

Stewart, E.L., Liu, Z., Crous, P.W., and Szabo, L.J. 1999. Phylogenetic relationships among some cercosporoid anamorphs of *Mycosphaerella* based on rDNA sequence analysis. Mycol. Res. 103:1491–1499.

Turgeon, B.G. 1998. Application of mating type gene technology to problems in fungal biology. Annu. Rev. Phytopathol. 36:115–137.

Turgeon, B.G., Bohlmann, H., Ciuffetti, L.M., Christiansen, S.K., Yang, G., Schafer, W., and Yoder, O.C. 1993. Cloning and analysis of the mating type genes from *Cochliobolus heterostrophus.* Mol. Gen. Genetics 238:270–284.

Turgeon, B.G., and Yoder, O.C. 2000. Proposed nomenclature for mating type genes of filamentous ascomycetes. Fungal Genet. Biol. 31:1–5.

Waalwijk, C., Mendes, O., Verstappen, E.C., de Waard, M.A.D., and Kema, G.H.J. 2002. Isolation and characterization of the mating-type idiomorphs from the wheat septoria leaf blotch fungus *Mycosphaerella graminicola*. Fungal Genet. Biol. 35:277–286.

Ware, S.B. 2006. Aspects of sexual reproduction in *Mycosphaerella* species on wheat and barley: genetic studies on specificity, mapping, and fungicide resistance. PhD thesis, Wageningen University, Netherlands.

Whitfield, L.S., Lovell-Badge, R., and Goodfellow, P.N. 1993. Rapid sequence evolution of the mammalian sex-determining gene SRY. Nature 364:713–715.

Yun, S.H., Berbee, M.L., Yoder, O.C., and Turgeon, B.G. 1999. Evolution of the fungal self-fertile reproductive life style from self-sterile ancestors. P. Natl. Acad. Sci. USA 96:5592–5597.

Yun, S.H., Arie, T., Kaneko, I., Yoder, O.C., and Turgeon, B.G. 2000. Molecular organization of mating type loci in heterothallic, homothallic, and asexual *Gibberella*/*Fusarium* species. Fungal Genet. Biol. 31:7–20.

Zhan, J., Kema, G.H.J., Waalwijk, C., and McDonald, B.A. 2002. Distribution of mating type alleles in the wheat pathogen *Mycosphaerella graminicola* over spatial scales from lesions to continents. Fungal Genet. Biol. 36:128–136.

APPENDIX II

Summary in English

Samenvatting (Summary in Dutch)

Opsomming (Summary in Afrikaans)

Acknowledgements

About the Author

List of Publications

Education Statement of the EPS Graduate School

SUMMARY

The genus *Cercospora* is one of the largest and most heterogeneous genera of hyphomycetes. Generally species of *Cercospora* are considered to be host-specific at the level of the plant genus or family, and this concept has led to the description of more than 3,000 species. Currently only between 600–700 species of *Cercospora* are recognized, while an additional 281 species are treated under *C. apii sensu lato*, which is the oldest name for a large complex of morphologically indistinguishable *Cercospora* species occurring on a wide range of host plants. In this thesis I have performed a molecular phylogenetic analysis to better distinguish species belonging to the *C. apii* species complex. **Chapter 1** gives an introduction to the genus *Cercospora,* with specific reference to the *C. apii* species complex. Furthermore, fungal reproduction strategies are discussed, along with different molecular markers that are commonly used in fungal genetic diversity studies.

Cercospora beticola, a species also belonging to the *C. apii* complex, causes Cercospora leaf spot on sugar beet and has a huge impact on the yield and quality of *Beta vulgaris* (sugar beet) production worldwide. *Cercospora apii*, which is morphologically identical to *C. beticola*, causes Cercospora leaf spot on *Apium graveolens* (celery) and it has been proposed that *C. beticola* and *C. apii* are synonymous. In **Chapter 2**, multilocus sequence data, amplified fragment length polymorphism (AFLP) analyses and cultural characteristics were used to identify and characterize morphologically similar *Cercospora* species that occur on celery and sugar beet. During this study, an undescribed *Cercospora* species was isolated from celery and it was shown that these three species, although morphologically very similar, are distinct functional species that should be retained as separate entities. A PCR-based diagnostic protocol was developed to distinguish between the three *Cercospora* species. In **Chapter 3** it is illustrated that *C. apii* and *C. beticola* can be isolated from each others' host and that the third species of *Cercospora* is thus far only known from celery. This third species is formally described as *C. apiicola* in this chapter.

