The Taxonomy, Phylogeny and Impact of *Mycosphaerella* species on Eucalypts in South-Western Australia

By

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Declaration

I declare that the work in this thesis is of my own research, except where reference is made, and has not previously been submitted for a degree at any institution

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Abstract

Plantation eucalypts are a recent and rapidly growing industry in Australia, and will eventually replace the logging of old-growth forests. Over 40% of these plantations have been established in south-western Australia, where more than 160 000 ha of *Eucalyptus globulus* plantations now occur. In the early 1900's, this species was widely planted as an exotic in South Africa, but succumbed to severe pest (*Gonipterus* sp.) and disease (*Mycosphaerella* sp.) problems. Similarly, in south-western Australia *E. globulus* is an exotic species, but with the additional threat that it is planted adjacent to indigenous eucalypts, which increases the possibility of pests and pathogens switching between closely related eucalypt hosts. Over the past ten years, there have been anecdotal reports of increasing levels of Mycosphaerella leaf disease (MLD) in *E. globulus* plantations in south-western Australia. This increase in disease level is of concern to the industry. To date there have been no comprehensive studies into the taxonomy, biogeography and population genetics of MLD in south-western Australia. This thesis investigated the impact of MLD in south-western Australia with a focus on its impact, taxonomy, biogeography and population genetics. It is the first study worldwide to quantify the relative impact of different *Mycosphaerella* species in a regional plantation estate.

A survey of pest, disease and nutritional disorders (Chapter 2) found that MLD was the most severe and frequently occurring, single taxonomic health threat to 1 and 2-year-old *E. globulus* plantations in south-western Australia. For the first time, this survey identified and quantified the impact of pest and disease damage to *E. globulus* plantations in the region. There were differences in the disease levels between plantations and this was due to initial *Mycosphaerella* species composition and inoculum level, and local climatic conditions favourable for disease, rather than to the provenance planted or the nutritional status of the individual plantations.

The survey for *Mycosphaerella* pathogens of eucalypts (Chapter 3) identified two new species of *Mycosphaerella* (*M. ambiphylla* and *M. aurantia*) and extended the known geographic range of eight other species (*M. cryptica, M. gregaria*, *M. lateralis, M. marksii, M. mexicana* , *M. nubilosa, M. parva* and *M. suberosa*). Of these: *M. lateralis* and *M. mexicana* were new records for Australia; and *M. gregaria, M. nubilosa* and *M. parva* were new records for Western Australia. A new anamorph, *Phaeophloeospora ambiphylla* was described and linked to *M. ambiphylla*. The occurrence of these new species and disease records in south-western Australia is significant for the plantation-eucalypt industry worldwide. The finding of two new species highlighted the need to quantify the disease impact of these on eucalypt plantations; and the extension of the range the remaining species raised important quarantine issues, concerned with the movement of plant material between regions and countries.

The biogeographical investigation of *Mycosphaerella* (Chapter 4) identified that the most widespread and serious cause of MLD in south-western Australia is *M. cryptica.* In addition to occurring on the exotic *E. globulus*, it also occurs on two of the three important indigenous forestry eucalyptus species in this region. That is, on *E. diversicolor,* and *E. marginata,* but not on *Corymbia calophylla.* In terms of the plantation estate of *E. globulus,* however, *M. nubilosa* is the most widespread pathogen. The current study found that MLD on *E. globulus* is a complex of several different species, whereas, on *E. diversicolor* and *E. marginata* it is caused by only *M. cryptica.* Two species, *M. cryptica* and *M. marksii* were found commonly on adult *E. globulus* leaves. Although *M. cryptica* was the most frequent and serious cause of disease on adult leaves, *M. marksii* levels appear to be increasing and the future epidemiology of this pathogen should be closely monitored. There is some concern that these two MLD species could become an economically important problem on adult leaves of *E. globulus.* At present severe levels of MLD is significantly more common on juvenile than on adult foliage.

The phylogenetic analysis (Chapter 5), based on ITS rDNA sequences from the present study and those obtained from GenBank accessions, found that

Mycosphaerella is an assemblage of largely polyphyletic anamorph genera. Ten distinct clades emerged from the analysis. With the exception of the *Dissoconium* and the *M. recutita* clade, which comprised of two and one species respectively, none were comprised entirely of one anamorph genus alone. The anamorph genera represented were often dispersed across more than one clade, indicating that these anamorphs have arisen separately in different phylogenetic lineages. *Cercospora*, *Stenella* and *Uwebraunia* anamorphs each occurred in more than one separate clade. Although on the basis of rDNA sequence data *Mycosphaerella* appeared mostly monophyletic there was some evidence that the *Mycosphaerella* genus may be polyphyletic. This was particularly evident from the *Dissoconium* clade which grouped as closely to the outgroup *Botryosphaeria* taxon as it did with the remaining *Mycosphaerella* species. It was argued that a multi-gene phylogeny, which includes sequencing many species in other genera aligned with *Mycosphaerella,* is required in order to satisfactorily answer the question of whether *Mycosphaerella* is truly monophyletic.

The phylogenetic analysis also showed that the taxonomy of *Mycosphaerella* based on ITS sequence data needs further clarification. Some species that are morphologically distinct, such as *M. vespa* and *M. molleriana,* shared identical ITS sequences. Other morphologically distinct species differed by as little as one or two nucleotides. Yet in other cases, the sequence variation amongst isolates from the same species differed substantially. Much of this variation in *M. cryptica* and other species was attributed to poorly edited sequences that had been lodged with GenBank. It was postulated that although a part of the remaining variation reflected the existence of cryptic species, some was likely to be genuine intra-species differences. It was concluded that further genes need to be sequenced, and more standardised cultural studies conducted in order to define species boundaries within *Mycosphaerella*.

Based on the ITS rDNA sequence data, two different molecular methods for the identification of *Mycosphaerella* species from eucalypts were developed (Chapter 6). The first of these was a PCR-RFLP method that enabled the identification of

Mycosphaerella species present on eucalypts in south-western Australia. A key is provided, which enabled the identification of species on a combination of PCR-RFLP DNA fragment migration patterns and a small number of morphological features. This key enables the identification of *Mycosphaerella* species more easily than keys that rely on morphological features alone. Therefore, this has made it easier for non-*Mycosphaerella* specialists to identify species from this genus. The second molecular method developed for the identification of *Mycosphaerella* species was that of primers that selectively amplify the DNA of *M. cryptica* and *M. nubilosa,* the two most important causes of MLD (Chapter 6). This will allow the rapid identification of these two species by non-specialists in *Mycosphaerella* taxonomy. The primers from the current study will also enable early diagnosis of the possible causal organism of MLD in a plantation. Once the use of these primers for amplifying DNA from leaf tissue has been optimised, they will also facilitate studies into the early infection process of *M cryptica* and *M. nubilosa*. For example, the presence of the pathogen may be detected prior to the appearance of symptoms. Studies may be conducted to determine the length of a hemi-biotrophic phase, and the extent of tissue colonisation both spatially and temporally, beyond the necrotic lesion in these two *Mycosphaerella* species. Previously, such studies have been hampered by the slow growth rate of these fungi in culture and the lack of media that would allow their selective isolation and detection by directly plating diseased and non-diseased host tissue.

This study has clearly indicated that *Mycosphaerella* species are the major disease threat to *E. globulus* plantations in Western Australia. It has also shown that over the relatively short period of time of less than ten years the number of species recorded has increased from three to ten, and that disease severity has increased in plantations. It is therefore critical to continue the research on this genus in order to understand the biology, epidemiology and population genetics of this pathogen. This is necessary in order to inform tree selection and silvicultural practise that will minimise the future impact of MLD. This is particularly important if the industry moves towards clonal and hybrid forestry as has occurred elsewhere in the world. This study has laid the foundations for

future research on this disease through the elucidation of the taxonomy of *Mycosphaerella* in south-western Australia and by providing some important molecular tools for its diagnosis and further study.

Publications arising from the current thesis

Peer reviewed journals

Jackson, S.L., Maxwell, A., Neumeister-Kemp, H.G. & Hardy, G.E.StJ. (2004). Infection, hyperparasitism and conidiogenesis of *Mycosphaerella lateralis* on *Eucalyptus globulus* in Western Australia. *Australasian Plant Pathology* **33:** 49–53

Maxwell, A., Dell, B., Neumeister-Kemp, H. G. and Hardy, G. E. (2003) *Mycosphaerella* species associated with *Eucalyptus* in south-western Australia: new species, new records and a key. *Mycological Research* **107**(3): 351-359.

Maxwell, A., Hardy, G.E.St.J, Wingfield, M. J. & Dell, B. (2000). First record of *Mycosphaerella lateralis* on Eucalyptus in Australia. *Australasian Plant Pathology.* **29**:279.

Maxwell, A., Hardy, G.E.St.J, & Dell, B. (2001). First record of *Mycosphaerella nubilosa* in Western Australia. *Australasian Plant Pathology.* **30**:65.

Neumeister-Kemp, H. G., Maxwell, A., Dell, B. and Hardy, G. E. St. J. (2003). An advanced slit-type-volumetric-spore sampling device for monitoring fungal spores in remote plantations. *Australasian Plant Pathology* (In Press).

Conference papers

Jackson, S.L., Maxwell, A., Neumeister-Kemp H. G. & Hardy, G.E.St.J, (2001). Infection Process of *Mycosphaerella* species on *Eucalyptus globulus* in Western Australia. In Proceedings of the *13th Biennial Conference for the Australasian Plant Pathology Society*, Cairns, Qld., Australia.

Maxwell, A., Dell, B., Jackson, S. L. & Hardy, G. E. St. J. (2003) Is *Mycosphaerella* a threat to the *Eucalyptus* estate in Western Australia? A bio-geographical perspective In Proceedings of the *8th International Congress of Plant Pathology,* New Zealand.

Maxwell, A., Hardy, G.E.St.J, & Dell, B. (2002). New *Mycosphaerella* species associated with *Eucalyptus* in Western Australia. In Proceedings of the *40th Congress of the Southern African Society of Plant Pathology*, Pretoria, South Africa.

Maxwell, A., Hardy, G.E.St.J, & Dell, B. (2002). *Mycosphaerella nubilosa*: a recent pathogen incursion into Western Australia? In Proceedings of the *40th Congress of the Southern African Society of Plant Pathology*, Pretoria, South Africa.

Maxwell, A., Hardy, G.E.St.J, & Dell, B. (2001). *Mycosphaerella* species associated with *Eucalyptus* in Western Australia. In Proceedings of the *13th Biennial Conference for the Australasian Plant Pathology Society*, Cairns, Qld., Australia.

Maxwell, A., Hardy, G.E.St.J, & Dell, B. (2001). *Mycosphaerella nubilosa*: a recent invasion into Western Australia? In Proceedings of the *13th Biennial Conference for the Australasian Plant Pathology Society*, Cairns, Qld., Australia.

Maxwell, A., Hardy, G.E.St.J., Wingfield, M.J. & Dell, B. (1999). A New Australian Record: *Mycosphaerella lateralis* isolated from eucalypt host. In Proceedings of the *12th Biennial Conference for the Australasian Plant Pathology Society*, Canberra, A.C.T., Australia.

Workshop papers

Mycosphaerella Leaf Blotch: an emerging problem in West Australian eucalypt plantations. At the *Tree Pathology Cooperative Program meeting.* Pretoria, South Africa, April 1999.

Jackson S. L., Maxwell, A., Neumeister-Kemp, H. G., Dell, B. and Hardy, G. E. St. J. (2003). *In-vitro* study into the role of *Dissoconium dekkeri* in Mycosphaerella leaf disease of *Eucalyptus globulus* in Western Australia. *Australasian Plant Pathology.*

Recently submitted papers

Maxwell, A., Dell, B., Jackson, S. L, and Hardy, G. E. (submitted 2004). Anamorph plasticity: an obstruction to a coherent taxonomy and phylogeny of *Mycosphaerella*. *Mycological Research* **(In Review)**.

Maxwell, A., Hardy, G. E., Jackson, S. L, and Dell, B. (submitted 2004). A PCR method for the identification of *Mycosphaerella* species on *Eucalyptus*. *Mycological Research* **(In Review)**.

Statement on the formatting of the current thesis and authorities for species

The formatting of the current thesis follows that for Mycological Research where possible. The authorities of scientific names of pathogens and their plant hosts will not be presented in this thesis unless they form part of a taxonomic study. In this regard, the current thesis adopts the policy of Mycological Research (Hawksworth, 2000: 124).

List of Abbreviations

acceleration due to gravity	*g*
Analysis of variance	ANOVA
day	d
deoxyribonucleic acid	DNA
diameter	diam
et alia	*et al.*
hour	hr
Internal transcribed spacer	ITS
large subunit of DNA	LSU
malt extract agar	MEA
minute	min
near ultra violet	nuv
per cent	%
polymerase chain reaction	PCR
randomly amplified polymorphic DNA	RAPD
restriction fragment polymorphism	RFLP
ribosomal DNA	rDNA
scanning electron microscopy	SEM
small subunit of DNA	SSU
species	sp., spp. (plural)
standard error of the mean	SE
subspecies	subsp., subspp. (plural)

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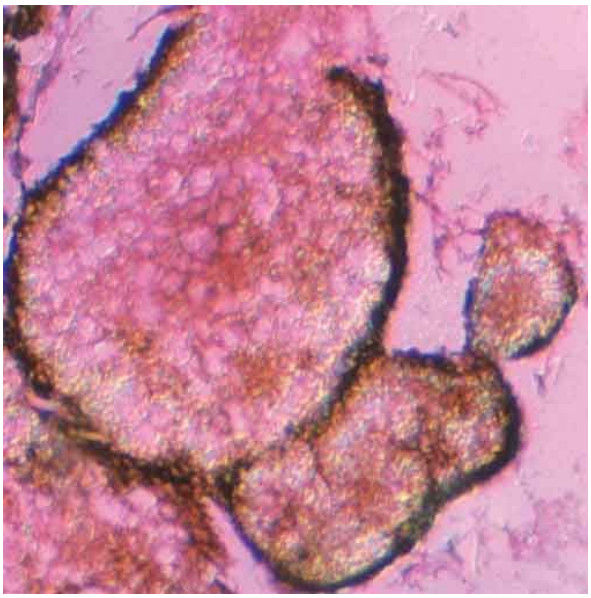
Chapter 1

Introduction



Chapter 1 Introduction

1.1 General introduction to the research

Eucalypts are a significant and expanding resource world-wide, with 14 million ha currently planted on a commercial basis, in addition to their natural occurrence in Australia where they form 124 million ha of forest and woodland (Anonymous 1997a, Turnbull 2000). Eucalypt plantations predominantly provide pulp for the rapidly expanding paper industry and saw-logs for building (Eldridge 1993). In Australia, there are 390 thousand ha of plantations, 14 million ha of native forest managed for wood products and a further 110 million ha of native stands that serve important environmental, social and ecological functions. These functions include watershed protection, flora and fauna conservation and recreation (Turnbull 2000). The native forest estate dedicated to timber production in Australia is diminishing, as logging is restricted in response to the need to maintain the biodiversity of these habitats. Thus, the plantation eucalypt industry is becoming increasingly important in supplying Australia's demand for wood products.

One of the main threats to the successful establishment of eucalypt plantations is that of disease. Plantations are typically monocultures of even-aged, single species, provenance or even clonal trees. It is widely accepted that monocultures, whether as crop plants or tree plantations, are more susceptible to pest and disease epidemics than heterogenous crops or forest (Jones 2001, Stone 2001, Strauss 2001, Zhu *et al.* 2000). Worldwide, many pest and disease outbreaks have been reported in eucalypt plantations (Park *et al.* 2000). Amongst these, Mycosphaerella leaf disease (MLD) has caused extensive damage in Australasia, South America, Western Europe, Southern Africa and South-East Asia (Park *et al.* 2000). Its impact was so severe in South Africa in the 1930's, that the establishment of *E. globulus* plantations was discontinued there (Crous 1998). As *E. globulus* is the only commercially grown plantation eucalypt

in the newly emerging industry in south-western Australia, it is critical that the impact of this disease is determined there. In addition, the potential for disease inoculum to spread from exotic eucalypt plantations to native eucalypt forest needs to be examined in south-western Australia.

Previous work on the impact of MLD has been regionally based, focussing on the situation in South Africa, New Zealand and in eastern Australia. This work has been of a taxonomic and epidemiological nature. The south-west region of Australia has not yet been studied in detail, and no work has been done anywhere on the population genetics of these pathogens. This thesis investigates the impact of MLD in Western Australia with a focus on its taxonomy, biogeography and population genetics.

1.2 Thesis outline

A review of MLD on eucalypts is firstly presented (Chapter 1). Then a survey comparing the level of impact of MLD with other diseases and pests on the *E. globulus* plantations in the south-west of Australia is made (Chapter 2). Taxa of *Mycosphaerella* present in *E. globulus* plantations and surrounding eucalypt forest are described (Chapter 3). The impact and biogeography of the *Mycosphaerella* species found is then considered (Chapter 4). This includes host, geographic and leaf stage distribution as well as level of impact. Sequencing of the ITS region of rDNA is performed in order to answer questions relating to the phylogeny of *Mycosphaerella*, including; intra- and inter-species variation for the delimitation of taxa; and whether *Mycosphaerella* is polyphyletic (Chapter 5). Molecular methods for reliably and rapidly identifying species of *Mycosphaerella* are developed, based on the ITS rDNA sequence information (Chapter 6). The population genetics of *M. nubilosa* are investigated using RAPD's (Chapter 7). Finally, the main findings from each of the chapters are treated in an overall General Discussion (Chapter 8).

1.3 Review of MLD on eucalypts

1.3.1 Eucalypts in commercial forestry

Eucalypt taxa important for forestry

Eucalyptus and the related genera of *Corymbia* and *Angophora* are collectively known as eucalypts, a convention that is followed in this thesis. Although they dominate the Australian landscape and occupy many environmental niches in Australia, parts of Papua New Guinea and Indonesia, they are not endemic outside of this range. There are more than 700 species of eucalypt (Brooker & Kleinig 1990) of which 113 belong to the genus *Corymbia*, 13 to *Angophora* and 600 to *Eucalyptus* (Potts & Pederick 2000). Within *Eucalyptus sensu stricto* 120 species belong to the sub-genus *Monocalyptus*, and 300 to the sub-genus *Symphomyrtus*. Phylogenetic affiliations reflect some functional, ecological and geographic differences between these taxa, which are relevant to plantation forestry in terms of site suitability, growth requirements and pest and disease susceptibility. Some generalisations regarding these attributes may be made at a generic and sub-generic level.

Generally, species of *Corymbia* are highly tolerant of soil pathogens, species of *Symphomyrtus* are moderately tolerant and species of *Monocalyptus* are susceptible (Florence 1996). Variation in resistance to stem and foliar pathogens occurs between eucalypt genera and sub-genera (Potts & Pederick 2000). *Sporothrix pitereka* causes disease only on *Corymbia,* the related *Angophora* and some *Eucalyptus* species within the section *Adnataria* (Walker & Bertus 1971). *Mycosphaerella* species are pathogenic on *Eucalyptus* species but not on *Corymbia* species (Potts & Pederick 2000), although there are some exceptions to this, such as *gregaria*, which occurs on *C. maculata* (Carnegie & Keane 1997).

Most plantation species grown worldwide are from the *Symphomyrtus* sub-genus (Potts & Pederick 2000), chosen for their high growth rate and pulp or saw-log suitability. Inter-species variability of the mostly *Symphomyrtus*

eucalypts has been used to match species to appropriate sites on the basis of their preferred climatic conditions (Florence 1996). Of the 700 species of eucalypt only a few have been grown in plantations. The more important of these include: *C. citriodora, E. camaldulensis, E. globulus, E. grandis, E. nitens, E. robusta, E. tereticornis, E. urophylla,* and *E. viminalis* (Florence 1996).

While inter-species genetic variability may be exploited in selecting species for growth under particular conditions and sites, there is also enormous genetic variability within species of eucalypt (Moran 1992). Eldridge (1972, in Potts & Pederick, 2000) found that the growth rate of *E. regnans* progeny, planted on a test site of uniform altitude, decreased with increasing altitude of parental origin. Other eucalypt species for which provenance variation has been demonstrated include *E. globulus* (Carnegie *et al.* 1994, Dungey *et al.* 1997) *E. delegatensis* (Dick & Gadgil 1983) and *E. grandis* (Florence 1996).

World-wide, *E. globulus* is the most extensively grown plantation species in Mediterranean climates, in the absence of frost (Florence 1996). It is valued because of its superior pulping qualities and fast growth rate (Williams *et al.* 1995). Variation within this species has been demonstrated for desirable traits such as growth rate (Beadle *et al.* 1995, Turnbull *et al.* 1993), form, pulp yield and quality (Washusen & Ilic 2001, Williams *et al.* 1995) and disease resistance (Dungey *et al.* 1997). Kirkpatrick (1974) identified four sub-species of *E. globulus; E. globulus* ssp. *bicostata, E globulus* ssp. *globulus, E. globulus* ssp. *maidenii, E. globulus* ssp. *pseudoglobulus.* Carnegie *et al.* 1994 found that provenances of *E. globulus* ssp. *globulus* and *E. globulus* ssp. *bicostata* were more susceptible to MLD than provenances of *E. globulus* ssp. *maidenii* and *E. globulus* ssp. *pseudoglobulus*. There were also significant differences among provenances within *E. globulus* ssp. *globulus* and *E. globulus* ssp. *pseudoglobulus*. Variation in the onset of adult foliage also occurs between provenances and families of *E. globulus.*

Eucalyptus globulus undergoes a heteroblastic ontogeny, where changes in leaf anatomy, morphology, surface waxes and orientation clearly differentiate the seedling, juvenile and adult stages of the tree (Brooker & Kleinig 1990). These

differences between leaf stages have consequences for pest and disease susceptibility. Park 1988b demonstrated that *M. nubilosa* is able to infect juvenile, but not adult leaves of *E. globulus.* As a consequence, the severity of MLD on *E. globulus* is correlated with delayed transition from juvenile to adult foliage (Dungey *et al.* 1997). Heather 1967a, Heather 1967b showed that the juvenile leaves of *E. globulus* ssp. *bicostata* have a double layer of wax that confers them with increased resistance to *Phaeophleospora epicoccoides* (as *Phaeoseptoria eucalypti*. Older leaves are more susceptible to infection by *P. epicoccoides* because, as the leaf ages, the upper 'rod -wax layer' is eroded. In contrast to this, (Park 1988b found that younger *E. globulus* ssp *pseudoglobulus* juvenile leaves, are more susceptible to infection by *M. nubilosa* than older juvenile leaves, presumably because of the increased lignification of older leaves.

Eucalypt plantations in south-western Australia

In south-western Australia forestry has been pursued in native stands dominated by *E. diversicolor* and *E. marginata* since about 1880 (Anonymous 2002). There are 1.2 million ha of forest that may be subject to timber harvest, of which 44 300 ha is currently dedicated to this use, and a further 1.2 million ha in forest conservation areas not available for future timber harvest (Anonymous 2002). Current silvicultural practice is to clear fell in *E. diversicolor* sites to a maximum of 40 ha, and to pursue a mixed regime for *E. marginata,* that includes cutting to gap (0.2 to 10 ha), shelter wood cutting, thinning and single tree selection (Anonymous 2002). The average annual timber yields is 182,000 m^3 of sawlogs (from a cut of approximately 18 000 ha) of *E. marginata* and 42, 000 $m^3$of *E. diversicolor* (Anonymous 2002). These levels will not be increased into the future according to the recent draft forest management plan for the region (Anonymous 2002). Hence, any increase in timber harvest will need to come from plantation timbers.

Over 40% of the eucalypt plantation area in Australia is located in the south-western region, which is currently planted to 152 000 ha of mostly *E. globulus.* Eucalypt plantations were first established in the south-west in 1980, and more

than 90% of the estate has been established post 1990 (Bailey & Dunconson 1998). The area of eucalypt plantations in Australia was over 389 000 ha in 2000 (Anonymous 2000) and is projected to reach 3.3 million ha by the year 2020 (Anonymous 1997b). This is necessary in order to meet increasing demand for timber product whilst fulfilling Australia's commitments to minimise global warming (Kyoto protocols) and maintain biodiversity in native forests (Montreal summit).

The spatial distribution of eucalypt plantations in Australia differs somewhat from commercial plantations in South Africa and South America. This may also have implications for disease development. In Argentina, Brazil, Chile and South Africa eucalypt plantations occur as continuous monocultures with no other forest species present for 10's or 100's of square kilometres (Young pers. comm.). Whereas, in Australia eucalypt plantations occur as a patchwork of smaller areas of 0.2–10 square kilometres (50–1000 ha) amongst pasture, viticulture, and importantly, mixed native forest, heath and woodlands.

The major disease threat to forestry in south-western Australia has been that of *Phytophthora cinnamomi* in the *E. marginata* forest. Although foliar disease impacts of up to 15% have been recorded on *E. marginata* (Abbott *et al.* 1993), no major epidemics have been reported in the forests of south-western Australia.

1.3.2 Foliage diseases occurring on eucalypts

The aetiology of foliar diseases in Australia differs in some respects to that elsewhere. While eucalypts planted outside of Australasia escape many of the pests and diseases endemic to the region, they may become exposed to new pathogens, which are pre-adapted to these myrtaceous hosts and have no resistance to such pathogens (eg. *P. psidii* (Park *et al.* 2000). Also, eucalypt plantations outside of Australia may be established beyond their optimal environmental range, such as in conditions of high humidity, which may favour disease development, as has occurred with *Cylindrocladium* outbreaks in Brazil (Ferreira *et al.* 1995), and Vietnam (Old *et al.* 1999).

Plantation eucalypt forestry has a longer history in countries such as South Africa where exotic eucalypts have been grown in extensive monocultures since the 1930's (Poynton 1979 in Hunter 2002), than in Australia. Thus, the role of the lower genotypic diversity present in eucalypt plantations in contributing to disease has been a factor in exotic plantations for over 50 years. Up until the 1980's the predominant silvicultural practice in Australia, has been the harvest of naturally occurring eucalypt stands (Turnbull 2000). Under these conditions foliar disease outbreaks have been rare. Presumably this is because of a long association between the host and pathogens in a relatively stable ecosystem. In this situation, disease may arise if the system is disturbed through a combination of events favourable for its development, such as a flush of juvenile foliage after logging, followed by a warm wet period (Park *et al.* 2000).

There are two known epidemics of foliar pathogens in native eucalypt forest (Carnegie 2000). These have both been attributed to *Aulographina eucalypti* with other pathogens implicated in a secondary role. The first of those epidemics was a defoliation of *E. nitens* in Victoria in 1974 (Neuman & Marks 1976). In this case *Alternaria, Harknessia* and *Mycosphaerella* species were also involved. The second case of severe defoliation due to *A. eucalypti* was that of *E. obliqua* in north-west Tasmania in 1973, where *Vermisporium fulcatum* [Sutton (Naj Raj)] [as *Seimatosporium falcatum* (Sutton) Shoemaker] was the secondary pathogen implicated (Palzer 1978 in Carnegie 2000).

Epidemics in plantation forestry are much more common than in native eucalypt forest (Carnegie 2000). Worldwide the following pathogens have been major causes of foliar disease of plantation eucalypts: *Aulographina eucalypti* (Cooke & Masse) Arx & E. Mül.; *Puccinia* psidii Winter; *Phaeophleospora epicoccoides* (Cooke & Massee) Crous, F.A. Ferreira & Sutton; *Phaeophleospora eucalypti* (Cooke & Massee) Crous, F.A. Ferreira & Sutton; *Phaeophleospora destructans* (M.J. Wingfield & Crous) Crous, F.A. Ferreira & Sutton; *Cryptosporiospsis eucalypti* Sankaran & B. Sutton; and *Cylindrocladium* spp. Many of these pathogens, such as the guava rust, *Puccinia psidii*, do not occur in Australia (Park *et al.* 2000). The most serious foliar pathogens of eucalypt

plantations in Australia are *Mycosphaerella* species (Carnegie 2000, Park 1984, Park 1988a, Park *et al.* 2000). Prior to the commencement of this study little was known of the impact of MLD on eucalypt plantations in south-western Australia, apart from the three disease records of *M. cryptica, M. marksii* and *M. suberosa* (Carnegie, Keane & Podger 1997).

The taxonomy and biology of *Mycosphaerella* have important implications in terms of understanding and managing the disease they cause.

1.3.3 Taxonomy and biology of *Mycosphaerella*

Taxonomically, *Mycosphaerella* is an extremely large genus and the subject of much debate upon the merits of splitting it into separate genera or sub-genera (Barr 1972, Crous 1998, Goodwin & Zismann 2001). The rationale for any changes to the current taxonomic structure depends ultimately on biological and genetic aspects of species within this taxonomic scheme.

Taxonomy

The first *Mycosphaerella* species described on eucalypts in Australia were *M. cryptica* and *M. nubilosa,* as *Sphaerella cryptica* and *Sphaerella nubilosa* by Cooke (Park & Keane Keane 1982a). Hansford (1952) transferred these fungi to the genus *Mycosphaerella*. Members of this genus are ascomycetes, a fungal division that has undergone considerable revision (Arx 1949, Arx 1983, Barr 1979).

Although the Ascomycota has been separately revised a number of times over the past 60 years, the genus *Mycosphaerella* is consistently placed in the family Mycosphaerellaceae within the order Dothideales (Arx 1983, Arx & Müller 1975, Barr 1972, Barr 1979, Barr 2001, Luttrell 1955). This placement is not altered in the revised taxonomy of Barr (2001). The genus *Mycosphaerella* is placed within the Dothideales in the taxonomic schemes of Barr (2001) and Erikson (1999) (Table 1.1).

Table 1.1 Comparison of recent taxonomic schemes for the classification of *Mycosphaerella*

Phylum	**Barr (2001)**	**Erikson (1999)**
Phylum	Ascomycota	Ascomycota
Subphylum	-	Pezizomycotina
Class	Loculoascomycota	Dothideomycetes
Order	Dothideales	Dothideales
Family	Mycosphaerellaceae	Mycosphaerellaceae
Genus	*Mycosphaerella*	*Mycosphaerella*

The ascomycetes occupy a broad range of ecological habitats as: hyperparasites, lichenised fungi, parasites and saprophytes. To some extent, these different habitats or trophic levels are recognised in the taxonomies of the ascomycetes. For example, the lichenised fungi are often placed into separate orders or families (Barr 1983, Barr 2001). However, the separation of taxa at the higher ranks, such as class and sub-class, relies more on phylogenetically conserved features of a developmental nature. Barr (2001) recognises four classes of ascomycetes: Saccharomycetes; Plectomycetes; Hymenoascomycetes; and Loculoascomycetes.

Mycosphaerella falls within the Loculoascomycota based on the formation of bitunicate asci and ascocarps of an ascolocular origin Barr (2001). One important feature in separating this class from other classes of the Ascomycota is the chronology of dikaryon formation in relation to ascocarp development. The dikaryon may either precede the development of a peridium or it may occur after the formation of a parenchymous stromatic 'peridium' (Barr 1983). In the first instance, the perithecium is derived from the dikaryotic tissue and always gives rise to unitunicate or prototunicate asci ('ascohymenial'). The latter gives rise to bitunicate asci in a 'pseudothecium' ('ascolocular' - so termed because the ascocarp is not formed from the dikaryotic hyphae, but rather precedes it). Luttrell (1955) was the first to differentiate the bitunicate ascomycetes from the unitunicate Pyrenomycetes. This split was based on the recognition that this trait was linked to a fundamentally different ascostromal ontogeny.

Barr's (2001) classification of orders within the Loculoascomycetes is based on twelve different character states, the majority of which relate to ascomal development. The presence or absence of a hamathecium, and it's nature if present, are important criteria in this taxonomic scheme. This classification is based on earlier work, which recognises different types of locule development. These were first described by Luttrell (1951) as the: *Elsinoe* type; *Dothidea* type; *Pleospora* type. In the *Elsinoe* type the ascocarp is indeterminate, irregular in shape and the wall is not clearly differentiated. Asci occur singly in monoascous cavities. In the *Dothidea* type, the ascocarp is determinate. The asci are arranged in a layer in monoascal cavities separated by interthecial tissues or without such tissue and occurring in a layer or fascicle. The *Pleospora* type of ascocarp is determinate, and the asci develop in a layer amongst pseudoperiphyses, that remain at maturity. *Mycosphaerella* species form the *Dothidea* type of ascocarp.

A range of characteristics is important for delimiting families and genera such as; ascus shape, ascospore shape, pigmentation and septation, and mitosporic state. The list and nature of these are long and complex and will not be discussed further, except to describe the genus *Mycosphaerella*. Members of this genus are characterised by the following (Arx & Müller 1975, Barr 1972, Sivanesan 1984). ü

> Parasitic on plants not on lichen. Ascomata brown to black, globose conic or depressed, ostiolate; immersed in host tissue, sometimes stromatic and becoming erumpent; scattered or grouped. Asci bitunicate; oblong, elongate, saccate, ovoid, rarely clavate; fasciculate, without filamentous paraphyses, few or many; 8-spored. Ascospores overlapping, biseriate or conglomerate; 'small' or 'narrow'; medianly or near-medianly one-septate; hyaline or light brown. Anamorph states may be hyphomycetous, pycnidial or acervulus. Some synonyms of *Mycosphaerella* include: *Sphaerella* (Fr.) Rabenh. (1856); *Cyclodothis* Syd. (1915); *Didymellina* Hšhnel (1918); and *Cynadothea* Wolf (1935). Some commonly occurring anamorph states of *Mycosphaerella* include: *Cladosporium*, *Ramularia*, *Cercospora*, *Cercosporella*, *Passolora*, *Phaeoisaria*, *Septoria*, *Aschochyta*, and *Phoma*.

Mycosphaerella was divided into three sections by Arx (1949): *Eu-Mycosphaerella,* with immersed non-stromatic ascomata and numerous, narrow asci on a fascicle; *Didymellina*, with non-stromatic ascomata and few saccate asci; and *Cymadothea*, with stromatic ascomata and cylindrical asci in a fascicle. Barr

(1972), later substituted the three sections with two sub-genera, namely: *Mycosphaerella* and *Didymellina*, that were differentiated on the basis of asci shape and conidial states; and nine sections, that were separated on habit and on ascospore morphology. She suggested that the sub-genera of *Mycosphaerella* might warrant the rank of separate genera. This suggestion has been since been revisited by Crous and other authors (Barr 1972, Crous 1998, Goodwin & Zismann 2001).

There are 1800 published names for species of *Mycosphaerella* or its synonym *Sphaerella* (a name which had to be abandoned as it was previously assigned to an algal genus) (Corlett 1991). The taxonomy of *Mycosphaerella* is largely host based (Corlett 1991) and this may account for the large size of the genus. Even the cosmopolitan *M. cryptica*, which infects in the order of 50 Eucalyptus species, does not attack the closely related *Corymbia* genus (Park *et al.* 2000). Nonetheless, it has been suggested that the number of species in this genus is inflated (Barr 1972). One reason for this is that the same species occurring on different hosts may be assigned to different taxa. Resolution of this will come with the standardisation of traditional identification methods, such as the wider application of ascospore germination pattern as proposed in Crous (1998) and in Park & Keane (1982a).

More importantly, molecular techniques for genome sequencing are now commonly available and affordable. Comparison of sequences for such regions as the internally transcribed spacer regions (ITS) 1 and 2 of the rRNA genes has proved very useful in delimiting and differentiating species (Beck & Ligon 1995, Crous *et al.* 2001a, Crous *et al.* 1999, Gardes & Bruns 1993, Goodwin & Zismann 2001). RAPD markers were used to differentiate species of *Mycosphaerella* on eucalypts in south-eastern Australia (Carnegie, Ades & Ford 2001).

Anamorph states remain important for differentiating species within the *Mycosphaerella* genus. Additional characters include: ascospore germination pattern; growth rates in culture; ascomatal, ascal and ascospore size and morphology; and symptomology. Ascospore germination was introduced by Park & Keane Keane (1982a) in order to differentiate two similar species of

Mycosphaerella occurring on eucalypts. This feature has been greatly elaborated in Crous (1998) where he outlines 14 germination patterns for distinguishing species of *Mycosphaerella*.

Biology

Mycosphaerella is a large genus, occurring on a broad range of hosts encompassing monocotyledonous and dicotyledonous plants. A full range of trophic habits is shown in *Mycosphaerella* from saprotrophy, to parasitism on leaves and hyperparasitism on other fungi. The typical lifecycle of the teleomorph is simple although a number of anamorph states may occur.

Mycosphaerella species are haploid for the majority of their lifecycle with only a short dikaryotic then diploid phase, which is restricted to that part of the hyphae which develops into an ascus within a pseudothecium. This sexual cycle gives rise to haploid ascospores, however, some species of *Mycosphaerella* also produce haploid conidia in an asexual reproductive cycle. There are few studies into the sexual development of *Mycosphaerella* species. However, Barr (1958) compared the development of *M. tassiana* and *M. typhae* ascoma in culture. Although similar in many respects she found that *M. tassiana* forms a multinucleate ascospore whereas *M. typhae* from a uninucleate ascospore. Both of these species are homothallic, however, many species of *Mycosphaerella*, such as *M. graminicola* are heterothallic (McDonald *et al*. 1996, McDonald *et al*. 1995) and each species must be studied individually before conclusions can be drawn on the details of its life cycle.

Attributes, such as heterothallism versus homothallism and reproduction by an asexual state, carry important implications in terms of the epidemiology of pathogens. These may determine a pathogen's population genetic structure, which in turn is implicated in the pathogen's ability to overcome host resistance through the production of novel combinations of alleles (McDonald & McDermott 1993, McDonald *et al.* 1996, McDonald *et al.* 1995).

Host range

Mycosphaerella species occur on monocot and dicot hosts ranging from wheat (*M. tritici*), banana (*M. fijiensis*) as well as coniferous (*M. pini*) and non-coniferous tree species including *Eucalyptus. Mycosphaerella* species attacking eucalypts display a range of degrees of host specificity. *Mycosphaerella nubilosa* has a relatively narrow host range and is only able to parasitise *Eucalyptus* species within the viminales series of the *Symphomyrtus* sub-genus. In contrast, *M. cryptica* has a broad host range and causes disease on 50 species across the *Eucalyptus* sub-genera *Monocalyptus* and *Symphomyrtus,* but is not recorded on the eucalypt genus *Corymbia*.

1.3.4 Epidemiology and pathology of *Mycosphaerella* species in Australia and world-wide

Symptoms and impact

The general symptoms and impact of MLD, caused by various species of *Mycosphaerella* are similar in many ways. Often the impact of each species cannot be separated as they occur as a disease complex, with one or a number of other species of *Mycosphaerella* involved. MLD causes loss in photosynthetic area and can lead to defoliation, resulting in reduced growth rates and wood volume. It may also cause poor tree form and in some cases, tree death (Carnegie 1991, Carnegie 2000, Park & Keane 1982b). Defoliation levels of 25% led to reductions in wood volume of *Eucalyptus nitens* infected with *Mycosphaerella* in South Africa (Lundquist & Purnell 1987). Carnegie *et al.* (1994) showed that MLD causes a negative effect on growth rate in *E. globulus* and more recently Carnegie *et al.* (1998) report that levels of diseased leaf area as low as 10% result in up to a 17% reduction in height of *E. globulus* in plantations. Defoliation due to MLD may be substantial and sustained over long periods. Heavy defoliation has been reported on *E. globulus* in Victoria, Australia, with levels up to 90% being recorded (Reinoso 1992). Carnegie (1991) reported that a plantation first assessed by Marks (Marks 1979 in Carnegie 2000) as heavily defoliated in its juvenile phase and later by Park & Keane (1982b) in

its adult phase, continues to be highly infected, with 70% of the crown area affected, 16 years after planting (Carnegie 1991, Carnegie 2000).

The symptoms of MLD range from small necrotic lesions on leaves of 1-2 mm (*M. parkii*) to larger blighting lesions occupying and distorting the entire leaf (*M. cryptica*, *M. gregaria*). Defoliation and shoot dieback may result, and when severe, impact on growth rate and tree form (Dick 1982). Differences in lesion form such as size, colour, texture, pseudothecial density and distribution and occurrence on the abaxial or adaxial leaf surface are helpful features for identifying the species of *Mycosphaerella* involved in causing disease.

1.3.5 Species causing MLD and their distribution worldwide

Outline of species and distribution

There are over 30 species of *Mycosphaerella* associated with diseased eucalypt foliage worldwide (Table 1.2). The origin, distribution, and impact of many of these species are poorly understood. Over 50% of these *Mycosphaerella* species are not recorded in Australia (Table1.2) and their origin is generally unknown. They may have moved from Australia along with eucalypt seedlings exported to these countries or they may have crossed from related Myrtaceous hosts occurring in areas where the eucalypts have been planted.

The most serious disease-causing species vary according to geographic region. *Mycosphaerella molleriana* was widely identified as the species causing disease in many places throughout the world (Crous 1998, Hedgecock 1926, Mirabolfathy 1990, Ruperez & Munoz 1980, Wallace 1947; cited in Carnegie 2000,). However, it has since been confirmed only from Portugal (where it was originally described) and California in the USA (Crous 1998, Crous & Wingfield 1997b). *Mycosphaerella heimii* and *M. suttoniae* are the most prevalent species in South America, although *M. cryptica* is also common in Chile (Table1.2). *Mycosphaerella cryptica* is the major cause of MLD in New Zealand (Beresford 1978, Cheah 1977, Cheah & Hartill 1987, Crous & Wingfield 1997, Dick 1982,

Dick & Gadgil 1983), although more recently *M. nubilosa* has been validly recorded (Dick 1982, Dick & Gadgil 1983). Up until recently, *M. juvenis* (originally misidentified as *M. molleriana*) was the predominant cause of MLD in South Africa (Crous 1998, Lundquist 1987, Lundquist & Purnell 1987). However, Hunter (2002) has shown that *M. nubilosa*, previously not validly recorded in South Africa, is now the most widespread *Mycosphaerella* pathogen of eucalypts in that country. Along with *M. cryptica* these two species are the most serious cause of MLD in Australia (Carnegie 2000, Carnegie *et al.* 1998, Park 1988a, Park *et al.* 2000, Park & Keane 1982b).

Comprehensive studies of the epidemiology and biology of species causing MLD on eucalypts have only been made for *M. cryptica* and *M. nubilosa* (Beresford 1978, Carnegie 2000, Carnegie *et al.* 1998, Cheah 1977, Cheah & Hartill 1987, Park 1988a, Park *et al.* 2000, Park & Keane 1982b). Park (1988a) showed that epidemics of *M. nubilosa* in south-eastern Australia are mono or bicyclic whereas those of *M. cryptica* are poly-cyclic. A comparison of the lifecycle and disease development of these two species is made below.

1.3.6 Comparison of the epidemiology of *M. cryptica* and *M. nubilosa*

Disease cycle

The disease cycle of *M. cryptica* is polycyclic and involves infection by ascospore and conidia, whereas that of *M. nubilosa* is mono or bicyclic and infection only occurs via ascospores. Warm wet conditions are important for disease development in terms of facilitating leaf infection, hyphal growth and ascospore release. Disease development is also dependent on the growth cycle of the tree, as flushes of new shoots that are susceptible to infection, must coincide with conditions favourable for infection.

Conditions for infection

Disease is encouraged by warm wet conditions that occur over summer in south-eastern Australia. Newly emerged or recently expanded foliage (less than 46 days-old for *M. nubilosa* on *E. globulus)* is more susceptible to infection (Park

1988b). *Mycosphaerella cryptica* is able to penetrate both adult and juvenile foliage, whereas *M. nubilosa* is only able to penetrate juvenile leaves (Park 1988b). Ascocarps surviving on necrotic lesions from the previous season's disease cycle, act as inoculum for the following epidemic cycle. Ascospore germination and subsequent leaf infection is optimal for both species under warm wet conditions of 5-7 days leaf wetness at 15-20 °C (Park 1988b). Leaf penetration may be direct, via the formation of an appressoria, or indirect via stomata. *Mycosphaerella cryptica* infects both directly and indirectly on either leaf surface, whereas *M. nubilosa* infects only indirectly on the abaxial leaf surface (Jackson 2001, Jackson *et al.* 2001, Jackson *et al*. 2002, Park 1988b).

Following this, hyphae ramify intercellularly within the leaf tissue, behaving as a hemi-biotroph (Luttrell 1974) before causing necrotic lesions upon which fruiting structures develop. After three weeks, lesions formed on *E. globulus* seedlings inoculated with *M. nubilosa* ascospores (Park 1988b). However, in older resistant leaves the incubation period increased to 24 weeks. Spermatia are extruded onto immature lesions in the presence of free water (Ganapathi 1979) and fertilise ascogonia, eventually giving rise to asci in mature ascocarps. Alternatively, ascocarps may result from the anastomosis of hyphae within the leaf, as occurs for *M. brassicicola* (Dring 1961). Acervuli develop after 4–6 weeks and then ascocarps with viable ascospores at 10–12 weeks on leaves infected at any age by *M. cryptica* (Cheah 1977, Park 1988b). Under optimal conditions disease develops at a similar rate for *M. nubilosa* (Park 1988b) but without the formation of an anamorph state. Unlike *M. cryptica*, disease development is slower for *M. nubilosa* on older juvenile leaves. At low temperature or moisture conditions, disease development slows for both *M. cryptica* and *M. nubilosa* (Park 1988b).

Formation of ascocarps and acervuli occurs on both attached and abscised leaves. The tendency for *M. cryptica* to form ascocarps on both leaf surfaces has been attributed to its ability to penetrate the leaf both directly and indirectly and to grow between tightly packed palisade mesophyll cells of the isobilateral adult foliage (Park 1988b). On the other hand, *M. nubilosa* predominantly forms

ascocarps on the abaxial surface, where stomata are more numerous, due to its inability to penetrate the leaf directly (Park & Keane 1982b). Following infection through stomata, the hyphae of *M. nubilosa* are restricted to the spongy mesophyll cells on the abaxial side of the juvenile leaves and hence pseudothecial development takes place there.