For most *Cercospora* species, including *C. beticola*, the sexual stage is unknown. Phylogenetic analyses of a variety of *Cercospora* species have placed them as a well-defined clade in the teleomorph genus *Mycosphaerella*. Therefore, if a sexual stage does exist for *C. beticola*, it would be a species of *Mycosphaerella*. In **Chapter 4** degenerate primers were developed and used to isolate the mating type genes of several *Cercospora* species. The *MAT1-1-1* and *MAT1-2* genes were characterized and both genes were found to be present in *C. beticola*, *C. zeae-maydis* and *C. zeina*. As only one of the two genes was found to be present in an isolate it is proposed that these *Cercospora* species are heterothallic. The mating types were approximately evenly distributed in field populations of these three species, a phenomenon that is often observed for actively sexually reproducing species. The degenerate primers developed in this study have also been successfully used to amplify the mating type genes of other *Mycosphaerella* species, and will be used as a basis for the characterization of these genes in future studies on additional related species.

Previous studies showed that genetic variation exists within *C. beticola,* but it was not known whether this variation was due to chromosomal rearrangements, asexual or sexual

recombination. Data obtained from AFLP analyses conducted during this study showed that the genetic variation observed in this species is most likely caused by recombination events occurring in natural populations (**Chapter 5**). The high level of genetic variation, high number of distinct genotypes and the equal distribution of the mating types within *C. beticola* populations are clear indications that sexual recombination events most likely play an important role in the reproductive cycle of this genetically diverse and heterogeneous species.

The AFLP technique is expensive and laborious and therefore additional molecular markers (microsatellites and SNPs) were developed in **Chapter 6** and tested on *C. beticola* populations. These markers were highly polymorphic and showed high levels of genetic variation as well as a high number of haplotypes. The markers can therefore be used for future studies to quantify the genetic diversity within *C. beticola*.

Two relatives of *C. beticola,* namely *Dothistroma septosporum* and *D. pini,* cause red band needle blight on pines. A sexual stage is known but rarely found, and therefore it has been assumed that sexual reproduction seldom occurs. The *MAT1-1-1* and *MAT1-2* genes were isolated from both of these species (**Chapter 7**) using the degenerate primers that were developed in Chapter 4. *Dothistroma* mating type-specific primers were developed during this study and these primers can distinguish between the two *Dothistroma* species as well as identify which mating type is present.

The results presented in this thesis are discussed in **Chapter 8**. The data presented in this study significantly improve our understanding on the taxonomic relationship between *C. beticola* and closely related species, the level of genetic variation that exists within and between geographically distinct *C. beticola* populations and the possible reproduction strategies that are present within *C. beticola* and close relatives.

Appendix I is a review of data obtained during this PhD study on mating type genes in *Cercospora* species and represents an expanded version of Chapter 4 of this thesis that will appear as a chapter in a book.

SAMENVATTING

Het geslacht *Cercospora* is een van de grootste en meest heterogene geslachten binnen de hyphomyceten. Over het algemeen wordt aangenomen dat *Cercospora* soorten waardplant-specifiek zijn op het niveau van plantengeslachten of plantenfamilies, een concept dat heeft geleid tot de beschrijving van meer dan 3000 soorten. Momenteel worden slechts 600 tot 700 *Cercospora* soorten onderscheiden. Bevendien worden 281 soorten behorend tot *C. apii sensu lato*, de oudste naam voor een groot complex van morfologisch niet te onderscheiden *Cercospora* soorten met een brede waardplant specificiteit, gerekend. In dit proefschrift heb ik een moleculair phylogenetische analyse uitgevoerd om soorten behorend tot het *C. apii* soortencomplex beter te onderscheiden. **Hoofdstuk 1** geeft een inleiding over het geslacht *Cercospora* met speciale aandacht voor het *C. apii* soortencomplex. Verder worden in dit hoofdstuk voortplantingsstrategieën van schimmels besproken evenals diverse moleculaire merkers die algemeen gebruikt worden in studies naar de genetische diversiteit van schimmels.

Cercospora beticola, één van de soorten behorend tot het *C. apii* complex, veroorzaakt de Cercospora bladvlekkenziekte op *Beta vulgaris* (suikerbiet) en heeft wereldwijd een groot effect op de opbrengst en kwaliteit van de suikerbiet productie. *Cercospora apii* de veroorzaker van Cercospora bladvlekkenziekte op *Apium graveolens* (selderij) is morfologisch identiek aan *C. beticola* en er is gesuggereerd dat *C. beticola* synoniem is aan *C. apii*. In **Hoofdstuk 2** wordt beschreven hoe multilocus sequentie data, Amplified Fragment Length Polymorphism (AFLP) analyses en kweek karakteristieken gebruikt zijn om morfologisch gelijkaardige *Cercospora* soorten voorkomend op selderij en suikerbiet te identifiseren en te karakteriseren. Tijdens deze studies werd van selderij een nog onbeschreven *Cercospora* soort geïsoleerd en aangetoond werd dat deze drie soorten, hoewel morfologisch gelijkaardig, verschillende functionele entiteiten zijn die beschouwd moeten worden als afzonderlijke soorten. Een PCR-protocol werd ontwikkeld om deze drie *Cercospora* soorten te kunnen onderscheiden. In **Hoofdstuk 3** wordt aangetoond dat *C. apii* en *C. beticola* voorkomen op elkaars waardplant en dat het voorkomen van de derde soort zich beperkt tot selderij en nu formeel beschreven is als *Cercospora apiicola*.

Voor de meeste *Cercospora* soorten, waaronder *C. beticola*, is het seksuele stadium onbekend. Na fylogenetische analyses zijn *Cercospora* soorten te plaatsen in een goed te onderscheiden tak van het teleomorfe geslacht *Mycosphaerella*. Daarom kan worden aangenomen dat een mogelijk geslachtelijk stadium van *C. beticola* tot *Mycosphaerella* zal behoren. In **Hoofdstuk 4** wordt beschreven hoe gedegenereerde primers zijn ontwikkeld en gebruikt om de mating-type genen van diverse *Cercospora* soorten te isoleren. De *MAT1-1-1* en *MAT1-2* genen werden gekarakteriseerd van *C. beticola*, *C. zeae-maydis* en *C. zeina*. Aangezien slechts één van beide genen werd aangetroffen per isolaat kan worden aangenomen dat deze *Cercospora* soorten heterothallisch zijn. In natuurlijke populaties van deze drie soorten waren de mating-type genen ongeveer gelijk verdeeld, hetgeen kenmerkend is voor soorten die zich actief seksueel voortplanten. De gedegenereerde primers ontwikkeld voor deze studie zijn inmiddels ook gebruikt om mating-type genen van andere *Mycosphaerella* soorten te amplificeren en

zullen ook in toekomstige studies gebruikt worden als basis voor de karakterisering van deze genen in gerelateerde soorten.

Eerdere studies hadden al laten zien dat binnen *C. beticola* genetische variatie bestaat. Het was echter niet bekend of deze variatie veroorzaakt wordt door chromosomale herschikkingen, aseksuele of seksuele recombinatie. Gegevens verkregen met AFLP-analyses (**Hoofdstuk 5**) toonden aan dat de genetische variatie in natuurlijke populaties zeer waarschijnlijk veroorzaakt wordt door recombinatie. Het hoge niveau van de waargenomen genetische variatie, het grote aantal verschillende genotypes, en de gelijke verdeling van mating-type genen in *C. beticola* populaties zijn duidelijke aanwijzingen dat seksuele recombinatie een belangrijke rol speelt in de voortplantingscyclus van deze genetisch diverse en heterogene soort.

De AFLP techniek is duur en arbeidsintensief en daarom werden additionele moleculaire merkers (microsatellieten en SNPs) ontwikkeld en getest op *C. beticola* populaties (**Hoofdstuk 6**). Deze merkers zijn zeer polymorf en toonden een hoog niveau van genetische variatie en kunnen een groot aantal haplotypen onderscheiden. Deze merkers kunnen dus gebruikt worden in toekomstige studies om de genetische diversiteit binnen *C. beticola* te meten.

Dothistroma septosporum en *D. pini*, twee soorten verwant aan *C. beticola*, veroorzaken rode band naalden schroei ziekte op pijnbomen. Een seksueel stadium is bekend maar wordt zelden gevonden en daarom wordt aangenomen dat seksuele voortplanting zeldzaam is. Gebruikmakend van de in Hoofdstuk 4 beschreven gedegenereerde primers werden de *MAT1-1-1* en *MAT1-2* genen van deze beide soorten geïsoleerd (**Hoofdstuk 7**). Tijdens dit onderzoek werden ook *Dothistroma* specifieke primers ontwikkeld die de de soort evenals het mating-type van de twee *Dothistroma* soorten kunnen onderscheiden.

In **Hoofdstuk 8** worden de resultaten van dit proefschrift bediscussieerd. De resultaten van dit proefschrift hebben geleid tot een duidelijke verbetering van het inzicht over de taxonomische relaties tussen *C. beticola* en verwante soorten, de mate van genetische variatie die bestaat in en tussen geografisch verschillende *C. beticola* populaties en de mogelijke voortplantingsstrategieën van *C. beticola* en zijn naaste verwanten.

Appendix I, is een overzichtsverhaal dat overzicht geeft van de gegevens verkregen tijdens dit promotieonderzoek aan mating-type genen in *Cercospora* soorten en is een uitgebreide versie van Hoofdstuk 4 van dit proefschrift dat als hoofstuk in een boek zal worden gepubliceerd.