Ascospore release requires the presence of free water (Beresford 1978, Cheah 1977, Park 1988b). However, discharge is only triggered as lesions dry, following their immersion in free water for periods over 15 seconds (Beresford 1978). According to Beresford (1978), lesions with mature ascocarps must be soaked for at least 15 minutes for maximum ascospore discharge. In a field trial of *M. cryptica* on *E. delegatensis* in New Zealand, air-borne ascospores were released immediately after rainfall and their numbers peaked 1–2 hours after commencement of precipitation (Cheah 1977). The same study also found that exposure to high humidity for at least 5–7 days was necessary to stimulate ascospore release and is therefore a requirement for ascocarp maturation. Temperatures in the range 10–30 °C were also needed for ascospore release. Ascospores are discharged up to 15 mm above the lesion surface (Park 1988b) and will therefore be wind dispersed over some distance. Conidia of *M. cryptica* however, are not actively discharged and are therefore largely splash dispersed over a shorter distance (Beresford 1978, Park 1988b). Thus, ascospores are implicated in the long-distance dispersal of propagules between trees and plantations whereas conidia are implicated in the spread of disease within a tree or between densely spaced trees.

Ascospores of *M. cryptica* and *M. nubilosa* can withstand up to 4 and 7 days, respectively, of drying after deposition (Park 1988b). Thus, infection may still take place if a second rainfall event follows that which stimulated the ascospore release within 4 days for *M. cryptica* and 7 days for *M. nubilosa*. Infection is then able to take place and the cycle repeated. The number and length of each cycle is dependent on the weather and growth conditions for that site and year.

The epidemic cycles of *M. cryptica* and *M. nubilosa* differ, and this may be explained in terms of differences in the biology of these two organisms. There is

no obligate alternation of the anamorph and teleomorph state for *M. cryptica,* enabling multiple disease cycles within a season in what is termed an unbranched, heterogeneous infection chain (Park 1988a). Following the initial infection, bursts of conidial release may take place in 4-week cycles and ascospore release in 10–12 week cycles that overlap. In the study at Nowa Nowa in Victoria four peaks in lesion development were recorded for *M. cryptica* and two were recorded for *M. nubilosa* over the 1980/1981 growing season (Park 1988a). Epidemic progress of *M. nubilosa* was bicyclic or monocyclic over the three-year period of this study (Park 1988a). Epidemic progress is slower and results in more pronounced peaks in disease expression and ascospore release for *M. nubilosa* because of the different biology of this species compared to *M. cryptica.* Lesion maturation is much slower for *M. nubilosa* when infection takes place on older juvenile leaves. The absence of a quickly maturing conidial state for *M. nubilosa* also results in a longer period for infection propagules to be released and hence initiate a new round of infection events.

Table 1.2 Distribution of *Mycosphaerella* species recorded on eucalypt hosts

***Mycosphaerella* species**	**Anamorph**	**Eucalypt host**	**Occurrence**	**Reference**
africana		*E. deanii, E. globulus, E. grandis, E. radiata, E. viminalis*	Colombia, Portugal, South Africa, Zambia	Crous & Wingfield 1996, Crous 1998
colombiensis	*Pseudocercospora colombiensis*	*E. urophylla*	Colombia	Crous 1998
cryptica	*Colletogloeopsis nubilosum*	Over 50 species from the *Monocalyptus* & *Symphomyrtus* sub-genera	Australia, Chile, New Zealand	Crous 1998, Crous *et al*. 1995, Wingfield *et al.* 1995, Dick 1982, Ganapathi & Corbin 1979, Park & Keane 1982, Keane 2000
crystallina	*Pseudocercospora crystallina*	*E. bicostata* & *E. grandis* x *camaldulensis*	South Africa	Crous & Wingfield 1996, Crous 1998
delegatensis	*Phaeophleospora delegatensis*	*E. delegatensis, E. obliqua*	Australia	Crous 1998, Park & Keane 1984
ellipsoidea	*Uwebraunia ellipsoidea*	*E. cladocalyx*		Crous 1998, Crous & Wingfield 1996
endophytica	*Pseudocercosporella endophytica*	*Eucalyptus* sp. *E. grandis, E. nitens*	South Africa	Crous 1998
flexuosa	*Unknown*	*E. globulus*	Colombia	Crous 1998
gracilis	*Pseudocercospora gracilis*	*E. globulus, E. urophylla*	Indonesia	Crous 1998, Crous & Alfenas 1995
grandis	*Unknown*	*E. grandis, E. nitens, E. globulus*	Australia	Carnegie & Keane 1994 Milgate 2001
gregaria	*Unknown*	*E. botryoides, E. grandis, C. maculata, E. saligna*	Australia	Crous 1998, Carnegie & Keane 1997
heimii	*Pseudocercospora heimii*	*E. obliqua, E. urophylla, Eucalyptus* sp.	Brazil, Indonesia, Madagascar	Crous 1998, Crous 1995, Park & Keane 1984, Crous & Swart 1995, Hunter 2002
heimioides	*Pseudocercospora heimioides*	*Eucalyptus* sp.	Indonesia	Crous 1998, Crous & Wingfield 1997b
intermedia	*Unknown*	*E. saligna*	New Zealand	Dick & Dobbie 2001

Table 1.2 Distribution of *Mycosphaerella* species recorded on eucalypt hosts

***Mycosphaerella* species**	**Anamorph**	**Eucalypt host**	**Occurrence**	**Reference**
irregularimosa	*Pseudocercospora irregularimosa*	*E. saligna*	Indonesia, South Africa	Crous 1998, Crous & Wingfield 1997b
juvenis	*Uwebraunia juvenis*	*E. globulus, E. grandis, E. nitens*	Kenya, South Africa, Tanzania, Zambia	Crous 1998, Crous & Wingfield 1996, Lundquist, 1987
keniensis	*Unknown*	*E. grandis*	Kenya	Crous 1998
lateralis	*Dissoconium dekkeri*	*E. globulus, E. saligna, E. nitens, E. grandis* x *saligna, E. grandis, E. maidenii*	Australia, South Africa, Zambia	Crous 1998, Crous & Wingfield 1996, Maxwell *et al*. 1999
longibasilis	*Unknown*	*E. grandis*	Colombia	Crous 1998,
marksii	*Unknown*	*E. botryoides, E. fraxinoides, E. globulus, E. grandis, E. nitens, E. quadrangulata, E. saligna*	Australia, Indonesia, Portugal South Africa	Crous 1998, Carnegie & Keane 1994, Crous & Wingfield 1996
mexicana	*Unknown*	*Eucalyptus* sp. *E. globulus*	Mexico, Australia	Crous 1998, Chapter 3
molleriana	*Colletogloeopsis molleriana*	*E. globulus*	USA**	Crous 1998, Crous & Wingfield 1997a, Park & Keane 1984
nubilosa	*Unknown*	*E. bridgesiana, E. cypellocarpa, E. globulus, E. gunnii, E. quadrangulata, E. viminalis*	Australia, New Zealand	Carnegie 1991, Dick 1982, Dick & Gadgil 1983, Park 1984
parkii	*Stenella parkii*	*E. grandis, E. saligna, E. globulus,*	Brazil, Colombia, Indonesia	Crous 1998, Crous *et al.* 1993
parva	*Unknown*	*E. globulus, E. grandis*	Australia	Crous 1998, Park & Keane 1982

Table 1.2 Distribution of *Mycosphaerella* species recorded on eucalypt hosts

***Mycosphaerella* species**	**Anamorph**	**Eucalypt host**	**Occurrence**	**Reference**
suberosa	*Unknown*	*E. dunnii, E. globulus, E. grandis, E. muelleriana, E. molluccana, E. viminalis*	Australia, Brazil, Colombia, Indonesia, New Zealand	Crous 1998, Crous *et al.* 1993, Carnegie *et al.* 1997, Dick 2001
suttoniae	*Phaeophleospora epicoccoides*	*Eucalyptus* sp., *E. grandis, E. smithii* x *grandis,*	Argentina, Australia, Bhutan, Brazil, Ethiopia, Hong Kong, Indonesia, Italy, Madagascar, Malawi, New Zealand	Crous 1998, Crous & Wingfield 1997b
***** *swartii*	*Sonderhenia eucalyptorum*	*E. coccifera, E. delegatensis, E. dives, E. elata, E. fastigata, E. globoidea, E. leucoxylon, E. nitens, E. obliqua,*	Australia, New Zealand	Crous 1998, Park & Keane 1984, Dick 1990
tasmaniensis	*Mycovellosiella tasmaniensis*	*E. nitens*	Australia	Crous 1998
vespa	*Coniothryium ovatum*	*E. globulus, E. viminalis*	Australia	Carnegie 2000, Carnegie & Keane 1998, Milgate *et al.* 2001
walkeri	*Sonderhenia eucalypticola*	*E. globulus*	Australia, Chile, New Zealand	Crous 1998, Park & Keane 1984, Wingfield, Crous & Peredo 1995

***** Includes hosts for the teleomorph only. There are several more species reported as hosts for the anamorph of *M. swartii*.
** Recorded elsewhere but only validly recorded in this region.

1.3.7 Control of MLD

Mycosphaerella leaf disease has been a problem for growing eucalypts in plantations since the 1930's when it caused the cessation of the planting of *E. globulus* in South Africa. The solution to the problem at that time was to switch to other more resistant eucalypt species. The options for disease control today are much the same as they were then, but with the benefit of a more detailed understanding of host resistance and the biology and epidemiology of the disease. Hence, in addition to exploiting host resistance, there is an increased potential for the use of cultural, biological and fungicidal methods of control. The tools of molecular biology available today enable an understanding of the population genetics of the pathogen, which can inform decisions on the best mode of disease resistance to develop, as well as assisting with issues relating to quarantine. Molecular markers may be enlisted to aid in the selection of resistant genotypes. There is also the promise of introducing disease resistance genes into trees grown in clonal forestry. In addition, increased computing power offers the exciting possibility of developing integrated control options based on modelling disease epidemiology. The costs and benefits of these potential control strategies are discussed.

Although fungicides are useful for the control of MLD on eucalypt seedlings in a nursery environment (Sanberg & Ray 1976 in Carnegie 2000; Dick & Gadgil 1983), they are not an economically viable option in large-scale plantations. Biological control with hypo-virulence factors could be an option if the *Mycosphaerella* species of interest readily anastomose, and if the appropriate viruses can be found. Hyperparasites of *Mycosphaerella* species could also be released to reduce the impact of MLD, but again this is reliant on discovering candidate mycoparasites. Hypo-virulence strategies have been successful against *Cryphonectria parasitica* (Heiniger & Rigling 1994, Liu & Milgroom 1996, Zhang *et al.* 1998). However, the most effective methods in the short term are likely to be selection for host resistance and cultural means of control, such as optimising stocking rates and fertiliser inputs.

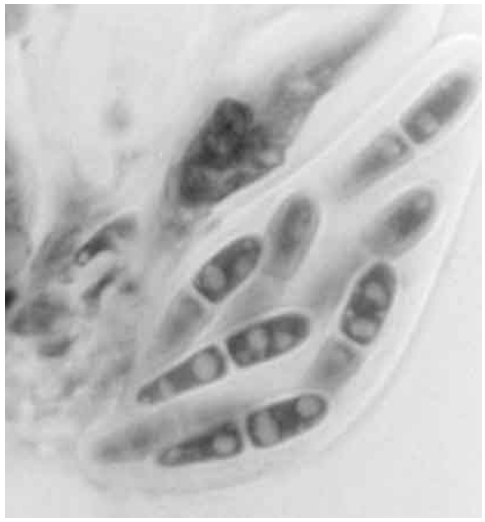
Fast growing species are thought to resist pest and disease attack by outgrowing their impact (Stone 2001). Therefore by reducing external stress to the tree this form of resistance is optimised. Carnegie (2000) found that application of phosphorous at a rate of 200 kg ha^{-1} reduced the severity of MLD on *E. globulus* over control plots with no added phosphorous. Similar reductions in MLD severity were achieved by reducing stocking densities.

Resistance to MLD has a moderate level of narrow sense heritability (Carnegie 2000, Carnegie *et al.* 1994, Dungey *et al.* 1997), and hence disease resistance can be selected for. Such selection will need to encompass resistance to a number of different species of *Mycosphaerella*. The biogeography of *Mycosphaerella* pathogens will need to be factored into any breeding programme. The scope of resistance, in terms whether resistance is effective across a range of *Mycosphaerella* species or is limited to one or a few, should also be considered. If resistance is limited then trees will be selected for the suite of *Mycosphaerella* pathogens present in a particular region.

The aim of this thesis was to determine the taxonomy and impact of *Mycosphaerella* species causing MLD in *E. globulus* plantations in south-western Australia. Phylogenetic and population genetics questions were also addressed for the taxa identified. These investigations were pursued in order to facilitate further studies into the epidemiology and population genetic structure of *Mycosphaerella* species on eucalypts and thereby devise strategies for its long-term control.

Chapter 2

Pests and diseases of *Eucalyptus globulus* plantations in south-western Australia



Chapter 2 Pests and diseases of *Eucalyptus globulus* plantations in south-western Australia

2.1 Introduction

Little work has been done to identify and rank the relative impact of health disorders in the *E. globulus* plantations of south-western Australia. Soils in south-western Australia are often deficient in a range of micronutrients (Chamberlain 2002). These deficiencies may be widespread, or locally distributed, and are especially common in areas of high rainfall (> 990 mm) and on coastal sites of highly leached sands (Chamberlain 2002). Although these plantations are often established on ex-pasture sites that have a history of regular fertiliser application for annual cropping, they may be susceptible to nutritional disorders, particularly in tree crops. This is especially the case for micronutrients, as these were often neglected in the fertilizer application to pasture sites (Chamberlain 2002). Evidence of deficiencies of copper, nitrogen, manganese, phosphorous, potassium and iron has been found at some plantations in south-western Australia (Chamberlain 2002). Such deficiencies may occur throughout a plantation, or in isolated pockets affecting as few as one or two trees in an otherwise nutritionally adequate landform. Micronutrient deficiencies such as copper or manganese impact on tree growth, form and biotic disease resistance. For example, copper deficiency in *Corymbia maculata* and other eucalypts, affects tree form, establishment, growth rates and wood quality through reduced lignification (Dell & Bywaters 1989, Dell, Malajczuk & Grove 1995). Nutrient deficiency is known to pre-dispose *Eucalyptus* to pest and disease attack (Ishaq 1999).

In 1998, at the time of this study, there were few studies of pest or disease problems in *E. globulus* plantations in south-western Australian. Published accounts of pest incidence were of a descriptive nature (Abbott 1993, CALM 1990), and the only other reference was that of Shea & Hewitt (1990) in which the unsupported observation that 'there was no significant damage due to insects' was made. Prior to 1998, there were no published records of fungal parasites in *E.*

globulus plantations in south-western Australia, other than the three *Mycosphaerella* species described by Carnegie, Keane & Podger (1997). Although significant in describing three *Mycosphaerella* species present on eucalypts in WA for the first time, the surveys were of a limited extent and intensity. Moreover, the interaction of pest, pathogen and nutritional deficiency on eucalypt plantations has not yet been studied in south-western Australia. Hence, there is a need for more comprehensive pest and disease surveys of the south-western Australian plantation estate.

The aims of this study were firstly, to compare the incidence and severity of pests and diseases of *E. globulus* plantations in south-western Australia with a focus on the greater Albany region. Secondly, to determine whether there were differences between sites in their pest and disease profiles. Finally, to test for interactions between Mycosphaerella leaf disease (MLD), and other diseases, pests and nutritional deficiencies.

2.2 Methods

2.2.1 Sampling design

In April 1998, one and two-year-old plantations, were assessed for pest, disease and nutritional disorders. Six plantation locations were surveyed between Albany and Manjimup (Figure 2.1). Three and six transects were made at one and two-year-old-sites, respectively. Symptoms of pest, disease and nutritional deficiencies were rated for each of 20 trees per transect. Statistical analyses were made to determine if there were significant differences between plantations in their symptom profiles, and whether there were significant interactions between symptom categories.

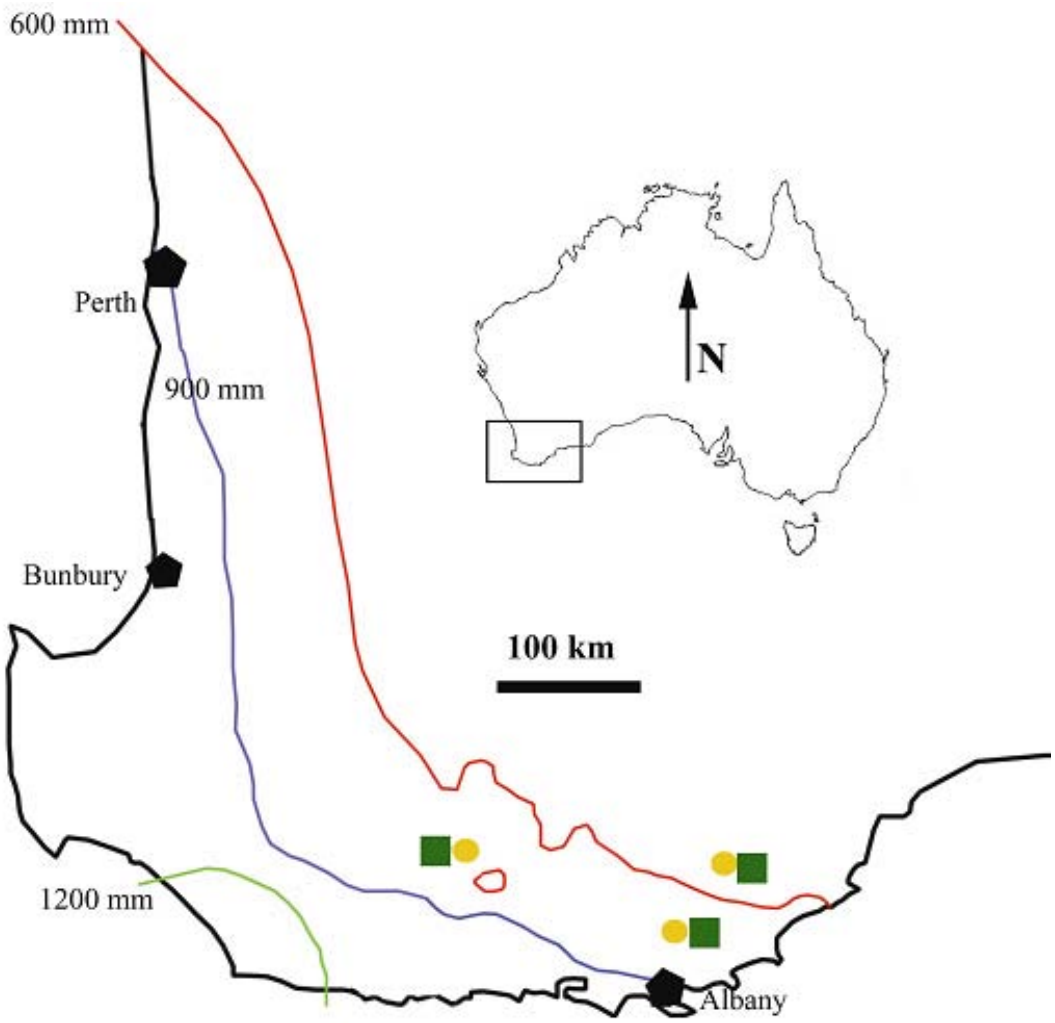


Figure 2.1 Location of *Eucalyptus globulus* plantations surveyed for incidence and severity of pest, disease and nutritional disorders in south-western Australia. One-year old (□) and two-year old (○) plantations indicated

2.2.2 Plantation location and description

The six plantations surveyed were selected on the basis of their: young ages to ensure the presence of juvenile foliage; uniform provenance planted, soil type and fertilizer history; and their geographically dispersed locations (Figure 2.1). Trees at sites 1–3 were one-year-old, and at sites 4–6 were two-years-old at the time of sampling. All trees sampled were of the same provenance (King Island = KI) except for compartments 5 and 1 of plantations 4 and 5, respectively. The provenances in these compartments are designated (B) and (C) at Chelgiup (4) and Range-Montanna south (5), respectively. All plots sampled were located over gravely duplex soils. All sites were subject to the standard Integrated Tree Cropping (ITC) fertiliser regime: addition of NPK (rate withheld) and a micronutrient mix (3% Cu, 5% Mn, 12% Fe, 3% Zn, and 1% Bo) at 28 kg/ha (Chamberlain 2002).

Adequate plant nutrient levels were confirmed through foliar analysis as described by Chamberlain (2002). Briefly, at each site 5–10 trees were sampled at random from each of 4 compartments. For each tree the youngest fully expanded leaves (YFEL) were sampled from actively growing branches in the top third of the canopy and combined into 1 sample. Samples were oven-dried at 70 °C to constant weight (3–4 days), and ground in a stainless steel mill for acid digestion. Total N was determined as outlined by Shedley, Dell & Grove (1995) using semimicro Kjeldahl digestion (Fleck 1974) of approximately 100 mg (dry weight) plant material, followed by colorimetric determination using a modified Berthelot reaction (Searle 1984). For other elements (P, K, S, Mg, Ca, Fe, Mn, Zn, B, and Cu), samples (approximately 600 mg dry weight), were digested in nitric acid (Zarcinas, Cartwright & Spouncer 1987), and analysed by inductively coupled plasma spectrometry (ICP-AES). In each digest a recovery test was included using standard reference material obtained from the State Chemistry Laboratory, Victoria. Values were accepted within one standard deviation of the certified values. Trees were classed as deficient if nutrient concentrations in the YFEL were within the deficient concentration range defined by Dell *et al.* (2001).

All plantations had adequate levels of macro and micronutrients with the exception of some compartments at Frankland and Range-Montana that were low in potassium (Appendix 2.1). One compartment was rated and sampled for pests and diseases per 1-year-old planting. In order to compare site variation, two compartments were sampled per 2-year-old planting. In each compartment, three plots of 20 plants each were sampled.

2.2.3 Sampling and disease assessment procedure

Sample plot locations for each compartment were chosen randomly, prior to visiting the site. Plots were located at least 50 metres into the plantation from any compartment boundary in order to minimise any potential 'perimeter effects'. On site, the south-west corner of each rectangular sample plot was marked and then 4 rows of 5 trees were rated for pest and disease symptoms.

Based on preliminary surveys, symptoms (Table 2.1) were identified and subsequently rated for incidence and for severity. The presence or absence of each symptom was recorded for each tree, and the severity of the more damaging health problems (Table 2.1), rated according to a log-linear scale (Figure 2.2). Ten leaves from the mid portion of a single branch were rated for each tree (detailed procedures in Appendix 2.2). Thus, comparisons between different causes of leaf damage were possible.

Insects were collected from 5 randomly selected trees at each plantation and returned to the laboratory for identification according to Naumann *et al*. (1991). The pattern of damage attributed to weevil and chrysomelid species was confirmed by maintaining the insects in glass jars containing *E. globulus* leaves, for up to 14 days. The presence of *Mycosphaerella* was confirmed from a random sample of ten diseased leaves from each plantation as described in detail in Chapter 3.

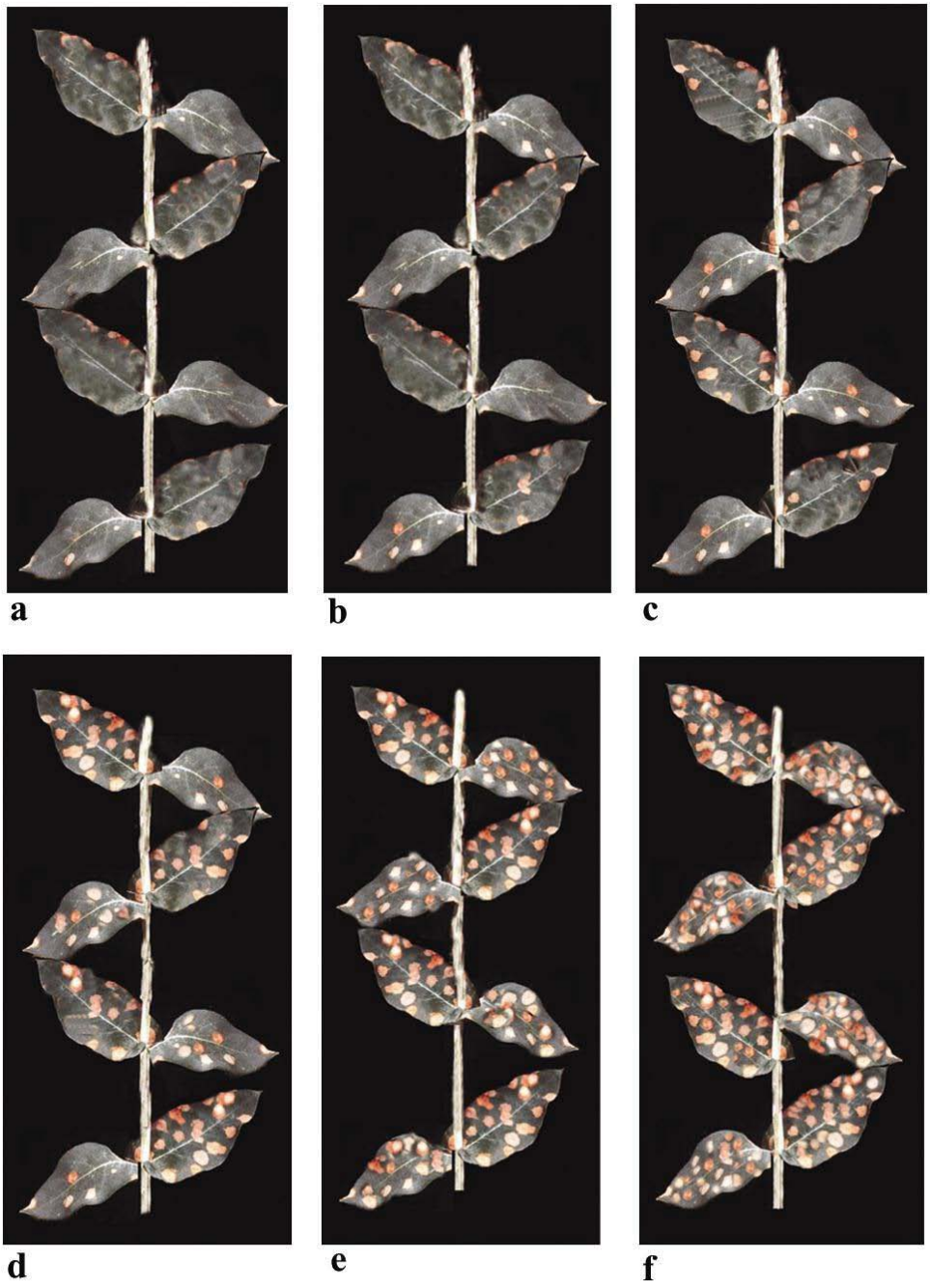


Figure 2.2 Rating diagrams used to assess severity of Mycosphaerella leaf disease symptoms showing logarithmic increase in lesion severity. (a) 3 %; (b) 6 %; (c) 12.5 %; (d) 25%; (e) 50 %; (f) 75 %.

Table 2.1 Nutrient deficiency, pest and disease symptoms assessed in a survey of *Eucalyptus globulus* plantations in south-western Australia

Nature of symptom	Symptom category	Causal agent	Assessment: incidence (I) & severity (S)
Nutrient deficiency	Stem distortion	micronutrient deficiency	S
	Crown decline	micronutrient deficiency & chewing insects	S
Insect pest	Grazed leaves	Insects	I & S
	Unevenly grazed leaf margins	Adult weevils	I & S
	Scalloped leaf margins	Adult chrysomelid beetles	I & S
	Etched leaf grazing	Weevil larvae	I
	Evenly grazed leaf margin	Chrysomelid larvae	I
	Leaf blister	Leaf blister sawfly	I & S
	Chlorotic spots	Sap sucking insects	I
	Leaf bud necrosis	Psyllids	I & S
	'Shot-hole'	Jarrah leaf miner	I
	Leaf and stem galls	Parasitic wasps	I
	Leaf skeletalisation	Autumn Gum Moth & other moths	I
Fungal disease	Trunk cankers	*Cryphonectria* sp.	I & S
	Foliage necrosis	*Mycosphaerella* spp., *Harknessia* spp., *Aulographina* sp.	I & S

2.2.4 Statistical analysis

Data were recorded on a data logger (Psion Workabout 1Mb Model), downloaded into an Excel spreadsheet and analysed statistically. The analyses used were a χ^2,

log-linear, ANOVA and ANCOVA analysis in STATISTICA version 5 (1995, StatSoft). Firstly, the data obtained were screened for their fit to assumptions of normality, linearity and homogeneity of variance. Then, analyses of variance were performed to test for differences between sites, and for correlations between *Mycosphaerella* damage and other pest or disease symptoms.

2.3 Results

The pest and disease symptoms observed in *E. globulus* plantations of south-western Australia (Illustrated in Figure 2.3) were compared in terms of frequency (Figure 2.4) and relative severity (Table 2.2). The following insect pests were identified: *Ardozyga* sp. (leaf tier caterpillar) *Cadmus excremntarius* (chrysomelids), *Catasarcus* sp. (weevils) *Ctenarytaina eucalypti* (Psyllids), *Chrysophtharta* sp. (chrysomelids), *Gonipterus scutellatus* (weevil) *Mnesampela privata* (autumn gum moth; AGM), *Oxyopsis* sp. (weevil), *Paropsis* sp, *Phylacteophaga froggattii* (leaf blister sawfly; LBSF). The following fungal disease species were identified: *Aulographina* (target spot), *Cryphonectria* (canker), *Harknessia* (leaf spot) and *Mycosphaerella* (leaf spot). The following nutritional disorders were identified: copper, phosphorous, potassium.

2.3.1 Comparison of pest and disease incidence

The most frequently occurring pests and diseases were MLD, weevil and chrysomelid grazing, psyllid damage and LBSF (Figure 2.4). The most severe health disorders were MLD (1–3%) crown decline (2–3% of whole tree) and leaf chewing (1–3%) (Table 2.2). The greatest contributors to the leaf-chewing category were chrysomelid beetle and weevils (Table 2.2). Although the modal damage severity of LBSF was the lowest of the causes identified (0%) the range of damage was greatest for this disorder (0–75%). At some plantations damage caused to juvenile foliage by LBSF was uniformly high (Kelora and Chelgiup) whereas at others it was uniformly low (Cobertup and Frankland).

Figure 2.3 Signs and symptoms of pests and diseases observed in *Eucalyptus globulus* plantations in south-western Australia: (a) Mycosphaerella leaf spot; (b) Leaf blister sawfly; (c) Crescent shaped grazing on leaf caused by chrysomelid beetle shown; (d) Leaf chewing caused by chrysomelid larvae; (e) Uneven leaf chewing caused by the weevil species *Gonipterus* and *Oxyops*; (f–g) leaf etching caused by the weevil larvae; (h) Autumn gum moth larvae grazing the leaf surface; (i) Crown decline caused by insect defoliation or nutrient deficiency; (j) Canker caused by *Cryphonectria* species

Table 2.2 Severity of pests diseases and nutritional disorders in *Eucalyptus globulus* plantations in south-western Australia

Nature of symptom	Symptom category	Severity range	Modal severity
Nutrient deficiency	Stem distortion	1–3*	1*
	Crown decline	0–50% (of crown)	7–12% (2–3% of whole tree)
Insect pest	Grazed leaves (combined insects)	0–50%	1–3%
	Unevenly grazed leaf margins (weevils)	0–50%	1–3%
	Scalloped leaf margins (chysomelids)	0–25%	1–3%
	Leaf blister saw fly	0–75+%	0%
Fungal disease	Trunk cankers (*Cryphonectria*)	1–3*	1*
	Foliage necrosis (*Mycosphaerella*)	0–50%	1–3%

* Ranking scale: 1= not severe; 2= moderately severe; 3= very severe.

Based on the severity and incidence data, the common insect pest and fungal disease threats to *E. globulus* plantations are ranked in order of decreasing importance as follows: *Mycosphaerella,* weevils, chrysomelid beetles, LBSF, AGM and psyllids.

2.3.2 Comparison of site profiles in terms of MLD impact

Differences in the severity of MLD on different provenances were tested at the two site-age combinations where there were different provenances present. ANOVA showed a highly significant (p= 0.003) difference in severity of MLD between the KI and B provenances at Chelgiup (Table 2.3). Similarly, a highly significant (p= 0.001) difference was found between provenances KI and C at Range-South (Table 2.3). No difference was found between the two different compartments of the KI provenance at Cobertup (Table 2.3). Therefore, the data from Provenance B and C

at Chelgiup and Range-South, respectively, were not used in the subsequent ANCOVA's in which interactions amongst site, MLD, and other symptoms were tested (Tables 2.4 &2.5). However, data from both of the compartments of the KI provenance from Cobertup were included in subsequent analyses.

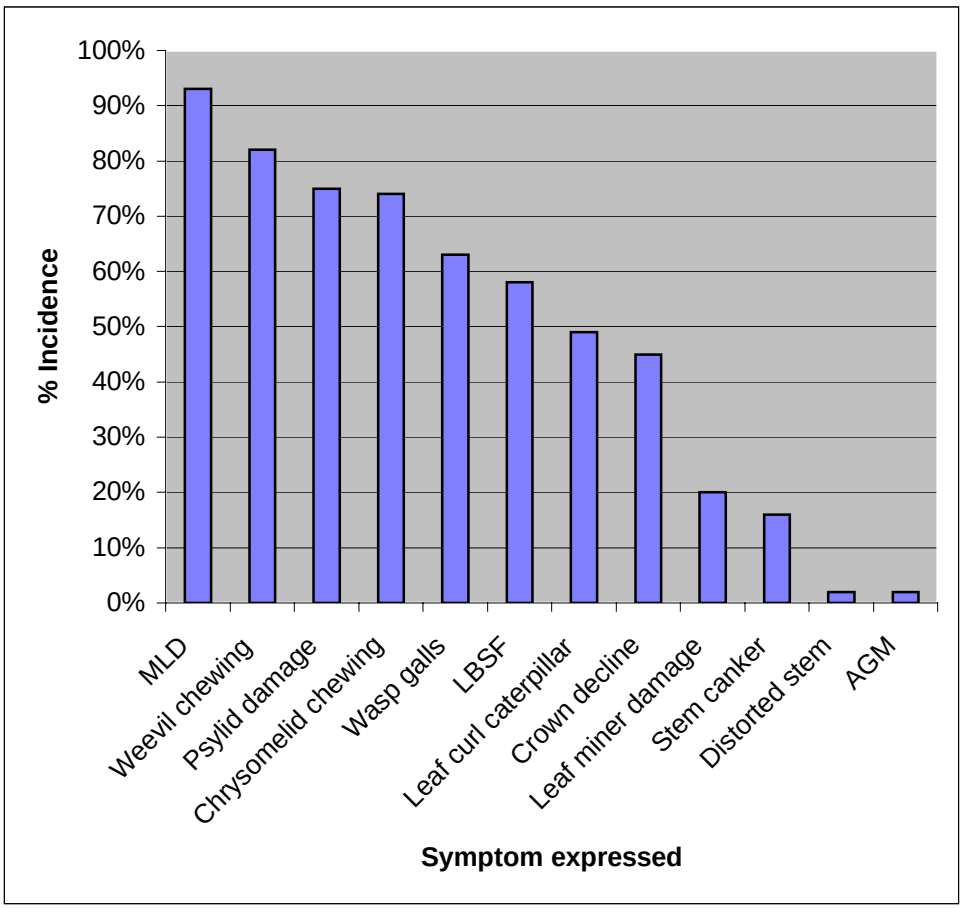


Figure 2.4 Comparison of the frequency of pest and disease symptoms occurring on *Eucalyptus globulus* trees grown in plantations in south-western Australia. Abbreviations indicated MLD (Mycosphaerella leaf disease), LBSF (leaf blister sawfly), AGM (autumn gum moth).

Table 2.3 Summary of ANOVA comparing the effect of Mycosphaerella leaf disease damage to different provenances of 2-year-old *Eucalyptus globulus* trees grown in plantations in south-western Australia. Provenances indicated KI (King Island) B (provenance confidential), C (provenance confidential). DF (degrees of freedom), MS (mean square)

Provenance	Effect	DF Effect	MS Effect	DF Error	MS Error	F	p-level
KIvB	1*	1*	4.52*	92*	0.48*	9.40*	0.003*
KIvC	1*	1*	3.10*	101*	0.28*	11.16*	0.001*
KIvKI	1	1	0.47	102	0.26	1.84	0.177

*significant effects at alpha = 0.01

The ANCOVA (Table 2.4) comparing severity of MLD damage amongst sites, whilst treating leaf chewing and leaf blister sawfly symptoms as covariates, indicates that there were significant differences between sites in terms of MLD severity. Differences were highly significant ($p < 0.001$) amongst plantations of all ages, as are differences amongst 1-year-old plantations; differences amongst 2-year old plantations are significant ($p = 0.013$). Symptoms of MLD were most severe at Chelgiup (one-yr-old) with a mean value of 3.2% damage to juvenile leaves and damage was least at Frankland (two-yr-old) where the damage severity was 1% (Figure 2.5).

Table 2.4 Summary of all effects from ANCOVA comparing Mycosphaerella leaf disease severity amongst one and two-year old *Eucalyptus globulus* plantations in south-western Australia.

Plantation age (yr)	Effect	Df Effect	MS Effect	Df Error	MS Error	F	p-level
1 & 2*	1*	5*	8.42*	356*	0.204*	41.29*	0.00*
1 only*	1*	2*	5.76*	166*	0.124*	46.51*	0.00*
2 only**	1**	2**	1.19**	188**	0.267**	4.45**	0.013**

** significant effects at p = 0.05
* significant effects at p = 0.01

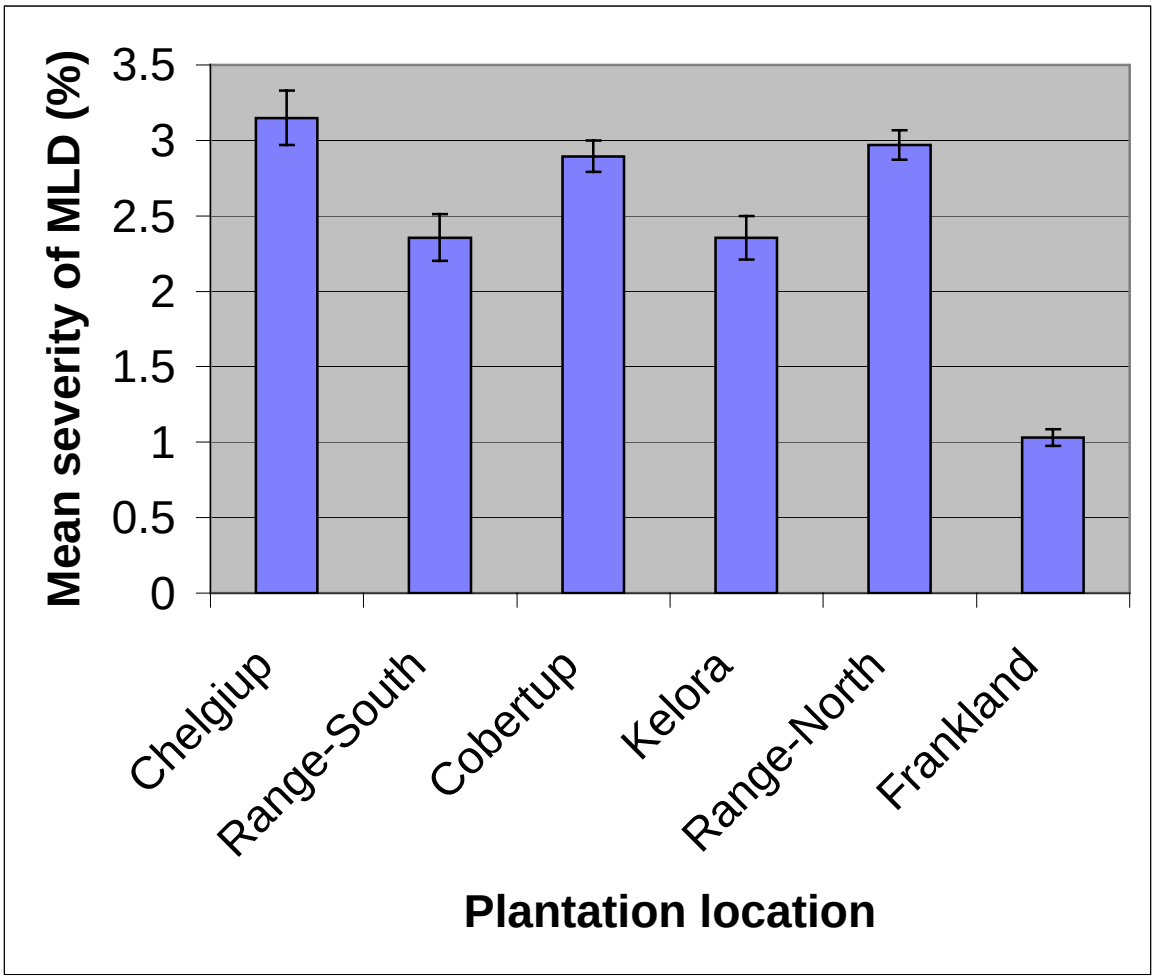


Figure 2.5 Comparison of mean severity of Mycosphaerella leaf disease amongst one and two-year old *Eucalyptus globulus* plantations in south-western Australia

2.3.3 Interactions between MLD and other pest and disease variables

A comparison of beta values for the covariates in this analysis indicate that there were no significant interactions between MLD damage and either LBSF or leaf chewing symptoms where data for one and two-year-old trees were combined (Table 2.5). However, as there was a trend for one-year-old trees to be less affected than two-year old trees, the analyses of variance were repeated separately, for one and two-year-old trees. These showed that there was a significant ($p = 0.012$) interaction of MLD damage with leaf chewing for the two-year but not for the one-year-old trees. There were no significant interactions between leaf blister sawfly and MLD damage for either one or two-year-old plantations. There was a significant correlation ($r^2=0.62$) between leaf chewing and MLD on two-year old trees.

Table 2.5 Within cells regression beta values of covariates from an ANCOVA comparing interactions amongst pest and disease symptoms with Mycosphaerella leaf disease severity at 1 and 2-year old *Eucalyptus globulus* plantations in south-western Australia.

Plantation ages (year)	****Covariate**	**B-weight**	**Error**	**beta**	**t(356)**	**p-level**
1&2	LBJ	-0.025	0.030	-0.043	-0.818	0.413
1&2	LC	0.052	0.038	0.072	1.356	0.175
1	LBJ	0.077	0.054	0.114	1.411	0.160
1	LC	0.021	0.035	0.049	0.614	0.540
2	LBJ	-0.056	0.039	-0.101	-1.42	0.155
2*	LC*	0.241*	0.096*	0.180*	2.525*	**0.012***

* significant effects at p=0.05

** Covariates are: Leaf blister sawfly on juvenile leaves (LBJ); leaf chewing (LC)

2.3.4 Effect of plantation age on pest and disease symptoms

A comparison of pest and disease symptoms affecting one and two-year-old plantations, found a higher incidence of MLD and LBSF in two-year-old plantations, than in one-year-old plantations. The converse was true for leaf curl caterpillar (Table 2.6).

Table 2.6 Pest and disease symptoms that are correlated with tree age in one and two year-old *Eucalyptus globulus* plantations in South-western Australia

Symptom	Tree age (years)	Pearson c^2	df	p
Mycosphaerella leaf disease	2	45	2	0
Leaf blister sawfly juvenile leaves	2	110	5	0
Leaf curl caterpillar	1	176	3	0

2.4 Discussion

MLD is the most severe and frequently occurring, single taxonomic health threat to one and two-year-old *E. globulus* plantations in south-western Australia. This was recorded far more frequently than any other foliar or stem pathogen and was more ubiquitous than any of the remaining pest or nutritional problems noted. The individual *Mycosphaerella* species causing this disease were not treated in this chapter, but are considered in Chapter 3. Other frequently occurring health problems included leaf grazing by weevils (*Gonipterus* & *Oxyops* spp.) and chrysomelids (*Chryophtharta* & *Paropsis* spp.), leaf blistering (LBSF: *Phylacteophaga froggatti*), leaf curl (*Ardozyga* sp.) and crown decline due to a combination of nutrient deficiency and leaf grazing by insects. Boland *et al.* (1998), in a study of the Manjimup area of south-western Australia, also found MLD to be the most widespread threat to *E. globulus* plantations.

For the first time, this survey identified and quantified (rated) the impact of insect damage to *E. globulus* plantations in the region. Although Loch & Floyd (2001)

recently ranked the importance of 21 insect pest species from *E. globulus* plantations in south-western Australia, they provide no data to support their assessment. The current study identified ten insect pests on *E. globulus* and rated their impact at a range of plantations in south-western Australia. Of the 85 species of insect pests that have been recorded on eucalypt plantations in Australia (Strauss 2001), 21 of these have been recorded in *E. globulus* plantations (Loch & Floyd 2001).

There were significant variations in the impact of MLD between plantations. This variation was not due to the provenance planted, nutrient status, or general soil type. Previous studies have shown a variation in MLD susceptibility amongst *E. globulus* provenances (Carnegie 2000, Carnegie *et al.* 1994) and in the current study, between different provenances at one site. However, the effect of provenance variation was eliminated in the current study by comparing geographically dispersed sites that were planted to the same provenance on similar soil types of a nutritionally adequate landform. Similarly, nutrient status, particularly phosphorus (Carnegie 2000), may influence the susceptibility of a plantation to MLD. In the current study, micro and macronutrient levels were generally adequate across plantations as substantiated by foliar analysis. Therefore, the difference in MLD level between the plantations studied is likely to be due to initial *Mycosphaerella* species composition and inoculum level, and local climatic conditions favourable for disease. It is likely that with increasing volume age of the plantation estate in south-western Australia that the level of disease will increase over time. This is because more inoculum may accumulate both with the volume of susceptible species and with the length of time that these have been established in south-western Australia. Studies have been made on the optimal condition for disease development, and on disease epidemics in individual plantations in eastern Australia (Park 1988a, Park 1988b, Park & Keane 1987). However, there are no investigations relating local weather conditions to differences in disease epidemics between plantations. Although, the optimal conditions for disease development is known for *M. cryptica* and *M. nubilosa*, interactions between these two and other *Mycosphaerella* species in causing disease has not been investigated.