OPSOMMING

Die genus *Cercospora* is een van die grootste en mees ongelyksoortige genera van hifomisete. In die algemeen word spesies van *Cercospora* gesien as gasheerspesifiek op plant genus of familie vlak, en dit het gelei tot die beskrywing van meer as 3,000 spesies. Op die oomblik word slegs tussen 600 en 700 species van *Cercospora* erken, waarby ‘n verdere 281 spesies as *C. apii sensu lato* gesien word; wat die oudste naam is vir ‘n groot kompleks van morfologies-identiese *Cercospora* species wat voorkom op ’n wye gasheer reeks. Ek het in hierdie tesis ‘n molekulêre filogenetiese analise uitgevoer om spesies wat aan die *C. apii* spesie kompleks behoort, beter te onderskei. **Hoofstuk 1** gee ‘n inleiding tot die genus *Cercospora* met spesifieke fokus op die *C. apii* spesie kompleks. Hiermee saam word swamme se voortplantingstrategiëe bespreek, tesame met verskillende molekulêre merkers wat algemeen gebruik word vir genetiese diversiteit studies van swamme.

Cercospora beticola, ‘n spesie wat behoort tot die *C. apii* kompleks, veroorsaak Cercospora blaarvlekke op suikerbeet en dit het ‘n groot effek op die hoeveelheid en kwaliteit van die produksie van *Beta vulgaris* (suikerbeet) regoor die wêreld. *Cercospora apii*, wat morfologies identies is aan *C. beticola*, veroorsaak Cercospora blaarvlek op *Apium graveolens* (seldery) en dit is voorgestel dat *C. beticola* en *C. apii* sinonieme is. In **Hoofstuk 2** word deoksi-ribonukleïnsuur (DNS) volgorde data van meerdere lokusse, analises van geamplifiseerde fragment lengte polimorfismes (“AFLP”) en kultuur eienskappe gebruik om morfologies identiese *Cercospora* spesies te karakteriseer wat voorkom op seldery en suikerbeet. Gedurende hierdie studie is ’n voorheen-onbeskryfde *Cercospora* spesie vanaf seldery geïsoleer en daar word aangetoon dat die drie spesies, al is hulle morfologies baie dieselfde, verskillende funksionele spesies is en dat hulle as aparte identiteite behou moet word. ’n Polimerase ketting reaksie (PKR)-gebaseerde diagnostiese protokol is ontwikkel om die drie *Cercospora* species te onderskei. In **Hoofstuk 3** word aangetoon dat *C. apii* en *C. beticola* van mekaar se gashere geïsoleer kan word en dat die derde spesie van *Cercospora* tot op hede nog net op seldery voorkom. Hierdie derde spesie word formeel in hierdie hoofstuk beskryf as *C. apiicola*.

Vir die meeste *Cercospora* spesies, insluitend *C. beticola*, is die geslagtelike stadium nog onbekend. Filogenetiese analises van ‘n verskeidenheid van *Cercospora* spesies het hulle in ’n goed-gedefinieerde groep in die teleomorf genus *Mycosphaerella* geplaas. Daarom, indien ‘n geslagtelike stadium wel teenwoordig is vir *C. beticola*, sal dit ‘n *Mycosphaerella* spesie wees. In **Hoofstuk 4** is gedegenereerde inleiers ontwikkel wat gebruik is om die gene wat die paringstipe van verskeie *Cercospora* spesies bepaal, te isoleer. Die *MAT1-1-1* en *MAT1-2* gene is gekarakteriseer en daar is gevind dat beide teenwoordig is in *C. beticola*, *C. zeae-maydis* en *C. zeina*. Omdat slegs een van die twee gene gevind word in ’n isolaat word daar voorgestel dat hierdie *Cercospora* spesies heterotallies is. Die paringstipes was ongeveer gelykop verdeel in veld populasies van die drie spesies, ‘n verskynsel wat gereeld waargeneem word in spesies wat aktief geslagtelik voortplant. Die gedegenereerde inleiers wat ontwikkel is tydens hierdie studie was ook suksesvol om die gene wat die paringstipes bepaal van addisionele *Mycosphaerella* spesies te amplifiseer, en dit sal gebruik word as basis vir die karakterisering van hierdie gene in toekomstige studies van addisionele verwante spesies.

Vorige studies het aangetoon dat daar genetiese variasie in *C. beticola* bestaan, maar dit was nie duidelik of dit as gevolg van chromosoom herrangskikking, ongeslagtelike of geslagtelike rekombinasie was nie. Data wat verkry is van die AFLP analises wat uitgevoer is gedurende die studie het aangetoon dat die genetiese variasie wat gevind is in die spesie heel moontlik veroorsaak is deur rekombinasie gebeure in natuurlike populasies (**Hoofstuk 5**). Die hoë vlak van genetiese variasie, groot aantal verskillende genotipes en die gelyke verspreiding van die paringstipes in *C. beticola* populasies is 'n duidelike aanduiding dat geslagtelike rekombinasie gebeure heel moontik 'n groot rol speel in die voortplantingsiklus van hierdie geneties diverse en ongelyksoortige spesie.

Die AFLP tegniek is duur en tydrowend en daarom is addisionele molekulêre merkers (mikrosatelliete en enkel nukleotied polimorfismes) ontwikkel in **Hoofstuk 6** en getoets op *C. beticola* populasies. Hierdie merkers was hoogs polimorfies en het 'n hoë vlak van genetiese variasie sowel as 'n groot aantal haplotipes getoon. Daarom kan hierdie merkers gebruik word om die genetiese diversiteit van *C. beticola* in toekomstige studies te meet.

Twee species verwant aan *Cercospora beticola,* naamlik *Dothistroma septosporum* en *D. pini*, veroorsaak Rooiband naald skroeisiekte op dennebome. 'n Geslagtelike stadium is bekend, maar word selde gesien en daarom word dit aanvaar dat geslagtelike voortplanting seldsaam is. Die *MAT1-1-1* en *MAT1-2* gene van beide spesies word geïsoleer (**Hoofstuk 7**) deur die gedegenereerde inleiers wat in Hoofstuk 4 ontwikkel is, te gebruik. *Dothistroma* paringstipe-spesifieke inleiers wat kan onderskei tussen die twee *Dothistroma* spesies, sowel as die paringstipe, word ontwikkel gedurende hierdie studie.

Die resultate wat in hierdie tesis gepresenteer is word in **Hoofstuk 8** bespreek**.** Die data verkry in die studie verbeter ons begrip van die taksonomiese verwantskap tussen *C. beticola* en naverwante spesies, die vlak van genetiese variasie wat bestaan binne en tussen geografies-verwyderde *C. beticola* populasies en die moontlike voortplantingstrategie van *C. beticola* en naverwante spesies.

Bylaag I is 'n oorsig van die data wat verkry is gedurende hierdie PhD studie op die paringstipe gene in *Cercospora* spesies en verteenwoordig 'n uitgebreide weergawe van Hoofstuk 4 van die tesis en sal as 'n hoofstuk van 'n boek gepubliseer word.

ACKNOWLEDGEMENTS

It seems like only yesterday that my husband (Ewald) and I left our family and friends behind in South Africa to come to the Netherlands, a country that was unfamiliar to both of us. The first year was not easy as we tried hard to find our feet and were met with people not understanding our "grappige leuke taaltje – Zuid Afrikaans". Luckily we made dear friends here in Holland over the past few years and I therefore would like to thank all of them (both Dutch and South African) who made it so much easier for us to continue our life here. It is impossible to thank everyone personally, but you have all touched my life in some way, be it with a card or SMS on a birthday, a social visit or "gezellig steengrillen". However, I do want to make some exceptions by thanking the Kirkenir family (Carl, Anneline, Elsje and Anja) thank you for your friendship, "Holland tips" and encouragement from the first day onward; Marius and Amanda Storm for their spiritual guidance and interest; the Holsters (Eddy, Mariette and Victor) and Peeters (Armand, Ansie, Maricelle and Tianda) families for their friendship.

The main reason for me being here in Holland was to obtain a PhD and that I did. I would like to thank Prof. dr. Pedro Crous, my study supervisor, for giving me the opportunity to do my PhD at the Centraalbureau voor Schimmelcultures (CBS). Pedro, your enthusiasm, motivating ideas, constructive suggestions and great guidance were a great help and allowed me to produce the data presented here. Although you have a very busy schedule, you always made time to review all drafts of my manuscripts as quickly as possible or to discuss important issues regarding my PhD. It has been a privilege to work in your research group.

To everyone who is (and was) part of the Evolutionary Phytopathology (EP) group during my PhD training, thank you for your help, friendship and especially your encouragement during the times I needed it most. To the "Evolutionary Phytopathology girls" (Arien and Mieke), thank you for all the interesting conversations and your friendship - I will miss you girls! The rest of the PhD students in the EP group, Lizel (who obtained her PhD and left us), Mahdi and Maikel, who know all about the ups and downs that come with being a PhD student, thank you for your support and useful discussions. Mahdi, good luck – I hope that in a few months time you will also be writing this part (acknowledgements) of your thesis, meaning that everything else is finished! And, of course, that Maikel will follow soon thereafter! My best wishes go out to Hans, who recently started with his PhD.