Furthermore, the population genetic structure of *M. cryptica* and *M. nubilosa* has not been studied. The former is a heterothallic fungus with a broad host range and the latter a homothallic fungus with a narrow host range (Park 1984), therefore, they are likely to have differing population genetic structure, that will vary according to geographic region and over time. Thus, separate populations within each of these two species may behave differently in terms of their disease epidemiology. Thus, the role of host provenance, weather conditions, initial inoculum composition and level, need to be further examined across a range of plantation locations in order to develop a model that may predict epidemic outbreaks.

One factor that clearly influenced the level of disease was plantation age. Two year-old plantations were more greatly affected by MLD than one-year-old plantations. This is likely to be due to the longer period of time for disease establishment. Assessment of older plantations is required.

There were high-level interactions between some variables such as MLD, site and leaf grazing insects. One important positive correlation was that between MLD and leaf grazing. This finding is complemented by evidence from pesticide trials in *E. globulus* plantations, which indicate that insecticides reduce damage by leaf chewing insects and the level of MLD (Neumeister-Kemp *et al.* 2002). There are competing explanations for the correlation between leaf grazing and MLD damage. Firstly, the chewing insects may transmit fungal spores, which are deposited into open wounds during feeding, and thereby facilitate infection by *Mycosphaerella*. However, this is unlikely to be a factor as *Mycosphaerella* conidia and ascospores are widely splash and wind dispersed (Beresford 1978, Cheah 1977, Park 1988b, Park & Keane 1987), and they do not require wounds for infection as they are able to infect through stomata (*M. nubilosa*) or epidermal cells (*M. cryptica*) of leaves (Jackson 2001, Jackson *et al.* 2002, Jackson *et al.* 2001) (Park 1988b). A second explanation is that stressed trees may be more susceptible to both insect and fungal attack. Thirdly, *Mycopshaerella* infected trees may be more palatable to leaf grazing insects. Finally, host resistance to chewing insects may be functionally related, or genetically linked, to MLD resistance. The final

explanation would mean that selection for MLD resistant trees, could also lead to trees more resistant to insect grazing by weevils and chrysomelids. Clearly therefore, it is important to determine the nature of the correlation between MLD and insect susceptibility, as this information will assist in devising control strategies in commercial forestry operations.

In the current study, estimates of leaf infection appear low in comparison with studies elsewhere, in which defoliation levels of up to 100% have been recorded (Beresford 1978, Carnegie 2000, Carnegie *et al.* 1998, Cheah 1977, Lundquist & Purnell 1987). There are three reasons for this. Firstly, in selecting plantations with similar soil and provenance profiles, more severely damaged plantations with juvenile leaf defoliation levels over 75% were ignored (This study, unpublished data). Secondly, because the method of measuring MLD only accounted for the middle section of an outer branch (3–6 month old leaves), and the inner highly infected and often defoliated branches (7–18 month old leaves) were ignored, disease severity was under-estimated. Thus, it is not possible from this study to make direct comparisons with defoliation levels at plantations from studies by other authors. This also highlights the difficulty in directly comparing the severity ratings of MLD amongst different studies, given this subjective element that will vary between assessors. Thirdly, the plantation estate in south-western Australia is still relatively young with 90% of establishment occurring over the past ten years (Anonymous 2000, Bailey & Dunconson 1998). Thus, there may be a lag period because inoculum levels are slow to increase. Nonetheless, MLD is known to reduce growth rates in plantations (Carnegie *et al.* 1994, Lundquist & Purnell 1987), with defoliation events as low as 10% resulting in tree height reductions of 17% (Carnegie *et al.* 1998). Severe infections disturb the dominance of the apical shoot leading to poor tree form, and have caused tree death in New Zealand (Beresford 1978, Cheah 1977). The level of leaf infection due to MLD is strongly correlated with the severity of leaf defoliation (Carnegie *et al.* 1994). Thus, in the future, this disease may pose a serious threat to the plantation eucalypt industry in south-western Australia.

The method used in the current study to assess pest and disease severity differs from that which has been developed by Stone *et al.* (2003) in terms of the amount of canopy assessed and the assessment of defoliation. In the current study only a section of a branch was chosen rather than sections of crown as has been done in the Crown Damage Index (CDI) within the National Standards for assessment set out on the Bureau of Rural Science (BRS) website. The single branch option was chosen because it is easier to reliably estimated damage levels from diagrams on this scale than on a whole crown scale. If adopted, the standard procedure set out by the BRS will help to compare studies between workers. However, it will not eliminate the subjective element in assessing crown damage that will lead to differences between individuals in their rating assessment and even variation between different days by the same individual. This is something that can be guarded against by adopting some form of calibration, which could be a set of computer-based images that the assessor could calibrate their judgement against prior to rating canopies.

The second difference between the current study and the BRS standard was that in the current study there was no specific rating scale for defoliation levels. Rather this was incorporated into the damage rating system in that severe defoliation was given the highest rank along with severe chewing or leaf infection. This was a limitation in the current study and could be improved by adopting a specific scale to rate defoliation.

Similar effects on tree form may occur from other problems noted in the plantations in these surveys. Crown decline, due to insect attack or nutrient deficiency is one such factor. This results in bushy trees (Stone, Simpson & Eldridge 1998) that may have reduced wood volume at harvest. Therefore, the potential outcomes of MLD, leaf grazing insects and micronutrient deficiency are similar. Based on incidence and severity data from this study, MLD and leaf chewing insects are the two causes most likely to lead to this problem in WA plantations. Potential nutritional deficiencies were successfully managed in these plantations. There was little evidence of tree malformation due to nutritional

deficiency, and the problem of crown decline was likely to be caused mostly by insect attack.

Potential management options for pest and disease symptoms require a good understanding of their causes. Options include breeding for pest or disease resistance, application of fertilizer and the application of fungicide or insecticide sprays. Control of pest or disease through aerial spraying is problematic. The expense involved would require a good epidemiological model that can predict damage and economic thresholds in order to inform the optimal spray regime. Insecticides have been sprayed in plantations in south-western Australia to control outbreaks of weevils for example (Fremlin 2002, Australian Forest Grower). However, the use of insecticides is unpopular in the local community and needs to be minimised where companies are seeking to meet ISO environmental standards. Aerial spraying of fungicides may carry the additional disbenefit of reducing the effectiveness of mycorrhizal fungi associated with plantation eucalypts

Conclusion

The most widespread and potentially damaging disease of *E. globulus* plantations in south-western Australia is MLD. At present little is known of the taxonomy or biogeography of the organisms involved in this disease in this region. These important aspects of the disease must be considered before meaningful epidemiological studies can be conducted. Therefore, the following chapters will investigate aspects of the taxonomy and biogeography of the *Mycosphaerella* species involved in this disease in south-western Australia.

Chapter 3

Mycosphaerella species associated with *Eucalyptus* in south-western Australia: new species, new records and a key

Publications from Chapter 3

Maxwell, A., Dell, B., Neumeister-Kemp, H. G. and Hardy, G. E. (2003) *Mycosphaerella* species associated with *Eucalyptus* in south-western Australia: new species, new records and a key. *Mycological Research* **107**(3): 351-359 .

Maxwell, A., Hardy, G.E.St.J, Wingfield, M. J. & Dell, B. (2000). First record of *Mycosphaerella lateralis* on *Eucalyptus* in Australia. *Australasian Plant Pathology*. **29**:279.

Maxwell, A., Hardy, G.E.St.J, & Dell, B. (2001). First record of *Mycosphaerella nubilosa* in Western Australia. *Australasian Plant Pathology*. **30**:65.

Chapter 3 *Mycosphaerella* species associated with *Eucalyptus* in south-western Australia: new species, new records and a key

3.1 Introduction

Mycosphaerella leaf disease (MLD) is a widespread disease occurring in natural *Eucalyptus* forests and in plantations (Crous 1998). It poses an economic threat to *Eucalyptus* plantations. For example, outbreaks of MLD led to the cessation of *Eucalyptus globulus* plantings in South Africa in the 1930's (Crous 1998). MLD causes loss in photosynthetic area and can lead to defoliation, resulting in reduced growth rates and wood volume. It may also cause poor tree form. Defoliation levels of 25 % led to reductions in wood volume of *Eucalyptus nitens* infected with *Mycosphaerella* in South Africa (Lundquist & Purnell 1987). Carnegie *et al.* (1994) showed that MLD causes a negative effect on growth rate in *E. globulus* and more recently Carnegie *et al.* (1998) report that levels of diseased leaf area as low as 10 % result in a 17 % reduction in height of *E. globulus* in plantations.

There are 29 *Mycosphaerella* species associated with eucalypts recognised in Crous (1998) and two additional species; *M. vespa* (Carnegie & Keane 1998) and *M. intermedia* (Dick & Dobbie 2001). The origin, distribution, and impact of many of these species are poorly understood. Many are only recorded outside of Australia in *Eucalyptus* plantations established as exotics (Crous 1998) but the origin of these *Mycosphaerella* species is unknown. In Australia, *M. cryptica* and *M. nubilosa* are the most serious disease-causing species (Carnegie *et al.* 1998, Park 1988a, Park & Keane 1982) whereas in southern Africa, *M. juvenis*, which is not recorded elsewhere, is responsible for most disease (Crous 1998). Recent papers have extended the known geographic and host range of some *Mycosphaerella* spp. associated with diseased *Eucalyptus* foliage (Carnegie, Keane & Podger 1997, Crous *et al.* 1998). Further intensive surveys of plantation and native eucalypt

forest are required in order to determine the full range and possible origin of *Mycosphaerella* species associated with *Eucalyptus*.

Investigations into *Mycosphaerella* on eucalypts in Australia have emphasised the south-eastern region with little consideration given to the south-western region. The 1994 survey of Carnegie *et al.* (1997) was the first to describe species of *Mycosphaerella* associated with disease on *Eucalyptus* in Western Australia (WA). They identified *M. cryptica* associated with *E. globulus*, *E. marginata* and *E. patens*; and *M. marksii* and *M. suberosa* associated with *E. globulus*. The former two fungi are common in eastern Australia and *M. suberosa* was previously known only from *Eucalyptus* in Brazil, Colombia (Crous *et al.* 1993, Crous 1998) and Indonesia (Crous & Wingfield 1997b). This extension in the known range of *Mycosphaerella* species underscores the need for a comprehensive survey of *Mycosphaerella* on *Eucalyptus* plantations in WA.

During the period 1998-2001, the *E. globulus* estate in south-western Australia was systematically sampled for leaf pathogens. Additional material was opportunistically collected from Queensland (QLD), New South Wales (NSW) and Victoria (Vic.) in eastern Australia. The results of this survey for *Mycosphaerella* are reported here.

3.2 Materials and methods

3.2.1 Collection of samples

Diseased *E. globulus* leaf material was collected from 30 plantations from Esperance in the south-east of WA to Albany in the south, inland to Manjimup and north to Bunbury (Figure 3.1). Single ascospore isolations were made from lesions on 50 leaves selected randomly from each of two 100 m transects at opposite ends of each plantation. Further diseased leaves from eucalypt species were opportunistically collected from QLD, NSW and Vic. in eastern Australia. Fungi were isolated, cultured and identified.

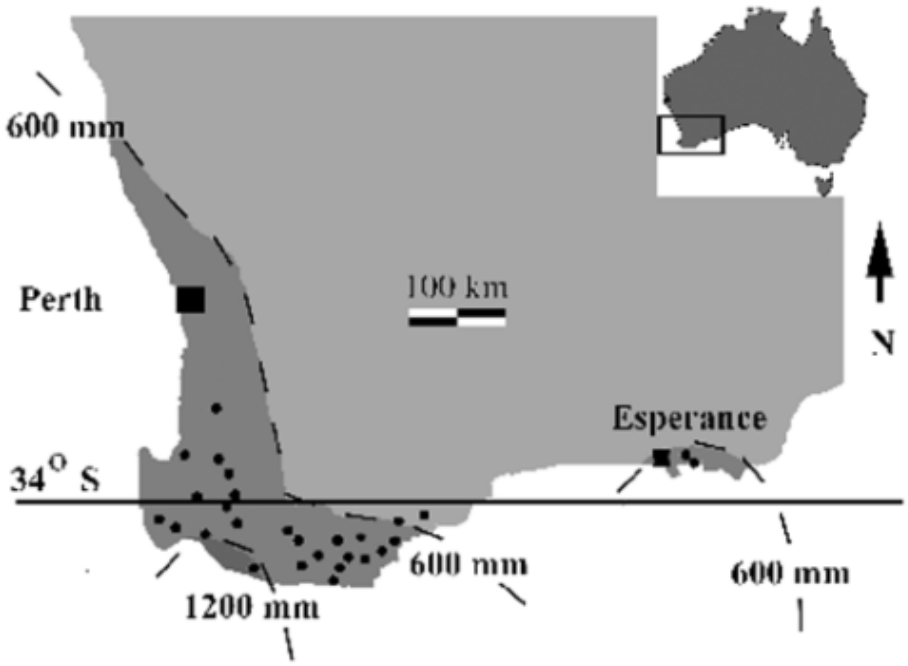


Figure 3.1 Location of *Eucalyptus globulus* plantations in south-western Australia from which *Mycosphaerella* species were isolated. Plantation locations (●), populations centres (■).

3.2.2 Species identification

Ascospores were discharged from mature lesions as described by Crous (1998), except that the Petri-dishes with adherent lesions were inverted in order to favour the attachment of actively discharged spores to the agar. Ascospore germination patterns were measured, drawn and recorded after 24 h of incubation at 20 °C, from a piece of agar that had been transferred to a slide and viewed under an Olympus BH2 light microscope.

Under a dissecting microscope (x70), single ascospore germinants were transferred to 90mm Petri-dishes containing 20 ml of 2 % Difco Malt Extract Agar (MEA) and maintained in pure culture. These plates were incubated at 25 ° in the dark and radial diameter of cultures measured after 4 and 8 wk. The range of growth rates from at least five different isolates was recorded for each species. After 8 wk, the plates were incubated under *nuv* light to induce the formation of fruiting structures. In addition, representative isolates from each species were sub-cultured onto Carnation Leaf Agar (CLA; Fisher *et al*. 1982) and grown at 25 ° under continuous *nuv* in order to promote the formation of fruiting structures. Colony colour was recorded for the upper and lower mycelial surface after 8 wk growth on MEA at 25 ° in the dark with the aid of the Methuen Handbook of Colour (Kornerup & Wanscher 1967). This was recorded as a Methuen colour name followed by the Methuen colour notation with the revised (as of 1967) Munsell re-notation of hue, value and chroma in parentheses. Mycelial scrapes were made from cultures on MEA and CLA after 4, 8 and 12 wk growth under *nuv*, mounted under acidified glycerol blue (0.05 % aniline blue (Gurr) in 50 % acidified (0.1 % HCl) glycerol) and investigated under an Olympus BH2 light microscope for the formation of anamorph states. Other features recorded include the formation of pigment and crystals in the agar.

Leaf symptoms were recorded. Ascomata were described from squash mounts and hand sections of lesions from which ascospores had recently discharged. In order to relate germination patterns to ascomata characteristics, hand-sections

were made from the area of lesion corresponding to that below the spores on the Petri-plate. Sectioned ascomata were mounted, stained with acidified glycerol blue and investigated under an Olympus BH2 light microscope on normal or phase contrast settings (100–1000x). Thirty measurements were made of ascus, ascospore and conidium dimensions under phase-contrast. From these, the 95 % confidence intervals were calculated and are presented with extremes in parentheses. All drawings were made with the aid of an Olympus drawing tube.

3.3 TAXONOMY

Mycosphaerella aurantia A. Maxwell, **sp. nov**. (Figures 3.2–3.8, 3.14–3.15)

Etym.: aurantius, Latin for orange coloured (Stearn, 1973), named for the orange-grey colouring of the culture surface on 2 % MEA.

Laesiones amphigenes, brunneae, semi-circulares, 1—8 mm diam. Ascomata amphigena, dispersa, nigra, globosa, 87—105 × 83—102 μm, ostiolata; parietes brunnei, e 3—5 stratis texturae angularis compositi. Asci bitunicati, fasciculati, obovoidei ad ellipsoidei, recti ad incurvati, 8–spori, (22–) 30—49 (–85) × (8–) 11—13 (–16) μm. Ascosporae bi- vel triseriatae, imbricatae, hyalinae, guttulatae, fusiformi-ellipsoideae extremitatibus rotundatis, non constrictae, per medianum 1-septatae, ad extremitatem basalem angustatae, (9–) 11—12 (–15) × 2—2.5 (–3) μm. Ascosporae germinatio ab extremitatibus ambitis ad axem longum sporae parallela; ascosporae hyalinae sed post 24 horas parum constrictae et subtiliter verruculosae, tum post 36 horas ramulos laterales facientes. Culturae post octo hebdomadum in 2 % MEA ad 25 °C in tenebris, pagina supera brunneo-aurantiaca, 7C6 (1.5YR: 5.7: 6.7), infera cinereo-brunnea, 7F3 (5R: 3.3: 0.9). Crystalla rufa in agaro crescentes. Velocitas incrementi post mensam unam sub 25 °C 16—24 mm. Anamorphasum non visum.

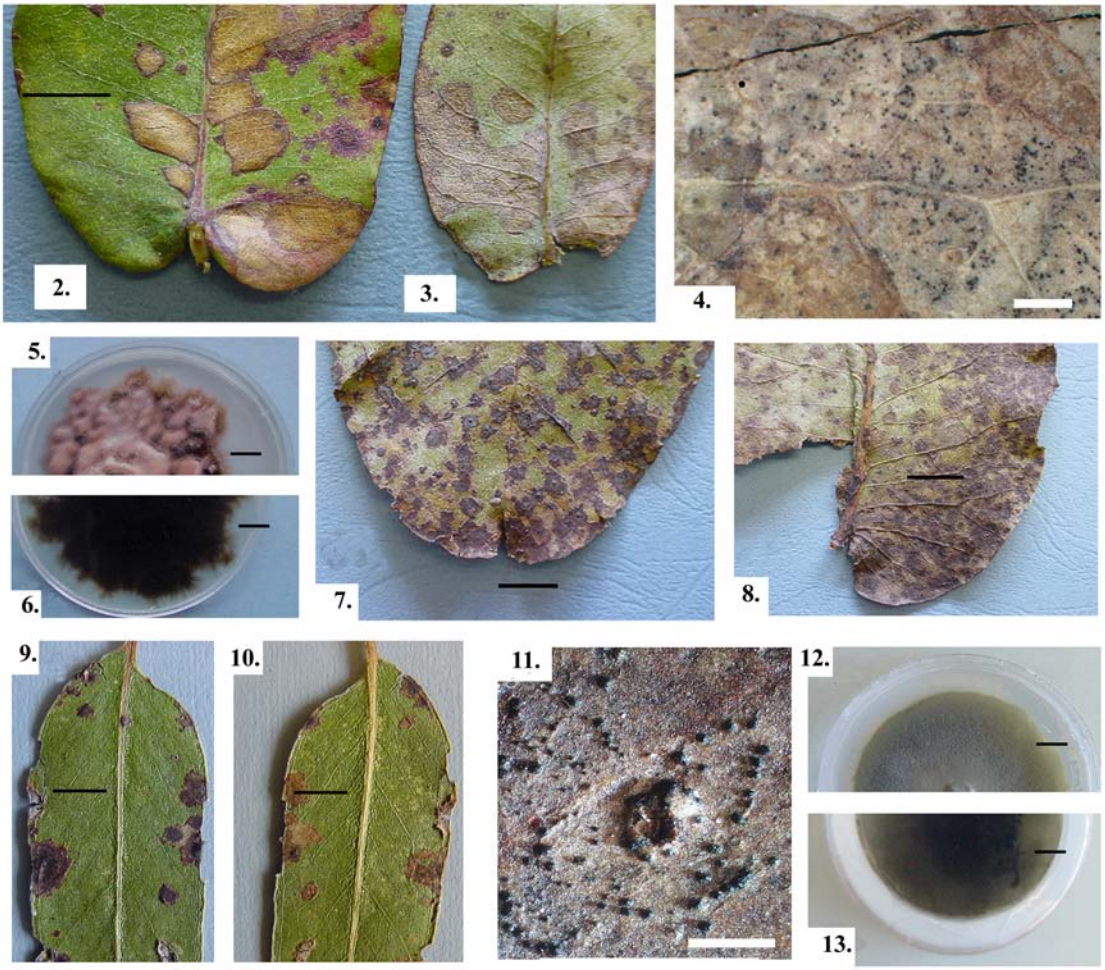
Typus: **Australia**: Western Australia: Bunbury, Summerlea plantation of Western Australian Chip and Pulp (WACAP) 115°37'E, 33°40'S, on *Eucalyptus globulus*, 1 May 2000, *A. Maxwell* (PERTH 05849543 **–** holotypus, MURU0001 – isotypus ex-type culture CBS 110500); Albany, Callistemon plantation of Integrated Tree Cropping (ITC), on *E. globulus*, 11 April 2000 *A. Maxwell* (MURU0002 paratypus). GenBank sequence ex-type AY 150331.

Lesions amphigenous, brown, sub-circular, 1–8 mm diam. *Ascomata* amphigenous, sparse, black, globose, 87–105 x 83–102 μm, ostiolate, walls brown comprising 3–5 layers of *textura angularis*. *Asci* bitunicate, fasciculate, obovoid to ellipsoid, straight to incurved, 8-spored, (22–) 30–49 (–85) x (8–) 11–13 (–16) μm. *Ascospores* bi- to

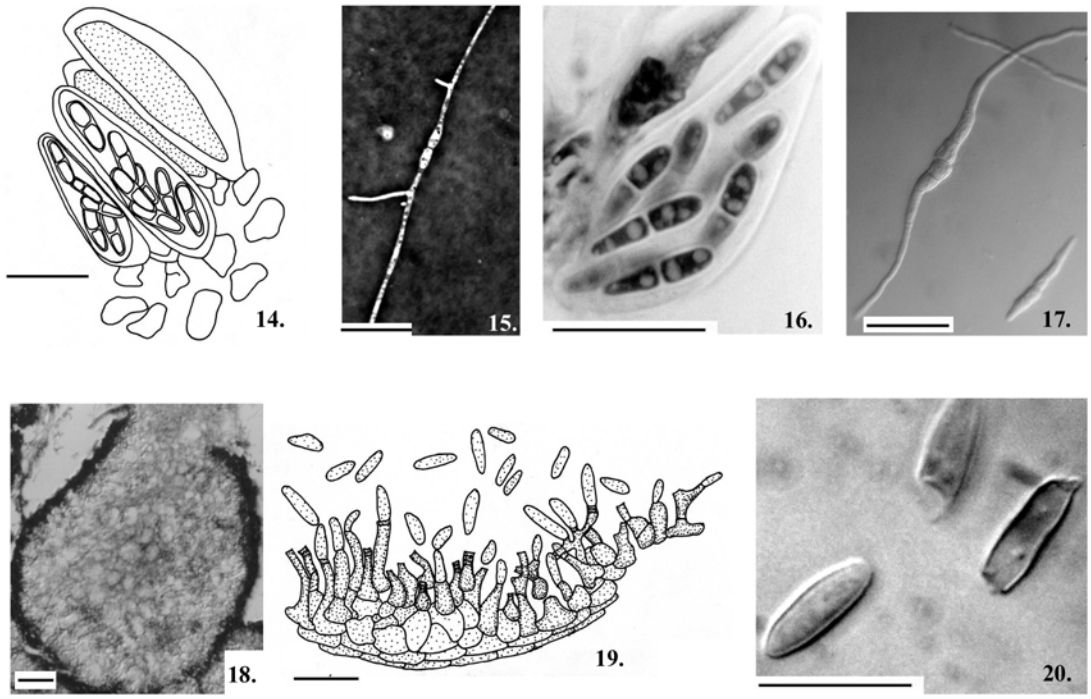
triseriate, overlapping, hyaline, guttulate, fusoid-ellipsoid, ends rounded, medianly 1-septate not constricted, tapering toward basal end, (9–) 11–12 (–15) x 2–2.5 (–3) µm. *Ascospore germination* from both ends parallel to the long axis of the spore, remaining hyaline but becoming slightly constricted and finely verruculose at 24 h then forming lateral branches after 36 h. *Culture colour* on 2 % MEA after 8 wk at 25 ° in the dark, surface brownish orange, 7C6 (1.5YR: 5.7: 6.7); reverse greyish brown, 7F3 (5R: 3.3: 0.9). Red crystals form in agar. *Growth rate* 16–24 mm after 1 month at 25°. *Anamorph* not seen.

Habit: Host *Eucalyptus globulus*. Occurring on juvenile leaves only. Found throughout the south-west of Australia. Isolated alone or with *M. cryptica*, *M. nubilosa*, *M. parva* or *M. gregaria* on the same lesion.

Notes: This species can be differentiated from other similar *Mycosphaerella* species on the basis of a combination of characteristics. It is most clearly different from other species of *Mycosphaerella* isolated from *Eucalyptus* in culture, as its upper surface becomes greyish orange on 2 % MEA. The spores are similar to those of *M. cryptica* in morphology except that they are smaller and not (or only rarely) constricted. The germination pattern of this species differs from that of *M. cryptica,* as do the cultural characteristics. *Mycosphaerella aurantia* is most similar to *M. tasmaniensis* and the *M. heimii* complex. However, it differs from the former as it has thick-walled not thin-walled ascospores and it does not form a *Mycovellosiella* anamorph in culture. *M. aurantia* is unlike *M. heimii* in ascospore shape, culture colour and it does not produce a *Pseudocercospora* anamorph on 2 % MEA or CLA in culture under *nuv*. The new species also differs from the other small-spored species because it has slightly larger ascospores, and in *M. keniensis* the ascospores do not become constricted upon germination; *M. parva* has constricted ascospores which darken and become prominently verruculose upon germination; *M. heimioides* germinates perpendicular to the ascospore and forms a *Pseudocercospora* anamorph on 2 % MEA under *nuv*.



Figures 3.2–3.6. *Mycosphaerella aurantia* (holotype). **Figure 3.2.** Lesion on adaxial surface of leaf. **Figure 3.3.** Lesion on abaxial surface of leaf. **Figure 3.4.** Distribution of ascomata on lesion. **Figure 3.5.** Surface of culture on MEA showing distinctive apricot colour formed after 8 wk. **Figure 3.6.** Reverse surface of culture on MEA. **Figures 3.7–3.13.** *Mycosphaerella ambiphylla* (holotype). **Figure 3.7.** Adaxial surface of juvenile leaf showing corky appearance of lesions. **Figure 3.8.** Abaxial surface of juvenile leaf showing lesion form. **Figure 3.9.** Adaxial surface of adult leaf showing corky appearance of lesions. **Figure 3.10.** Abaxial surface of adult leaf showing corky appearance of lesions. **Figure 3.11.** Lesion showing distribution of the ascomata. **Figure 3.12.** Surface of culture on MEA. **Figure 3.13.** Reverse surface of culture on MEA. Bars: Figures 3.2–3, 3.5–10, 3.12–13 = 10 mm; Figure 3.3 = 5 mm; Figure 3.11 = 2 mm.



Figures 3.14–15. *Mycosphaerella aurantia* (holotype). **Figure 3.14.** Asci containing ascospores. **Figure 3.15.** Germinating ascospore on MEA after 24 hours. **Figures 3.16–20.** *Mycosphaerella ambiphylla* (holotype). **Figure 3.16**. Asci containing ascospores. **Figure 3.17.** Germinating ascospores after 24 h on 2 % malt extract agar. **Figure 3.18.** Pycnidia (anamorph *Phaeophleospora*) formed on CLA. **Figure 3.19.** Conidia produced percurrently and sympodially in pycnidia formed on CLA. **Figure 3.20.** Conidia formed from pycnidia on CLA. Bars = 20 μm.

Mycosphaerella ambiphylla A. Maxwell, **sp. nov**. (Figures 3.9–3.13, 3.16–3.20)

Etym.: *ambiphyllus* named for the formation of ascomata on both surfaces of the leaf, *ambi* (Latin) 'both' and *phyllus* (Latin) 'leaf'.

Anamorph: *Phaeophleospora ambiphylla*

Laesiones elevatae, parum subereae, amphigenes, atro-rufo-brunneae marginibus rufis, irregulares ad circulares, 1—8 mm diam. Ascomata amphigena, dispersa, nigra, globosa, (60–) 86—96 (–110) × (60–) 88—100 (–120) μm; parietes brunnei, e 2—3 stratis texturae angularis compositi. Asci bitunicati, fasciculati, obovoidei ad ellipsoidei, recti ad incurvati, 8-spori, (30–) 55.5—64.5 (–80) × (7–) 9—11 (–16) μm. Ascosporae bi- vel triseriatae, imbricatae, guttulatae, fusiformes ad fusiformi-ellipsoideae apice obtuso, ad basin parum angustatae, per medianum 1-septatae, parum constrictae parietibus crassis, laevibus, hyalinae ad sub-hyalinae, (12–) 14—15 (–22) × (3.5–) 4.5—5.0 (–6) μm. Ascosporae germinatio post 24 horas ab extremitatibus ambitis ad axem longum sporae parallela; sporae 3-septatae et gradatim constrictae, subhyalinae, parietibus laevibus. Velocitas incrementi post unam mensam ad 25 °C 35—45 mm. Culturae in 2 % MEA pagina supera olivaceo-flava, 3C6 (6.5YR: 6.8: 6.8), infera olivacea, 3F6 (8.5Y: 3.4: 2.7). Pycnidia in 2 % MEA et CLA post octo *nuv* hebdomadum formantia, globosa, 80—300 × 60—270 μm, sine basi pulvinata, unilocularia, ostiolata; parietes brunnei, e 3 vel 4 stratis texturae angularis compositi. Conidiophora absentia. Cellulae conidiogenae ampulliformes, percurrenter et inerdum sympodialiter prolificantes, parietibus subtiliter verruculosis, pallide brunneis. Conidia holoblastica, (3–) 3.5—4.5 (–5) × (5–) 10—15 (–20) μm, pallide brunnea, parietibus tenuibus, subtiliter verruculosa, recta vel parum curvata, cylindrica ad ellipsoidea, ad apicem obtuso, ad basin truncata margine segmentato.

Typus: **Australia**: Western Australia: Manjimup, Boorara plantation of WACAP 116°10'E, 34°45'S, on *Eucalyptus globulus*, 16 Feb. 2000, *A. Maxwell* (PERTH 05849608 – holotypus, ex-type culture CBS 110499; MURU0003, MURU0004, MURU0005 – isotypus). GenBank sequence ex-type AY150675.

Lesions raised, slightly suberised with red margin, amphigenous, dark red-brown, irregular to rounded, 1–8 mm diam. *Ascomata* amphigenous, sparse, black, globose, (60–) 86–96 (–110) x (60–) 88–100 (–120) μm, brown walls comprising 2–3 layers of *textura angularis*. *Asci* bitunicate, fasciculate, obovoid to ellipsoid, straight to incurved, 8-spored, (30–) 55.5–64.5 (–80) x (7–) 9–11 (–16) μm. *Ascospores* bi- to triseriate, overlapping, guttulate; fusoid to fusoid-ellipsoid with obtuse apical end and slightly tapered basal end; medianly 1-septate slightly constricted, thick-walled, hyaline to sub-hyaline, smooth-walled (12–) 14–15 (–22) x (3.5–) 4.5–5.0 (–6) μm. *Ascospore germination* from both ends after 24 h, parallel to the long axis of

the spore, which becomes 3-septate and increasingly constricted, remaining sub-hyaline and smooth-walled. *Culture colour* on 2 % MEA, surface olive - yellow, 3C6 (6.5Y: 6.8: 6.8); reverse olive, 3F6 (8.5Y: 3.4: 2.7). *Growth rate*, 35–45 mm after 1 month at 25 °. *Anamorph* not seen on leaf. *Pycnidia* forming on 2 % MEA and on CLA after 8 wk under *nuv*, globose, 80–300 x 60–270 µm lacking pulvinate base, unilocular, ostiolate, wall brown, comprising 3–4 layers of *textura angularis*. *Conidiophores* absent. *Conidiogenous cells* ampulliform, proliferating percurrently and occasionally sympodially, walls finely verruculose, pale brown. *Conidia* holoblastic, pale brown, thin-walled, finely verruculose, straight or slightly curved, cylindrical to ellipsoidal, apex obtuse, base truncate with marginal frill. Conidial dimensions (3–) 3.5–4.5 (–5) x (5–) 10–15 (–20) µm.

Habit: Host *Eucalyptus globulus*. Occurring on adult and juvenile leaves. Occurring alone or with *Mycosphaerella cryptica*, *M. nubilosa*, *M. parva* or *M. suberosa* on the same leaf. Isolated alone or along with *M. cryptica*, *M. nubilosa*, *M. parva*, or *M. suberosa* from a single lesion.

Notes: Lesions of *Mycosphaerella ambiphylla* are similar to those of *M. suberosa* in that they are suberised, although not to the same degree. In culture, *M. ambiphylla* is flat, olive-yellow and comparatively fast growing (40 mm month^{-1}) whereas *M. suberosa* is compact, raised, black and very slow growing (2–5 mm month^{-1}). Also, *M. suberosa* ascospores germinate from several germ-tubes after 24 h, and become dark, verruculose and distorted (type E; Crous 1998) whereas *M. ambiphylla* has only one germination-tube at each end of the ascospore (type C; Crous 1998). The ascospores of *M. ambiphylla* most resemble those of *M. molleriana*, *M. nubilosa* and *M. vespa* in terms of size, morphology and germination pattern. All of these species germinate from both ends with slight constriction of the septum. In *M. ambiphylla*, the ascomata are amphigenous, the ascospores are thick-walled and slightly larger and wider (14–15 x 4.5–5 µm), whilst in *M. molleriana* the ascomata are mostly hypophyllous, the ascospores are thin-walled and slightly smaller and narrower (12–14 x 3–3.5 µm) in length (Crous 1998). Importantly, *M. molleriana* forms a *Colletogloeopsis* anamorph, whereas *M. ambiphylla* forms a *Phaeophleospora* anamorph. *M. ambiphylla* occurs on juvenile and adult leaves, is fast growing (35–

45 mm month^{-1}) and readily forms the *Phaeophleospora* anamorph in culture, whereas *M. nubilosa* occurs almost exclusively on juvenile leaves, is slow growing (10 mm month^{-1}) and does not form an anamorph in culture. *Mycosphaerella ambiphylla* is most clearly differentiated from the recently described *M. vespa* on the basis of the anamorph formed. The former develops a *Phaeophleospora* anamorph whereas the latter develops a *Coniothyrium* anamorph (Milgate *et al.* 2001). In addition *M. ambiphylla* is faster growing (35–45 mm month^{-1} compared to 20–35 mm month^{-1}) and forms ascomata on both surfaces of the leaf as opposed to *M. vespa*, which is hypophyllous (Carnegie & Keane 1998).

Phaeophleospora accommodates pycnidial fungi forming brown, rough-walled, cylindrical, holoblastic conidia with obtuse apices and truncate bases with a marginal frill. The conidia are produced from brown, ampulliform, lageniform or short cylindrical, rough-walled conidiogenous cells with several proliferations. In the neotype designation for the type species, the conidiogenous cells of *Phaeophleospora eugeniae* are described as percurrent (Crous, Ferreira & Sutton 1997). However, sympodial conidiogenesis is not precluded for this genus. Similar genera to *Phaeophleospora* are *Microsphaeropsis*, *Colletogloeopsis, Readeriella,* and *Coniothyrium*. *Microsphaeropsis* conidia lack ornamentation and the conidiogenous cells are enteroblastic, 'phialidic' and hyaline (Sutton 1980). This differs from the present anamorph, which forms finely verruculose holoblastic conidia, from percurrent or sympodially proliferating, finely verruculose brown conidiogenous cells. In *Readeriella*, conidiogenesis is exclusively percurrent and the conidia produced are deltoid, thick-walled with three lateral obtuse projections (Sutton 1980) unlike the thin-walled, cylindrical to elliptical conidia of the present anamorph. *Colletogloeopsis* differs from the anamorph under consideration in that it forms thick-walled spores in an acervulus, not in a pycnidium. The anamorph of *M. ambiphylla* differs from the genus *Coniothyrium* in the following respects: the conidia of *Coniothyrium* are verruculose and the conidiogenous cells are hyaline and smooth-walled (Sutton 1980) whereas in this anamorph both the conidia and the conidiogenous cells are pale brown and finely verruculose; also, conidiogenesis in *Coniothyrium* is characterised by percurrent proliferation only. In

contrast, conidiogenesis in the present anamorph is both percurrent and sympodial.

Presently, all of the fungi accommodated within *Phaeophleospora* have septate conidia. The conidia of *M. ambiphylla* are aseptate, therefore this species is clearly different from previously described species within the genus *Phaeophleospora*. However, in a recent re-examination of the holotype of *Coniothyrium ovatum,* Milgate *et al.* (2001) disagree with the original description of the conidiogenous cells as hyaline and smooth-walled (Swart 1986), finding that they were brown and verruculose; ie similar to *Phaeophleospora.* Therefore, a comparison is made between *C. amhadii*, *C. eucalypticola*, *C. kalgurensis*, *C. ovatum* and *M. ambiphylla* (Table 1). It is evident that the conidia and pycnidia of *M. ambiphylla* are larger than those of the four *Coniothyrium* spp. on eucalypts.

Table 3.1. Comparison of the pycnidial and conidial dimensions of *Coniothyrium* species associated with *Eucalyptus* species and the *Phaeophleospora* anamorph of *Mycopshaerella ambiphylla* from *Eucalyptus globulus.*

Species	**Pycnidia (diameter μm)**	**Conidia (length x width μm)**	**Source**
C. amhadii	Not given	6–7 x 3.5–4.5	Sutton (1974)
C. eucalypticola	Very small	8.5–10 x 6–7.5	Sutton (1980)
C. kallangurense	To 250	4–7 x 2.5–5	Sutton (1980)
C. ovatum	To 80	(6–) 7–9 (–11) x 3–3.5 (–4)	Crous (1998)
C. ovatum	32–75	(7.5–) 9 (–12) x (2.5–) 3 (–5)	Milgate *et al.* (2001)
C. ovatum	40–70 (–80)	(6–) 7–11 x 3–4.5 (–5)	Swart (1986)
M. ambiphylla	80–300	(5–) 10–15 (–20) x (3–) 3.5–4.5 (–5)	This study

Mycosphaerella mexicana Crous, *Mycologia Memoir* **21**: 81 (1998). (Figure 3.21)

The specimens of *M. mexicana* isolated in the present survey agree with that of the type description (Crous 1998) except for the following small differences: *Asci* were shorter and wider in the present study (52–60 x 16.5–19 μm) than in the type description (50–80 x 10–15 μm). Cultural features are not described in the type description; therefore these features are described here. *Culture colour* on 2 % MEA surface olive grey, 3F2 (-: 3.5: 0.2); reverse olive grey, 3F2 (but 'darker') (-: 3.5: 0.2). *Mycelia* a dense, aerial form. *Growth rate* 12–18 mm month^{-1}.

Habit: Host *Eucalyptus globulus.* Observed on older juvenile leaves, occurring alone or with a combination of *Mycosphaerella cryptica*, *M. marksii, M. nubilosa* or *M. parva* on the same lesion.

Specimens examined: **Australia**: Western Australia: Manjimup, Darling View plantation (WACAP), 116°00'E, 33°10'S, *Eucalyptus globulus*, 2 May 2000, *A. Maxwell* (PERTH 05849632, MURU0006, MURU0007, MURU0008). Culture CBS 110502.

Mycosphaerella gregaria Carnegie & Keane, *Mycol. Res.* **101**: 843 (1997). (Figure 3.21)

The specimens of *Mycosphaerella gregaria* from the present study agreed with the type description of Carnegie & Keane (1997) except for the following small differences. *Asci* were smaller in the present study (28–32 x 5.5–7 μm) than in the type (37.5–47.5 x 6.5–8.5 μm). *Ascospores* were smaller in the present study (9.5–11 x 2–2.5 μm) than in the type description (10.5–15.5 x 2.5–3.5 μm). *Culture colour* is described in the type but not with reference to standardised colour charts. The current study on 2 % MEA, surface greyish rose 11B6 (10RP: 5.5: 8.5) becoming olive brown 4E4 (5Y: 4.8: 3.1) towards the margin. Reverse, brownish grey, 4F2 (-: 3.5: 0.3). Forms sclerotia, and unlike the type description, does not form a red or red-brown pigment in the agar.

Habit: Host *Eucalyptus globulus*. Observed on older juvenile and leaves intermediate between their juvenile and adult phase. Widespread in south-

western Australia. Occurring alone or with one or a combination of *Mycosphaerella cryptica*, *M. marksii, M. nubilosa* or *M. parva* on the same leaf.

Specimens examined: **Australia:** Western Australia: Bunbury, Summerlea plantation (WACAP) 115°37'E, 33°40'S, *Eucalyptus globulus*, 1 May 2000, *A. Maxwell* (PERTH 05849551); Manjimup, Channeybearup plantation (WACAP), on *E. globulus*, 16 Feb. 2000, *A. Maxwell* (MURU0009); Busselton, Reid plantation (WACAP), on *E. globulus*, 2 May 2000, *A. Maxwell* (MURU0010); Esperance, Chips plantation of ITC, on *E. globulus*, 15 Dec. 2000, *A. Maxwell* (MURU0011). Culture CBS 110501.

Mycosphaerella lateralis Crous & M.J. Wingfield, *Mycologia* **88**: 454 (1996). (Figure 3.21)

The specimens of *Mycosphaerella lateralis* from the present study agreed with the type description of Crous & Wingfield (1996) except for the following small differences. *Ascomata* width was 45–65 µm (not 40–60) and 60–80 µm (not 50–70) high. *Asci* were 40–60 x 7–10 µm (not 30–50 x 6–10). The anamorph formed was *Dissoconium dekkeri* after 6 weeks at 25° under *nuv* light. This was characterised by 1-septate obclavate primary conidia, 20–30 x 3–4 µm, discharged from light brown conidiogenous cells. Cultures were fast growing, 15–25 mm per month on MEA at 25° in the dark; even or uneven edged, cream aerial mycelium, yellow to light brown reverse.

Specimens examined: **Australia:** Western Australia: Esperance, Chips plantation of ITC, on *E. globulus*, 1998, *A. Maxwell* (MURUAM98-147); Albany, Callistemon plantation of Integrated Tree Cropping (ITC), on *E. globulus*, 11 April 2000 *A. Maxwell* (MURUAM99-46); Queensland, Gympie, 1998 *A. Maxwell* (MURUAM98-163).

Mycosphaerella nubilosa (Cooke) Hansf. *Proceedings of the Linnean Society of New South Wales* **81**: 36 (1965). (Figure 3.21)

The specimens of *Mycosphaerella nubilosa* from the present study agreed with the type description of Park & Keane (1982a) except for small differences. The morphology of this species in south-western Australia is described. *Lesions* occurring predominantly on juvenile leaves, but also (rarely) on adult foliage. *Pseudothecia* mostly hypophyllous, black, becoming erumpent, globose, 60-120 µm in diameter, containing, 8-spored, bitunicate *asci* 35-65 x 10-15 µm. *Ascospores* 2–3 seriate,

thin-walled, colourless, guttulate, straight to slightly curved, medianly 1-septate, septum slightly constricted, 11–16 x 3–4 μm. Ascospores distorting and becoming increasingly constricted at germination. After 24 h at 25°C, *ascospores germinating* from each end. *Anamorph* not seen. *Cultures* slow growing, 10–15 mm per month on MEA at 25°C in the dark; even edged, olivaceous grey both surfaces with sparse aerial mycelia.

Specimens examined: **Australia:** Western Australia: Bunbury, Summerlea plantation (WACAP) 115°37'E, 33°40'S, *Eucalyptus globulus*, 1 May 2000, *A. Maxwell* (CBS 110500, MURU0002; GenBank AY 150331); Manjimup, Woodraka plantation (WACAP) 116°05'E, 34°30'S, 29 Feb 2000, *A. Maxwell* (MURU0103); Manjimup, Channeybearup plantation (WACAP), on *E. globulus*, 16 Feb. 2000, *A. Maxwell* (MURU0127); Busselton, Reid plantation (WACAP), on *E. globulus*, 2 May 2000, *A. Maxwell* (MURU0122); Esperance, Chips plantation of ITC, on *E. globulus*, 15 Dec. 2000, *A. Maxwell* (MURU0101).

Mycosphaerella parva R.F. Park & Keane, *Tran. Bri. Mycol. Soc.* **79**: 99 (1982a).

(Figure 3.21)

The specimens of *Mycosphaerella parva* from the present study agreed with the type description of Park & Keane (1982a) except for the following small differences. *Ascomata* narrower size range in the present study (56–68 μm), than in the type (42–91 μm) diameter. *Asci* were smaller in the present study (30–38 x 8.5–10.5 μm) than in the type (29–48.5 x 6–13 μm). Cultural features are not given in the type description. This study: *Culture colour* on 2 % MEA: Surface, olive, 3D5 (5.5Y: 5.9: 4.1); Reverse, goose-turd, 3F3 (7.5Y: 3.5: 0.6).

Habit: Host *Eucalyptus globulus*. Observed on older juvenile leaves. Widespread in south-western Australia. Occurring alone or with one or a combination of *Mycosphaerella cryptica*, *M. gregaria*, *M. marksii, M. nubilosa* or *M. mexicana* on the same lesion.

Specimens examined: **Australia**: Western Australia: Bunbury, Darling View plantation (WACAP), 116°00'E, 33°10'S, on *Eucalyptus globulus*, 2 May 2000, *A. Maxwell* (PERTH 05849586; MURU0012); Manjimup, Woodraka plantation (WACAP) 116°05'E, 34°30'S, 29 Feb 2000, *A. Maxwell* (MURU0013). Culture CBS 110503.

Other *Mycosphaerella* species isolated in this survey: *M. cryptica, M. marksii,* and *M. suberosa* (Figure 3.21). These have been previously recorded in south-western Australia and are therefore not discussed in this Chapter. However, they are included in the following key for the identification of *Mycosphaerella* species

present on *E. globulus* in WA. Additional foliar pathogens that were recorded on eucalypts in this study were *Aulographina eucalypti*, *Coniochaeta* sp., *Harknessia* spp., *Microthyrium eucalypticola* and *Vermisporium* sp. on *E. globulus* in south-western Australia; *Dissoconium aciculare* on *Eucalyptus maidenii* x *E. grandis* in Qld; and *Phaeothyriolum microthyrioides* on *Corymbia calophylla* in south-western Australia. These were not widespread causes of disease on eucalypts in this survey and are not described in this Chapter.

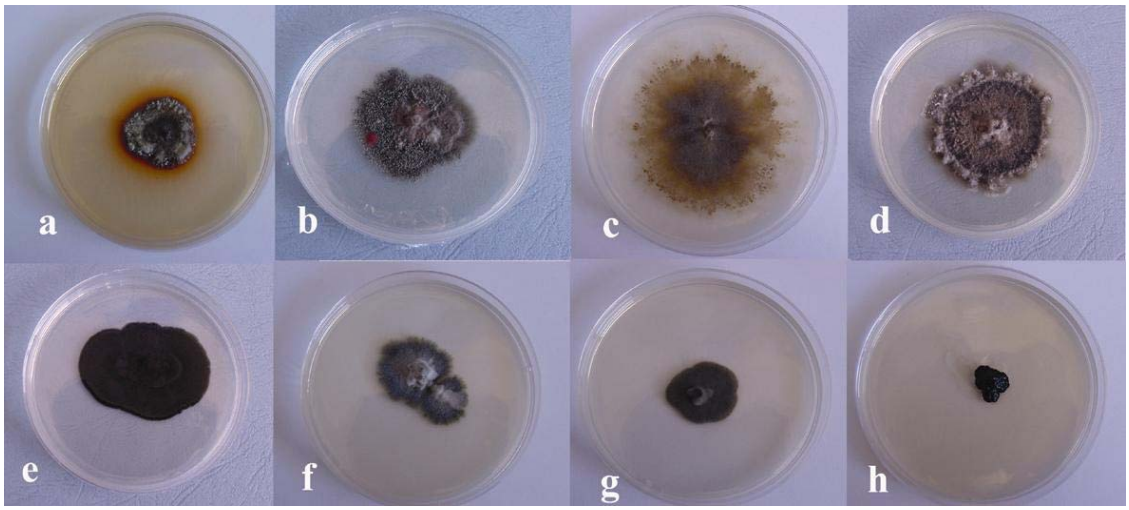


Figure 3.21 Cultures of Mycosphaerella spp. present in Western Australia grown on 2% MEA in the dark. M. cryptica (a), M. gregaria (b), M. lateralis (c), M. marksii (d), M. mexicana (e), M. nubilosa (f), M. parva (g), M. suberosa (h).

Germination patterns described in the following key refer to the typical pattern seen after 24 h on 2 % MEA at 20 °, and reference letter where given, is according to the scheme of Crous (1998). Cultural feature such as surface colour, pigment formation and anamorph formed refer to growth on 2 % MEA after 2 months under *nuv*. Growth rates refer to growth rate on 20 ml of 2 % MEA in 90 mm plates at 25 °.

3.4 Key to Mycosphaerella *species occurring on* Eucalyptus globulus *in Western Australia*

1	Lesions corky, more prominent on one side of the leaf than the other; ascomata in concentric rings……	2
	Lesions not corky, not more prominent on one side of the leaf than the other; ascomata not in concentric rings……	5
2(1)	Ascospore germination from each end parallel to the long axis of the spore, spores becoming constricted at the median septum, not verruculose, not darkening or distorting, type C; cultures fast growing (40 mm month^{-1}); *Phaeophleospora* anamorph……	**ambiphylla**
	Ascospore germination parallel or perpendicular long axis from one or both ends, constricting at the median septum, ascospores and the adjacent germ tube cells becoming darker and verruculose, slow growing (<20 mm month^{-1})…	3
3(2)	Ascospores (6–) 8–9 (–11) μm; germination from one end perpendicular to the long axis of the spore, sometimes both ends, spores distorting, ascospores and the adjacent germ tube cells becoming slightly darker and verruculose, type N or L……	**parva**
	Ascospores (11) 12–18 (20) μm; germination from one or both ends, ascospores distorting or constricting at the median septum, ascospores and the adjacent germ tube cells becoming markedly dark and verruculose, type E or H…	4

3.4 Key to Mycosphaerella *species occurring on* Eucalyptus globulus *in Western Australia*

4(3)	Ascospores (11–) 13–15 (–16) μm; ascospore germination from several germ tubes, ascospores becoming markedly distorted; cultures very slow growing (<5 mm month^{-1}); culture surface black; mycelia raised in folded mounds and also deeply embedding into and distorting the agar……	**suberosa**
	Ascospores (15–) 17–18 (–20) μm; ascospore germination from each end parallel to the long axis of the spore, ascospores becoming constricted at the median septum but not markedly distorted; cultures slow growing (<18 mm month^{-1}); culture surface dark olivaceous grey; mycelia not raised and folding, not deeply embedding or distorting the agar……	**mexicana**
5(1)	Ascomata amphigenous or epiphyllous……	6
	Ascomata hypophyllous……	15
6(5)	Ascomata amphigenous……	7
	Ascomata epiphyllous……..	13
7(6)	Ascomata densely distributed over the lesion; ascospore germination from one end, perpendicular to the long axis of the spore, not distorting or constricting, type A; in culture forms red-brown diffusible pigment; *Colletogloeopsis* anamorph……..…….	**cryptica**
	Ascomata not densely distributed over the lesion; ascospore germination parallel or perpendicular, not type A; not producing a red-brown diffusible pigment on MEA; not forming a *Colletogloeopsis* anamorph on MEA……	8

3.4 Key to Mycosphaerella *species occurring on* Eucalyptus globulus *in Western Australia*

8(7)	Ascospore germination parallel or perpendicular, ascospores becoming dark and verruculose, constricted at the median septum or distorted…	9
	Ascospore germination parallel to the long axis of the spore, not becoming dark or verruculose, slightly constricted at the median septum but not distorted……	10
9(8)	Ascospores (6–) 8–9 (–11) μm; germination from one end perpendicular to the long axis of the spore, sometimes both ends; ascospores distorting, spores and adjacent germ tube cells becoming slightly darker and verruculose, type N or L…….……..	**parva**
	Ascospores (15–) 17–18 (–20) μm; ascospore germination from each end parallel to the long axis of the spore, ascospores constricting at the median septum but not distorting, spores and adjacent germ tube cells becoming markedly darker and verruculose, type H……	**mexicana**
10(8)	Lesions often forming along leaf margins; ascomata aggregated in clumps of 3–10; cultures forming sclerotia & red-pink patches on MEA…	**gregaria**
	Lesions may or may not form along leaf margins; ascomata not aggregated in clumps of 3–10; not forming sclerotia or red-pink patches on MEA..	11
11(10)	Cultures fast growing (40 mm month^{-1}); culture surface olive grey; *Phaeophleospora* anamorph….	**ambiphylla**
	Medium growth (15–30 mm month^{-1}), culture surface pale olive brown or orange grey; not forming *Phaeophleospora* anamorph……	12
12(11)	Culture surface pale olive brown; *Dissoconium* anamorph……	**lateralis**
	…	**aurantia**
	Culture surface orange grey; no anamorph……	

3.4 Key to Mycosphaerella *species occurring on* Eucalyptus globulus *in Western Australia*

13(6)	Ascomata sparse (1–20 per lesion); ascospores (15) 17–18 (20) μm, constricted at the median septum, slightly olivaceous and verruculose; ascospore germination from each end parallel to the long axis of the spore, ascospores and adjacent germ tubes becoming darker and more verruculose……..	**mexicana**
	Ascomata not sparse (>20 per lesion); ascospores <15 μm, not constricted at the median septum, not pigmented or verruculose; ascospore germination from each end parallel to the long axis of the spore but not darkening or becoming verruculose…….	14
14(13)	Ascospores with an asymmetrical apical cell; on germination ascospores not becoming constricted at the median septum, not developing lateral branches; culture surface olivaceous grey; no anamorph……	**marksii**
	Ascospores with or without an asymmetrical apical cell; on germination ascospores becoming constricted at the median septum and developing lateral branches; culture surface olivaceous grey; *Dissoconium* anamorph……	**lateralis**
15(5)	Ascospores (11) 12–14 (16) μm; germination from each end parallel to the long axis of the ascospore, becoming constricted at the median septum, not becoming verruculose or distorted..	**nubilosa**
	Ascospores (6) 8–9 (11) μm; germination from one end sometimes each end, perpendicular to the long axis of the ascospore, becoming constricted at the median septum, verruculose and distorted…….	**parva**

3.5 DISCUSSION

This survey has identified two new species of *Mycosphaerella* (*M. ambiphylla* and *M. aurantia*) and extended the known geographic range of five other species (*M. gregaria*, *M. lateralis, M. mexicana, M. nubilosa* and *M. parva*). A new anamorph, *Phaeophleospora ambiphylla*, is described & Linked to *M. ambiphylla*. All of the three previously described species of *Mycosphaerella* from south-western Australia (*M. cryptica, M. marksii, M. suberosa*) were also identified in the current survey. Thus, this study identified the presence of ten *Mycosphaerella* species associated with MLD in south-western Australia. The occurrence of two new species and five new disease records in WA is significant for the plantation-eucalypt industry worldwide. The finding of two new species brings with it the need to quantify the disease impact of these and previously recorded *Mycosphaerella* species on eucalypt plantations; and the extension of the range of five species has quarantine implications.

The appearance of two new *Mycosphaerella* species increases the number of *Mycosphaerella* species associated with eucalypts to 33. This includes the 29 species recognised in Crous (1998) and the newly described *M. vespa* (Carnegie & Keane 1998) and *M. intermedia* (Dick & Dobbie 2001).

M. ambiphylla and *M. aurantia* were the only *Mycosphaerella* species present on some lesions, suggesting that they are primary pathogens. However, they frequently occurred in association with other *Mycosphaerella* species. The role of these new species in causing disease needs to be examined. Epidemiological and pathogenicity studies have been conducted on *M. cryptica* and *M. nubilosa* (Park 1988a, b), some limited infection work conducted on *M. parva* (Park & Keane 1982) and *M. vespa* (Milgate *et al.* 2001), but not on any of the remaining 27 species occurring on eucalypts. Infection studies and pathogenicity tests need to be conducted with these little understood species in order to understand their role in the disease syndrome.

Quarantine issues are raised by the extension of the geographic range of *M. gregaria, M. lateralis*, *M. mexicana*, *M. nubilosa* and *M. parva*. *Mycosphaerella nubilosa*

is a major cause of MLD in eastern Australia and New Zealand and its occurrence in south-western Australia is of considerable concern. This pathogen was not found in the survey of Carnegie *et al.* (1997) and so the origin and impact of this species in south-western Australia should be examined.

The rapid appearance and spread of *M. nubilosa* in Western Australia, since the 1994 survey of Carnegie *et al.* (1997), is of great interest. It is unlikely that *M. nubilosa* would have been missed in their survey if it had been present at the current level. There are two alternative explanations for the rapid establishment of this pathogen since 1994. Firstly, *M. nubilosa* may be a recent introduction from eastern Australia, probably arriving with *E. globulus* seedlings or seed material. Alternatively, *M. nubilosa* may have already existed at low levels in the indigenous eucalypt community and only recently become apparent in response to the mass planting of *E. globulus* which is particularly susceptible to this pathogen. The impact of *M nubilosa* is considered in Chapter 4, and its origin is considered in Chapter 7 of the current thesis.

The origin of species formerly known only outside of Australia is of particular relevance. *Mycosphaerella mexicana*, isolated in this study, was previously known only from Mexico (Crous 1998). The known geographic range of other species of *Mycosphaerella* such as *M. suberosa* have also recently been extended, from South America (Crous *et al.* 1993, Crous 1998) and Indonesia (Crous & Wingfield 1997b), to now include south-western Australia (Carnegie *et al*. 1997). The biogeography of these and many other species occurring on eucalypts is not well known. It may be that these species occur on a range of hosts scattered across many continents. When eucalypts are established in new areas, inoculum on host trees already present in these areas may then infect these newly established trees. Alternatively, inoculum may travel with eucalypt seed or seedlings into the new areas of establishment. A third, less likely, alternative is that spores are able to travel vast distances in wind currents from their centre of origin and infect hosts where they occur in new areas. It is important to determine how these pathogens are spreading in order to inform quarantine policy decisions.

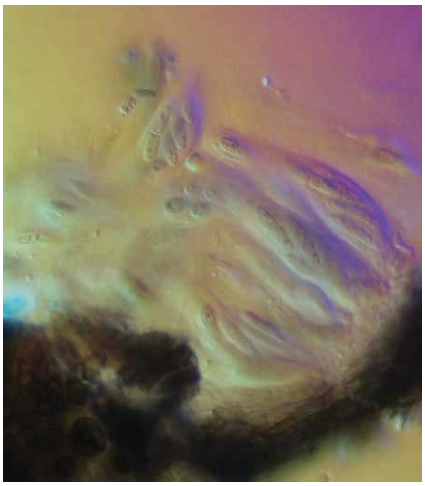
The impact of the non-*Mycosphaerella* foliar pathogens that were recorded on eucalypts in this study needs to be monitored. Particular attention should be paid to *Aulographina eucalypti,* which has previously reached epidemic levels in eastern Australia (Carnegie 2000), defoliating *E. nitens* in Victoria (Neuman & Marks 1976) and *E. obliqua* (Palzer 1978 in Carnegie 2000). In the first of these examples a *Harknessia* sp. was also involved. Of the remaining species, *Coniochaeta* and *Dissoconium aciculare* have not previously been recorded from eucalypt foliage, although *C. ligniaria* has been recorded from *E. pellita* seeds (Yuan *et al.* 1997). Whereas, *D. aciculare* has been recorded from a range of non-eucalypt host plants, in association with powdery mildews (Erysiphaceae) which *Dissoconium* is thought to parasitise (Hoog, Oorschot and Hijwegen 1983, Hijwegen & Buchenauer 1984). The ability of this and the other *Dissoconium* species (*Mycosphaerella lateralis*) to parasitise MLD causing species such as *M. cryptica* and *M. nubilosa,* is a future avenue of research.

Microthyrium eucalypticola, Vermisporium sp. and *Phaeothyriolum microthyrioides* are biotrophic pathogens generally regarded as having a minor impact on their host (Park *et al.* 2000).

The distribution of different *Mycosphaerella* species may be determined through more extensive disease surveys on eucalypts and adjacent myrtaceous hosts in areas where plantations occur. The centre of origin of a given *Mycosphaerella* species may be determined from population level studies using molecular markers. Work comparing the population of *M. nubilosa* in south-western Australia with that in eastern Australia is reported in Chapter 7. Further work of this nature needs to be made to investigate the likely origin of other *Mycosphaerella* species recently isolated in south-western Australia and elsewhere.

Chapter 4

Biogeography of *Mycosphaerella* spp. in south-western Australia



Chapter 4 Biogeography of *Mycosphaerella* spp. in south-western Australia

4.1 Introduction

There are 33 *Mycosphaerella* species reported as parasites of eucalypts (Chapter 3). The three most important disease causing species (*M. cryptica, M. juvenis, M. nubilosa*) are well studied in terms of their host range, epidemiology and global distribution (Carnegie *et al.* 1998, Crous 1998, Park 1988a, Park *et al.* 2000). However, comprehensive biogeographical investigations into the complex of species causing Mycosphaerella leaf disease (MLD) on a regional basis are lacking. Detailed assessments that have been made were restricted to single plantations (Carnegie 2000) or were unsupported generalisations about regional occurrence of pathogens (Crous 1998, Dick 1982, Dick 1990, Dick & Gadgil 1983, Park 1984). The occurrence, distribution and relative impact of the many different species of *Mycosphaerella* that contribute to MLD is not well documented in terms of the composition of species occurring within a plantation, on a leaf or a single lesion.

Worldwide, the main focus of research into MLD has been on the epidemiology of the three major disease causing species. These investigations have been restricted to a few individual plantations either in south-eastern Australia (Carnegie *et al.* 1998, Park 1988a, Park & Keane 1987), southern Africa (Crous 1998, Lundquist & Purnell 1987) or New Zealand (Beresford 1978, Cheah 1977). In southern Africa, *M. juvenis* (misidentified as *M. nubilosa*) is the major disease causing species (Crous & Wingfield 1996) and epidemiological data is available for this (Lundquist & Purnell 1987). Although a comprehensive inventory of the 10 species occurring in South Africa, providing taxonomic descriptions and species distribution has been published (Crous & Wingfield 1996), there are no studies available on the fine scale distribution and importance of those species. Similarly, in New Zealand the epidemiology of *M. cryptica* (misidentified as *M. nubilosa*) has been

investigated in detail at two sites (Beresford 1978, Cheah 1977). Although there are taxonomic descriptions that include broad scale distributions of the three predominantly occurring *Mycosphaerella* species in New Zealand (Dick 1990) there are no quantitative data of the fine-scale distribution and impact of these species on eucalypt plantations in New Zealand.

At the time of the present study, the recorded distribution of the eleven species of *Mycosphaerella* in Australia was as follows (Figure 4.1): *M. cryptica, M. marksii* occurred throughout southern Australia, *M. tasmaniensis* occurred only in Tasmania, *M. gregaria, M. nubilosa, M. parva, M. suttoniae, M. swartii, M. vespa* only in south-eastern Australia and *M. suberosa* only in south-western Australia (Park *et al.* 2000) (Figure 4.1). Studies in Australia have been largely restricted to Victoria and New South Wales (Carnegie *et al.* 1998, Park 1988a, Park & Keane 1982a, Park & Keane 1982b, Park & Keane 1982c, Park *et al.* 2000) with only recent publications reflecting the situation in Tasmania (Milgate *et al.* 2001) and Western Australia (Chapter 3) (Carnegie *et al.* 1997). These more recent publications reflect the increasing importance and rapid expansion of the plantation eucalypt industry in these regions. A comprehensive study is required to determine the level of disease occurring across the *E. globulus* estate in Western Australia. This needs to identify the *Mycosphaerella* species, their host range, leaf phase occurrence and geographical distribution.

Aims of this study

The aims of this study were threefold. Firstly, to compare the host range and impact of different *Mycosphaerella* species, identified in Chapter 3, on the major forestry eucalypt species (*E. globulus E. marginata*, *E. diversicolor*, and *Corymbia calophylla*) in south-western Australia. Secondly, to establish whether there was a different composition in *Mycosphaerella* species present on juvenile and adult leaf phases of *E. globulus*. Thirdly, to ascertain the geographic range of *Mycosphaerella* species occurring in plantations of *E. globulus,* in south western Australia.

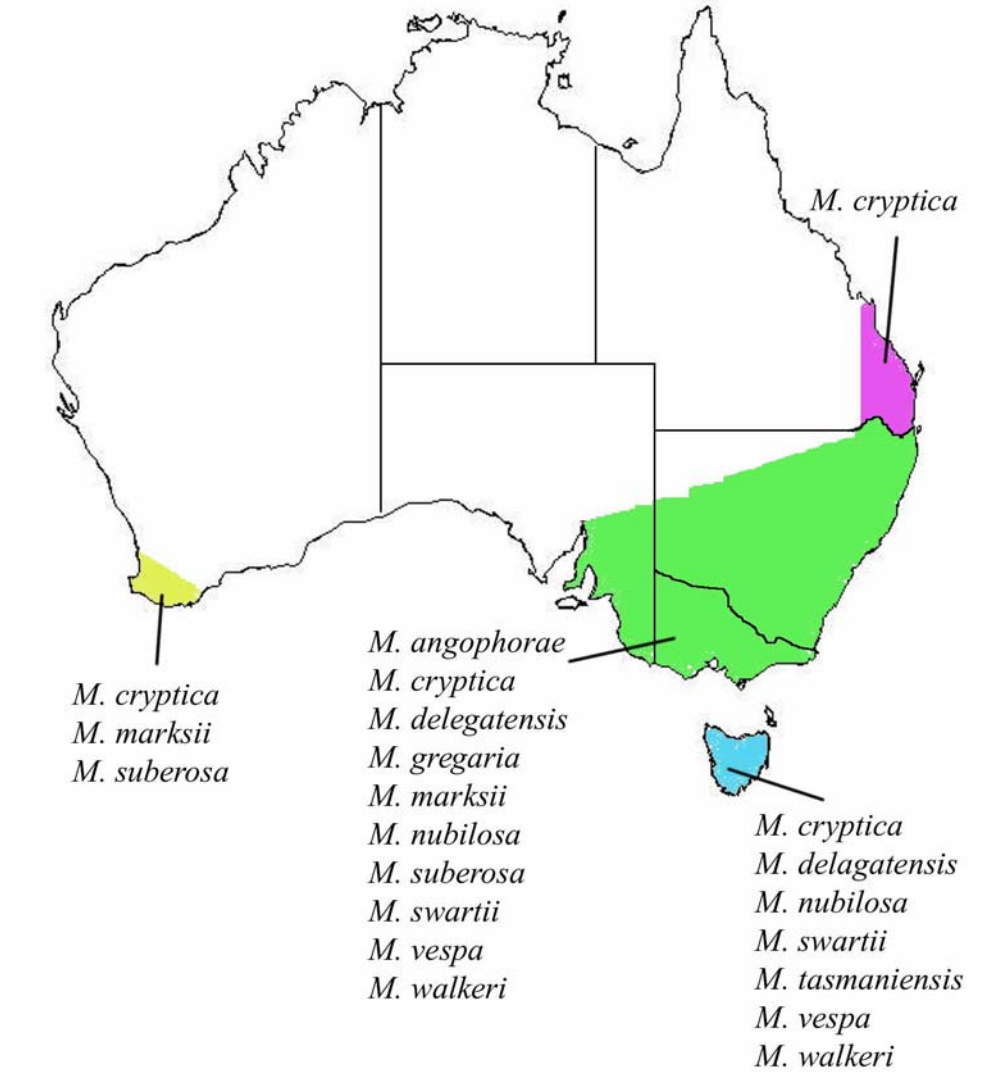


Figure 4.1 Distribution of *Mycosphaerella* species parasitising eucalypts in Australia prior to Jan 2000.

4.2 Materials and Methods

4.2.1 Experimental design

Surveys were conducted in order to satisfy the three aims of this study. Firstly, geographically dispersed *E. globulus, E. marginata*, and *E. diversicolor*, sites were selected and surveyed. Disease incidence and severity was rated in two 100m transects at each *E. globulus,* and one 200m transect at each native forest, location. Diseased leaves were randomly collected from ten trees at each site, returned to the laboratory for disease assessment and pathogen isolations. The % composition of each MLD symptom category (Chapter 3.3) was assessed for each leaf. The causative organisms were then isolated and identified from these leaves. Statistical analyses were conducted to determine if there were significant differences in the host preferences, leaf stage preference and geographic locations of each of the *Mycosphaerella* species identified.

4.2.2 Site selection

The locations of *E. globulus* plantation and native eucalypt forest sites, were selected in the following manner. A map of south-western Australia was divided into 6 regions based on evaporation and rainfall isohyets (Figure 4.2). Each of these regions was then further divided into 400 km^2 grids. Then, where possible, a 2–3 year old *E. globulus* and a 1–4 year old *E. marginata* or *E. diversicolor* location was randomly selected from within each grid. Thus, the full range of environmental conditions under which forestry eucalypts growing in this area, was represented. A total of 33 *E. globulus* plantations, 22 *E. diversicolor* locations and 25 *E. marginata* locations were surveyed (Figure 4.2). Most native forest sites were mixed stands, and where *C. calophylla* was present, this species was also rated and sampled at the rate of 10 trees per location. Diseased leaf material from other eucalypt species (*Eucalytus jacksonii*) that had symptoms of MLD, were also opportunistically collected and the causative organism isolated.

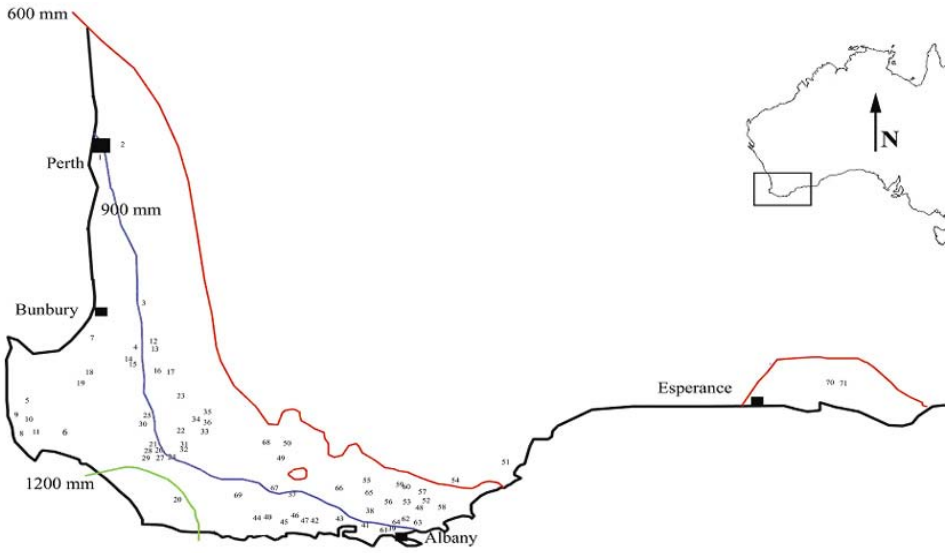


Figure 4.2 Locations of *Eucalyptus globulus* plantations, *Eucalyptus diversicolor* and *Eucalyptus marginata* forest sites surveyed for incidence and severity of MLD in south-western Australia. *Eucalyptus globulus* plantations, 1, 3–8, 20–25, 37–43, 48–58, 70–71; *Eucalyptus diversicolor* forest, 9–11, 26–30, 44–45, 50–61; and *Eucalyptus marginata* forest, 2, 12–19, 31–36, 46–47, 62–69. Mean annual rainfall isohyets 600, 900, 1200 mm indicated.

4.2.3 Survey and transects

At each *E. globulus* location two transects were made. Transects were randomly chosen at each plantation. Each transect was initiated at the fifth tree in from the plantation boundary, in order to minimise potential perimeter effects. Every 10th tree was rated and sampled from each transect of 50 trees.

At each native forest location, a single transect was made after walking 5m into the canopy area. Each transect was made into the forest, perpendicular to the stand boundary. Every ten metres, the nearest sapling was rated and diseased leaf material collected, until a total of ten trees were assessed for each forestry species present.

For both plantation and native forests, trees were rated and sampled as follows. Incidence of MLD was recorded for the whole tree as either present or absent. Severity of MLD was assessed on a randomly chosen branch at a height of 1.5 m as described in Chapter 2.1. Where present, five diseased juvenile phase and five diseased adult phase leaves were collected randomly from each sample tree, placed in plastic bags, then returned to the laboratory where they were stored at 4 °C until being processed (4.2.4).

At all sites, general observations were made on the overall health of the stand. These included symptoms of nutritional deficiency and insect attack as well as other disease symptoms such as stem canker and *Harknessia* leaf disease.

4.2.4 Isolation and identification

Isolation and identification of *Mycosphaerella* species were made from lesions that were dissected from the leaves collected from each tree sampled. Single ascospore isolations were made onto 2% MEA according to a method modified from Crous (1998). Briefly, lesions were soaked for 2 h in sterile water, dried with paper towel, and attached with double sided adhesive tape to the lid of Petri-plates containing 2% MEA. The leaf surface upon which the ascomata occurred was directed towards the media. Where ascomata were amphigenous, then the lesion was cut in two and the adaxial side of one half and the abaxial side of the other half of the lesion, was directed towards the medium. The plates were inverted in order to reduce contamination from phyllosphere fungi that are unable to discharge spores forcibly, and incubated for 24–48 h at 24 ° in the dark. Ascospore germination patterns were recorded, single ascospore cultures established and fungi identified as described in Chapter 3. The species present on each of five diseased juvenile (and where present, five adult) leaves were recorded. Also, the percentages of necrotic area due to each species, was recorded for the leaves collected. This assessment was made according to the lesion-type's described in the Key in Chapter 3 and then compared to the species identification based on the complete set of morphological data.

4.2.5 Statistical analysis

Prior to analysis, data for parametric tests were screened for assumptions of homoscedasticity, normality, non-correlations of means and variances and presence of outliers (Tabachnick & Fidell 1996). Where data did not fit these assumptions, they were transformed using accepted functions (Tabachnick & Fidell 1996). Where the transformed data did not fit the above assumptions then both parametric and non-parametric statistical analyses were conducted. Host range data (4.3.1) was analysed via a χ^2 statistic; leaf phase and leaf surface data (4.3.2) via ANOVA and the Mann-Whitney U test (as some assumptions required for ANOVA were not met); geographic difference in disease intensity (4.3.3) was compared via ANOVA and Kruskal-Wallis ANOVA by ranks (as some assumptions of ANOVA were violated).

4.3 Results

4.3.1 Host range of MLD species on forestry eucalypts in WA

MLD occurred on *E. diversicolor, E. globulus* and *E. marginata*, but not on *C. calophylla.* Of 840 trees assessed, MLD was present on 99% of *E. globulus*, 67 % of *E. diversicolor,* 35 % of *E. marginata* and 0 % of *C. calophylla* trees (Table 4.1). Of all sites examined, 100% of *E. globulus* plantations, 100% of *E. diversicolor* locations and 80% of *E. marginata* locations were affected by MLD (Table 4.1). The level of disease incidence is significantly different between these host species ($p<0.01$, Table 4.1).

Table 4.1 Proportion of eucalypt species locations at which MLD was present and statistical significance of difference. (Pearson Chi-square: 508, df=3, p<0.001)

Host species	% of sites affected	% of trees affected
Corymbia calophylla	0	0
Eucalyptus diversicolor	100	67
Eucalyptus globulus	100	99
Eucalyptus marginata	80	35

Two previously undescribed *Mycosphaerella* species were isolated in the course of the current Chapter. Full taxonomic descriptions of these species are not included in this thesis, but they were provisionally designated as *Mycosphaerella kalima* nom. prov. and *Mycosphaerella kempii* nom. prov. The first of these, *M. kalima* nom. prov. was found at one location near Esperance and is morphologically similar to *M. marksii* in most respects except that it forms a flatter mycelial growth in culture and a brown pigment on MEA. This has since been sequenced and found conspecific with *M.* marksii. The second of these, *M. kempii* nom. prov. is similar to *M. mexicana,* except that it forms larger ascospores and a very strong red diffusible pigment on MEA. Further work is required on the taxonomy of *M. kempii* nom. prov.

M. cryptica was isolated from *E. diversicolor, E. globulus, E. marginata* and *E. jacksonia*. The remaining 10 *Mycosphaerella* species (including the two provisionally new species) were only isolated from *E. globulus.*

4.3.2 Leaf phase preference of MLD species on *E. globulus*

ANOVA comparison of mean proportion of species occurrence on adult or juvenile foliage indicates that *M. cryptica* occurred significantly ($p<0.01$) more often on diseased adult leaves than on diseased juvenile leaves (Figure 4.3). *M. nubilosa, M. parva* and *M. marksii* occurred significantly ($p<0.01$) more often on

juvenile than on adult foliage (Figure 4.3). There was no significant difference in the leaf phase preference of the remaining species. *M. cryptica* was isolated from 38% of trees with diseased adult canopy and 15 % of trees with diseased juvenile foliage, whereas, *M. nubilosa* was isolated from less than 1% of diseased adult foliar samples and 20% of diseased juvenile canopy trees. Both *M. marksii* and *M. parva* were isolated from 9% of diseased juvenile foliage trees, and from 3% and less than 1%, respectively, of diseased adult foliage trees (Figure 4.3). More than one species was frequently isolated from a single lesion, leaf and diseased canopy area of a tree. In some instances no *Mycosphaerella* species were isolated from the diseased canopy of a tree.

Non-parametric comparisons of leaf phase occurrence (Table 4.2) supported the parametric statistical findings. The Mann-Whitney U test indicated that *M. cryptica* occurred significantly ($p<0.01$) more frequently on diseased adult than on diseased juvenile foliage (Table 4.2), whereas, *M. nubilosa* occurred significantly more often on juvenile foliage ($p<0.01$).

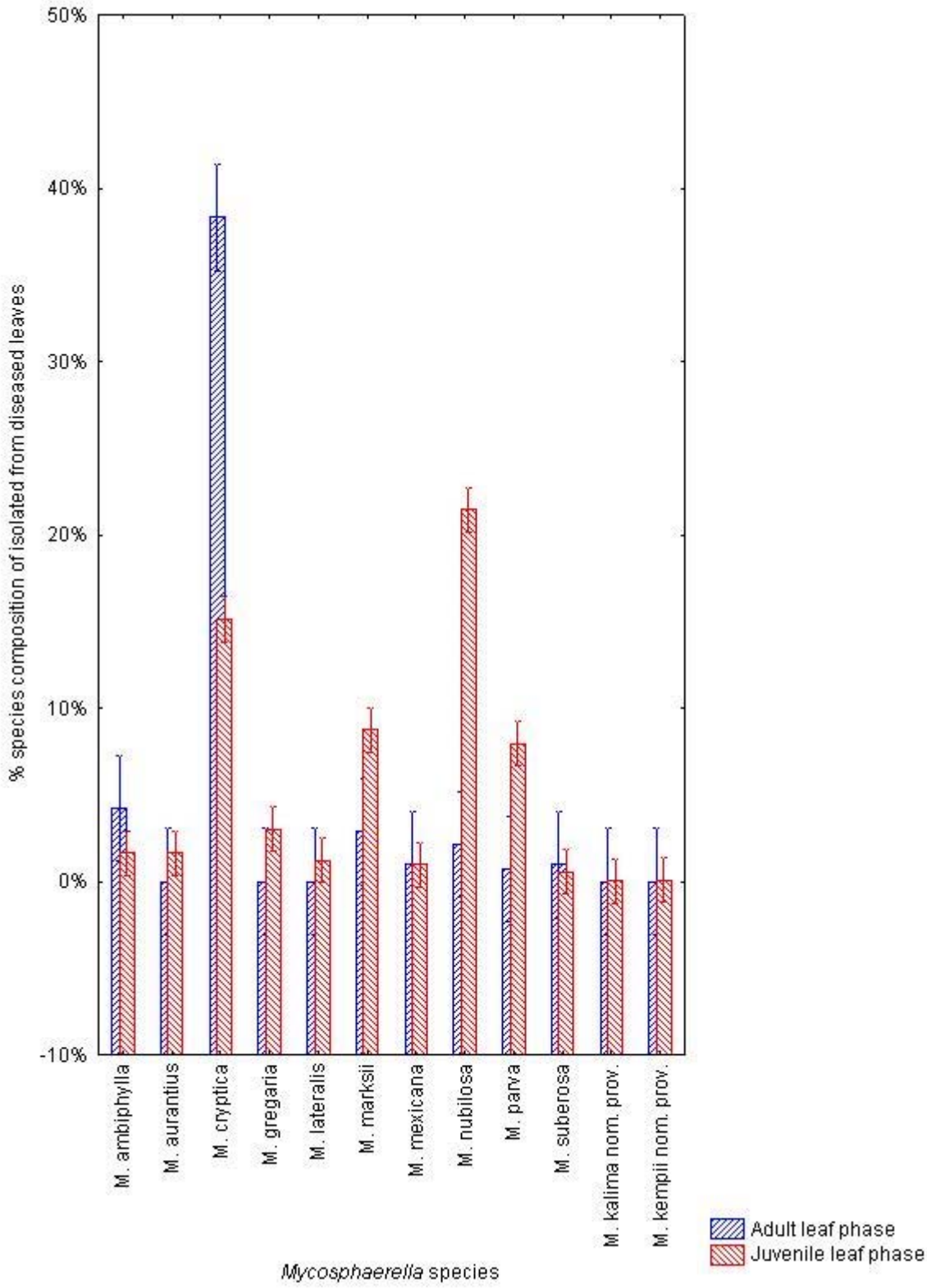


Figure 4.3 Comparison of the leaf phase preference amongst 12 *Mycosphaerella* species on the basis of the mean percentage of diseased leaves from which each species was isolated. $P<0.01$. Vertical bars denote 0.95 confidence intervals of the mean.

Table 4.2 Mann-Whitney U Test comparing occurrence of different *Mycosphaerella* species on adult and juvenile leaf phase of diseased *Eucalyptus globulus.*

Species	Adult Rank Sum	Juvenile Rank Sum	U	Z	p-level	Valid N
M. ambiphylla	25404	133925	18965	1.00	0.31	85
M. aurantia	22865	136465	19210	-0.83	0.41	85
M. cryptica	32242	127087	11647	6.18	**0.00**	84
M. gregaria	21966	137364	18396	-1.28	0.20	84
M. lateralis	22932	136398	19362	-0.58	0.56	84
M. marksii	21291	138039	17721	-1.77	0.077	84
M. mexicana	23542	135787	19972	-0.14	0.9	84
M. nubilosa	15927	143402	12357	-5.66	**0.00**	84
M. parva	20133	139197	16563	-2.61	**0.009**	84
M. suberosa	23834	135495	20055	0.07	0.94	84
M. kalima	23688	135642	20118	-0.03	0.97	84
M. kempii	23688	135642	20118	-0.03	0.97	84

Tests in bold are significant at p < 0.01

ANOVA comparison of mean rank of species occurrence on abaxial or adaxial foliage indicates that *M. marksii* ascomata occurred significantly ($p<0.01$) more often on the adaxial than the abaxial surface of diseased leaves (Figure 4.4). *M. nubilosa* ascomata occurred significantly ($p< 0.01$) more often on the abaxial than the adaxial surface of diseased foliage (Figure 4.4). There was no significant difference in the leaf surface occurrence of the remaining species. *M. cryptica* was isolated from the adaxial leaf surface for 18% of diseased trees and similarly from 18% of trees from the abaxial leaf surface. *M. nubilosa* was the species most frequently isolated from the abaxial leaf surface (37% of trees), and aside from *M. cryptica, M. marksii* was most frequently isolated species from the adaxial leaf surface (15% of trees).

Non-parametric comparisons of ascomata leaf surface occurrence (Tables 4.4) supported the parametric statistical findings. The Mann-Whitney U test indicated that *M. marksii* ascomata occurred significantly ($p<0.01$) more often on the adaxial surface of diseased leaves (Table 4.3), whereas, *M. nubilosa* ascomata occurred significantly ($p<0.01$) more often on the abaxial surface of diseased foliage (Table 4.3).

Table 4.3 Mann-Whitney U Test comparing the rank some of different *Mycosphaerella* species on the abaxial and adaxial surface of diseased *Eucalyptus globulus* leaves

Species	Adult Rank Sum	Juvenile Rank Sum	U	Z	p-level	Valid N
M. ambiphylla	79514	79816	39611	-0.08	0.94	282
M. aurantia	80360	78970	39067	0.36	0.72	282
M. cryptica	79132	80197	39229	-0.27	0.78	282
M. gregaria	79987	79343	39440	0.17	0.87	282
M. lateralis	79231	80099	39328	-0.22	0.82	282
M. marksii	92834	66496	26593	6.80	**0.00**	282
M. mexicana	79947	79382	39479	0.14	0.88	282
M. nubilosa	52474	106856	12571	-14.000	**0.00**	282
M. parva	78046	81284	38143	-0.84	0.40	282
M. suberosa	79527	79803	39624	-0.07	0.94	282
M. kalima	79806	79524	39621	0.07	0.94	282
M. kempii	79806	79524	39621	0.07	0.94	282

Tests marked in bold are significant at p <.01

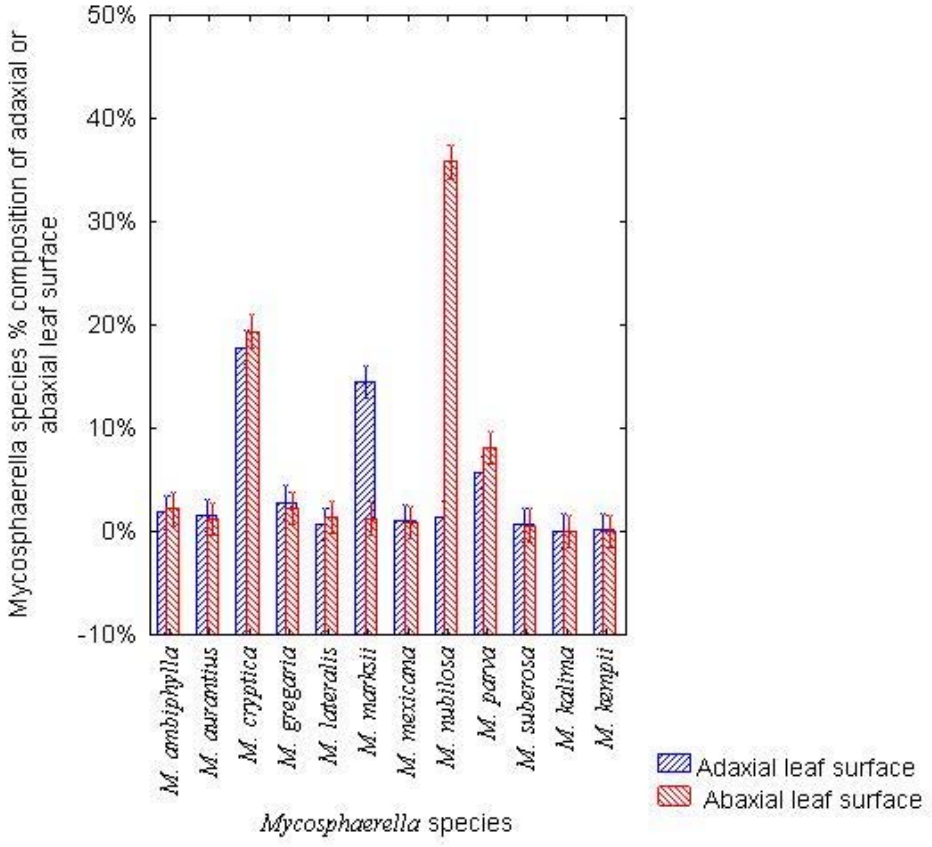


Figure 4.4 Comparison of the leaf surface occurrence of ascomata, amongst 12 *Mycosphaerella* species on the basis of the mean proportion of diseased leaves from which each species was isolated.

4.3.3 Geographic distribution of MLD impact and species composition of *E. globulus* plantations in south-western Australia

ANOVA amongst *E. globulus* trees indicated that there were significant differences in the mean severity ranking (log_{10} transformed) amongst plantations ($p<0.01$). Sites: Gerner, Henwood, Thomas, Boorara and Darling View were the most severely affected, with mean MLD ratings of 8, 8, 7.9, 7 and 5.5 respectively (Figure 4.5). At each of these sites MLD frequently caused 100 % defoliation of the

trees juvenile foliage. Sites: Hamilton, Wren and Lamberti were the least affected, all having a mean MLD rating of 1 (Figure 4.5). Non parametric analysis (Kruskal-Wallis ANOVA by ranks) supported the parametric analysis in showing a significant ($p<0.01$) difference amongst sites in terms of MLD severity (Table 4.4). Gerner, Henwood and Thomas were the highest ranking (most severely effected) and Lamberti, Wren and Hamilton the lowest ranking (least severely effected) plantations.

Table 4.4 Kruskal-Wallis ANOVA by ranks comparing occurrence of different *Mycosphaerella* species on the abaxial and adaxial surface of diseased *Eucalyptus globulus* leaves (Kruskal-Wallis test: H (29, N= 300) = 228.8; $p<0.0005$).

Plantation	Valid N	Sum of Ranks
Gerner	10	2825
Henwood	10	2825
Thomas	10	2805
Boorrarra	10	2695.5
Darling View	10	2338
Chelgiup	10	2297
Napier Creek	10	2228
Summerlea	10	1960.5
Channeybearup	10	1858.5
Range-Montanna	10	1763
Kemp	10	1652.5
Warrenella	10	1518
Dudijup	10	1518
Cobertup	10	1518
Chips	10	1465
Detri	10	1448.5
Kelora	10	1448.5
Callistemon	10	1417
StWherberg	10	1342.5
Reid	10	1256.5
Jindalee	10	1139
Kalima	10	1072
Woodrakkarra	10	1069.5
Shedley	10	1000
Murdoch	10	727
Bentink	10	528.5
Blight	10	459
Lamberti	10	325
Wren	10	325
Hamilton	10	325

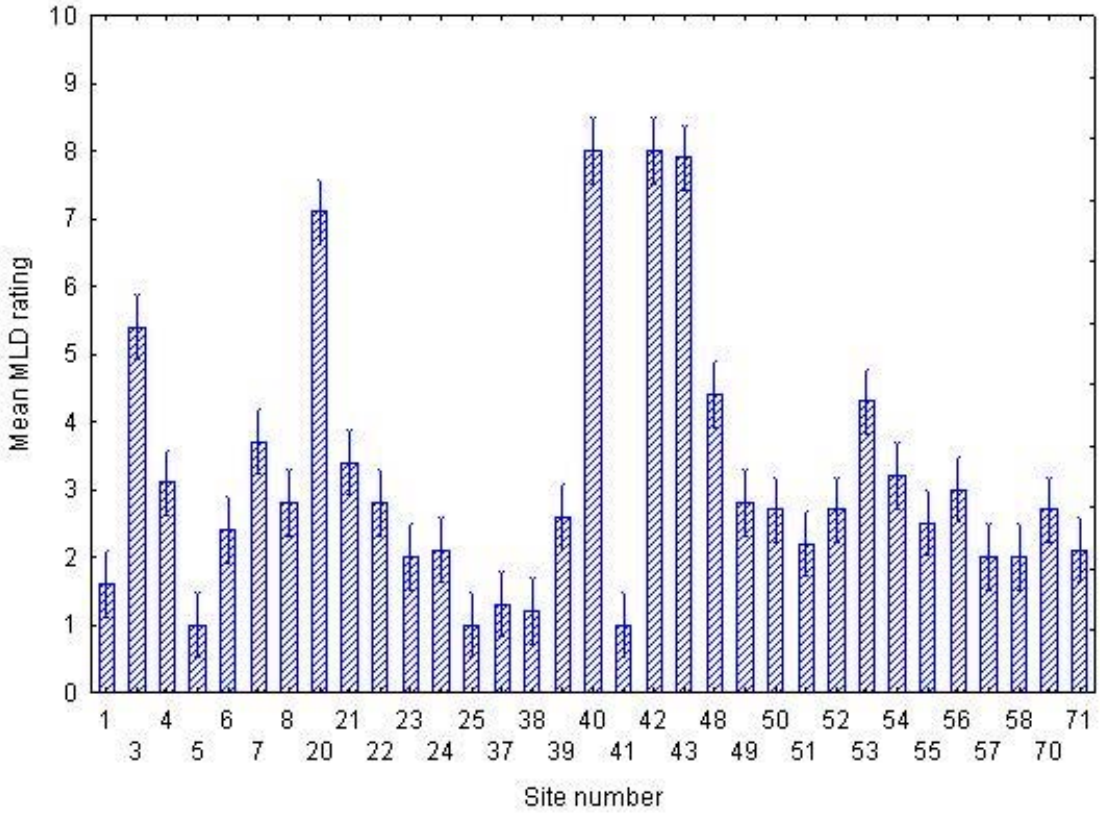


Figure 4.5 Comparison of mean Mycosphaerella leaf disease severity ratings amongst *Eucalyptus globulus* plantations in south-western Australia. Non-transformed data. Vertical bars denote 0.95 confidence intervals of the mean. $F(32, 297)=42$, $p<0.05$.