A special thanks to all my colleagues at CBS who contributed to the success of this study, whether it was by giving scientific input, encouraging words or just a smile that brightened up my day. There are so many of you that I would like to express my gratitude to. I would like to thank Jan Dijksterhuis for making the SEM photos of *C. beticola* infections of sugar beet leaves. I also thank all the people from the collection for helping me lodging or obtaining cultures; members of the different research groups for interesting discussions and suggestions; everybody in the media kitchen for always having the solutions and media available for use and sterilizing the items that are so essential in the lab; the guys at the IT department for keeping the computer running and the e-mails flowing; the crew from civil services for keeping the building clean and functioning; and the administrative personnel for providing helpful advice from ordering to getting my residence permit renewed. I want to thank each and every one of

you for making working here at CBS a time to remember with joy. A special thanks to Janny and Trix; you went the extra mile to make me feel at home.

To the members of the Dutch *Mycosphaerella* Group, thank you for your support and all the Tuesday morning discussions we had during my PhD research. I am very grateful to all the co-authors of the published and submitted manuscripts that form the chapters in my thesis; your input and expertise taught me a lot. This study would not have been possible without fungal strains; I can therefore not neglect to thank everybody who provided me with diseased plant material or fungal cultures. My gratitude also goes out to the Odo van Vloten Stichting and the Royal Netherlands Academy of Arts and Sciences (KNAW) for their financial support.

I would like to thank our families for their support. Each telephone conversation, SMS and e-mail message made the 9,648 km between us seem a lot less. The Moolmans and Groenewalds, I thank you for every prayer, word of encouragement and thinking of me, especially before I had to give a talk or during the nerve-wrecking wait for reviewers' comments. Mom, thank you for all your love and for being there for me during the ups and downs of my PhD. The weekly telephone call (usually at least an hour long), was always a time that I looked forward to very much. You are a great mother and friend!

Ewald, no words can describe the gratitude that I feel when I think of everything you meant to me in the almost 10 years that we are together; especially the last four that were mostly spent behind a pipet or in front of a computer. Thank you for all your help, understanding, support and love during this study period. It was not uncommon to see you helping out with a dish wash cloth or a vacuum cleaner; you cannot know how much those little things meant to me. It was a privilege that it was possible for us to work and publish together as the Groenewald team!

I also want to thank my God for giving me the opportunity and strength to do this PhD and who made it possible for me to carry on even in times when things did not work out as planned.

ABOUT THE AUTHOR

Marizeth Groenewald was born on the 19th of August 1975 in Somerset West, a town that is surrounded by mountains and the beautiful beaches of the south western coast of South Africa. Her main interest in school was Biology, therefore she did her undergraduate study (BSc) in Biochemistry, Genetics and Microbiology at the University of Stellenbosch, South Africa, and completed this degree *Cum Laude* (1994–1996). She continued with her postgraduate studies at the Department of Microbiology at the same university, and obtained the BSc (Honnours) degree *Cum Laude* (1997) and the MSc degree (Thesis title: Regulated expression of the *Schizosaccharomyces pombe* malic enzyme gene) *Cum Laude* (1998–2000). During her postgraduate studies, she worked as a student assistant of the undergraduate practical courses at the Department of Genetics and Microbiology. After finishing her postgraduate studies, she got a permanent position as a technical officer at the Department of Genetics where she worked for two years. Part of her job description was to tutor undergraduate and post graduate practical courses. At the end of December 2002 she went to the Netherlands with her husband and started working in the Evolutionary Phytopathology Group at the Centraalbureau voor Schimmelcultures (CBS Fungal Biodiversity Centre), Utrecht, as an "Onderzoeker in Opleiding" (OIO). She started her PhD on the 1st of January 2003 under the supervision of Prof. dr. P.W. Crous and intended to graduate at the University of Wageningen in February 2007. This dissertation presents the results of her PhD study on the molecular characterization of *Cercospora beticola* and its relatives. After finishing her PhD, she continued her scientific career as a postdoctoral researcher in the Yeast Division at CBS.

LIST OF PUBLICATIONS

Full papers and book chapters

Viljoen, M., **van der Merwe, M.,** Subden, R.E., and van Vuuren, H.J.J. 1998. Mutation of Gly-444 inactivates the *S. pombe* malic enzyme. FEMS Microbiol. Let. 167:157–162.

Groenewald, M., and Viljoen-Bloom, M. 2001. Factors involved in the regulation of the *Schizosaccharomyces pombe* malic enzyme gene. Curr. Gen. 39:222–230.

Groenewald, M., Groenewald, J.Z., and Crous, P.W. 2005. Distinct species exists within the *Cercospora apii* morphotype. Phytopathology 95:951–959.