On the basis of mean severity rankings (Figure 4.5) each plantation was assigned a category of severe (>5), moderate (2–5) or low (<2) MLD damage and plotted on a map along with rainfall and evaporative demand (Figure 4.6). The more severely affected plantations are clustered in the Denmark to Manjimup region, in the over 1000 mm rainfall area.

The distribution of each of the *Mycosphaerella* species isolated in south-western Australia was mapped (Figure 4.7). The geographic impact of species in decreasing order of site frequency (in parentheses) was: *M. cryptica* (43), *M. nubilosa* (32), *M. parva* (23), *M. marksii* (20), *M. gregaria* (8), *M. aurantia* (6), *M. mexicana* (5), *M. suberosa* (4), *M. lateralis* (3), *M. ambiphylla* (1), *M. kalima* nom. prov. (1), *M. kempii* nom. prov. (1). However, when *E. globulus* plantations only are compared, then *M. nubilosa* is the most widespread *Mycosphaerella* species.

Other leaf disease fungi isolated in the current study were *Aulographina eucalypti*, *Coniochaeta* sp., *Harknessia* sp., *Microthyrium eucalypticola* and a *Vermisporium* sp. These were minor and caused disease at only a limited number of locations.

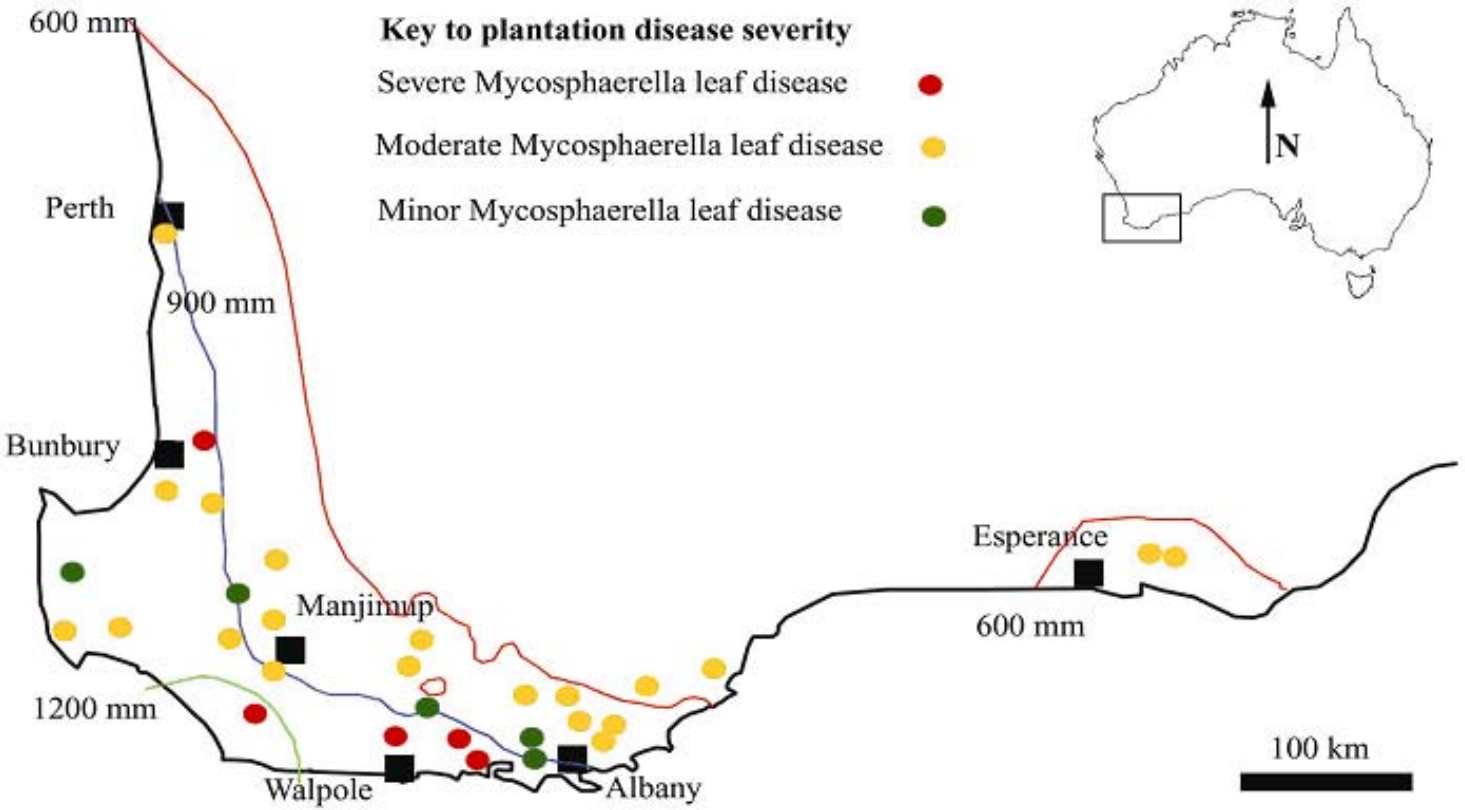


Figure 4.6 Geographic variation in the severity of Mycosphaerella leaf disease on *Eucalyptus globulus* plantations in south-western Australia.

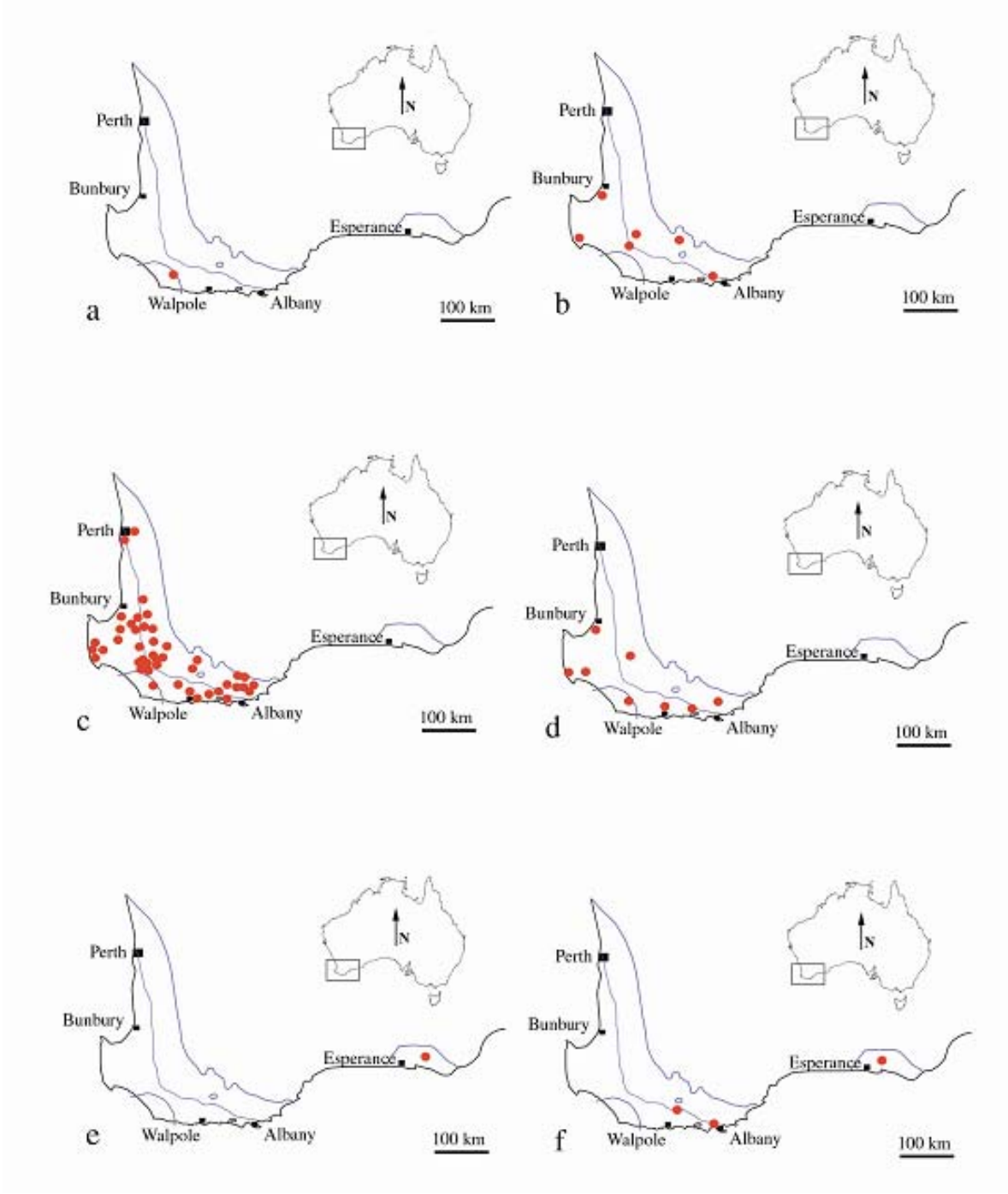
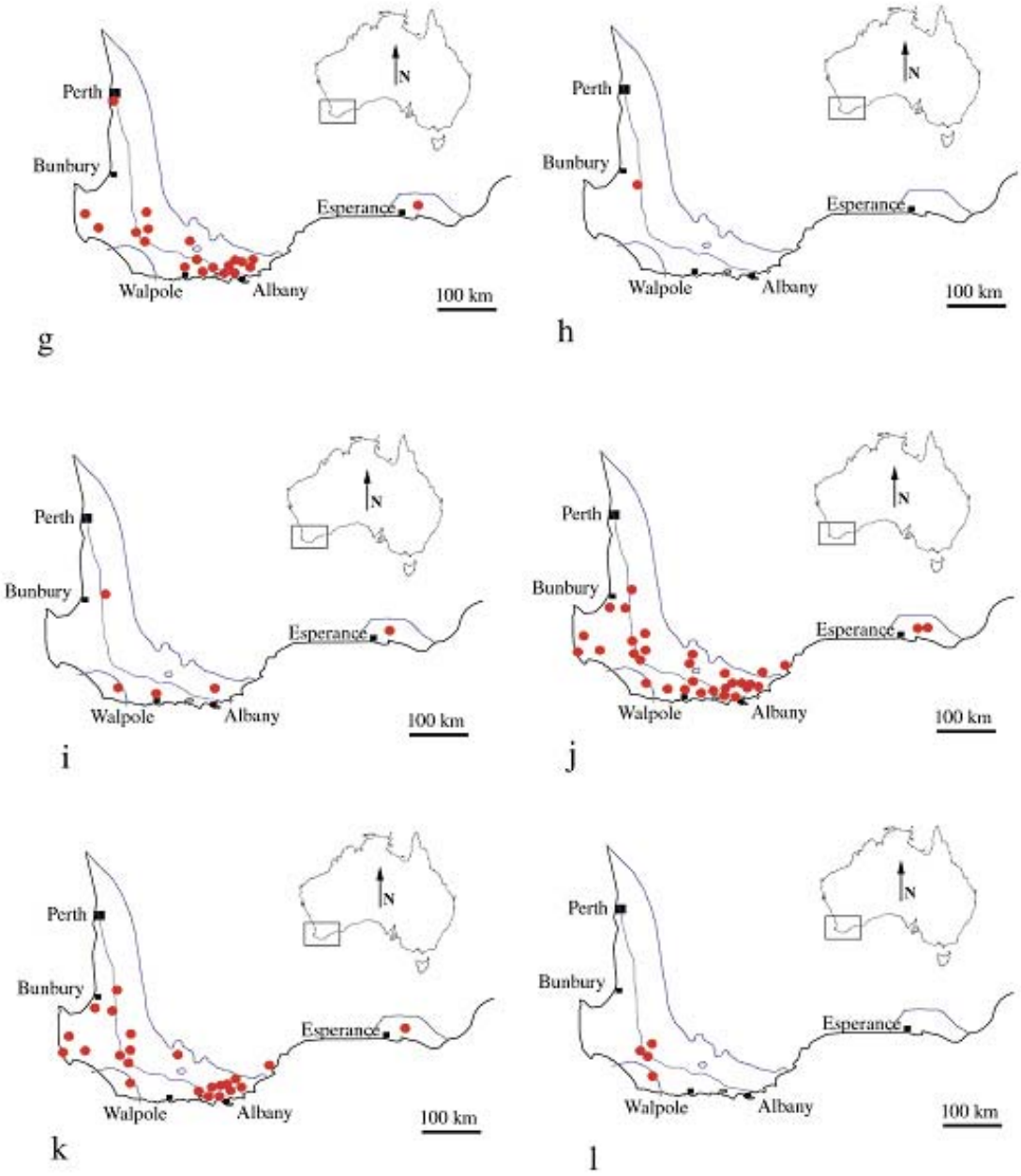


Figure 4.7 Distribution of *Mycosphaerella* species isolated from eucalypts in south-western Australia. *M. ambiphylla* (a); *M. aurantia* (b); *M. cryptica* (c); *M. gregaria* (d); *M. kalima* nom. prov. (e); *M. lateralis* (f); continued overleaf…



...Figure 4.7 (continued) Distribution of *Mycosphaerella* species isolated from eucalypts in south-western Australia. *M. marksii* (g); *M. kempii* nom. prov. (h); *M. mexicana* (i); *M. nubilosa* (j); *M. parva* (k); *M. suberosa* (l).

4.4 Discussion

This study has clearly identified that the most widespread and serious cause of MLD in south-western Australia is *M. cryptica.* This species occurred on three of the four important forestry eucalypt species in this region. In terms of the plantation estate of *E. globulus,* however, *M. nubilosa* was the most widespread pathogen. The current study indicates that MLD on *E. globulus* is a complex of several different species, whereas, on *E. diversicolor* and *E. marginata* it was caused exclusively by *M. cryptica.*

For the first time, the leaf phase and leaf surface occurrence of those *Mycosphaerella* species present in south-western Australia, has been quantitatively determined. *Mycosphaerella cryptica* occurred on both juvenile and adult phase foliage of *E. globulus.* This species was the major contributor to disease on adult leaves of *E. globulus*, whereas *M. nubilosa* was responsible for the majority of disease on juvenile foliage. *M. nubilosa,* previously only recorded on juvenile foliage (Park *et al.* 2000, Park & Keane 1982a), was isolated from adult foliage on rare occasions in the current study. The other species that contributed to disease on adult leaves were *M. marksii, M. ambiphylla, M. mexicana, M. parva,* and *M. suberosa*. However, these were minor in comparison to *M. cryptica*. All species were present on juvenile phase leaves.

All except two species were equally likely to be isolated from the abaxial or adaxial leaf surface. *M. marksii* was isolated significantly more often from the adaxial surface and *M. nubilosa* from the abaxial surface. Therefore, the current study has quantitatively confirmed observations from previous studies (Park 1988b, Park *et al.* 2000). Although Park (1984) and Park & Keane (1982a) indicated that *M. nubilosa* ascomata form predominantly on the abaxial leaf surface, this observation has not been quantified or confirmed as statistically significant till now.

Two provisionally new species, *M. kalima* nom. prov and *M. kempii* nom. prov., were isolated in the current work. These species occurred at only one location each

and did not appear to be significant contributors to MLD. Sequencing of the rDNA ITS region of *Mycosphaerella kalima* nom. prov has since shown this species to be *M. marksii.* Further work is required on the taxonomy of *M. kempii* nom. prov. to confirm its status as new a species.

MLD is widespread in *E. globulus* plantations throughout south-western Australia. The level of disease impact varies considerably between plantations, from a mean severity of 1.5% necrotic leaf area up to 100% defoliation of juvenile foliage across a plantation. Geographically, the most severely diseased plantations were clustered in the region between Manjimup and Walpole. It is likely that this is due to the higher level of rainfall of the region. These rainfall conditions are more conducive for leaf infection than the drier regions of the northern and the eastern range of the plantation estate. Also, the inoculum levels of *M. cryptica* are higher in the surrounding *E. diversicolor* forests of this region, than other more northern and eastern areas of *E. marginata* forest.

Further work on the biogeography of *Mycosphaerella* in south-western Australia should compare changes in species composition with season. Work by Carnegie and Ades (2002) found a change in the relative proportions of *M. cryptica* and *M. nubilosa* between spring summer and autumn in eastern Australia. Assessments late in the season may need to take account of defoliated leaves. Plantations that are more affected by more pathogenic species may be heavily defoliated than plantations with less pathogenic species.

Chapter 5

Molecular taxonomy of *Mycosphaerella* species on eucalypts

Chapter 5 Molecular taxonomy of *Mycosphaerella* species on eucalypts

5.1 Introduction

Historically, the taxonomy of *Mycosphaerella* has been based on a combination of morphological traits and on the underlying assumption of restricted host ranges of *Mycosphaerella* species (Barr 1972; Barr 1983; Sivanesan & Shivas 2002).

There are more than 1800 species of *Mycosphaerella* described (Corlett 1991) from several hundred different host species (Corlett 1991) and the taxonomy of this genus is therefore largely host based. Although it is assumed that *Mycosphaerella* species are very host specific few cross-inoculation experiments have been conducted to verify this (Crous 1998, Sivanesan & Shivas 2002). Thus, the size of this genus may be artificially inflated and some species described separately from different hosts, may in fact be conspecific. Sequencing of the ITS regions of the rDNA is emerging as a reliable tool for determining phylogenetic relatedness and consequently for differentiating species (Crous *et al.* 2000, Crous *et al.* 2001a, Crous *et al.* 1999, Crous, Kang & Braun 2001b, Foster *et al.* 1993, Myburg, Wingfield & Wingfield 1999). The ITS rDNA region of some of the more important *Mycosphaerella* species have been sequenced (Crous *et al.* 2001a, Crous *et al.* 2001b, Crous & Mourichon 2002, Stewart *et al.* 1999), and these data used to test hypotheses concerning the phylogeny of *Mycosphaerella*. There is a need for more putative species to be sequenced in order to determine whether each of the 1800 described species of *Mycosphaerella* are in fact distinct species, and in order to resolve phylogenetic questions.

The aims of the present study were to:

1) determine the ITS sequence for all *Mycosphaerella* species occurring on eucalypts in south-western Australia and thereby verify the morphology based identification;

2) compare the ITS sequences of *Mycosphaerella* species from eucalypts in south-western Australia to all other available sequences of *Mycosphaerella* species from eucalypts in order to determine their intra and inter-specific variation at this locus; and

3) investigate broader phylogenetic concepts: a) that host is a predictor of phylogenetic closeness; b) that anamorph state reflects phylogenetic closeness; and c) that speciation is occurring in geographically separated populations of some *Mycosphaerella* species.

5.2 Materials and Methods

5.2.1 Experimental design

Single spore isolates of *Mycosphaerella* were obtained from diseased eucalypts throughout Australia. These were identified morphologically. The DNA from at least three randomly selected isolates of each morphological species (Table 5.1) was extracted and the ITS region amplified and sequenced. These sequences were aligned and compared amongst each other to measure inter and intra-species variation. The sequences were further compared with other *Mycosphaerella* species on databases obtained from BLAST search (Table 5.1). Phylogenetic trees were constructed with the software package Phylogenetic Analysis using Parsimony (PAUP, v. 4.1b; Swofford 1998).

5.2.2 Isolation, identification, culture and harvest of fungi

Single spore isolates of *Mycosphaerella* species were obtained from lesions as described in Chapter 3.2.2. These were identified on morphological characters (Chapter 3.3) and maintained on 2% MEA. Multiple hyphal fragments of three fungal isolates from each morphological species (Table 5.1), were inoculated into 80ml of V-8 juice broth (Stewart *et al.* 1999) (100ml filtered V-8 juice (Campbell's), 5g malt extract, 1g KH_2PO_4, 1g K_2HPO_4; made up to 1000ml with distilled water and adjusted to pH 5.5). Flasks were incubated for 14-21 days at 21 °C after which the mycelia were harvested by filtering through Whatmans (number 5) filter paper. Mycelia were transferred with a sterile scraper to petri plate, frozen at –70 ° for 1 h and then freeze-dried. The lyophilised mycelia were stored in sterile 1.5 ml microfuge tubes (Eppendorph) at –20 ° until needed for DNA extraction.

Table 5.1 Cultures and sequence accession numbers of sequences compared in the current study. Isolates sequenced as part of the current study indicated by an asterisk (*). CBS = Culture collection Centraalbureau voor Schimmelcultures. CMW = culture collection Mike Wingfield, University of Pretoria, South Africa; R & '98' prefix = Culture collection Aaron Maxwell, Murdoch University, Australia; STEU = Culture collection Stellenbosch University, South Africa.

Species	Isolate number	GenBank accession	Host	Origin	Anamorph
M. africana	STEU794	AF173314	*Eucalyptus*	RSA	Unknown
M. allii-cepae		AB026162	*Allium cepa*	?	*Cladosporium*
M. ambiphylla	*R210		*E. globulus*	SW Aust	*Phaeophleospora*
M. ambiphylla	*R221		*E. globulus*	SW Aust	*Phaeophleospora*
M. ambiphylla	*R222		*E. globulus*	SW Aust	*Phaeophleospora*
M. ambiphylla	*R211		*E. globulus*	SW Aust	*Phaeophleospora*
M. arachidis		AF297224	*Arachis hypogaea*	?	*Cercospora*
M. arbuticola	CBS355.86	AF362063	*Arbutus meziesii*	?	Unknown
M. asterinoides		AF222850	Solonaceae	?	Unknown
M. aurantia	*R151		*E. globulus*	SW Aust	Unknown
M. aurantia	*R152		*E. globulus*	SW Aust	Unknown
M. berberidis	CBS342.52	AF362062	*Berberis* sp.	Pakistan	Unknown
M. bixae	STEU2554	AF362056	?	?	Unknown
M. brassicicola	CBS228.32	AF362052	Brassicaceae	?	*Asteromella*
M. brassicicola	IPO95510	AF297223	*Brassica olarecea*	Neth.	*Asteromella*
M. citri		AF181703	*Citrus sinensis*	?	*Stenella*
M. colombiensis	STEU1106	AF309612	*E. urophylla*	Colombia	*Pseudocercospora*
M. confusa	CBS256.35	AF362058	?	?	*Pseudocercospora*
M. cruenta	CBS462.75	AF362065	*Vigna* sp.	?	*Pseudocercospora*
M. cryptica	*R089		*E. globulus*	SW Aust	*Colletogloeopsis*
M. cryptica	*R090		*E. globulus*	SW Aust	*Colletogloeopsis*
M. cryptica	*R091		*E. globulus*	SW Aust	*Colletogloeopsis*
M. cryptica	*R101		*E. globulus*	SW Aust	*Colletogloeopsis*
M. cryptica	*R110		*E. globulus*	SW Aust	*Colletogloeopsis*

…Table 5.1 Cultures and sequence accession numbers of sequences compared in the current study.

Species	Isolate number	GenBank accession	Host	Origin	Anamorph
M. cryptica	*R114		*E. diversicolor*	SW Aust	*Colletogloeopsis*
M. cryptica	*R115		*E. globulus*	SW Aust	*Colletogloeopsis*
M. cryptica	*R118		*E. delegatensis*	Vic Aust	*Colletogloeopsis*
M. cryptica	*98125		*E. grandis x*	Qld, Aust	*Colletogloeopsis*
M. cryptica	*98191		*E. globulus*	SW Aust	*Colletogloeopsis*
M. cryptica		AY045494	*Eucalyptus*	NZ	*Colletogloeopsis*
M. cryptica		AY045495	*Eucalyptus*	SE Aust	*Colletogloeopsis*
M. cryptica		AY045496	*Eucalyptus*	Tas, Aust	*Colletogloeopsis*
M. cryptica		AY045498	*Eucalyptus*	SE Aust	*Colletogloeopsis*
M. cryptica	STEU936	AF309585	*Eucalyptus*	Australia	*Colletogloeopsis*
M. cryptica	CMW2732	AF309622	*Eucalyptus*	Chile	*Colletogloeopsis*
M. cryptica	CMW3279	AF309623	*Eucalyptus*	SE Aust	*Colletogloeopsis*
M. crystallina		AF222839	*Eucalyptus*	?	*Pseudocercospora*
M. dearnessii		AF260817	*Pinus* sp	Nth USA	*Lecanosticta*
M. dearnessii	STEU3391	AF362070	*Pinus* sp	?	*Lecanosticta*
M. ellipsoidea	STEU1225	AF173303	*Eucalyptus*	?	*Uwebraunia*
M. fijiensis		AF181705	*Musas* sp	Fiji	*Paracercopora*
M. flexuosa	CMW5224	AF309603	*Eucalyptus*	Colombia	*Uwebraunia*
M. fori	CMW9095	AF468869	*Eucalyptus*	RSA	*Pseudocercospora*
M. frageriae	STEU656	AF173312	*Fragaria vesca*	?	*Ramularia*
M. graminicola	STEU658	AF362068	*Triticum*	?	*Septoria*
M. graminicola		AJ300330	*Triticum*	?	*Septoria*
M. graminicola		MGU77363	*Triticum*	?	*Septoria*
M. grandis		AY045516	*Eucalyptus*	Australia	Unknown
M. gregaria	*R237		*E. globulus*	SW Aust	Unknown
M. gregaria	*R240		*E. globulus*	SW Aust	Unknown
M. heimii		AF222841	*Eucalyptus*	?	*Pseudocercospora*
M. heimioides		AF222842	*Eucalyptus*	?	*Pseudocercospora*
M. intermedia		AY045517	*Eucalyptus*	NZ	Unknown
M. intermedia		AY045518	*Eucalyptus*	NZ	Unknown
M. irregulariramosa		AF222843	*Eucalyptus*	?	*Pseudocercospora*
M. juvenis	CMW4937	AF309604	*Eucalyptus*	RSA	*Uwebraunia*
M. juvenis	STEU1005	AF173299	*Eucalyptus*	RSA	*Uwebraunia*
M. keniensis	STEU 1084	AF173300	*E. grandis*	Kenya	Unknown
M. latebrosa	CBS183.97	AF362051	*Acer*	?	Unknown
M. lateralis	*R257		*E. globulus*	SW Aust	*Dissoconium*
M. lateralis	*R258		*E. globulus*	SW Aust	*Dissoconium*
M. lateralis	*98133		*E. maidenii*	Qld, Aust	*Dissoconium*
M. lateralis	*98148		*E. globulus*	SW Aust	*Dissoconium*
M. lateralis	*98149		*E. globulus*	SW Aust	*Dissoconium*
M. lateralis	*98163		*E. maidenii*	Qld, Aust	*Dissoconium*
M. lateralis	STEU1532		*?*	RSA	*Dissoconium*
M. lateralis	STEU1535	AF173309	*E. grandis x saligna*	RSA	*Dissoconium*
M. lateralis	STEU825	AF309624	*E. grandis x saligna*	RSA	*Dissoconium*
M. lupini	STEU1661	AF362050	*Humulus lupulus*	?	Unknown
M. macrospora		AF297231	*Iris gernanica*	Nth USA	*Cladosporium*
M. marasasii	STEU348	AF309591	*Syzygium*	RSA	*Stenella*

…Table 5.1 Cultures and sequence accession numbers of sequences compared in the current study.

Species	Isolate number	GenBank accession	Host	Origin	Anamorph
M. marksii	STEU935	AF173316	*Eucalyptus*	?	Unknown
M. marksii	STEU982		*Eucalyptus*	?	Unknown
M. marksii		AF309588	*Eucalyptus*	?	Unknown
M. marksii		AF468873	*Eucalyptus*	?	Unknown
M. marksii	*R234		*E. globulus*	SW Aust	Unknown
M. marksii	*R242		*E. globulus*	SW Aust	Unknown
M. marksii	*R243		*E. globulus*	SW Aust	Unknown
M. marksii	*R247		*E. globulus*	SW Aust	Unknown
M. mexicana	*R215		*E. globulus*	SW Aust	Unknown
M. mexicana	*R216		*E. globulus*	SW Aust	Unknown
M. mexicana	*R216Y		*E. globulus*	SW Aust	Unknown
M. mexicana	*SJ5		*E. globulus*	SW Aust	Unknown
M. molleriana	STEU784	AF309619	*Eucalyptus*	USA	*Colletogloeopsis*
M. molleriana	STEU1214		*Eucalyptus*		*Colletogloeopsis*
M. musicola		AF181706	*Musa*	?	*Pseudocercospora*
M. nubilosa	*R001		*E. globulus*	SW Aust	Unknown
M. nubilosa		AY045506	*Eucalyptus*	SE Aust	Unknown
M. nubilosa		AY045507	*Eucalyptus*	SE Aust	Unknown
M. nubilosa		AY045508	*Eucalyptus*	SE Aust	Unknown
M. nubilosa		AY045509	*Eucalyptus*	SE Aust	Unknown
M. nubilosa	*R002		*E. globulus*	SW Aust	Unknown
M. nubilosa	*R004		*E. globulus*	SW Aust	Unknown
M. nubilosa	*R051		*E. globulus*	SE Aust	Unknown
M. nubilosa	*R057		*E. globulus*	SE Aust	Unknown
M. nubilosa	*98-099		*E. globulus*	SW Aust	Unknown
M. nubilosa	*98-101		*E. globulus*	SW Aust	Unknown
M. nubilosa	CMW6211	AF449094	*E. globulus*	SE Aust	Unknown
M. nubilosa	CMW6210	AF449095	*E. globulus*	SE Aust	Unknown
M. nubilosa	CMW9000	AF449096	*E. nitens*	RSA	Unknown
M. nubilosa	CMW9001	AF449097	*E. nitens*	RSA	Unknown
M. nubilosa	CMW9002	AF449098	*E. nitens*	RSA	Unknown
M. nubilosa	CMW9003	AF449099	*E. nitens*	RSA	Unknown
M. nubilosa	CMW3282	AF309618	*E. globulus*	SE Aust	Unknown
M. nubilosa	106NZ	AY045505	*Eucalyptus*	NZ	Unknown
M. parkii	STEU353	AF173311	*E. saligna*	Brazil	*Stenella*
M. parva	*R248		*E. globulus*	SW Aust	Unknown
M. parva	*R249		*E. globulus*	SW Aust	Unknown
M. parva	*R250		*E. globulus*	SW Aust	Unknown
M. parva	*R251		*E. globulus*	SW Aust	Unknown
M. pini		AF013227	*Pinus nigra*	Nth USA	*Dothistroma*
M. populorum		AF243392	*Populus balsonifera*		Unknown

…Table 5.1 Cultures and sequence accession numbers of sequences compared in the current study.

Species	Isolate number	GenBank accession	Host	Origin	Anamorph
M. recutita	CBS287.49	AF362059	*Festuca rubra*		Unknown
M. rubella	CBS288.49	AF362060	*Angellica sylvestris*		Unknown
M. suberosa		AY045503	*Eucalyptus*		Unknown
M. suttoniae	STEU1346	AF309621	*Eucalyptus*	Indonesia	*Phaeophleospora*
M. tasmaniensis		AF310107	*E. nitens*	Tas, Aust	*Mycovellosiella*
M. tassiana	CBS111.82	AJ238469	Polyphagous		*Cladosporium*
M. vespa		AY045497	*Eucalyptus*		*Coniothyrium*
M. vespa		AY045498	*Eucalyptus*		*Coniothyrium*
M. vespa		AY045499	*Eucalyptus*		*Coniothyrium*
M. walkeri	STEU2769	AF309616	*Eucalyptus*		*Sonderhenia*
Dissoconium aciculare	*R262		*E. maidenii*	Qld, Aust	*Dissoconium*
Botryosphaeria rhodina	outgroup	AF243401	?		-
Mycovellosiella eucalypti	STEU1457	AF309617	*Eucalyptus*	Brazil	*Mycovellosiella*
Dothidea insculpta	outgroup	AF027764	?		-

5.2.3 DNA extraction

Microfuge tubes containing lyophilised mycelia were immersed in liquid nitrogen and the mycelia then ground into fine powder with an electric pellet mixer (Kontess). Extraction buffer (200 µl; 200 mmol Tris HCl pH 8.5, 250 mmol NaCl, 25 mmol EDTA and 0.5% SDS; (Raeder & Broda 1985) was added to each microfuge tube containing up to 200 µl volume of ground mycelia, and mixed with the homogenate by gentle inversion. This solution was incubated for 1–2 h at 65 ^{o} and then centrifuged at 13 200 *g* (Beckman Microfuge E) for 10 minutes The resulting supernatant was transferred into a microfuge tube containing 600 µl NaI solution and 7 µl of silica slurry. The solution was briefly vortexed, placed on ice for 10 min in order to precipitate the DNA onto the silica matrix under conditions of high salt and low temperature. This was then centrifuged for 10 sec and the resulting supernatant removed. The pellet was washed twice; firstly, with 600 µl of wash solution (50% ethanol, EDTA, Tris buffer; Bresawash) and secondly, with 600 µl of 100% ethanol. Each wash involved briefly vortexing the solution, followed by centrifugation (13 200 *g* for 5 sec) with the subsequent removal of the supernatant.

After aspiration, 25 µl of Tris EDTA (TE) buffer was added. The pellet was resuspended and incubated for 10 min at 50 ° in order to dissolve the DNA, under conditions of high temperature and low salt, into the TE buffer. The solution was centrifuged for 10 min at 13 200 ***g*** to pellet the silica matrix. Supernatant containing the genomic DNA was transferred into a sterile 0.5 ml microfuge tube to which 3 µl of 1 mg ml^{-1} RNAse (Boehringer Mannheim) was added and incubated for 60-90 min at 37 ° in order to digest any RNA present. The DNA concentration was determined using a Hoefer DyNA Quant 200 fluorometer according to the manufacturers instructions. The DNA was then stored at –20 ° until required for Polymerase Chain Reaction (PCR) amplifications.

5.2.4 DNA Amplification

The ITS1 and ITS 2 regions of the rDNA were amplified using the primers ITS1f and ITS 4 (Gardes & Bruns 1993, White *et al.* 1990). Amplification solutions (50 µl) were made aseptically in sterile 200µl microfuge tubes, containing; 5 ng genomic DNA, 0.2 µM primer, 2.5 mM $MgCl_2$ (Biotech International), 2.5 U *Tth* plus polymerase (Biotech International), 1x polymerisation buffer (Biotech International) equivalent to 67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg ml^{-1} gelatin, 0.2 mM dNTPs and sterile, deionised water (Astar) to make up the reaction volume of 50 µl. These solutions were vortexed for 1–2 s, then centrifuged for 5 s at 13 200 ***g***. The PCR's were performed (Applied Biosystems Gene Amp 9600 thermocycler) according to the following parameters: Initial denaturing step of 96 ° for 2 min; then 30 cycles of 94 ° (30 s) denaturing, 55 ° (30 s) annealing, 72 ° (2 min) extension; this was followed by a 7 min extension cycle at 72 °, then a hold cycle of 10 °. Products of the PCR reaction were stored at 4 ° prior to cleaning and sequencing.

The PCR products were electrophoresed on a 1% agarose gel (in TAE buffer) at 90V for 40 minutes. The size of the DNA bands was determined against a λDNA marker (restricted with *Hin*diii & *Eco*Ri; Fisher Biotec) as the molecular weight standard. DNA fragments were visualised under UV following gel staining with ethidium bromide (0.5 µg/ml) for 20 to 30 min and de-staining in 1x TAE buffer

for 10 minutes Where DNA bands were present, the remaining PCR product was purified using the silica binding method as outlined for the DNA extraction method described above, with the following modifications: The PCR product was not incubated in extraction buffer; the volumes of NaI salt, wash, and ethanol solutions were reduced from 600 to 200 µl; and the RNAse digestion step was omitted. The concentration of DNA in the clean PCR product was determined by comparing band intensity against a known amount of marker DNA, visualised on a 1% agarose gel stained with ethidium bromide as described previously. The DNA concentrations determined using this method, were verified by measuring a sub-set of PCR products fluorometrically (Hoefer DyNA Quant 200).

5.2.5 DNA sequencing

Double stranded ITS fragments were sequenced from each end using an ABI PRISM™ Rhodamine Dye Terminator Ready Reaction Kit in 10 µl sequence reactions according to the manufacturers instructions. Between 80 and 160 ng of purified PCR product and 1.6 pmol of primer (either ITS 1, ITS 2, ITS 3, ITS 4; (White *et al.* 1990) was added to each reaction. Sequencing reactions were performed according to the following parameters (Applied Biosystems GeneAmp 9600 thermocycler): Initial denaturing step of 96 ° for 2 min; then 25 cycles of 94 ° (30 s) denaturing, 50 ° (5 s) annealing, 60 ° (4 minutes) extension; then a hold cycle of 10 ° until collected and precipitated. The products of the sequence reaction were ethanol precipitated. Briefly, sterile 0.5 ml microfuge tubes were prepared with 24 µl of 100% ethanol and 1 µl of Sodium Acetate (10%; pH 5.2). The sequence product was added, then vortexed for 5 s and placed on ice for 20 minutes to precipitate the DNA. The tubes were centrifuged at 13 200 ***g*** for 30 min in order to pellet the DNA, then the supernatant removed. The DNA pellet was washed in 250 µl of 70% ethanol, centrifuged at 13 200 ***g*** for 5 min, then the supernatant removed. The tubes were blotted dry, then dried under vacuum in a rotor speedvac for 10 minutes

Sequence products were electrophoresed on 5% acrylamide gels, which were then washed for 10 minutes with 1000 ml of 20% ethanol, stained and exposed to

Kodak SB film. The DNA sequence data was read by an electronic digitizer and aligned by LaserGene version 1.60dz using the CLUSTAR V method. The sequencing of the DNA template was conducted using the software package, Sequencase v 2.0.

5.2.6 Phylogenetic analysis

The forward and reverse sequence data for each isolate were edited and aligned with SEQUED (v1.04, PE Applied Biosystems, Foster City, California) or GeneTool (Double Twist Inc., 2000) with manual adjustments where necessary. Additional *Mycosphaerella* sequence data, including that of *Mycovellosiella eucalypti,* which is presumed to have a *Mycosphaerella* teleomorph, were downloaded from GenBank, via a BLAST search, and saved as FASTA format in the text module of GeneTool. Sequence data from all of the different species were aligned in Clustal W, saved as Phylip format and optimised manually. Aligned sequence data were imported into PAUP (v. 4.1b; Swofford 1998) and phylogenetic trees constructed using the heuristic search option. The initial set of trees was obtained by the 'simple' option for the stepwise addition of taxa and these trees evaluated via the tree bisection reconstruction (TBR) method of branch swapping. Bootstap analysis (1000 replications) was used to evaluate the confidence of the branch nodes of the trees (Felsenstein 1985). *Botryosphaeria rhodina* and *Dothidea insculpta* were chosen as outgroups on the basis that they are sister taxa of differing degrees of affinity to *Mycosphaerella*, within the Dothideales. In addition, a neighbour joining tree was derived in PAUP for each analysis.

Separate analyses were performed in order to resolve: a) intra-specific variation; b) inter-specific variation; c) anamorph and host based clades. Firstly, the intra-species diversity of *M. cryptica* and *M. nubilosa* was compared amongst isolates of each species. Secondly, the phylogenetic structure of *Mycosphaerella* species on eucalypts in south-western Australia was investigated through a comparison of all isolates sequenced from this region with other species occurring on eucalypts elsewhere. Thirdly, *Mycosphaerella* species isolated off eucalypts from south-western Australia were compared with species from non-eucalypt hosts in order to resolve the phylogenetic issues. The analysis included all isolates of all species

from the current study, and all available isolates form the GenBank database. However, this analysis was prohibitively slow (not close to completion even after 1 week) and so two additional analyses were conducted. One that reduced the 'max trees' setting to 100 and the bootstrap replication to 100; and another that included a subset of isolates but retained the original higher bootstrap setting of 1000. The subset of isolates chosen for the final analysis included only one isolate from each species, and where species differed by less than 3 nucleotides (nt), only one of these species was retained for the analysis. The topology of both trees was similar, so only the simpler, but more stringent 1000 bootstrap version was retained in the results section of this thesis.

5.3 Results

5.3.1 Intra specific variation

ITS sequences of *Mycosphaerella* species from the present study show little intra-species variation (Table 5.2). The differences ranged from as low as 0 nucleotide (nt) amongst ten *M. cryptica* isolates from the diverse hosts *E. globulus*, *E. grandis*, *E. diversicolor* and *E. delegatensis* in eastern, southern and south-western Australia and up to a 4 nt difference between the eastern and south-western Australian isolates of *M. lateralis*. There was 1 nt intra-specific variation within *M. nubilosa* and 1 nt intra-specific variation within *M. marksii*; 3 nt differences within *M. parva*; and 0 nt differences within the remaining species sequenced. The *M. lateralis* isolates from south-western Australia all shared the same sequence, and those from eastern Australia were different from the south-western population.

There was a substantial increase in intra-specific variation when sequences on GenBank were added to the analysis (Table 5.2). The greatest increase in sequence variation from this inclusion occurs for *M. cryptica,* for which base substitutions and indels result in a 60 nt difference between isolates. The increase in difference between *M. cryptica* isolates is mostly due to three isolates. One from eastern Australia (AY045496), which has a 20 nt deletion three N's and several base substitutions; another isolated off *Eucalyptus urophylla* from Chile (AF309622), which has three, 1-3 nt insertions and several base substitutions; and an isolate

from south-eastern Australia (AF309623), which was poorly edited on GenBank. The latter of these sequences contained two complete repeats of the ITS region, that were different from each other, on GenBank. When these three sequences were removed from the comparison, then only 2 nt differences remained within *M. cryptica,* a transition mutation at position 461 and a single base deletion of nucleotide 171 (Appendix 5.1).

Variation of *M. nubilosa* increased from 1nt to 5 nt differences as a result of four substitutions that occur predominantly in the South African population isolated from *E. nitens*. The inclusion of *M. grandis* with *M. parva* sequences increased the number of nt difference from 3 to 4 for this species complex. The sequences of *M. molleriana* and *M. vespa* were added to the analysis of the sequence variation in *M. ambiphylla,* as these two former species, although morphologically distinct from *M. ambiphylla,* share considerable sequence homology (Figures 5.3–5.4; Tables 5.2–5.3). There were 4 nt differences within this species complex.

Table 5.2 Comparison of intra-species variation of *Mycosphaerella* species on eucalypts in terms of nucleotide site differences.

Species	ITS size range (ITS1/4 inclusive: this study)	Nucleotide differences this study (N sequences)	Nucleotide differences entire database (N sequences)
M. ambiphylla	538	0 (2)	4 (7)*
M. aurantia	534	0 (4)	0 (4)
M. cryptica#	539	0 (10)	60 (17)**
M. cryptica##	539	0 (10)	2 (14)
M. gregaria	727***	0 (4)	0 (4)
M. lateralis	564	4 (6)	4 (9)
M. marksii	536	1 (4)	9 (8)
M. mexicana	539	1 (4)	1 (4)
M. nubilosa	539	1 (7)	17 (19)
*M. nubilosa*****	539	1 (7)	5 (18)
M. parva	538	3 (4)	4 (7)*****

* Inclusive of the possibly con-specific *M. molleriana*, and *M. vespa.*
** Includes the deletion in GenBank accession AY045496
# Includes all isolates identified as *M. cryptica*
Excludes outlying isolates of *M. cryptica* AY045496, AF309622 and AF309623
*** Includes 180bp insertion
**** Excludes outlying sequence AF309618
***** Includes the probably conspecific *M. grandis.*

Sequences from the 36 isolates included in the comparison of intra-specific variation of *M. nubilosa* and *M. cryptica* were aligned (Appendix 5.1). The 20nt deletion from isolate AY045496 was excluded from the phylogenetic analysis. Of 561 total characters: 419 characters were constant; 91 variable characters were parsimony-uninformative and 51 characters were parsimony informative. Tree length = 179 Consistency index (CI) = 0.9050; Homoplasy index (HI) = 0.0950; CI excluding uninformative characters = 0.7952; HI excluding uninformative characters = 0.2048 Retention index (RI) = 0.9715. Rescaled consistency index (RC) = 0.8792, f value = 2776, f-ratio = 0.2278 51. A neighbour-joining tree (not shown) of similar topology was also derived.

Phylogenetic analysis revealed one major clade of *M. cryptica*, including isolates from four eucalypt species, from populations across Australia and New Zealand (Figures 5.1 and 5.2). There were three outlying isolates of *M. cryptica*, AY045496 from Tasmania Australia, AF309622 from Chile and AF309623 from south-eastern Australia.

Parsimony analysis revealed two major clades within *M. nubilosa* and one outlying isolate (Figures 5.1 and 5.2). The first major clade is comprised of isolates from *Eucalyptus* from New Zealand and south-eastern Australia, and *E. globulus* from south-western Australia. The second clade is comprised of four isolates from *E. nitens* in South Africa and two from *E. globulus* from south-eastern Australia. All of the South African isolates of *M. nubilosa* clustered in this second clade.

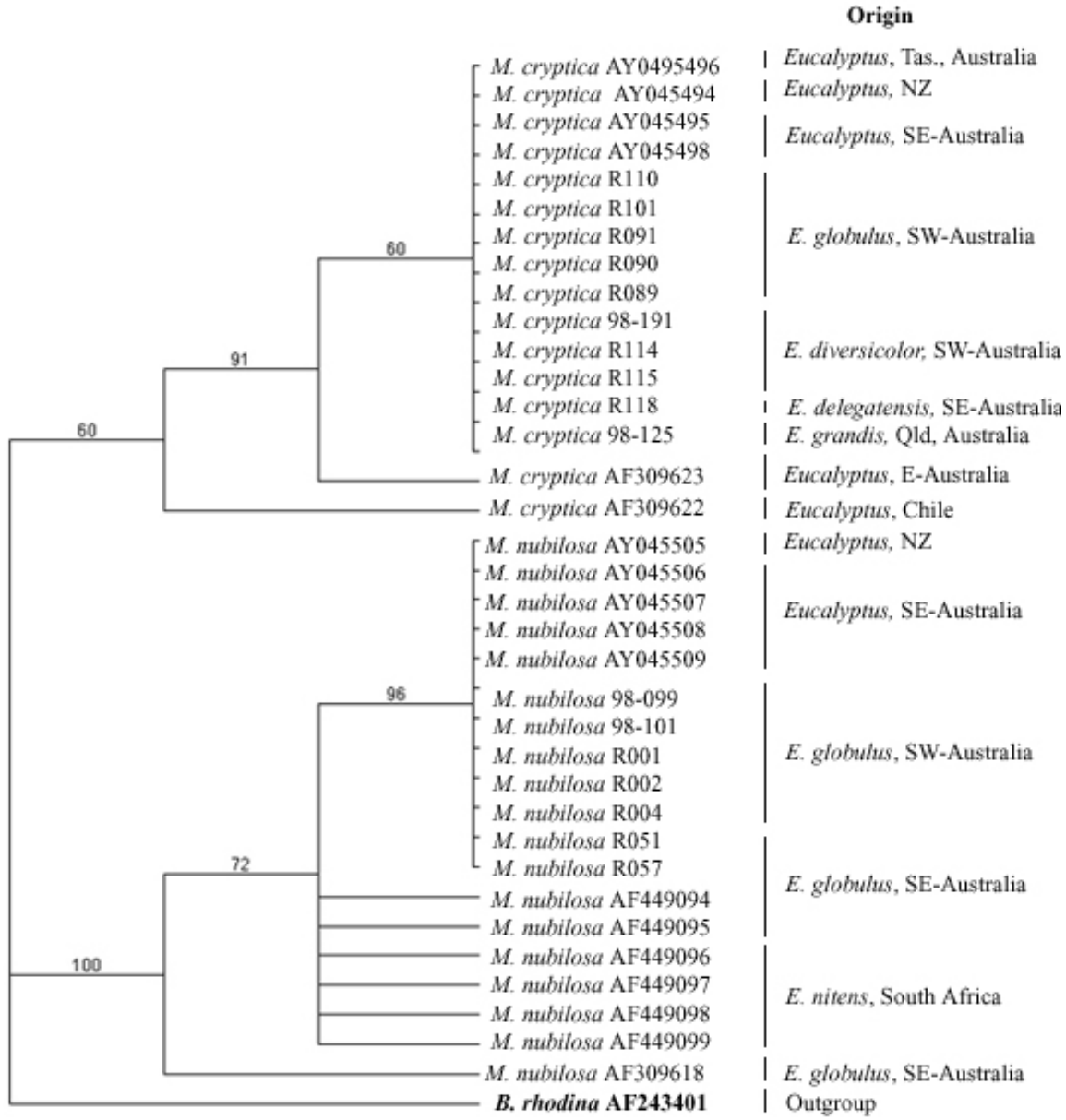


Figure 5.1 Phylogram, based on ITS rDNA sequences, indicating intra-species variation of *Mycosphaerella cryptica* and *Mycosphaerella nubilosa*. Bootstrap support from 1000 replicates indicated.

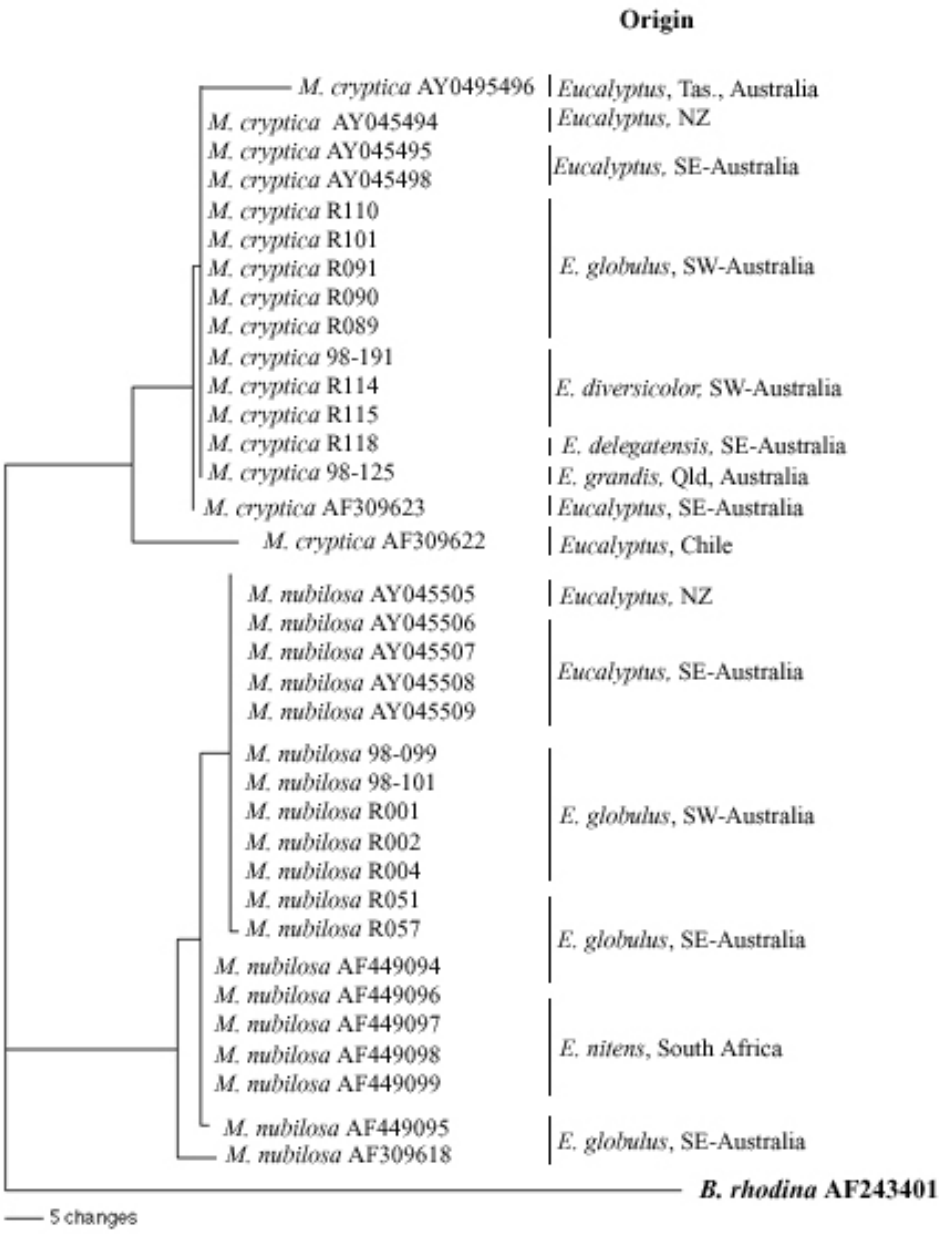


Figure 5.2 Phylogram, based on ITS rDNA sequences, indicating intra-species variation of *Mycosphaerella cryptica* and *Mycosphaerella nubilosa*. Branch lengths indicated.

5.3.2 Inter-specific variation of *Mycosphaerella* species isolated from eucalypts

The size of the ITS region of *Mycosphaerella* species isolated from eucalypts in south-western Australia ranged from 534 to 549 nt's except for *M. lateralis* and *M. gregaria* which were 564 and 727 nt long, respectively. *Mycosphaerella gregaria* contained a 180 nt insertion. Sequences from the 71 isolates included in the comparison of inter-specific variation of *Mycosphaerella* species isolated off eucalypts in south-western Australia were aligned (minus the 180 nt insertion of *M. gregaria*) (Appendix 5.2). Of 628 total characters, 322 characters were constant, 58 variable characters were parsimony-uninformative, 248 characters were parsimony informative. One most parsimonious tree was obtained after 1000 bootstrap replicates (Figures 5.3 and 5.4; Tree Length=1114 characters; CI=0.497; RI=0.851; RC=0.423; HI=0.503; G-fit=160.470). A neighbour-joining tree (not shown) of similar topology was also derived.

In the phylogenetic analysis of ITS sequences, the morphological species of *Mycosphaerella* isolated off eucalypts in the current study, grouped with their corresponding species, where they were available on GenBank (Figures 5.3 and 5.4). The ITS sequence variation between closely related species was typically 4–8 nt, but was as low as 0 nt. The sequences for *M. cryptica* clustered in a distinct group with strong bootstrap support (Figure 5.3). The *M. cryptica* isolates sequenced in the current study clustered with the *M. cryptica* isolate from Chile, which was the least similar sequence of *M. cryptica* available on GenBank (Figures 5.1 and 5.2). *Mycosphaerella cryptica* formed in a clade that included *M. ambiphylla, M. molleriana*, *M. vespa, M. nubilosa*, *M. suttoniae*, *M. mexicana, M. suberosa* and *M. tasmaniensis* (Figure 5.4).

The newly described *M. ambiphylla* clustered with *M. molleriana* and *M. vespa* with strong bootstrap support (Figures 5.3 and 5.4; and Table 5.3). *Mycosphaerella*

ambiphylla, which has a *Phaeophleospora* anamorph state, differed by 3 nt changes from *M. molleriana* which has a *Colletogloeopsis* anamorph state. The isolates of these three species were more than 99% similar (Table 5.3). One isolate of the recently described *M. vespa* was identical to one isolate of *M. molleriana.*

Three species clustered as a small sub-group within this larger clade. This subgroup was comprised of *Mycosphaerella mexicana* at 30 nt changes from a node that it shared with *M. suberosa* (40 changes), and *M. tasmaniensis* (over 60 changes) (Figure 5.4).

The south-western Australian *M. marksii* isolates clustered with the GenBank sequence of *M. marksii* and with the newly described *M. intermedia* with strong bootstrap support (Figure 5.3). The branch lengths of this phylogenetic tree showed that the *M. marksii* isolates from the current study were equally distant from the *M marksii* and the *M. intermedia* sequences on GenBank (Figure 5.4). The similarity matrix (Table 5.4) shows that *M. marksii* isolates are more similar to each other than to *M. intermedia.* However, the *M. intermedia* sequences on GenBank were incompletely edited in that they contained a number of unresolved nt's (N's). These three species formed in a clade that included species from *M. aurantia* to *M. walkeri* (Figures 5.3 and 5.4). The sequences of the newly described species, *M. aurantia* does not match any published species. However, in parsimony analysis it grouped closely to *M. africana* from which it differed by only 5 nt's. *Mycosphaerella gregaria* formed a distinct group and was most close to *M. ellipsoidea*. However, the placement of *M. gregaria* in this tree is in the absence of the 180 nt insert unique to this species. *Mycosphaerella crystallina, M. irregulariramosa, M. heimii* and *M. heimioides,* all clustered closely together, at less than 5 changes from their common node (Figure 5.4). This cluster has strong bootstrap support (Figure 5.3).

Mycosphaerella parva from the current study clustered with the published sequence for *M. grandis* with strong bootstrap support (Figure 5.3). There was greater than 99% similarity between all *M. parva* and *M. grandis* isolates (Table 5.5). These species formed a distinct clade, as did two of the species, which have an *Uwebraunia* anamorph, *M. flexuosa* and *M. juvenis.*

There were two non-*Mycosphaerella* teleomorphs available as out groups in this analysis and *Dothidea insculpta* proved most distant to the *Mycosphaerella* species. In fact, the *Dissoconium* clade grouped more closely to *Botryosphaeria rhodina* than to the other *Mycosphaerella* species in the current analysis. In this phylogram, *M. lateralis,* which has a *Dissoconium* anamorph state, clustered near *Dissoconium aciculare* (Figures 5.3 and 5.4).

In addition to *D. dekkeri* isolates from eastern and south-western Australia*, D. aciculare* was also sequenced in the current study. Although the species of *D. aciculare* sequenced in the current study was isolated from *E. grandis* in eastern Australia, it was more than 99% similar to GenBank sequences of *D. aciculare* from non-eucalypt hosts (data not shown).

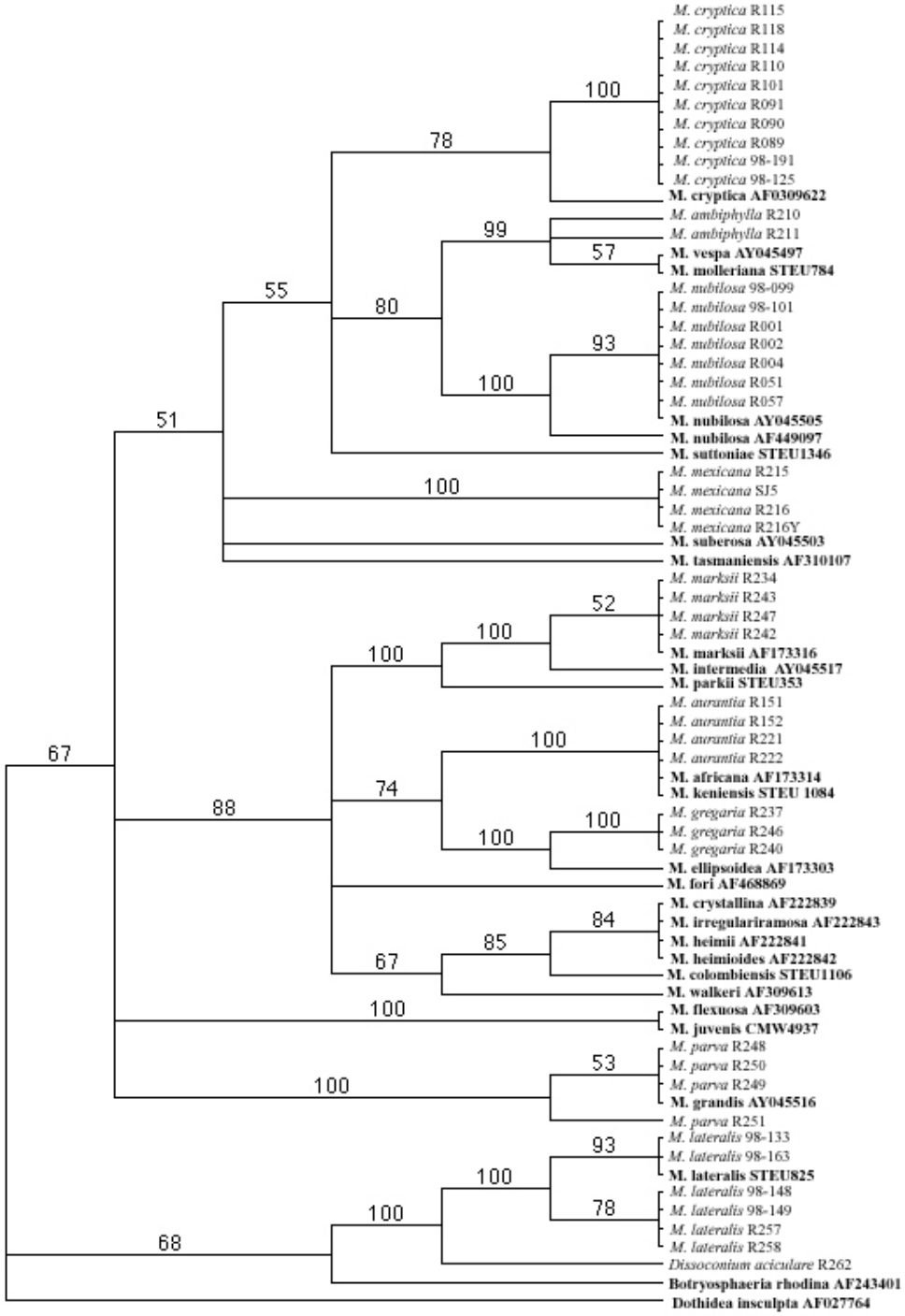


Figure 5.3 Phylogram based on ITS rDNA sequences of *Mycosphaerella* species, indicating the degree of closeness between isolates of *Mycosphaerella* from eucalypts in south-western Australia and those from eucalypts elsewhere. Bootstrap support from 1000 replicates indicated. Sequences from GenBank in bold.

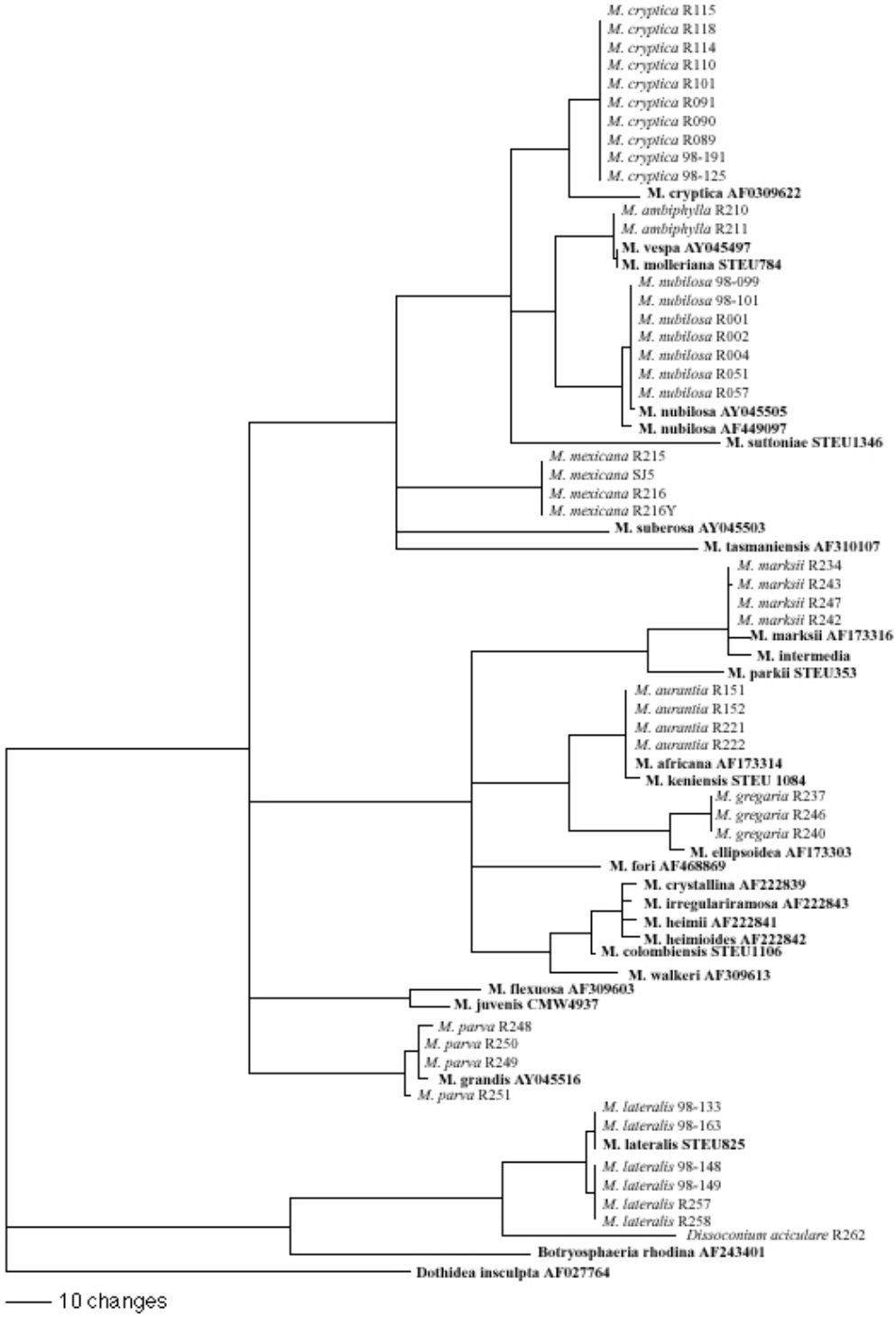


Figure 5.4 Phylogram based on ITS rDNA sequences of *Mycosphaerella* species, indicating the degree of closeness between isolates of *Mycosphaerella* from eucalypts in south-western Australia and those from eucalypts elsewhere. Branch lengths indicated. Sequences from GenBank in bold.

Table 5.3 Similarity matrix (%) between ITS rDNA sequences of *Mycosphaerella ambiphylla* and closely related *Mycosphaerella* species. 1–2 = *M. ambiphylla*; 3–4 = *M. molleriana*; 5–7 = *M. vespa*. (1 = R210, 2 = R211, 3 = STEU784, 4 = STEU1214, 5 = AY045497, 6 = AY045499, 7 = AY045498).

	1	**2**	**3**	**4**	**5**	**6**	**7**
1		100	99.8	99.4	99.8	99.8	99.8
2	100		99.8	99.4	99.8	99.8	99.8
3	99.8	99.8		99.6	100	100	100
4	99.4	99.4	99.6		99.6	99.6	99.6
5	99.8	99.8	100	99.6		100	100
6	99.8	99.8	100	99.6	100		100
7	99.8	99.8	100	99.6	100	100	

Table 5.4 *Mycosphaerella marksii* and *M. intermedia* multiple ITS rDNA sequence alignment % identity matrix. 1–8 = *M. marksii*; 9–10 = *M. intermedia*. (1=98-004, 2=98-129, 3=R234, 4=R247, 5=STEU 982, 6= AF173316, 7=AF309588, 8=AF468873, 9=AY045517, 10=AY045518).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1		99.8	99.8	99.8	99.6	99	99.4	99.8	94.5	94.5
2	99.8		100	100	99.8	99.2	99.6	100	94.7	94.7
3	99.8	100		100	99.8	99.2	99.6	100	94.7	94.7
4	99.8	100	100		99.8	99.2	99.6	100	97.4	94.7
5	99.6	99.8	99.8	99.8		98.6	99.4	99.8	90	90
6	99	99.2	99.2	99.4	98.6		99.6	99.2	89.4	89.4
7	99.4	99.6	99.6	99.6	99.47	99.6		99.6	89.6	89.6
8	99.8	100	100	100	99.8	99.2	99.6		90	90
9	94.5	94.7	94.7	94.7	90	89.4	89.6	90		100
10	94.5	94.7	94.7	94.7	90	89.4	89.6	90	100	

Table 5.5 *Mycosphaerella parva* and *M. grandis* multiple ITS rDNA sequence alignment % identity matrix. 1–4 = *M. parva*; 5–7 = *M. grandis.* (1=R248, 2=R249, 3=R250, 4=R251, 5=AY045516, 6= AY045514, 7= AY045513).

	1	**2**	**3**	**4**	**5**	**6**	**7**
1		100	100	99.6	99.3	99.3	99.3
2	100		100	99.6	99.4	99.4	99.4
3	100	100		99.6	99.4	99.4	99.4
4	99.6	99.6	99.6		99.1	99.1	99.1
5	99.3	99.4	99.4	99.1		100	100
6	99.3	99.4	99.4	99.1	100		100
7	99.3	99.4	99.4	99.1	100	100	

Table 5.6 Similarity matrix of ITS rDNA sequences of *Mycosphaerella lateralis* isolates from south-western and south-eastern Australia (1=98-133, 2=98-148, 3=98-149, 4=98-163, 5=R257, 6=R258, 7=STEU825, 8=STEU1232, 9=STEU1235).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1		99.3	99.3	100	99.3	99.3	100	100	100
2	99.3		100	99.3	100	100	99.2	99.2	99.2
3	99.3	100		99.3	100	100	99.2	99.2	99.2
4	100	99.3	99.3		99.3	99.3	100	100	100
5	99.3	100	100	99.3		100	99.2	99.2	99.2
6	99.3	100	100	99.3	100		99.2	99.2	99.2
7	100	99.2	99.2	100	99.2	99.2		100	100
8	100	99.2	99.2	100	99.2	99.2	100		100
9	100	99.2	99.2	100	99.2	99.2	100	100	

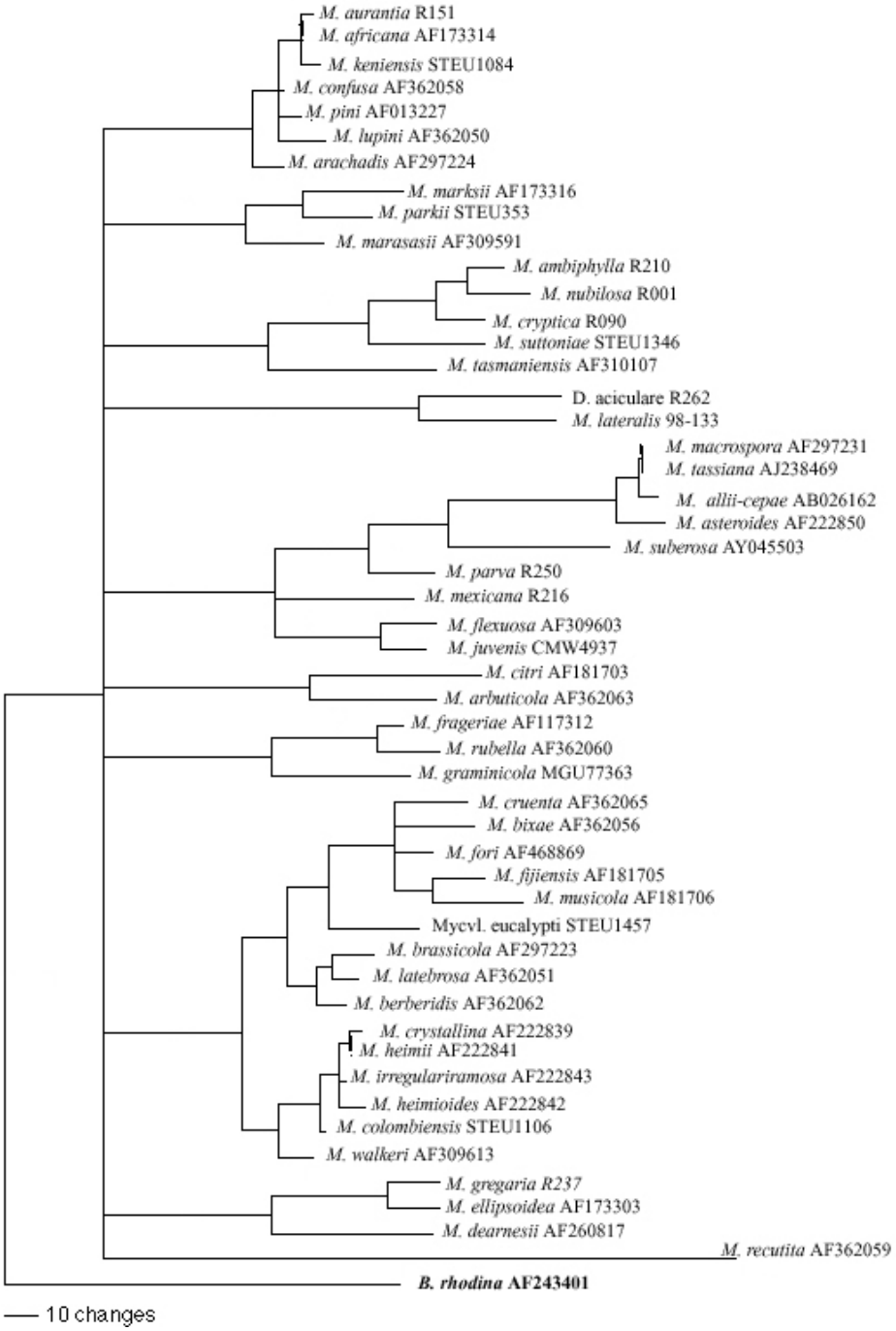


Figure 5.5 Phylogram indicating the relationship between *Mycosphaerella* species sequenced in the current study and all *Mycosphaerella* species sequences available on GenBank, irrespective of host. Branch lengths indicated. Bootstrap values as indicated in Figures 5.6 and 5.7. Anamorph species with an unknown *Mycosphaerella* teleomorph are not italicised; Mycvl. = *Mycovellosiella*; D. = *Dissoconium*.

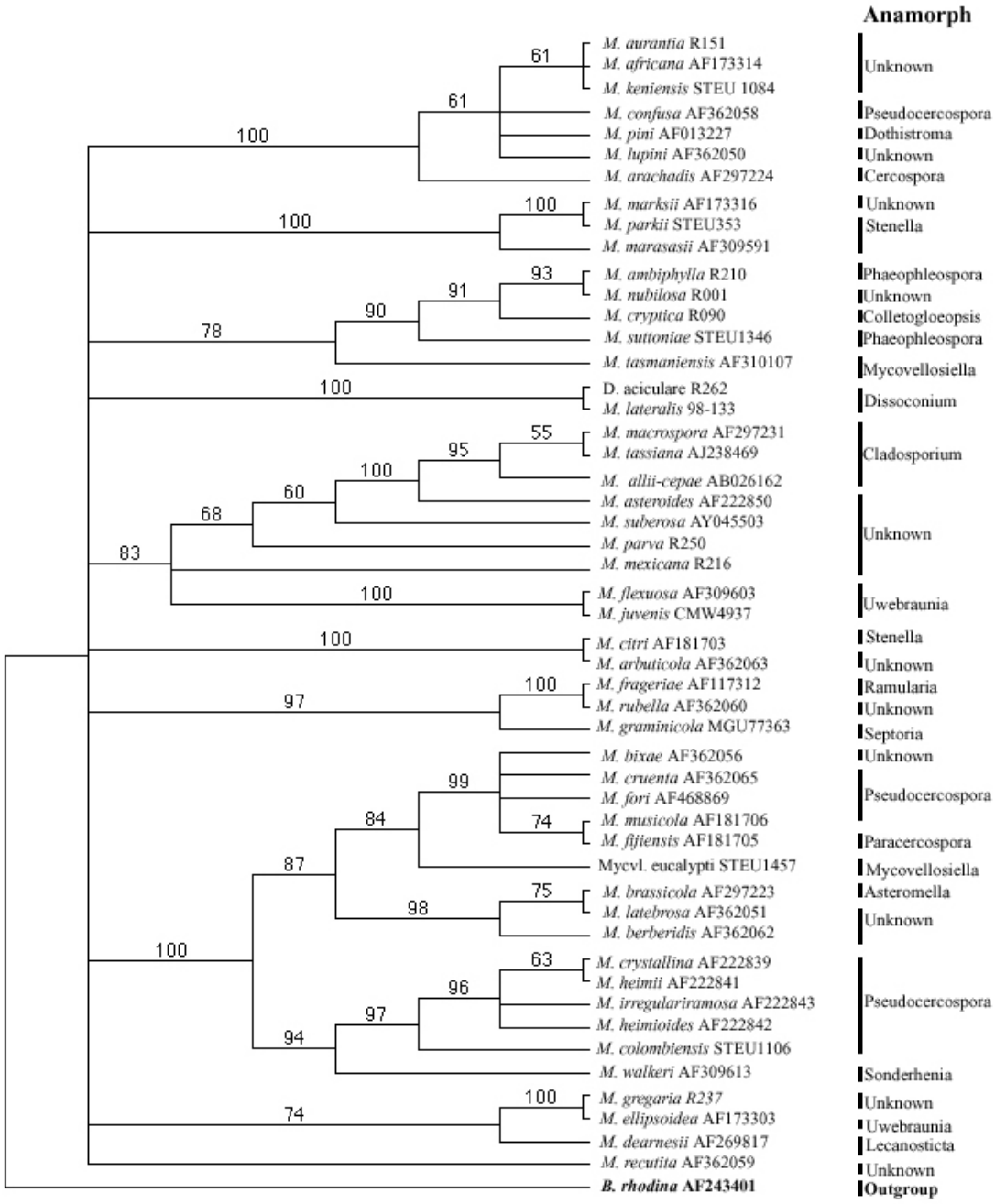


Figure 5.6 Phylogram indicating the relationship between clades of *Mycosphaerella* species and their anamorph state. Bootstrap values as indicated. Branch lengths as indicated in Figure 5.5. Anamorph species with an unknown *Mycosphaerella* teleomorph are not italicised; Mycvl. = *Mycovellosiella*; D. = *Dissoconium*.

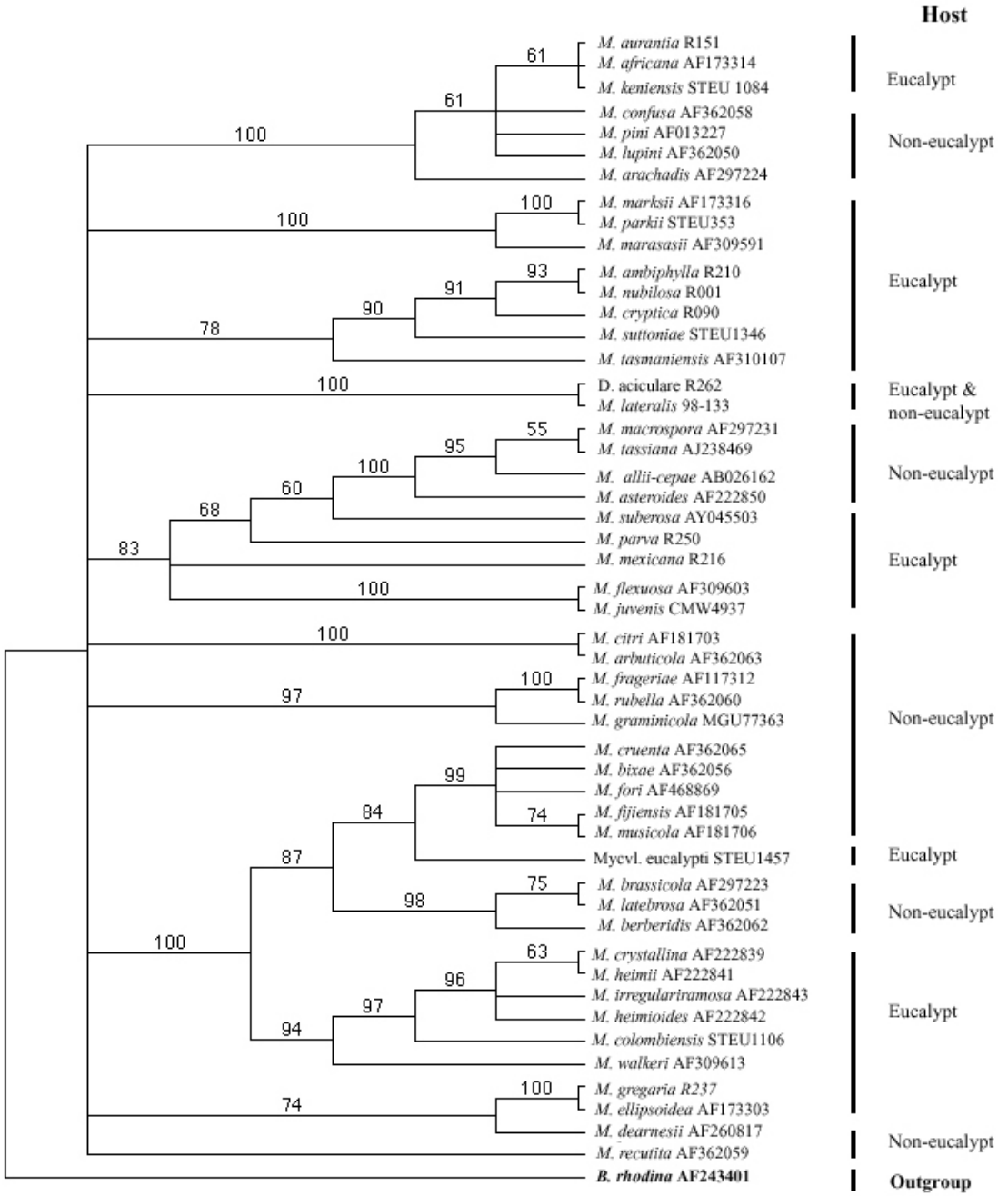


Figure 5.7 Phylogram indicating the relationship between *Mycosphaerella* species sequenced and host of origin. Bootstrap values as indicated. Branch lengths as indicated in Figure 5.5. Anamorph species with an unknown *Mycosphaerella* teleomorph are not italicised; Mycvl. = *Mycovellosiella*; D. = *Dissoconium*.

5.3.3 Phylogenetic comparisons

The ITS region of fifty *Mycosphaerella* taxa and *Botryosphaeria rhodina* that was used as an out-group, were trimmed and aligned (Appendix 5.3). These were subject to parsimony analysis. Of a total of 586 characters, 186 characters were constant, 102 variable characters were parsimony-uninformative and 298 characters were parsimony-informative. One most parsimonious tree (Figure 5.5) was obtained after 1000 bootstrap replications (Length=2224; CI=0.407; RI=0.606; RC=0.247; HI=0.593; G-fit=-149.890). A tree of similar topology was also obtained from an analysis of reduced stringency (100 bootstrap replications) involving all unique sequences from all of the species listed in Table 5.1. The phylogram, derived from the lower stringency but more isolate and species inclusive analysis, is not presented due to page space limitations. However, where relevant, aspects of that tree are referred to in the text of the results section 5.3.3 and in the discussion section 5.4 that follows. A neighbour-joining tree (not shown) of similar topology was also derived. Four species omitted from the phylogenies presented were: *M. molleriana* and *M. vespa* which clustered with the *M. ambiphylla* presented; *M. grandis* which clustered with the *M. parva* presented; *M. intermedia* which clustered with the *M. marksii* presented;

The species resolved into 10 distinct clades (Figures 5.5–5.7), with total branch lengths ranging from: 80 changes for the *M. lupini* cluster to 200 changes for the *M. asteroides* cluster; and 220 changes for *Mycosphaerella recutita* which resolved on a branch independent of all other species (Figure 5.5). The number of species represented in each clade ranged from one (*M. recutita*) to 15 (the clade including *M. cruenta* to *M. walkeri*). Bootstrap support for each of these clades was strong (Figure 5.6). The branch lengths separating species was as low as the one nt that separated *Mycosphaerella macrospora* and *M. tassiana* (Figure 5.5). In the lower stringency tree (not shown), isolates of *M. molleriana* and *M. vespa* were identical and clustered closely with *M. ambiphylla* at a distance of 3–5 nt changes, as is shown from earlier analyses in Figures 5.3 and 5.4. Therefore, only *M. ambiphylla* was retained in Figures 5.5–5.7.

There was no correlation between anamorph state and the clade into which a *Mycosphaerella* species clustered (Figure 5.6). Cercosporoid (*Cercospora, Paracercospora* and *Pseudocercospora*) anamorphs were present in two of the ten clades. They occurred along with non-cercosporoid anamorphs in each of the two clades. The three species with a *Stenella* anamorph, *M. citri, M. marasasii* and *M. parkii*, fell into two separate clades. Similarly, the three species with *Uwebraunia* anamorphs fell into two separate clades; and the *Mycovellosiella* species resolved into a separate clade. The three species, *M ambiphylla, M. molleriana* and *M. vespa* with *Phaeophleospora, Colletogloeopsis* and *Coniothyrium* anamorphs respectively, all clustered closely together in the same clade in the lower stringency tree (not shown) and in Figures 5.3 and 5.4.

There was no clear relationship between host genera and clade. *Mycosphaerella* species off eucalypts were present in 7 out of 10 of the clades that formed, usually along with species off other hosts (Figure 5.7, Table 5.1). Often, species that clustered very closely and were separated by few nt changes, were from the same host. This was true for: *M. aurantia, M. africana* and *M. keniensis* which were all isolated from eucalypts; *M. ambiphylla, M. molleriana* and *M. nubilosa* which were all from eucalypts; and *M. fijiensis* and *M. musicola* from bananas. However, in some cases *Mycosphaerella* species from widely different host genera clustered equally close together. For example: *M. pini* and *M. lupini*, which were isolated from *Pinus* and *Humulus* species, respectively; and *M. brassicicola* and *M. latebrosa* from *Brassica* and *Acer* hosts, respectively.

The *Mycosphaerella recutita* sequence from GenBank was significantly different from the other *Mycosphaerella* species compared in this analysis. In a subsequent blast search of the *M. recutita* sequence, it matched species of *Phaeosphaeria* more closely than it did species of *Mycosphaerella.* The closest match was with the *Phaeosphaeria triglochinicola* isolate CBS 600.86 (gi|17017220|gb|AF439507.1).

5.4 Discussion

The current study is the first to compare the intra-species variation in ITS rDNA sequences of *M. cryptica* and *M. nubilosa,* the two most important causes of MLD on eucalypts. It was also the first study to determine the ITS rDNA sequences of *M. ambiphylla, M. aurantia, M. gregaria, M. mexicana* and *M. parva;* and the first to compare the sequences of south-western Australian isolates of these species and of *M. lateralis* with sequences of isolates from elsewhere.

Ten *M. cryptica* and six *M. nubilosa* isolates off different host species from throughout Australia were sequenced in the current study in order to determine their intra-species variation. The intra-species variation for these was 0 for *M. cryptica* and 1 for *M. nubilosa*. The *M. cryptica* sequence from the current study, was also the one most commonly present on the GenBank database. The amount of intra-species variation of *M. cryptica* isolates from other studies lodged with GenBank was much greater (up to 60 nt changes more) than that from those sequenced in the current study alone. It is likely that those present on the GenBank database that had several base substitutions are different species within a *M. cryptica* species complex. However, as some of these differences were clearly due to errors in the editing of sequences, it is possible that these isolates do not represent cryptic species, but rather are the erroneous product of further undetected sequencing errors.

Although the sequence variation within *M. nubilosa* also increased when those sequences lodged with GenBank were added to the analysis, this variation did not increase to the same extent as occurred with *M. cryptica.* The most common *M. nubilosa* genotype sequenced in the current study was also the most frequently occurring on GenBank. Isolates with this sequence were present in NZ, south-eastern Australia and south-western Australia. The other common genotype of *M. nubilosa* on GenBank differed by 4-5 nt from those in the current study, and mostly were isolated off *E. nitens* in South Africa. This sequence was highly conserved in the South African population of *M. nubilosa.* This is likely due to founder effects in the South African population of *M. nubilosa.* The fact that this genotype was also present on *E. globulus* from south-eastern Australia may indicate that populations

of *M. nubilosa* are disjunct within Australia. This is explained by the limited host range and homothallic lifecycle of *M. nubilosa,* that leads to more isolated divergent populations of this species; than of *M. cryptica* which is heterothallic and present on a wide range of eucalypt hosts throughout Australia. Population level studies using variable neutral molecular markers are required to determine the population structure of these two pathogens. The population genetics of *M. nubilosa* is considered further in Chapter 7.

On the basis of the entire *Mycosphaerella* sequence database, the degree of nucleotide divergence necessary to define species boundaries within *Mycosphaerella* was unclear. The intra-specific variation within *Mycosphaerella* species diverged considerably. Some species appeared to have substantial intra-species variation, much greater than the 1–2 nt suggested as typical of *Mycosphaerella* by Goodwin *et al.* (2001). The high intra-species variation may be explained by the inclusion of some indels and poorly edited sequences in the database. However, even with the removal of the questionable sequences and indels, the sequence variation of *M. cryptica, M. lateralis, M. marksii, M. nubilosa* and *M. parva* remained at 2, 4, 9, 5 and 3 nt, respectively. It may be that some of these species represent complexes of more than one species. On the other hand some closely related species diverged by only a few nt, and in some cases were probably conspecific.

Phylogenetic analysis and sequence comparison detected some species that have been described as distinct, that may be conspecific. These species include the recently described *M. vespa* and the earlier recorded *M. molleriana,* for which identical sequences were present on GenBank. *Mycosphaerella ambiphylla* may also form part of this species, as sequences varied by only 2-3 nt from those of *M. molleriana* and *M. vespa.* On the basis of this evidence it is likely that *M. vespa* is synonymous with *M. molleriana*. However, further work is required to fully determine the status of these two species and the closely related *M. ambiphylla.* Each of these three species is described as having a different anamorph state; *Colletogloeopsis molleriana* for *M. molleriana, Coniothyrium ovatum* for *M. vespa* and *Phaeophleospora ambiphylla* for *M. ambiphylla.* Although they are all recognised as

coelomycetes, conidia are formed in an acervulus for *Colletogloeopsis,* and in a pycnidium for *Coniothyrium* and *Phaeophleospora*. Pycnidial and conidial dimensions of *P. ambiphylla* and *C. ovatum* were significantly different, as was their mode of conidiogenesis (Chapter 3). However, it is possible that the size and morphology of conidia and pycnidia and conidiogenesis itself are plastic in these species. The formation of an acervulus as opposed to a pycnidium may depend on substrate and environmental conditions. There are examples of this from other fungi, where conidia may form on a loose assemblage of conidiogenous cells, a sporodochia or an acervulus depending on the environmental conditions under which they occur (Crous *et al.* 2000, Verkley & Priest 2000, Verkley 1998). These three species must be grown on the same substrate and under the same conditions, in order to compare the morphology of the anamorph state between these three species. In addition further isolates of all these species should be sequenced in order to determine the species boundaries, if and where they occur, for this complex. Multi-gene sequence data would also be useful in clarifying the phylogeny of these taxa.