Crous, P.W., Groenewald**,** J.Z., **Groenewald, M.,** Caldwell, P., Braun, U., and Harrington, T.C. 2006. Species of *Cercospora* associated with grey leaf spot of maize. Stud. Mycol. 55:189–197.

Groenewald, M., Groenewald, J.Z., Braun, U., and Crous, P.W. 2006. Host range of *Cercospora apii* and *C. beticola*, and description of *C. apiicola*, a novel species from celery. Mycologia 98:275–285.

Groenewald, M., Groenewald, J.Z., Harrington, T.C., Abeln, E.C.A., and Crous, P.W. 2006. Mating type gene analysis in apparently asexual *Cercospora* species is suggestive of cryptic sex. Fungal Genet. Biol. 43:813–825.

Stergiopoulos, I., **Groenewald, M.,** Staats, M., Lindhout, P., Crous, P.W., de Wit, P.J.G.M. 2007. Mating-type genes and the genetic structure of a world-wide collection of the tomato pathogen *Cladosporium fulvum*. Fungal Genet. Biol. doi: 10.1016/j.fgb.2006.11.004.

Groenewald, M., Barnes, I., Bradshaw, R.E., Brown, A.V., Dale, A., Groenewald, J.Z., Lewis, K.J., Wingfield, B.D., Wingfield, M.J., and Crous, P.W. 2006. Characterization and distribution of mating type genes in the Dothistroma needle blight pathogens. (submitted).

Groenewald, M., Groenewald, J.Z., and Crous, P.W. 2006. Indirect evidence for sexual reproduction in *Cercospora beticola* populations from sugar beet. (submitted).

Groenewald, M., Groenewald, J.Z., and Crous, P.W. 2006. Development of polymorphic microsatellite and single nucleotide polymorphic markers for *Cercospora beticola*. (submitted).

Groenewald, M., Groenewald, J.Z., and Crous, P.W. 2006. Mating type genes in *Cercospora beticola.* Chapter in *Cercospora beticola* book. (submitted).

Groenewald, J.Z., **Groenewald, M.,** Braun, U., and Crous, P.W. 2006. *Cercospora* speciation and host range. Chapter in *Cercospora beticola* book. (submitted).

Abstracts

Groenewald, M., Groenewald, J.Z., and Crous, P.W. 2006. Characterization of *Cercospora* mating type genes. Page 43 in: Proc. Joint Meeting of the American Phytopathological Society / Canadian Phytopathological Society and the Mycological Society of America, Quebec, Canada.

Groenewald, M., Groenewald, J.Z., and Crous, P.W. 2006. Mating type genes in *Cercospora beticola:* does sex occur in apparently asexual species? Page 139 in: First International symposium on Cercospora leaf spot of sugar beet as part of the Joint Meeting of the APS/CPS/MSA, Quebec, Canada.

Groenewald, J.Z., **Groenewald, M.,** Braun, U., and Crous, P.W. 2006. *Cercospora* speciation and host specificity. Page 139 in: First International symposium on Cercospora leaf spot of sugar beet as part of the Joint Meeting of the APS/CPS/MSA, Quebec, Canada.

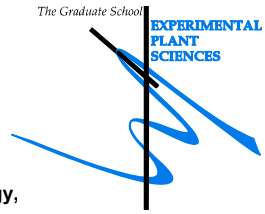
Groenewald, M., Groenewald, J.Z., and Crous, P.W. 2006. Characterization and frequency of mating type genes in *Cercospora.* Page 290 in: Proc. 8th International Mycological Congress, Cairns, Australia.

Groenewald, M., Groenewald, J.Z., and Crous, P.W. 2005. Distinct species exist within the *Cercospora apii* morphotype. Page 295 in: Proc. 15th Biennial Australasian Plant Pathology Conference, Geelong, Australia.

Groenewald, J.Z., **Groenewald, M.,** Braun, U., and Crous, P.W. 2005. Speciation in the *Cercospora apii* morphotype-is it really a complex issue? Page 378. Proc. 15th Biennial Australasian Plant Pathology Conference, Geelong, Australia.

Groenewald, M., and Crous, P.W. 2004. Genetic variation within populations of *Cercospora beticola*. Page 33 in: Proc. 7th Conference of the European Foundation for Plant Pathology & BSPP Presidential Meeting, Aberdeen, Scotland.