Another pair of species that may be conspecific is *M. grandis* and *M. parva.* Although Carnegie and Keane (1994) show small morphological differences between *M. grandis* and *M. parva*, Crous (1998) claimed that these species could not be differentiated on morphological grounds. Although type material was not examined in the current study, their descriptions from the literature overlap and the major criteria for differentiating these species appear to be that *M. grandis* is a pathogen and *M. parva* a saprophyte on old *M. nubilosa* lesions (Carnegie and Keane 1994). Evidence from Chapter 4 indicates that *M. parva* can be found on lesions without other species present (although typically it occurs as part of a disease complex), further blurring the separation of these two species. The molecular evidence from the current study supports the combination of *M. grandis* under *M. parva*. Alternatively, the sequence similarity between *M. parva* and *M. grandis* could also be explained if *M. parva* isolates have been mistakenly identified as *M. grandis*. Sequencing of DNA from type specimens of each of these two species could help clarify their status as separate species.

However, further work investigating the ability of *M. parva* ascospores to infect leaves and cause disease under controlled glasshouse conditions are required.

The sequences of *M. tassiana* and *M. macrospora* were identical. Both of these species have a *Cladosporium* anamorph and conidia and ascospores of similar dimensions. Therefore, it is likely that they are conspecific. Previously, (Crous *et al.* 2001b) have proposed that *M. heimii, M. heimioides* and *M. crystallina* may be conspecific. The phylogenetic data of the present study supports this proposal. Further work comparing several and diverse isolates of these species in culture should be made to clarify the status of this group.

Mycosphaerella lateralis was characterised by two distinct genotypes that differed at 4 nt sites. These were separated along geographic lines, with one genotype being restricted to eastern Australia and the other to south-western Australia. The eastern Australian genotype isolated off *E. grandis* and *E. maidenii* was identical to those lodged with GenBank. It is likely that these are two distinct populations of the same species, however, further work comparing their morphology under the same conditions is required to determine this.

Mycosphaerella lateralis clustered with the other *Dissoconium* anamorph species in this study, namely *D. aciculare* that was isolated off *E. grandis* and *E. maidenii* from Qld, Australia. These clustered separately from the other similar anamorph genus *Uwebraunia. Dissoconium* was first erected as a separate Genus from *Cordana*, based on the forcible discharge of macro and micro conidia in a droplet of fluid for *Dissoconium*. Crous & Wingfield (1996) later erected the Genus *Uwebraunia*, which accommodates fungi morphologically similar to *Dissoconium*. These two genera were separated on two criteria. Firstly, that *Uwebraunia* species are pathogens of eucalypts, whereas *Dissoconium* species are hyperparasites. Secondly, that conidiogenesis is percurrent in *Uwebraunia,* whereas it is sympodial in *Dissoconium.* Recently, Jackson *et al.* (2003) have shown that *D. dekkeri* is able to infect *E. globulus* leaves and that the conidiogenesis of *D. dekkeri* is both sympodial and percurrent. Therefore, the separation of these two anamorph genera based on morphological criteria needs to be reviewed. Studies on the molecular taxonomy of this group indicate that the anamorph *Uwebraunia* has arisen separately, at least

twice within the teleomorph genus *Mycosphaerella* (Crous *et al.* 2001a, Crous *et al.* 1999). Also, according to the sequence homology of the large subunit (28s) of the ribosomal RNA operon, *D. dekkeri* is more closely aligned with *Uwebraunia ellipsoidea* than *U. ellipoidea* is with *U. juvenis* (Crous *et al.* 2001a). Further morphological and molecular studies on *Dissoconium*, *Uwebraunia* and the related genera *Cordana* and *Dactylaria* are required to clarify the differentiation of these taxa

The phylogenetic data suggests that *Mycosphaerella* is an assemblage of largely polyphyletic anamorph genera. Ten distinct clades emerged from the analysis, none of which was comprised entirely of one anamorph genus alone. The anamorph genera represented were often dispersed across more than one clade, indicating that these anamorphs have arisen separately in different phylogenetic lineages. Thus the anamorph genera share derived (or apomorphic) traits, such as conidium morphology, that have arisen separately in different phylogenetic lineages. These anamorphs are therefore polyphyletic in their origin.

Cercosporoid (*Cercospora, Paracercospora* and *Pseudocercospora*), *Stenella* and *Uwebraunia* anamorphs each occurred in more than one separate clade. The largest clade contained *Pseudocercospora, Paracercospora*, *Mycovellosiella* and *Sonderheinia* anamorphs. It could be argued that this clade is dominated by the *Pseudocercospora* species. However, both *Pseudocercospora* and *Mycovellosiella* also occurred in at least one other clade. Furthermore, with the exception of *Pseudocercospora* and *Paracercospora,* these anamorphs are considered taxonomically distinct (Crous *et al.* 2000, Stewart *et al.* 1999).

Nonetheless, species that clustered closely together sometimes shared the same or a similar anamorph genus. These include: *M. ambiphylla*, *M. molleriana, M. cryptica* and *M. suttoniae* which have a *Colletogloeopsis* or the similar *Phaeophleospora* anamorph state; *M. macrospora, M. tassiana, M. alii-cepae* which all share a *Cladosporium* anamorph; *M. flexuosa* and *M. juvenis*, that share an *Uwebraunia* anamorph; *M. cruenta, M. fori, M. musicola* and *M. fijiensis* that all share a *Pseudocercospora* or *Paracercospora* anamorph; and the *M. heimii* complex that also share a *Pseudocercospora* anamorph. However, the expression of a particular

anamorph state did not predict phylogenetic closeness, as the same anamorph genus may occur on widely divergent branches of the phylogenetic tree. Thus, although anamorph state remains a useful feature in differentiating *Mycosphaerella* taxa, it cannot be claimed that anamorphs underpin monophyletic lineages within *Mycosphaerella,* nor that the anamorph genera considered in this study are monophyletic.

The question remains however, as to whether the *Mycosphaerella* teleomorph genus is monophyletic. There is some evidence from the current study that *Mycosphaerella* may be polyphyletic. There are two arguments that support this conclusion. The first relates to the placement of the *Dissoconium* clade in this and previous studies, and the second relates to branch lengths within *Mycosphaerella* clades. This latter argument reflects on the philosophical underpinnings of molecular phylogeny.

In the current analysis of *Mycosphaerella* species from eucalypts, *M. lateralis,* which has a *Dissoconium* anamorph, clustered more closely to the non-*Mycosphaerella* taxon *Botryosphaereia rhodina,* where two non-*Mycosphaerella* outgroup taxa were included. This suggests that some derived (apomorphic) characters that define *Mycosphaerella* may have arisen separately. If this is the case, then *Mycosphaerella* is polyphyletic. There are previous studies in which the *Dissoconium* clade grouped separately from the remaining *Mycosphaerella* species (Crous *et al.* 1999, Crous *et al.* 2001a, Crous *et al.* 2001b) as occurred in the current study. For example, the most parsimonious tree of Crous *et al.* (2001b) showed that the *Dissoconium* clade grouped more closely to the non-*Mycosphaerella* teleomorph *Didymella* than to the other *Mycosphaerella* species in their study.

Similarly, the *M. recutita* sequence was very different from the other *Mycosphaerella* species in the current study. In a blast search, this species was more closely aligned with *Phaeosphaeria* species than with *Mycosphaerella*. It may be that this species was incorrectly identified as a species of *Mycosphaerella,* however it could also be evidence that *Mycosphaella* is polyphyletic.

The second piece of evidence that suggests *Mycosphaerella* may be polyphyletic relates to the branch lengths that separate the clades within *Mycosphaerella.* In the current study, the ten clades are well supported by bootstrap analysis, and branch lengths are long in comparison to those separating teleomorph genera, such as between *Leptosphaereia, Phaeosphaeria* and *Ophiosphaeria* (Goodwin & Zismann 2001). These branch lengths indicate a long evolutionary divergence of taxa within *Mycosphaerella sensu stricto* and provide evidence that clades within *Mycosphaerella* are evolutionarily equivalent to entire genera in other groups. Although these phylogenetic lineages remain difficult to differentiate on the basis of one easily discernable morphological feature, such as anamorph affiliation, they are nonetheless evolutionarily distinct groups. Strictly speaking, this does not mean that *Mycosphaerella* is polyphyletic, but rather that it is a monophyletic lineage composed of more than one genus in evolutionarily genetic terms. It then remains a philosophical question as to whether these clades warrant the status of separate genera or sub-genera. More studies are required that compare *Mycosphaerella* with closely related genera using sequence information from other genes, before firm conclusions on the phylogeny of *Mycosphaerella* can be drawn.

Attempts to date to differentiate lineages within *Mycosphaerella* on the basis of molecular and traditional taxonomy have relied upon trying to fit the old schema to the molecular data. What is required is a new approach that defines the different groups according to the molecular data. This database must contain information from a number of non-linked, neutral, and appropriately informative genes, such as intron containing regions of beta-tubulin, histone, actin and other such loci. Such multi-gene phylogenies have been used successfully to redefine *Fusarium* (Geiser, Pitt & Taylor 1998). The clades derived from this type of study should then be compared against the stable well-documented morphological features of each species, in a correspondence type analysis, in order to determine which features are the most powerful in predicting group membership of the molecular based clades.

The placement of the *Dissoconium* clade in the current study (and in the previous studies mentioned above) was not always consistent. In the analysis of the full

Mycosphaerella data set, which included only one non-*Mycosphaerella* outgroup *Botryosphaereia rhodina*, *Dissoconium* did not cluster any closer to this outgroup than any of the other *Mycosphaerella* clades. It should be noted that all clades in this tree were equally distinct in terms of the arrangement of their nodes. However, in this tree, the presence of the highly variable sequence of *M. recutita* may have altered the topology of this tree significantly. The sequence of *M. recutita* was very different from that of the other *Mycosphaerella* species, and indeed this isolate may have been misidentified as a *Mycosphaerella* species. Also, the presence of only one out-group taxon may have forced an incorrect placement of the *Dissoconium* clade in this tree.

The selection of outgroup taxa must be carefully considered in answering phylogenetic questions, and this is a process that may be refined as more is known about the phylogeny of *Mycosphaerella* and related genera. For example, the use of *Cladosporium* as an outgroup by Crous *et al.* (2001a) may not have been the best choice in determining whether *Mycosphaerella* is polyphyletic, given that this anamorph is linked with *Mycosphaerella* and also that it is probably a polyphyletic genus itself (Crous *et al.* 2000, Crous *et al.* 2001b). Another aspect that has not been sufficiently covered in answering the question of whether *Mycosphaerella* is polyphyletic is that few isolates of species from related teleomorph genera have been sequenced and analysed in conjunction with *Mycosphaerella* species. If *Mycosphaerella* is polyphyletic, then different clades within *Mycosphaerella* will cluster more closely with morphologically different species from other teleomorph genera. If this hypothesis is to be adequately tested then many more species from related teleomorph genera must be included in phylogenetic studies that attempt to determine whether *Mycosphaerella* is polyphyletic in origin.

Previous authors have reached differing conclusions on the question of *Mycosphaerella* phylogeny. Crous *et al.* (2001b) in a study of *Mycosphaerella* on *Myrtaceae* concluded that this was a monophyletic assemblage of species within the *Mycosphaerella* section *Plaga* (as defined in Barr 1972 and in Crous *et al.* 2001a), and that this was comprised of two clades of *Pseudocercospora* species. The cladograms in their study did not indicate branch lengths and therefore it is not

possible to examine the degree of closeness between species and clades. However, where the species compared in that study are the same as the current investigation, the pattern of clustering was similar.

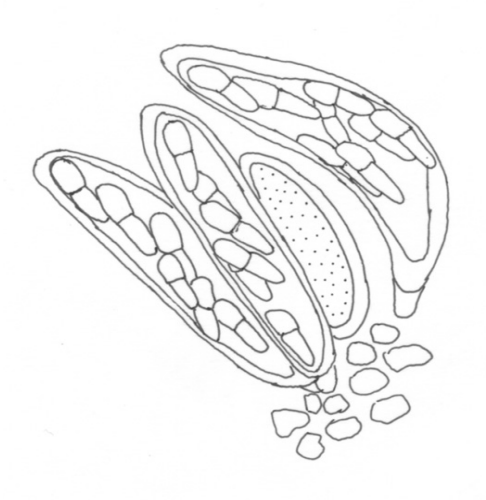
Goodwin, Dunkle & Zismann (2001) found that *Mycosphaerella* is a monophyletic assemblage of polyphyletic anamorph genera. The current study agrees with their finding that the anamorph genera within *Mycosphaerella* are polyphyletic. However, (Goodwin *et al.* 2001) have ignored the issue of the *Dissoconium* clade, and rely on the common node for all of the remaining species in reaching their conclusion. They do not believe that the long branch lengths between *Mycosphaerella* clades indicates that these clades are phylogenetically distinct lineages.

The current study provides strong evidence that there is no correlation between *Mycosphaerella* host and clade. *Mycosphaerella* species off eucalypts clustered with those from a range of other hosts. For example, *M. aurantia* off *E. globulus* clustered alongside *M. pini* off *Pinus*. Although the evidence for this is available in previous molecular studies of *Mycosphaerella* this is the first time that this hypothesis has been explicitly tested. Analogous to the pattern for anamorph affiliation, closely related fungi were often isolated from the same host genus, but this was not a predictor of phylogenetic closeness.

The ITS rDNA region was useful the resolution of species of *Mycosphaerella* on eucalypts in south-western Australia, in that they confirmed morphological separation of these species. Of all ten species found here, each formed a distinct group in the phylogenetic trees derived. The identification of the species also agreed with that of published species. Sequence data from the ITS rDNA region may be useful in developing rapid molecular based methods for the identification of species. This approach is developed in Chapter 6.

Chapter 6

Rapid and reliable molecular based identification of *Mycosphaerella* species



Chapter 6 Rapid and reliable molecular based identification of *Mycosphaerella* species

6.1 Introduction

Surveys of *E. globulus* plantations in south-western Australia have revealed two previously undescribed species and five new records of *Mycosphaerella* (Chapter 3). These findings are based on the morphological traits of these fungi on diseased foliage and in culture. However, the taxonomy of *Mycosphaerella* based on morphological features is difficult, in that species are differentiated on the basis of small differences in ascospore size, shape and germination pattern (Crous 1998). The formation of an anamorph state is also important in differentiating *Mycosphaerella* species, and these may not form readily in culture, or may require weeks or months of growth on specific media under specific conditions in order to develop.

There are numerous examples of mis-identification of *Mycosphaerella* species from eucalypts, such as studies on what was thought to be *M. nubilosa* in New Zealand (Beresford 1978, Cheah 1977) that was later identified as *M. cryptica* (Park & Keane 1982a). The naming of the anamorph state of *M. cryptica, Colletogloeopsis nubilosum*, was based on the misapprehension that this anamorph was connected to the teleomorph *M. nubilosa*. Early records have attributed MLD outbreaks to *M. molleriana*, when in fact other *Mycosphaerella* species were the cause (Crous 1998). Sequence information (Chapter 5) has revealed three examples of conspecificity of separately described *Mycosphaerella* species of eucalypts. These include *M. molleriana, M. parva* and *M. tassiana*. The species *M. ambiphylla* and *M. vespa* are conspecific with *M. molleriana; M. grandis* is conspecific with *M.* parva; and *M. macrospora* is conspecific with *M. tassiana.* These examples highlight the difficulty in the conventional taxonomy of this genus. Molecular-based methods therefore, provide an additional tool that can make species identification more certain.

Comparison of sequences for such regions as the internally transcribed spacer regions (ITS) 1 and 2 of the rRNA genes has proved very usefull in delimiting and differentiating species (Berbee *et al.* 1995, Chillali *et al.* 1998, Crous *et al.* 1999, Faris *et al.* 1996, Guo, Hyde & Liew 2000). Sequence comparison of this region has enabled a more secure differentiation of the species of *Mycosphaerella* on eucalypts in south-western Australia (Chapter 5). However DNA sequencing is a relatively expensive and time-consuming technique for identifying species. The data obtained from ITS sequences may be utilised to develop less expensive and more rapid molecular means for the identification of *Mycosphaerella* species. These include PCR-based restriction fragment length polymorphic DNA (PCR-RFLP's) and species-specific primers for DNA amplification and visualisation via agarose gel electrophoresis.

PCR-RFLP's have been utilized for the identification of eucalypt pathogens such as *Cryphonectria cubensis* and related species (Myburg *et al.* 1999) and *Mycosphaerella* species on other hosts (Ueng *et al.* 1998). Species -specific primers for the detection of *Mycosphaerella fijiensis* and *M. musicola* on banana (Johanson 1995, Johanson *et al.* 1994, Johanson & Jeger 1993) have also been developed. There is a need to develop fast molecular methods for identifying *Mycosphaerella* species associated with diseases of eucalypts.

Therefore, the aims of the present study were to:

1) Generate a PCR-RFLP based technique to identify species of *Mycosphaerella* from eucalypts.

2) Develop species specific primers for the detection of *M. cryptica* and *M. nubilosa.*

6.2 Materials and Methods

6.2.1 PCR-RFLP

ITS rDNA sequence data for *Mycosphaerella* species (Chapter 5) were analysed for potential restriction sites using the sequence editor module of Genetool. The consensus sequences of each of the 10 species of *Mycosphaerella* from eucalypts in south-western Australia were imported, along with all other available *Mycosphaerella* species on the National Centre for Biotechnology Information (NCBI) GenBank database, into Genetool (Table 6.1). These sequences were screened against the list of 'commercially available' enzymes in Genetool. A matrix of enzymes and the restriction sites for each of the 10 species most commonly causing disease in Australia was derived. From this list, the restriction enzymes that were potentially most informative for the greatest number of fungi were tested against the PCR products from the ITS1f and ITS4 primers (Gardes & Bruns 1993, White *et al.* 1990) for the rDNA region (ITS1f/4 rDNA).

Table 6.1 List of isolate sequences screened for restriction digestion of ITS rDNA PCR products and for the species specific primers MC2F/MC2R and MN1F/MN1R

Species	Isolate number	GenBank accession	Host	Origin	Anamorph
M. ambiphylla	R210		*E. globulus*	SW Aust	*Phaeophleospora*
M. ambiphylla	R221		*E. globulus*	SW Aust	*Phaeophleospora*
M. ambiphylla	R222		*E. globulus*	SW Aust	*Phaeophleospora*
M. ambiphylla	R211		*E. globulus*	SW Aust	*Phaeophleospora*
M. aurantia	R151		*E. globulus*	SW Aust	Unknown
M. aurantia	R152		*E. globulus*	SW Aust	Unknown
M. cryptica	R089		*E. globulus*	SW Aust	*Colletogloeopsis*
M. cryptica	R090		*E. globulus*	SW Aust	*Colletogloeopsis*
M. cryptica	R091		*E. globulus*	SW Aust	*Colletogloeopsis*
M. cryptica	R101		*E. globulus*	SW Aust	*Colletogloeopsis*
M. cryptica	R110		*E. globulus*	SW Aust	*Colletogloeopsis*
M. cryptica	R114		*E. diversicolor*	SW Aust	*Colletogloeopsis*
M. cryptica	R115		*E. globulus*	SW Aust	*Colletogloeopsis*
M. cryptica	R118		*E. delegatensis*	Vic Aust	*Colletogloeopsis*
M. cryptica	98125		*E. grandis x*	Qld, Aust	*Colletogloeopsis*
M. cryptica	98191		*E. globulus*	SW Aust	*Colletogloeopsis*
M. cryptica		AY045494	*Eucalyptus*	NZ	*Colletogloeopsis*
M. cryptica		AY045495	*Eucalyptus*	SE Aust	*Colletogloeopsis*

…Table 6.1 List of isolate sequences screened for restriction digestion of ITS rDNA PCR products and for the species specific primers MC2F/MC2R and MN1F/MN1R

Species	Isolate number	GenBank accession	Host	Origin	Anamorph
M. cryptica		AY045496	*Eucalyptus*	Tas, Aust	*Colletogloeopsis*
M. cryptica		AY045498	*Eucalyptus*	SE Aust	*Colletogloeopsis*
M. cryptica	STEU936	AF309585	*Eucalyptus*	Australia	*Colletogloeopsis*
M. cryptica	CMW2732	AF309622	*Eucalyptus*	Chile	*Colletogloeopsis*
M. cryptica	CMW3279	AF309623	*Eucalyptus*	SE Aust	*Colletogloeopsis*
M. crystallina		AF222839	*Eucalyptus*		*Pseudocercospora*
M. ellipsoidea	STEU1225	AF173303			*Uwebraunia*
M. flexuosa	CMW5224	AF309603	*Eucalyptus*	Colombia	*Uwebraunia*
M. fori	CMW9095	AF468869	*Eucalyptus*	RSA	*Pseudocercospora*
M. grandis		AY045516	*Eucalyptus*	Australia	Unknown
M. gregaria	R237		*E. globulus*	SW Aust	Unknown
M. gregaria	R240		*E. globulus*	SW Aust	Unknown
M. heimii		AF222841	*Eucalyptus*		*Pseudocercospora*
M. heimioides		AF222842	*Eucalyptus*		*Pseudocercospora*
M. intermedia		AY045517	*Eucalyptus*	NZ	Unknown
M. irregulariramosa		AF222843	*Eucalyptus*		*Pseudocercospora*
M. juvenis	CMW4937	AF309604	*Eucalyptus*	RSA	*Uwebraunia*
M. juvenis	STEU1005	AF173299	*Eucalyptus*	RSA	*Uwebraunia*
M. keniensis	STEU 084	AF173300	*E. grandis*	Kenya	Unknown
M. lateralis	R257		*E. globulus*	SW Aust	*Dissoconium*
M. lateralis	R258		*E. globulus*	SW Aust	*Dissoconium*
M. lateralis	98133		*E. maidenii*	Qld, Aust	*Dissoconium*
M. lateralis	98148		*E. globulus*	SW Aust	*Dissoconium*
M. lateralis	98149		*E. globulus*	SW Aust	*Dissoconium*
M. lateralis	98163		*E. maidenii*	Qld, Aust	*Dissoconium*
M. lateralis	STEU1535	AF173309	*Eucalyptus*		*Dissoconium*
M. lateralis	STEU825	AF309624	*Eucalyptus*	RSA	*Dissoconium*
M. marasasii	STEU348	AF309591	*Syzygium*	RSA	*Stenella*
M. marksii	STEU935	AF173316	*Eucalyptus*		Unknown
M. mexicana	R216		*E. globulus*	SW Aust	Unknown
M. molleriana	STEU784	AF309619	*Eucalyptus*	Nth USA	*Colletogloeopsis*
M. nubilosa	R001		*E. globulus*	SW Aust	Unknown
M. nubilosa		AY045506		SE Aust	Unknown
M. nubilosa		AY045507		SE Aust	Unknown
M. nubilosa		AY045508		SE Aust	Unknown
M. nubilosa		AY045509		SE Aust	Unknown
M. nubilosa	R002		*E. globulus*	SW Aust	Unknown
M. nubilosa	R004		*E. globulus*	SW Aust	Unknown
M. nubilosa	R051		*E. globulus*	SE Aust	Unknown
M. nubilosa	R057		*E. globulus*	SE Aust	Unknown
M. nubilosa	98-099		*E. globulus*	SW Aust	Unknown
M. nubilosa	98-101		*E. globulus*	SW Aust	Unknown
M. nubilosa	CMW6211	AF449094	*E. globulus*	SE Aust	Unknown
M. nubilosa	CMW6210	AF449095	*E. globulus*	SE Aust	Unknown
M. nubilosa	CMW9000	AF449096	*E. nitens*	RSA	Unknown

…Table 6.1 List of isolate sequences screened for restriction digestion of ITS rDNA PCR products and for the species specific primers MC2F/MC2R and MN1F/MN1R

Species	Isolate number	GenBank accession	Host	Origin	Anamorph
M. nubilosa	CMW9001	AF449097	*E. nitens*	RSA	Unknown
M. nubilosa	CMW9002	AF449098	*E. nitens*	RSA	Unknown
M. nubilosa	CMW9003	AF449099	*E. nitens*	RSA	Unknown
M. nubilosa	CMW3282	AF309618	*E. globulus*	SE Aust	Unknown
M. nubilosa		AY045505		NZ	Unknown
M. parkii	STEU353	AF173311	*E. saligna*	Brazil	Stenella
M. parva	R250		*E. globulus*	SW Aust	Unknown
M. parva	R251		*E. globulus*	SW Aust	Unknown
M. suberosa		AY045503			Unknown
M. suttoniae	STEU1346	AF309621	*Eucalyptus*	Indonesia	*Phaeophleospora*
M. tasmaniensis		AF310107	*E. nitens*	Tas, Aust	*Mycovellosiella*
M. vespa		AY045497			*Coniothyrium*
M. walkeri	STEU2769	AF309616	*Eucalyptus*		*Sonderhenia*
Dissoconium aciculare	R262		*E. maidenii*	Qld, Aust	*Dissoconium aciculare*

Single ascospore isolates of ten *Mycosphaerella* species were obtained from lesions as described in Chapter 4.2. These were identified on morphological characters (Chapter 4.3) and maintained on 2% MEA. Axenic mycelia, were obtained and DNA extracted as described in Chapter 5.2. The extracted DNA was stored at -20 °C until required for PCR's prior to restriction digestion or for the testing of species-specific primers. PCR reactions using the primer ITS1f and ITS 4 (Gardes & Bruns 1993, White *et al.* 1990) were conducted as described in Chapter 5.2, and the product then restricted. The isolates and species tested for PCR RFLP's and species specific primers are listed (Table 6.2).

Table 6.2 List of isolate sequences tested for restriction digestion of ITS rDNA PCR products and for the species-specific primers MC2F/MC2R and MN1F/MN1R

Species	Isolate number	Host	Origin	Anamorph
M. lateralis	R257	*E. globulus*	SW Aust	*Dissoconium*
M. lateralis	R258	*E. globulus*	SW Aust	*Dissoconium*
M. lateralis	98133	*E. maidenii*	Qld, Aust	*Dissoconium*
M. lateralis	98148	*E. globulus*	SW Aust	*Dissoconium*
M. lateralis	98149	*E. globulus*	SW Aust	*Dissoconium*
M. lateralis	98163	*E. maidenii*	Qld, Aust	*Dissoconium*
M. mexicana	R216	*E. globulus*	SW Aust	Unknown
M. nubilosa	R001	*E. globulus*	SW Aust	Unknown
M. nubilosa	R002	*E. globulus*	SW Aust	Unknown
M. nubilosa	R004	*E. globulus*	SW Aust	Unknown
M. nubilosa	R051	*E. globulus*	SE Aust	Unknown
M. nubilosa	R057	*E. globulus*	SE Aust	Unknown
M. nubilosa	98-099	*E. globulus*	SW Aust	Unknown
M. nubilosa	98-101	*E. globulus*	SW Aust	Unknown
M. parva	R250	*E. globulus*	SW Aust	Unknown
M. parva	R251	*E. globulus*	SW Aust	Unknown
M. suberosa	R237	*E. globulus*	SW Aust	Unknown
Dissoconium aciculare	R262	*E. maidenii*	Qld, Aust	

ITS1f/4 rDNA PCR products were restricted in 20 µl aliquots for 2–4 h at 37 ° according to the manufacturer's instructions (Promega Corporation, Australia). Restriction digests were performed for each species isolate combination as: 1) a single digest with *HaeII*; 2) a single digest with *ApaI*; 3) a double digest with both *HaeII* and *ApaI* present. Restriction products were electrophoresed on a 2% agarose gel (in TAE buffer) at 90V for 75 min using a mini sub-cell system (Biorad, Australia). The size of the DNA bands was determined against a 100 bp DNA marker (Gibco, Australia) as molecular weight standard. DNA fragments were visualised under UV following gel staining with ethidium bromide (0.5 µg/ml) for 20 to 30 min and de-staining in 1x TAE buffer for 10 minutes

A dichotomous key for the identification of the *Mycosphaerella* species based on restriction enzyme digest profiles was developed, based on the response of species tested in this study.

6.2.2 Species-specific primer development

The consensus sequences for the ITS1f/4 rDNA region for each of the 10 species of *Mycosphaerella* from eucalypts in south-western Australia were imported, along with all other available *Mycosphaerella* species on the NCBI GenBank database, into Genetool (Table 6.1). These sequences were assessed for forward and reverse primers in the ITS region of the rDNA, in the 'sequence editor' module of Genetool. Primer sites were chosen from the variable (ITS1 or ITS2) regions of the rDNA that were within the size range 17–23 nt and with a Tm of 55–65 °, that were free of structural impediments to annealing, and that would amplify a product of 200–400 nt. All other *Mycosphaerella* species on the database were then searched for matches to those primer sites to ensure species specificity. A BLAST search was also conducted to ensure that the primer sites were not present on other fungal species associated with eucalypts, or in the host plant DNA. Forward and reverse primers specific to *M. cryptica* (MC2F and MC2R) and *M. nubilosa* (MN1F and MN1R) (Table 6.3) were chosen and tested against DNA extracts of each of these species. PCR reactions were performed on DNA from 15 isolates of *M. cryptica* and 15 isolates of *M. nubilosa* from locations throughout Australia. The isolates of *M. cryptica* were off one of four different hosts, *E. globulus, E. diversicolor, E. grandis* and *E. maidenii*. Isolates of *M. nubilosa* were off either *E. globulus* or *E. nitens*. To ensure against 'false-positive' amplification of closely related species, the primer pairs, MC2F/MC2R and MN1F/MN1R were tested against three isolates of *M. nubilosa* and of *M. cryptica*, respectively. False positive amplification was also tested against DNA extracts from three isolates of each of the remaining eight species for each of the primer pairs (Table 6.2).

PCR reactions were performed as described in Chapter 5.2 with the exception that the primer pairs used were MC2F/MC2R, and MN1f/MN1R, respectively, and the annealing temperature was increased from 55 ° to 58 °. To confirm the presence and size of the PCR products, they were electrophoresed on a 1% agarose gel (in TAE buffer) at 90V for 40 minutes. The size of the DNA bands was determined against a λDNA marker (restricted with *Hin*diii & *Eco*Ri; Fisher Biotec) or a 100 bp DNA marker (Gibco) as molecular weight standard. DNA fragments were

visualised under UV following gel staining with ethidium bromide (0.5 μg/ml) for 20 to 30 min and de-staining in 1x TAE buffer for 10 minutes

Table 6.3 List of species-specific primers developed for *Mycosphaerella cryptica* and *Mycosphaerella nubilosa*

Species	**Direction**	**Sequence (5' - 3')**	**Length** (nt)	**Tm** (° C)
M. cryptica	Forward	cccgcccgacctccaacc	18	58
M. cryptica	Reverse	cggtcccggaagcgaaacag	20	58
M. nubilosa	Forward	gcgccagcccgacctcc	17	57
M. nubilosa	Reverse	ggtccccgtcagcgaaacagt	21	56

6.3 Results

6.3.1 PCR-RFLP

Based on the matrix of restriction enzymes versus the restriction size fragments obtained for each of ten *Mycosphaerella* species, the combination of *HaeII* and *ApaI* gave the greatest resolving power. The restriction sites for these enzymes on the ITS1f/4 rDNA region for each *Mycosphaerella* species (Figure 6.1) resulted in the following size fragments (Table 6.4).

Table 6.4 Predicted fragmented sizes for the restriction digest of ITS rDNA PCR products of *Mycosphaerella* species using the enzymes Hae II and *ApaI*.

Species	**Non-restricted PCR product**	***HaeII***	***ApaI***	***HaeII+ApaI***
M. ambiphylla	573	177+396	150+423	150+177+246
M. aurantia	569	152+417	569	152+417
M. cryptica	574	79+495	150+425	71+79+425
M. gregaria	763	338+425	763	338+425
M. lateralis	599	92+507	165+434	93+165+339
M. marksii	571	152+419	571	152+419
M. mexicana	577	177+400	78+497	78+177+320
M. nubilosa	574	574	150+424	150+424
M. parva	574	171+403	157+417	157+171+246
M. suberosa	577	175+402	89+488	89+175+313

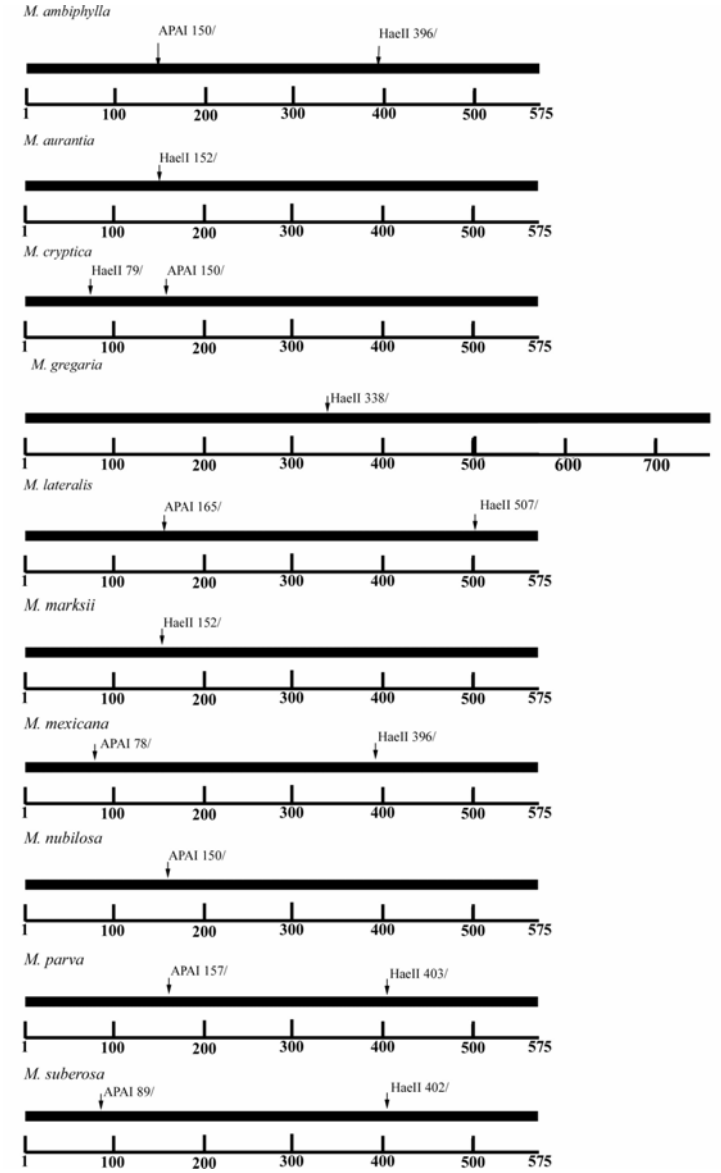


Figure 6.1 Predicted restriction sites for *HaeII* and *ApaI* enzymes for the PCR product of the ITS rDNA of ten *Mycosphaerella* species isolated from eucalypts.

Restriction digestion of the ITS1f/4 rDNA region of nine of the ten species tested gave the expected fragment size pattern (Figure 6.2, *M. lateralis* profile not shown). However, *ApaI* failed to restrict the PCR product of *M. suberosa*, thus giving a restriction digest profile similar to that of *M. marksii. HaeII* restricted all species except *M. nubilosa,* resulting in four identifiable banding profiles. Firstly, that of one 565–585 bp band (*M. nubilosa*); secondly that of 330–350 +415–435 bp (*M. gregaria*); thirdly that of 150–175 + 400–420 bp (*M. ambiphylla, M aurantia, M. marksii, M. mexicana, M. parva* and *M. suberosa*); fourthly that of 80–100 + 490–510 bp (*M. cryptica* and *M. lateralis*).

Restriction with *ApaI* gave four identifiable profiles: Firstly, one band of 765 bp (*M. gregaria*); Secondly, one band of 570 bp (*M. aurantia, M. marksii* and *M. suberosa*); Thirdly, two bands of 150–170 + 420–440 bp (*M. ambiphylla, M. cryptica, M. lateralis, M. nubilosa* and *M. parva*); Fourthly, two bands of 80 + 500 bp (*M. mexicana*).

Restriction with both enzymes simultaneously gave four unique banding profiles. Firstly, two bands 150–180 + 240–260 bp (*M. ambiphylla* and *M. parva*); Secondly, two bands of 150 + 420 bp (*M. aurantia, M. marksii, M. nubilosa* and *M. suberosa*); Thirdly, two bands 70–80 + 415–435 bp (*M. cryptica*); Fourthly, two bands 330–350 + 235–255 bp (*M. gregaria*); Fifthly, three bands 80–100 + 160–180 + 320–340 bp (*M. lateralis,* and *M. mexicana,*).

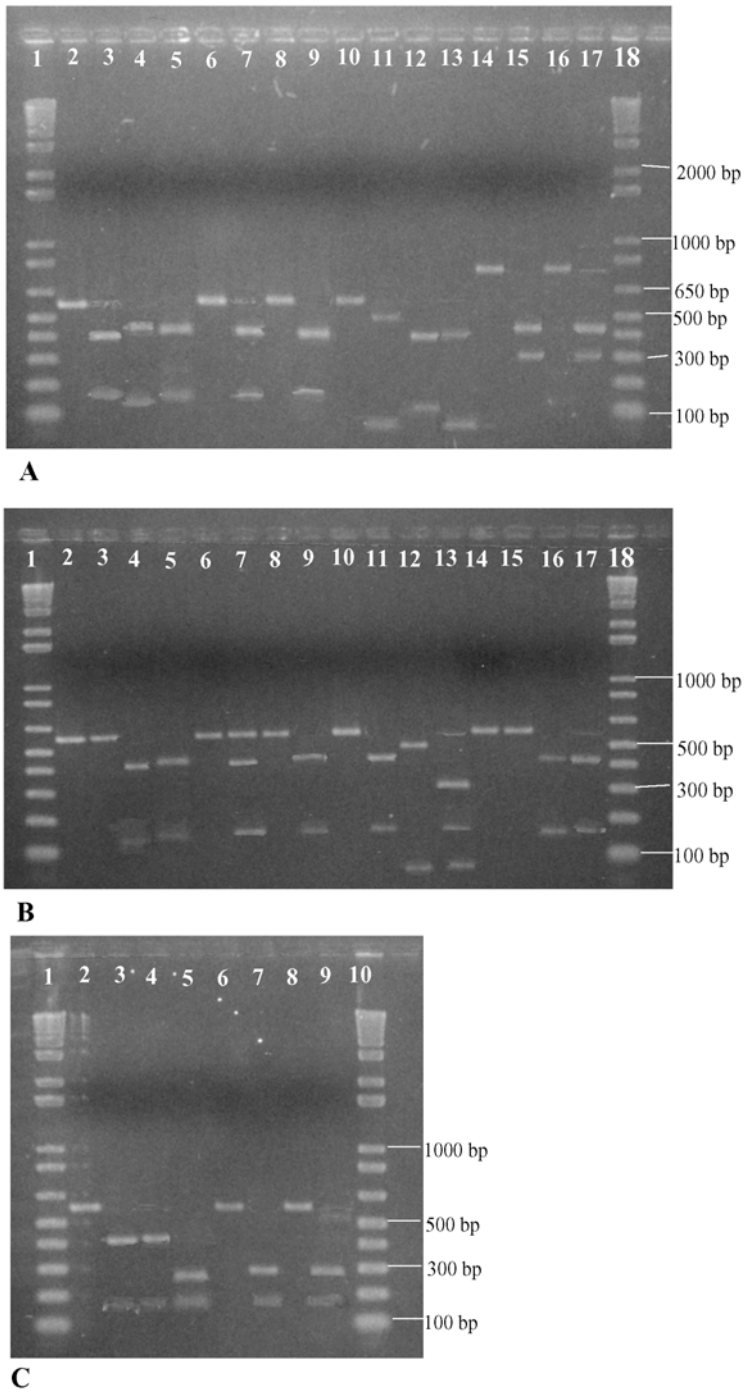


Figure 6.2 Restriction digest of *Mycosphaerella* species off eucalypts in south-western Australia. Gel A: L1 100 bp marker, L2-5 *M. ambiphylla*, L6-9 *M. aurantia*, L10-13 *M. cryptica*, L14-17 *M. gregaria*, L18 100bp marker. Gel B: L1 100bp marker, L2-5 *M. nubilosa*, L6-9 *M. marksii*, L10-13 *M. mexicana*, L14-17 *M. nubilosa*, L18 100bp marker. Gel C: L1 100bp marker, L2-5 *M. parva*, L6-9 *M. suberosa*, L10 100bp marker. Lane order of enzyme digests for each species, 1st uncut, 2nd *HaeII*, 3rd *APAI*, 4th *HaeII* and *APAI* simultaneously.

A dichotomous key for the identification of *Mycosphaerella* species based on restriction digest profiles of ITS1f/4 rDNA using *HaeII* and *ApaI* was developed (Table 6.5).

Table 6.5 Key to *Mycosphaerella* species occurring on *Eucalyptus globulus* in south-western Australia based on restriction digest profiles of PCR product (ITSif/4 primer pair) of the ITS region of the rDNA using *HaeII* and *ApaI*

1 Non restricted band and *ApaI* digestion gives one band > 700 bp, *HaeII* restriction and combined *HaeII* + *ApaI* restriction give 2 bands 330–350 +420–440 bp,….....	**gregaria**
Non-restricted band < 700 bp.....	**2**
2(1) *HaeII* restriction gives 1 band 550–600 bp, *ApaI* restriction and combined *HaeII* + *ApaI* restriction give 2 bands 140–160 + 420–440 bp…….	**nubilosa**
HaeII restriction gives 2 bands..…….....	**3**
3(2) *HaeII* restriction gives 2 bands 80–100 + 490–510 bp *ApaI* restriction gives 2 bands 150–170 + 420–440.....……	**4**
HaeII restriction gives 2 bands 150–180 + 400–420 bp …	**5**
4(3) Combined *HaeII* and *ApaI* restriction gives 2 bands 70–90 + 420–440 bp.....	**cryptica**
Combined *HaeII* and *ApaI* restriction gives 3 bands 80–100 + 160–180, 330–350 bp.....……	**lateralis**
5(3) *ApaI* restriction gives a band of 560–580 bp, combined *HaeII* and *ApaI* restriction gives 2 bands 140–160 + 410–430 bp.....…….	**6**
ApaI restriction gives 2 bands .....……	**7**

…Table 6.5 Key to *Mycosphaerella* species occurring on *Eucalyptus globulus* in south-western Australia based on restriction digest profiles of PCR product (ITSif/4 primer pair) of the ITS region of the rDNA using *HaeII* and *ApaI*

6(5)	Culture growth >15 mm/month, mycelia orange coloured after 2 months at 20 °C on 2% MEA…….....	**aurantia**
	Culture growth >15 mm/month, mycelia olivaceous (not orange-coloured) after 2 months at 20 °C on 2% MEA	**marksii**
	Culture very slow growing (< 5 mm/month), mycelia black, embedding in agar after 2 months at 20 °C on 2% MEA.....	**suberosa**
7(5)	*ApaI* restriction gives 2 bands 80–100 + 490–510 bp, combined *HaeII* and *ApaI* restriction gives 3 bands 70–90 + 170–190 and 310–330 bp……....	**mexicana**
	ApaI restriction gives 2 bands 140–160 + 420–440 bp, combined *HaeII* and *ApaI* restriction gives 2 bands 150–180 + 240–260	**8**
8(7)	Ascospores > 12 μm long, cultures fast growing (40 mm month^{-1}); *Phaeophleospora* anamorph.....	**ambiphylla**
	Ascospores (6–) 8–9 (–11) μm, type N or L germination pattern; cultures growth rate < 20 mm month^{-1}, no anamorph….....……...	**parva**

6.3.2 Species specific primer development

A BLAST search for the sequence sites for each of the primer pairs MC2F/ MC2R and MN1F/MN1R found no exact matches with non-target fungal or plant DNA, including closely related *Mycosphaerella* species.

PCR reactions resulted in a 400 bp product for the MC2 primer pair in the presence of *M. cryptica* DNA but not in the presence of any other of the nine *Mycosphaerella* species present (including *M. nubilosa* DNA). Similarly, the primer pair MN1 amplified a 400 bp product in the presence of *M. nubilosa* DNA, but not in the presence of DNA from the remaining nine species (Figure 6.3). Their corresponding species-specific primer pair amplified each of the 15 isolates of *M. cryptica* and *M. nubilosa*. None of the DNA from the three isolates each of the non-target *Mycosphaerella* species gave a product in the PCR reactions.

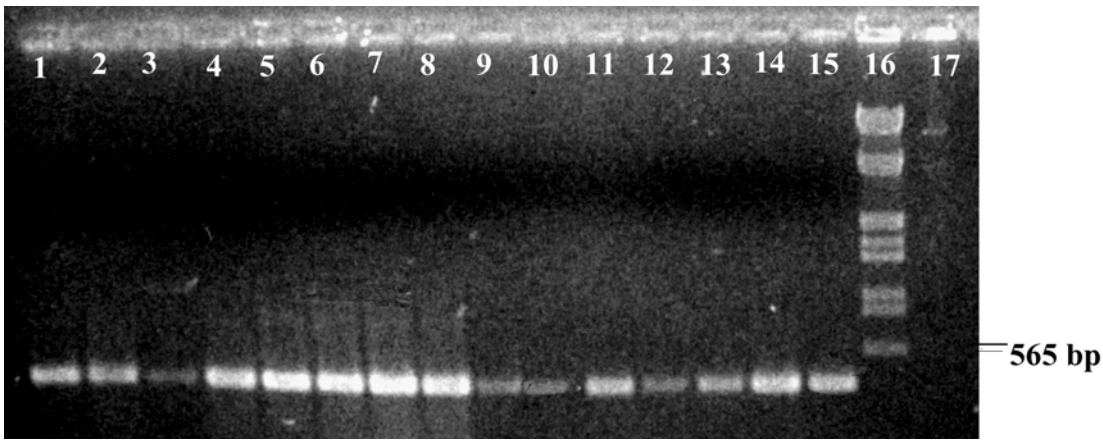


Figure 6.3 1% agarose Gel of PCR product from *Mycosphaerella* DNA using primers specific for either *M. cryptica* or *M. nubilosa*. Lanes 1-8 *M. nubilosa* amplified with MN1F and MN1R primers; L1 R001, L2 R002, L3 R004, L4 R051, L6 R055, L7 R056, L8 R057. Lanes 9-15 *M. cryptica* amplified with MN2F and MN2R primers; L9 R120, L10 R089, L11 R090, L12 R114, L13 R102, 14 R115, L15 R118. Lane 16 HindIII/EcoRI Lamba DNA marker. L17 *M. ambiphylla, M. aurantia, M. gregaria, M. lateralis, M. marksii, M. mexicana, M. nubilosa, M. parva, M. suberosa* combined with MC2F, MC2R primers; L18 *M. ambiphylla, M. aurantia, M. cryptica, M. gregaria, M. lateralis, M. marksii, M. mexicana, M. parva, M. suberosa* combined with MN1F, MN1R primers.