Education Statement of the Graduate School
Experimental Plant Sciences



Issued to: **Marizeth Groenewald**
Date: **19 February 2007**
Group: **Evolutionary Phytopathology (CBS, Utrecht) & Laboratory of Phytopathology, Wageningen University**

1) Start-up phase	*date*
► **First presentation of your project**	
Molecular characterization of *Cercospora beticola*	May 2003
► **Writing or rewriting a project proposal**	
Incorporated most of the molecular aspects of the project in the final proposal	2003
► **Writing a review or book chapter**	
"Mating type genes in *Cercospora beticola*" will be published as a chapter in *Cercospora beticola* book	2006
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*10.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD Student Days**	
Utrecht University	27 Mar 2003
Free University of Amsterdam	03 Jun 2004
Radboud University Nijmegen	02 Jun 2005
► **EPS theme symposia**	
Theme 4 'Genome plasticity', Nijmegen	2004
► **NWO Lunteren days and other National Platforms**	
Willie Commelin Scholten day 2003-2006	2003-2006
Annual PhD Student Day Graduate School Biodiversity	2003-2006
► **Seminars (series), workshops and symposia**	
Symposium 'Evolutionary Consequences of Life without Sex', UvA, Amsterdam	24 Nov 2003
CBS Seminar Series	2003-2006
CBS Symposium 'Fungal Phylogenomics', Utrecht	11-12 May 2004
GFP and LUC: applications of light reporters in biology workshop	11-12 Apr 2005
► **Seminar plus**	
► **International symposia and congresses**	
CBS Centenary, KNAW, Amsterdam, Netherlands	13-14 May 2004
7th Conference of the European Foundation for Plant Pathology & BSPP Presidential Meeting, Aberdeen, Scotland	05-10 Sep 2004
Mycosphaerella leaf diseases of Eucalypts workshop, Geelong, Australia	25-26 Sep 2005
15th Biennial Australasian Plant Pathology Conference, Geelong, Australia	26-29 Sep 2005
Joint Meeting of the APS/CPS/MSA, Quebec, Canada	29 Jul-02 Aug 2006
1st Int. *Cercospora beticola* symposia, Quebec, Canada	02 Aug 2006
► **Presentations**	
CBS Centenary, Amsterdam, Netherlands (poster)	13-14 May 2004
7th Conference of the EFPPF & BSPP Meeting, Aberdeen, Scotland (poster)	06 Sep 2004
Annual meeting of the Southern African Society for Plant Pathology, Hartenbos, South Africa (poster)	24 Jan 2005
15th Biennial Australasian Plant Pathology Conference, Geelong, Australia (poster)	29 Sep 2005
2006 Willie Commelin Scholten day, Utrecht, Netherlands (oral)	17 Feb 2006
Joint Meeting of the APS/CPS/MSA, Quebec, Canada (oral)	01 Aug 2006
1st International *Cercospora beticola* workshop, Quebec, Canada (oral)	02 Aug 2006
8th International Mycological Congress, Cairns, Australia (poster)	21 Aug 2006
Scientific symposium of the section Mycology of the NVVM, Utrecht, Netherlands (oral)	17 Nov 2006
► **IAB interview**	02 Jun 2005
► **Excursions**	
Subtotal Scientific Exposure	*22.8 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
EPS Spring School 'Functional Genomics: theory and hands-on data analysis'	23-28 Aug 2003
PhD Introduction to fungal biodiversity	02-13 Feb 2004
National Herbarium NL PhD Course 'Advanced Topics in Phylogenetic Analysis'	21-25 Feb 2005
► **Journal club**	
Monthly Dutch *Mycosphaerella* meeting	2003-2006
Biweekly evolutionary phytopathology group meeting	2003-2006
► **Individual research training**	
Subtotal In-Depth Studies	*7.2 credits**

4) Personal development	*date*
► **Skill training courses**	
Course 'Time planning' (KNAW)	Feb 2004
Course 'Project management' (KNAW)	Feb 2004
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Member of "Evenementen Commissie" CBS	Jul 2003 - Jun 2005
Subtotal Personal Development	*3.2 credits**

TOTAL NUMBER OF CREDIT POINTS*	**43.7**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

The research presented in this thesis was conducted at the Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT, Utrecht, Netherlands.

Financial support for the research was provided by the Odo van Vloten Stichting and the Royal Netherlands Academy of Arts and Sciences (KNAW).

The Training and Supervision Plan was completed at the Graduate School Experimental Plant Sciences.

Layout and design:
Ewald and Marizeth Groenewald.

Front and back cover:
Sugar beet leaf showing Cercospora leaf spot disease symptoms (Photographed by the author).

Front cover insets:
Scanning electron microscopy photographs of *Cercospora beticola* conidiophores emerging from the stoma (Photographed by Jan Dijksterhuis, CBS, Utrecht, Netherlands).

Back cover insets:
Left: Celery leaf infected with *C. apii* (Photographed by the author).
Right: Sugar beet plant infected with *C. beticola* (Photographed by the author).

Printed at:
Ponsen & Looijen B.V., Wageningen, Netherlands.