6.4 Discussion

Primers that selectively amplify DNA from *M. cryptica* and *M. nubilosa*, the two most important causes of MLD were successfully developed in the current study. This will allow non-*Mycosphaerella* -specialists with access to basic molecular laboratory facilities to identify of these two species. Although these primers were not tested for the amplification of *M. cryptica* and *M. nubilosa* DNA from infected host plant tissue, methods for detecting *Mycosphaerella* and other fungal DNA in plant tissue are well established (Beck & Ligon 1995, Johanson 1995, Johanson *et al.* 1994, Johanson & Jeger 1993, Dunstan *et al.* 2000). The primers from the current study will enable early diagnosis of the causal organism of MLD in a plantation, once a protocol for the direct amplification of *Mycosphaerella* DNA from host tissue has been finalised. The development of these primers will also facilitate studies into the early infection process of *M. cryptica* and *M. nubilosa*, in that the presence of the pathogen may be detected prior to the appearance of symptoms. Studies may be conducted to determine the length of a hemi-biotrophic phase, and the extent of tissue colonisation both spatially and temporally, beyond the necrotic lesion in these *Mycosphaerella* species. Previously, such studies have been hampered by the slow growth rate of these fungi in culture and the lack of media that would allow their selective isolation and detection by directly plating diseased and non-diseased host tissue.

Primers for the detection of other *Mycosphaerella* species are currently being tested (Jackson, S. pers. comm.) and will be utilised to investigate the disease aetiology of the MLD disease complex. Although *M. cryptica* and *M. nubilosa* are well studied, the role of other species in the MLD disease complex is not well understood. For example, *M. marksii* and *M. parva* are often isolated from diseased eucalypts but their ability to infect leaves and cause disease has not been proven (Park 1984, Park *et al.* 2000, Park & Keane 1982a). This is because these pathogens are often isolated from lesions that are also colonised by other *Mycosphaerella* species, and hence it is difficult to obtain pure ascospore suspensions of *M. parva* and *M. marksii* for pathogenicity studies (Chapter 4.3). The development of species-specific primers will enable the order and pattern of species colonisation of a leaf

to be established. It may be that some of the less commonly isolated species, or those that sporulate on older senescing leaves, infect and cause disease on healthy young leaves, but are slower than other *Mycosphaerella* species to sporulate. Hence, they may appear to be saprophytes, but are in fact slowly maturing pathogens.

This specific PCR technique offers advantages over randomly amplified polymorphic DNA (RAPDs), the previously published molecular method for the identification of *Mycosphaerella* species from eucalypts (Carnegie *et al.* 2001). The use of species-specific primers is more reliable and robust than RAPDs, which are often not reproducible between different labs (Brown 1996, McDonald & McDermott 1993). At present however, only two primers have been tested for the identification of *Mycosphaerella* species on eucalypts. Further primers need to be tested for the remaining species. These should be designed to give a different size PCR product for each species in order to enable multiplexing of the amplification mix so that the presence a number of *Mycosphaerella* species can be tested simultaneously in one PCR reaction. For the present, however, the PCR-RFLP method for discriminating between *Mycosphaerella* species on eucalypts will prove useful.

The PCR-RFLP system developed in the current study will make it easier to identify *Mycospaherella* species isolated off eucalypts. Also, it will facilitate the comparison of species between laboratories. These restriction profiles are consistent and reproducible, and will enable workers to confirm if their identification of a particular species on morphological traits, is the same as those of workers elsewhere.

Some possible discrepancies in *Mycosphaerella* identification that are apparent from the present study are those for *M. gregaria* and *M. suberosa*. The ITS rDNA PCR product from *M. gregaria* in the present study is 180 nt larger than all other species of *Mycosphaerella* identified from eucalypts, due to a large insertion. However, Hunter (2002) also amplified the ITS region of this species in South Africa, and does not mention this increased fragment size. Thus, one of these two species must have been misidentified as *M. gregaria*. Type material must be re-examined and the original type culture sequenced, in order to resolve this discrepancy.

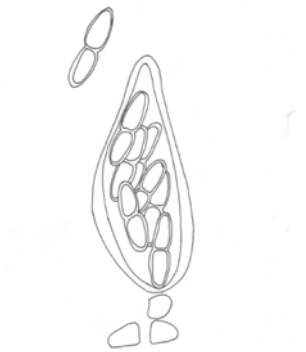
The actual restriction profile for *M. suberosa* differed from that which was predicted on the basis of sequences downloaded from GenBank. The GenBank sequence was used to screen for restriction sites because isolates in the current study were incompletely sequenced. Therefore, the sequence of *M. suberosa* from the current study must differ from that on GenBank. Thus, it may be that *M. suberosa* is variable at the *ApaI* restriction site, or that *M. suberosa* is a species complex. It is difficult to envisage that this species could have been misidentified, as it is the most distinctive species to occur on eucalypts, in terms of its suberised lesion, unique germination pattern, very slow growth rate and unique black, folded colony appearance.

The restriction site based key for identifying *Mycosphaerella* species developed in the current study, is simpler and requires less steps than the morphology based key presented in Chapter 3. However, morphological and cultural observations are still required to arrive at a species determination. Further restriction digests could be introduced to help discriminate between species without the need to refer to cultural and morphological descriptions. The enzymes Alu**,** RsaI, and AccIII could be used to discriminate between *M. marksii* and *M. aurantia*; *M marksii* and *M. suberosa; M. ambiphylla* and *M. parva* respectively. A restriction based system to identify all 33 species of *Mycosphaerella* from eucalypts would become large and procedurally clumsy, and the simplest method is likely to be one based on conserved and reliable morphological characters, with restriction site profiles used to discriminate between morphologically similar species.

These techniques for the identification and differentiation of *Mycosphaerella* species have important functions in helping to manage MLD. Different species may have differing climatic requirements for disease development and therefore it is important that the particular complex of species present in a plantation is known. These techniques may help in identifying spore loads of different species in epidemiological studies. It may be possible to utilise real-time PCR techniques to quantify the level of disease caused by particular species at different times of the season.

Chapter 7

Phenotypic and genotypic variation within *Mycosphaerella nubilosa* in south-western Australia



Chapter 7 Phenotypic and genotypic variation within *Mycosphaerella nubilosa* in south-western Australia

7.1 Introduction

Mycosphaerella cryptica and *M. nubilosa* are regarded as the most serious causes of MLD on eucalypts (Carnegie 2000, Park 1988a, Park & Keane 1987, Park & Keane 1982b). Although *M. nubilosa* is widespread in south-eastern Australia, (Carnegie 2000, Park 1988a, Park & Keane 1982b), it was only recently isolated in south-western Australia (Maxwell, Hardy & Dell 2001). *M. nubilosa* has been associated with a limited number of eucalypt hosts (*E. bridgesiana, E. cypellocarpa, E. globulus, E. gunii, E. quadrangulata, E. viminalis*) all within the series *Viminalis* (Park & Keane 1984, Park *et al.* 2000). In south-western Australia, *M. nubilosa* has only been isolated from the exotically planted *E. globulus* (Maxwell *et al.* 2001) and its origin here is not known. It may have spread from endemic eucalypts, or have been recently introduced from south-eastern Australia with infected *E. globulus* seedlings.

No work has been published on the population genetics of *M. nubilosa* in Australia or elsewhere. An investigation into the population genetic structure of *M. nubilosa* will help answer questions such as those pertaining to the origin of this pathogen in south-western Australia.

Two techniques that have been used to estimate genotypic diversity in pathogen populations are vegetative compatibility groupings (VCG's) and randomly amplified polymorphic DNA (RAPD's). VCG typing is a conventional method that has been successfully used to estimate genotypic diversity of fungal pathogens on eucalypts and other hosts (Heerden 2001, Powell 1995). The attraction of this method is that it is cheap, technically simple and is usually relatively quick. RAPD's analysis is a molecular approach that has been used to measure genotypic diversity in *Mycosphaerella* (Czembor & Arseniuk 1999, Hirst *et al.* 1999, Huang,

Smalley & Guries 1995, Yi *et al.* 2000) as well as other plant pathogenic fungi. The advantages of RAPD over other commonly used molecular-based markers such as random fragment length polymorphism (RFLP'), amplified fragment length polymorphism (AFLP's) and Microsatellite DNA, is that it is relatively cheap and quick to perform. Although it is a dominant marker system, this is not a major concern when the primary goal of a study is to identify clones from a haploid fungal population, such as *Mycosphaerella nubilosa*.

The current study investigates the phenotypic and genotypic variation of *M. nubilosa* in south-western Australia. The phenotypic variation was measured through a comparison of growth rate at different temperatures. The genotypic variation was determined on the basis of VCG and RAPD analyses.

7.2 Materials and Methods

7.2.1 Experimental overview

Phenotypic variation was compared amongst *Mycosphaerella nubilosa* populations from south-western Australia. This included a comparison of growth rate at three different temperatures.

The genotypic diversity (G^) amongst isolates of *M. nubilosa* from south-western Australia was measured using two different methods; firstly with RAPD's and secondly via VCG's. The value of G^ obtained from the RAPD experiment was compared with that from a south-eastern Australian sample of *M. nubilosa*, based on data of Carnegie *et al.* (2001) that were re-analysed for the present study. Carnegie *et al.* (2001) indicated the number of unique banding profiles (genotypes) associated with *M. nubilosa* in a UPGMA tree derived from his study. This information was used to calculate the genotypic diversity of *M. nubilosa* from the eastern Australian sample.

The value of G^ from the VCG data was compared with a value obtained from isolates of *M. nubilosa* from south-eastern Australia that were also tested in the current study. Similarly, VCG tests were made amongst *M. cryptica* isolates from south-western Australia and south-eastern Australia in order to determine the

genotypic diversity of *M. cryptica.* Details of the VCG methodology and calculations are given in section 7.2.5.

7.2.2 Fungal isolates

Single ascospore isolates of *M. nubilosa* were obtained from diseased *E. globulus* leaves as described previously (Chapter 4.2). Fungal cultures were maintained on 2% malt extract agar (MEA, Difco) at 25 °C in the dark. The identity of the isolates and their origin are outlined in Table 7.1.

Table 7.1a Identity of *Mycosphaerella* isolates, used in the present study.

Isolate MURU number	Species	Geographical origin	Experimental use (Growth [G], VCG [V], RAPD's [R])
40	*M. nubilosa*	Tasmania	V
41	*M. nubilosa*	Tasmania	V
42	*M. nubilosa*	Tasmania	V
43	*M. nubilosa*	Tasmania	V
44	*M. nubilosa*	Tasmania	V
45	*M. nubilosa*	Tasmania	V
46	*M. nubilosa*	Tasmania	V
47	*M. nubilosa*	Tasmania	V
51	*M. nubilosa*	Victoria	V
52	*M. nubilosa*	Victoria	V
53	*M. nubilosa*	Victoria	V
54	*M. nubilosa*	Victoria	V
55	*M. nubilosa*	Victoria	V
56	*M. nubilosa*	Victoria	V
57	*M. nubilosa*	Victoria	V
58	*M. nubilosa*	Victoria	V
59	*M. nubilosa*	Victoria	V
61	*M. nubilosa*	Victoria	V
62	*M. nubilosa*	Victoria	V
63	*M. nubilosa*	Victoria	V
64	*M. nubilosa*	Victoria	V
65	*M. nubilosa*	Victoria	V
66	*M. nubilosa*	Victoria	V
67	*M. nubilosa*	Victoria	V
68	*M. nubilosa*	Victoria	V
69	*M. nubilosa*	Victoria	V
71	*M. nubilosa*	Victoria	V
72	*M. nubilosa*	Victoria	V
76	*M. nubilosa*	Victoria	V
70	*M. nubilosa*	Victoria	V
71	*M. nubilosa*	Victoria	V
72	*M. nubilosa*	Victoria	V
73	*M. nubilosa*	Victoria	V
74	*M. nubilosa*	Victoria	V

…Table 7.1a Identity of *Mycosphaerella* isolates, used in the present study.

Isolate MURU number	Species	Geographical origin	Experimental use (Growth [G], VCG [V], RAPD's [R])
75	*M. nubilosa*	Victoria	V
76	*M. nubilosa*	Victoria	V
77	*M. nubilosa*	Victoria	V
155	*M. nubilosa*	Western Australia	V
156	*M. nubilosa*	Western Australia	V
157	*M. nubilosa*	Western Australia	V
158	*M. nubilosa*	Western Australia	V
160	*M. nubilosa*	Western Australia	V
161	*M. nubilosa*	Western Australia	V
162	*M. nubilosa*	Western Australia	V
163	*M. nubilosa*	Western Australia	V
164	*M. nubilosa*	Western Australia	V
165	*M. nubilosa*	Western Australia	V
166	*M. nubilosa*	Western Australia	V
167	*M. nubilosa*	Western Australia	V
301	*M. nubilosa*	Western Australia	G, V, R
302	*M. nubilosa*	Western Australia	G, V, R
303	*M. nubilosa*	Western Australia	G, V, R
304	*M. nubilosa*	Western Australia	G, V, R
305	*M. nubilosa*	Western Australia	G, V, R
306	*M. nubilosa*	Western Australia	G, V, R
307	*M. nubilosa*	Western Australia	G, V, R
308	*M. nubilosa*	Western Australia	V, R
309	*M. nubilosa*	Western Australia	G, V, R
310	*M. nubilosa*	Western Australia	G, V, R
311	*M. nubilosa*	Western Australia	G, V, R
312	*M. nubilosa*	Western Australia	G, V, R
313	*M. nubilosa*	Western Australia	V, R
314	*M. nubilosa*	Western Australia	G, V, R
315	*M. nubilosa*	Western Australia	G, V, R
316	*M. nubilosa*	Western Australia	G, V, R
317	*M. nubilosa*	Western Australia	G, V, R
318	*M. nubilosa*	Western Australia	G, V, R
319	*M. nubilosa*	Western Australia	G, V, R
320	*M. nubilosa*	Western Australia	G, V, R
321	*M. nubilosa*	Western Australia	G, V, R
323	*M. nubilosa*	Western Australia	V, R
325	*M. nubilosa*	Western Australia	G, V, R
85	*M. cryptica*	Queensland	V
86	*M. cryptica*	Queensland	V
101	*M. cryptica*	Western Australia	V
102	*M. cryptica*	Western Australia	V
103	*M. cryptica*	Western Australia	V
104	*M. cryptica*	Western Australia	V
105	*M. cryptica*	Western Australia	V
106	*M. cryptica*	Western Australia	V
107	*M. cryptica*	Western Australia	V
109	*M. cryptica*	Western Australia	V
110	*M. cryptica*	Western Australia	V
111	*M. cryptica*	Western Australia	V
112	*M. cryptica*	Western Australia	V
113	*M. cryptica*	Western Australia	V
114	*M. cryptica*	Western Australia	V
115	*M. cryptica*	Western Australia	V
116	*M. cryptica*	Western Australia	V
117	*M. cryptica*	Western Australia	V

…Table 7.1a Identity of *Mycosphaerella* isolates, used in the present study.

Isolate MURU number	Species	Geographical origin	Experimental use (Growth [G], VCG [V], RAPD's [R])
118	*M. cryptica*	Victoria	V
119	*M. cryptica*	Victoria	V
120	*M. cryptica*	Victoria	V
121	*M. cryptica*	Western Australia	V
122	*M. cryptica*	Western Australia	V
123	*M. cryptica*	Western Australia	V
124	*M. cryptica*	Western Australia	V
126	*M. cryptica*	Western Australia	V
127	*M. cryptica*	Western Australia	V
128	*M. cryptica*	Western Australia	V
129	*M. cryptica*	Western Australia	V
130	*M. cryptica*	Western Australia	V
132	*M. cryptica*	Western Australia	V
133	*M. cryptica*	Western Australia	V
134	*M. cryptica*	Western Australia	V
135	*M. cryptica*	Western Australia	V
136	*M. cryptica*	Western Australia	V
137	*M. cryptica*	Western Australia	V
141	*M. cryptica*	Victoria	V
142	*M. cryptica*	Victoria	V
143	*M. cryptica*	Tasmania	V
144	*M. cryptica*	Tasmania	V
145	*M. cryptica*	Western Australia	V

Table 7.1b Identity of *Cryphonectria* isolates, used in the present study.

Isolate number	Species	Geographical origin	Experimental use (Growth [G], VCG [V], RAPD's [R])
E2	*C. eucalypti*	Western Australia	V
E3	*C. eucalypti*	Western Australia	V
E4	*C. eucalypti*	Western Australia	V
E5	*C. eucalypti*	Western Australia	V
C1	*Cryphonectria cubensis*	South Africa	V

7.2.3 Phenotypic variation

Growth rate

The growth rate of twenty isolates of *Mycosphaerella nubilosa* was determined at three different temperatures (15, 25 and 28°) with three replicate plates per isolate-temperature treatment. Inoculum plugs of 5 mm diam were dissected with a sterile cork borer from the margin of an actively growing colony on MEA, and placed mycelial side down at the centre of a Petri-dish of MEA (20 ml; Difco).

These plates were sealed with Parafilm M (American National Can., Chicago, USA) incubated in the dark and the radial diam of the cultures measured after 4, 6 and 8 wk.

In addition to radial diameter, the dry weight of 15 *M. nubilosa* isolates was recorded after 8 wk growth at 18, 25 and 28°. These isolates were grown on a sterile cellophane membrane over the MEA plates, in order to facilitate the removal and assessment of mycelial weight. Prior to placing over the MEA, the cellophane was cut into 90 mm discs, and softened by boiling for 2 h in 1 L of water, amended with 1 g of EDTA. This was followed by boiling for 2 h in water, the water changed and boiled again for 2 h. The discs were then placed into a glass Petri-dish, sealed with aluminium foil, and sterilised by autoclaving for 20 min at 121 °C on three consecutive days. After the radial growth of the colonies was recorded, the mycelia was scraped from each plate and placed into a 1.5 ml microfuge tube. The mycelia were air dried at 60 °C for 2 d until there was no further reduction in dry weight.

Statistical analysis

Prior to analysis, data for parametric tests were screened for assumptions of homoscedasticity, normality, non-correlations of means and variances and presence of outliers (Tabachnick & Fidell 1996) using STATISTICA (v. 6). Where data did not fit these assumptions, they were transformed using accepted functions (Tabachnick & Fidell 1996). Significant main effects and interactions were compared with the Least Significant Difference (LSD) test.

7.2.4 Genotypic variation within *M. nubilosa* based on RAPD's

DNA extraction.

Multiple hyphal fragments of the *M. nubilosa* isolates (Table 7.1) were inoculated into 80 ml of V-8 juice broth (Stewart *et al.* 1999). Flasks were incubated for 14–21 d at 21 ° in the dark, after which the mycelia were harvested and DNA extracted using the silica binding method described previously (Chapter 5.2). The DNA concentration was determined using a Hoefer DyNA Quant 200 fluorometer

according to the manufacturer's instructions. The DNA was then stored at –20 ° until RAPD-PCR amplifications.

PCR amplification and gel electrophoresis.

Twelve different 10-mer oligonucleotide primers were used in this study (Table 7.2). All RAPD-PCR reactions were performed aseptically in sterile 200 µl microfuge tubes with a reaction volume of 25 µl, containing; 5 ng genomic DNA, 0.2 mM primer, 2.5 mM $MgCl_2$ (Biotech International), 1.1 U *Tth* plus polymerase (Biotech International), 1x polymerisation buffer (Biotech International) equivalent to 67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg ml^{-1} gelatin, 0.2 mM dNTPs and sterile, deionised water (Astar) to make up the reaction volume. PCRs were performed in an Applied Biosystems GeneAmp 9600 thermocycler programmed for 94 ° (2 min) followed by forty cycles of 94 ° (1 min), 36 ° (1 min) and 72 ° (2 min). A water control was run with each series of PCR reactions to check for DNA contamination of reagents. The PCR products were electrophoresed on 1.5% agarose in 1x TAE buffer at 80V for 1 hr and 20 min using 100 bp ladder (Fisher Biotec) as the molecular weight standard. Visualisation of DNA fragments was performed under UV lights following gel staining with ethidium bromide (0.5 µg ml^{-1}) for 15–30 min and de-staining in 1x TAE buffer for 10–15 minutes All PCR reactions were repeated to validate against false positive and negative bands.

Table 7.2 Sequence and source of RAPD primers.

Primer*	Sequence (5' - 3')
OPE-15	ACGCACAACC
OPI-2	GGAGGAGAGG
OPI-6	AAGGCGGCAG
OPI-9	TGGAGAGCAG
OPI-14	TGACGGCGGT
OPI-17	GGTGGTGATG
OPI-20	AAAGTGCGGG
OPV-8	GGACGGCGTT
OPV-18	TGGTGGCGTT
OPV-19	GGGTGTGCAG
OPX-1	CTGGGCACGA
OFW-6	AGGCCCGATG

*Primer reference numbers based on primer kits available from Operon Technologies, Inc., Alameda, California, USA

Genotypic data analysis

Reproducible DNA bands generated from agarose gel electrophoresis were scored either as present (1) or absent (0) in a binary matrix. These data were used for the estimation of genotypic diversity in the sample population. The genotypic diversity (G^) and its variance (*Var* (G^) were calculated from the frequency of *M. nubilosa* isolates occurring in each of the genotype groups using the following formulas (Stoddard & Taylor 1988): $G^{\wedge} = 1/\Sigma p_i^{\,2}$; $Var\ (G^{\wedge}) = 4/N(G^2)[G^{2\wedge}p_i^{\,3} - 1]$. Where p_i is the observed frequency of the ith of R genotypes, N is the sample size, G is the sample population genotypic diversity. The standard deviation of the sample genotypic diversity was calculated from the square root of the variance. The percentage of maximum diversity of the *M. nubilosa* sample population was calculated ((G/N)*100; (McDonald & McDermott 1993).

In addition the value of G for the south-western Australia population of *M. nubilosa* from the current study was compared with that from an eastern Australian population of *M. nubilosa* from a previous study (Carnegie *et al.* 2001). The data from the previous work of Carnegie (*et al.* 2001) utilised a subset of 10-

mer primers from the current study in order to differentiate *Mycosphaerella* species. Thus in the current study, Carnegies data was re-analysed for the purpose of determining the genotypic diversity in the eastern Australian sample of *M. nubilosa*.

7.2.5 Genotypic variation within *M. nubilosa* based on VCG's

VCG design

Twenty-seven *M. nubilosa* and thirty-two *M. cryptica* isolates from south-western Australia, and thirty four and nine isolates of each species respectively, from eastern Australia, were used to investigate the existence of VCG's in these two species (Table 7.1). At least three replicates for each of six isolates were inoculated per agar plate in a design, which ensured that there were three interactions between each isolate on a plate (Figure 7.2). All possible permutations of the isolates were tested against one another. Three replicate plates were inoculated for each of the permutations.

VCG media

The VCG tests were established on 45 mm Petri-plates containing one of the following media (7 ml), the first three of which have previously been used for the purpose of determining VCG's. These were oatmeal agar (OA) without bromecresol green and the following three media, each amended with the pH indicator bromocresol green, at 50 mg L^{-1}: MEAg (24 g L^{-1} Difco malt extract, 2 g L^{-1} yeast extract, 200 mg L^{-1} tannic acid, 100 mg L^{-1} methionine, 2 mg L^{-1} biotin, 2 mg L^{-1} thiamine, and 20 g L^{-1} agar) (Heerden 2001)), Potato dextrose agar G (PDAg: Difco potato dextrose agar supplemented with 7 g L^{-1} malt extract, 2 g L^{-1} yeast extract, 800 mg L^{-1} tannic acid, 100 mg L^{-1} methionine, 2 mg L^{-1} biotin, 2 mg L^{-1} thiamine, and 5 g L^{-1} agar) (Powell 1995); K-C agar (1 g. L^{-1} casamino acids, 1 g. L^{-1} yeast extract, 1 g L^{-1} Ca $(NO_3)2$, 200 mg L^{-1} KH2PO4, 250 mg L^{-1} MgSO4, 150 mg L^{-1} NaCl, Tan, pers. comm.).

The OA was prepared by simmering 100 g of oats in 1 L of water (stirring occasionally) for 1 h. After the porridge had cooled it was sieved through a single

layer of cheesecloth. This extract was then made up to 1 L with distilled water and 30 g agar, then autoclaved at 121 ° for 20 minutes As a positive control, six isolates of *Cryphonectria eucalypti* from different VCG's (Jackson, T. pers. comm., Venter *et al.* 2001) were tested against each other on three replicate plates of each of the preceding media.

VCG identification and data analysis

VCG's were identified according to whether they had merged, forming a confluent mycelium (compatible), or whether they developed a barrage reaction at the point of mycelial contact (Anagnostakis 1977). On media incorporated with the pH indicator bromocresol green, incompatible reactions were further characterised by the formation of a dark coloured line along the area of mycelial contact (Powell 1995) where cell death had resulted in leakage of acidic cellular contents and altered the pH of the media.

VCG's were tabulated for each species. Each VCG was considered a distinct genotype and the Genotypic diversity was calculated as described above for the RAPD data.

7.3 Results

7.3.1 Phenotypic variation

Growth rates

There was a strong correlation between growth rates of each isolate as determined by mycelial mass and radial diameter ($r^2 = 0.96$, $p<0.05$), therefore only the analysis of radial growth is presented because this involved more isolates. There were significant differences in growth rate amongst isolates of *M. nubilosa* ($p<0.01$) (Figure 7.1) at each of the three temperatures. The growth rate was fastest for all isolates at 25° and slowest at 15° (Figure 7.1). According to its growth at 25° each isolate was classified as fast (> 14mm/mnth), moderate (12–14 mm/mnth) or slow (< 12 mm/mnth). The occurrence of these growth rate phenotypes was compared amongst region of isolation. However, there was no obvious relationship between

region of origin and growth rate (Table 7.3) with isolates from each region ranging from slow to fast, except for Esperance which had only one isolate and that grew at a 'moderate' rate.

Table 7.3 Tabulation of isolate growth rates against their region of origin.

Isolate (MURU)	Region of origin	Growth rate
1 (301)	Esperance (Kalima)	Moderate
2 (302)	Albany (Napier Creek)	Moderate
3 (303)	Manjimup (Woodrakara)	Moderate
4 (304)	Albany (Cobertup)	Fast
5 (305)	Manjimup (Wren)	Moderate
6 (306)	Manjimup (Channeybearup)	Slow
7 (307)	Manjimup (Shedley)	Moderate
8 (309)	Manjimup (Boorara)	Moderate
9 (310)	Manjimup (Dudijup)	Fast
10 (311)	Denmark-Walpole (Gerner)	Moderate
11 (312)	Denmark-Walpole (Thomas)	Fast
12 (314)	Denmark-Walpole (Hamilton)	Fast
13 (315)	Denmark-Walpole (Bentink)	Slow
14 (316)	Bunbury – Augusta (Warranella)	Moderate
15 (317)	Bunbury – Augusta (Lamberti)	Fast
16 (318)	Bunbury – Augusta (Summerlea)	Moderate
17 (319)	Bunbury – Augusta (Kemp)	Slow
18 (320)	Bunbury–Augusta (Darling View)	Moderate
19 (321)	Denmark-Walpole (Blight)	Moderate
20 (325)	Bunbury – Augusta (Summerlea)	Fast

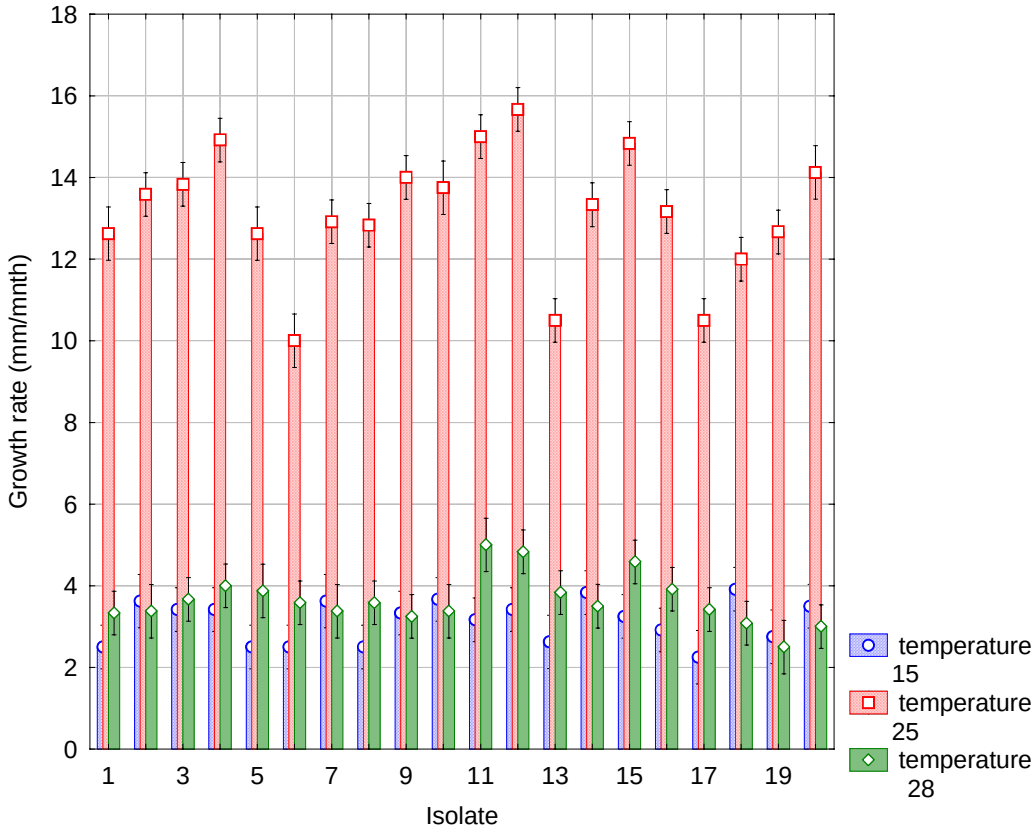


Figure 7.1 Comparison of radial growth (mm/month) amongst isolates of *Mycosphaerella nubilosa* at 15, 25 and 28 °C.

7.3.2 Genotypic variation

VCG data

No barrages formed between any combinations of *M. nubilosa* isolates on any of the four media tested and a confluent hyphal zone occurred between all isolates (Figure 7.2). However, on the positive control plates, *C. eucalypti* isolates of different VCG's did form barrages (Figure 7.2). The formation of barrage zones was evident after 2 wk growth of the *C. eucalypti* isolates. The barrage zones were most clear on OA, followed in order of clarity by PDAg, MEAg, K-CAg. The formation of confluent zones occurred after 3–6 months for *M. nubilosa* isolates. Many plates became contaminated with *Penicillium* and other species during this extended incubation time.

Tests between *M. cryptica* isolates also failed to induce barrage reactions. However, growth was extremely slow and mycelial contact only resulted from 10% of isolate pairings after 6 months of incubation. Therefore the genotypic diversity of *M. cryptica* was not calculated.

The genotypic diversity of the south-western Australian *M. nubilosa* population, as calculated from VCG data was 1 (Table 7.4). G^ for the south-eastern Australia population was also 1.

Table 7.4 Genotypic diversity of a *Mycosphaerella nubilosa* population from south-western Australia based on VCG data

Population	Genotype	Count	Frequency	*G^
south-western Australia	1	1	27/27	1
south-eastern Australia	1	1	34/34	1
Combined east and west	1	1	61/61	1

G^ = genotypic diversity; Var = variance; * Terms explained in text

RAPD Data

Eighty-two distinct, reproducible DNA bands were scored from all 12 primers, of which 3 (3.6%) were polymorphic. The DNA banding pattern was identical for all isolates, with all primers tested, except for isolate R4 (Figure 1, lane 5). OPE-15 was the only primer to reveal genetic polymorphism (Figure 7.3).

The genotypic diversity of *M. nubilosa* based on RAPD's data was greater than that obtained from VCG data. The **G^**, % maximum diversity and variance values for the Western Australian population (1.1, 5.5, 0.01) were smaller than the eastern Australian population (6.3, 48, 1.06) (Table 7.5).

Table 7.5 Comparison of the genotypic diversity of a *Mycosphaerella nubilosa* population from south-western Australia with that from south eastern Australia based on randomly amplified polymorphic deoxyribonucleic acid primer sites

Population	Genotype	Count	Frequency	G^	% Maximum diversity	Var (G^)
*Western Australia	1	1	1/20	1.1	5.5	0.01
	1	19	19/20			
*Eastern Australia	5	1	1/13	6.3	48	1.06
	1	2	2/13			
	2	3	3/13			

G^ = genotypic diversity; Var = variance; * Terms explained in text

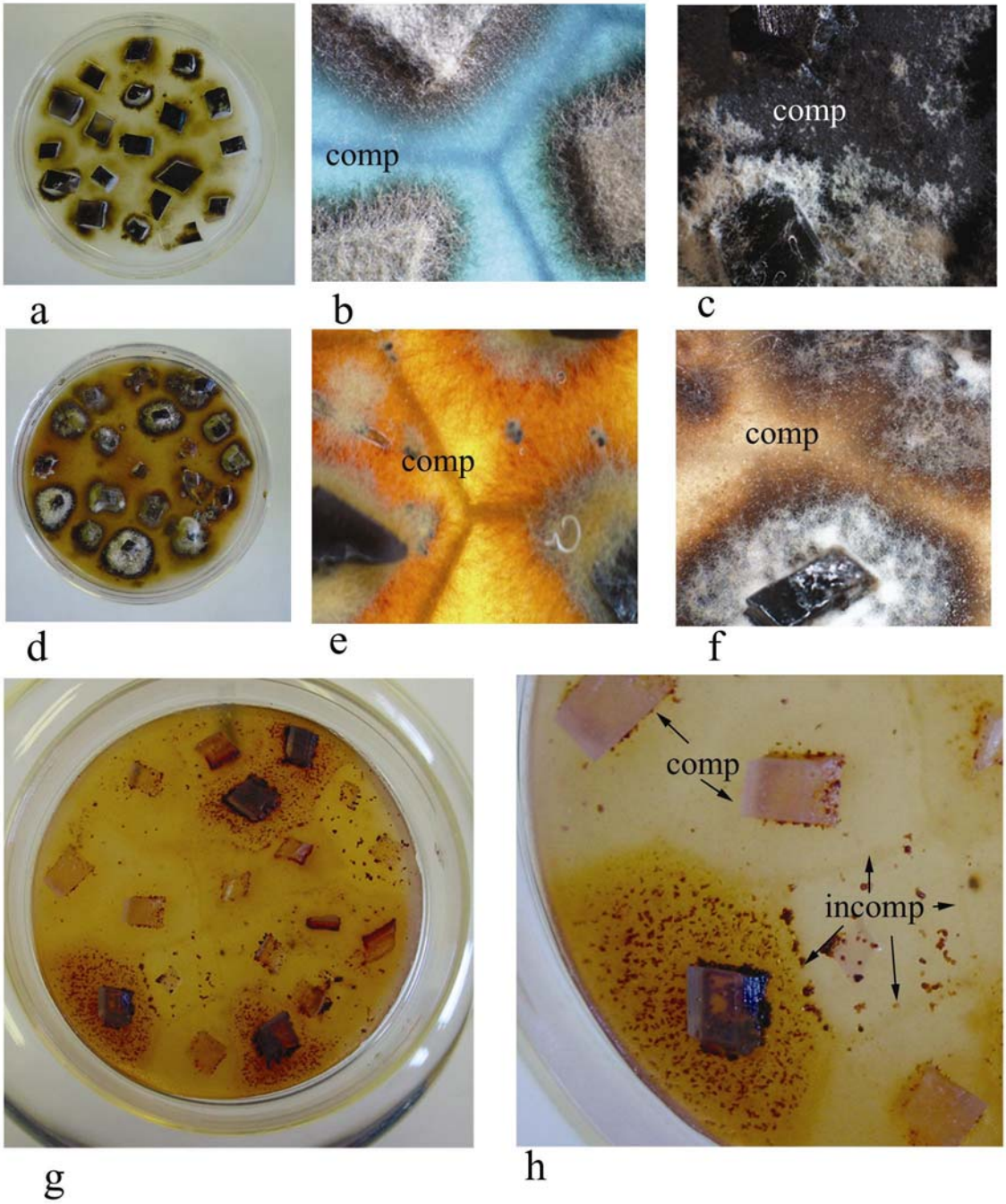


Figure 7.2 Vegetative compatibility reactions in *Mycosphaerella nubilosa* (a-c), *M. cryptica*(d-f) and *Cryphonectria* (g-h).Reactions are all compatible (comp) between different isolates of *M. nubilosa* and between different isolates of *M. cryptica* as no barrage zones are apparent. Some reactions are compatible and some reactions are incompatible (incomp) between isolates of *Cryphonectria.* Barrage zones are apparent between isolates from different VCG groups of *Cryphonectria.* The mycelia at the margins of the *Mycosphaerella* colonies is less dense than at the centre of the colonies due to nutrient depletion with time. Media was oatmeal agar (a, c, d, f, g, h), K-C (b), MEAg (e).

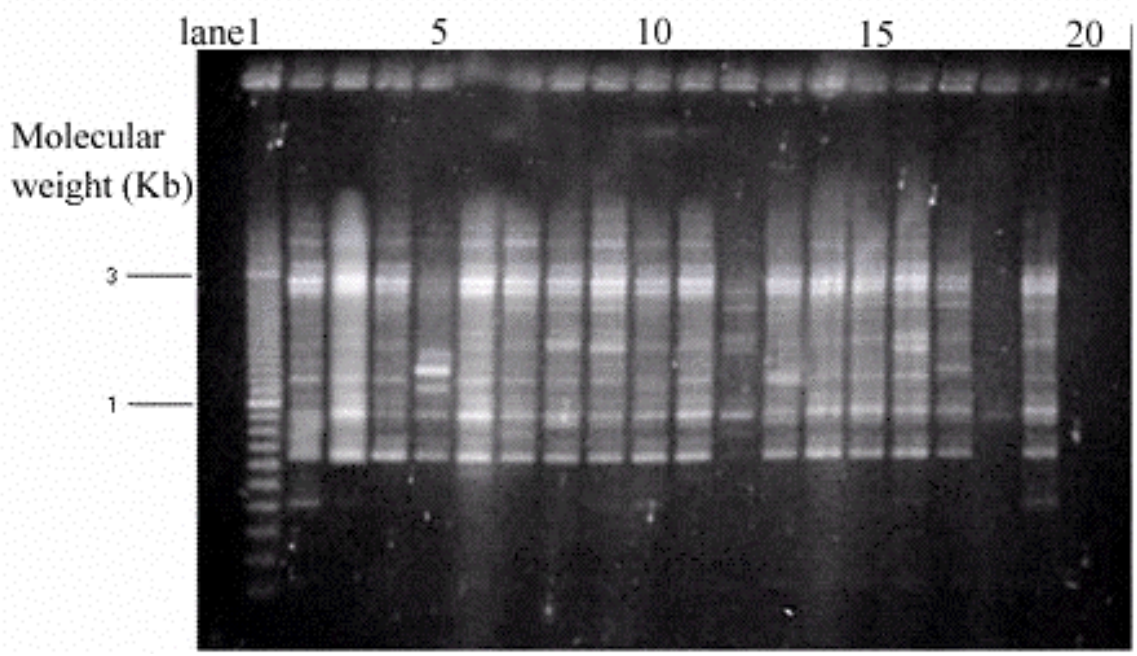


Figure 7.3 RAPD profile of *Mycosphaerella nubilosa* isolates obtained from primer OPE-15. Lane 1-20: 100 bp molecular weight ladder, R1, R2, R3, R4, R5, R6, R7, R9, R10, R11, R12, R14, R16, R17, R18, R19, R20, R22, negative control, 100bp ladder.

7.4 Discussion

The low level of genetic variation detected in the sample population suggests that *M. nubilosa* has spread from south-eastern Australia into Western Australia recently. Although *M. nubilosa* is widespread in south-eastern Australia, (Carnegie 2000, Park 1988a, Park & Keane 1982b), it was only recently isolated in south-western Australia (south-western Australia) (Maxwell *et al.* 2001). *M. nubilosa* has been associated with a limited number of eucalypt hosts (*E. bridgesiana, E. cypellocarpa, E. globulus, E. gunii, E. quadrangulata, E. viminalis*) all within the series *Viminales* (Dick & Gadgil 1983, Park & Keane 1984, Park *et al.* 2000). Of the eucalypts in WA that are within this series, none are forestry species or grow in high rainfall areas where MLD is likely to develop. Although there are now forestry eucalypts indigenous to south-western Australia that belong to the *Viminales* series, *E. diversicolor* is a closely related species in the same sub-genus, *Symphomyrtus*. Due to their phylogenetic closeness to the *Viminales*, these species may be potential hosts for *M. nubilosa*. Over time genetic mutation or sexual recombination in *M. nubilosa* could more easily result in adaptation to such related hosts than to less related hosts such as *E. marginata.*

Although Carnegie *et al.* (2001) concluded that a sample of *M. nubilosa* isolates from eastern Australia had a low degree of genetic variation with the unweighted pair-group mean method using arithmetic means (UPGMA) this was in comparison to *M. cryptica.* They calculated that 13 *M. nubilosa* isolates from diverse hosts in eastern Australia clustered at a simple matching distance of less than 0.115, compared with *M. cryptica* isolates which clustered at the greater distance of 0.3 (although *M. cryptica* was reduced to 0.064 when an outlying isolate from Tasmania was excluded). The genotypic diversity of *M. nubilosa* of this same sample (current study) is much lower than that for sample from south-western Australia. In the Carnegie *et al* (2001) study not more than three isolates shared an identical RAPD profile, whereas in the current study up 19 isolates shared the same identical RAPD profile.

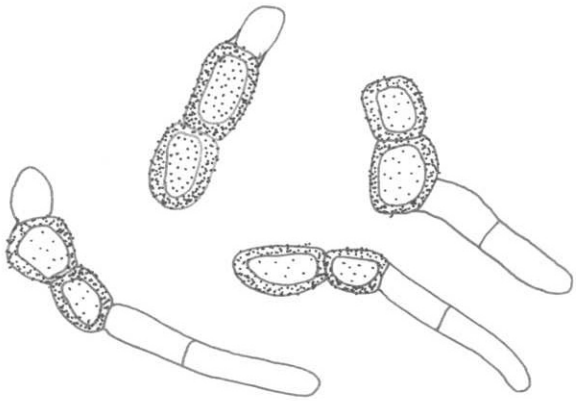
VCG data indicated that there is no genotypic diversity in either the eastern or the western populations of *M. nubilosa.* This conclusion is contradicted by the RAPD data, which detected some degree of diversity in both populations. Therefore it is likely that *M. nubilosa* forms few or no compatibility groupings, or that the media utilised were inappropriate for detecting incompatibility reactions in this species. This technique was a slow and inconclusive way to measure genotypic diversity in the two *Mycosphaerella* species tested. RAPD's were far superior for this purpose. This conclusion is particularly evident when considering the even more slow-growing *M. cryptica,* for which mycelial contact had not occurred between 90% of isolates tested, even after 6 months of growth. Thus, although VCG groups is a rapid and technically simple way of assessing genotypic diversity in relatively fast growing fungi such as *Cryphonectria* (Heerden 2001), it is inappropriate for studying large populations of slow growing species such as *M. nubilosa* or *M. cryptica.*

There were significant differences in growth rates amongst *M. nubilosa* isolates and this could have implications for the identification of species. It is important that the range of growth rate is clearly established when this is used as a criteria to help in the identification of taxa. The growth rate of isolates from diverse hosts from throughout the species occurrence should be used in order to establish reliable estimates of this parameter. Also, there were large differences in growth rates for the same isolates at different temperatures. An increase from 25 to 28 resulted in a large decrease in growth rate. This emphasises the importance of maintaining constant and accurate temperatures when conducting experiments to compare the growth rate between isolates, especially if this is used as a criteria to identify the isolate in question.

It is puzzling that although all isolates in south-western Australia are genotypically identical according to RAPD's data, they varied phenotypically in terms of their growth rates. This is an indication that the RAPD's method may not be sensitive to detect all of the genotypic variation of *M. nubilosa* in south-western Australia.

Chapter 8

General Discussion



Chapter 8 General Discussion

8.1 Summary of research findings

The key findings of this thesis were:

- MLD is the most widespread and severe disease in eucalypt plantations in south-western Australia
- The number of species associated with MLD in south-western Australia has increased from three to ten since 1994
- Importantly, *M. nubilosa,* which was absent in 1994 is now the greatest cause of MLD on young *E. globulus* in south-western Australia
- *M. cryptica* is the most significant cause of MLD on forestry eucalypts in general and on adult *E. globulus* foliage in particular
- The relative severity of MLD disease has increased in *E. globulus* plantations over the past ten years to the extent that it is now more severe in plantations than in traditional forestry areas in south-western Australia
- Phylogenetic evidence based on the ITS region of the rDNA is that anamorph genera associated with *Mycosphaerella* are polyphyletic
- *Mycosphaerella* as a teleomorph genus is probably mostly monophyletic, with the exception of the '*Dissoconium*' clade which may have a separate evolutionary origin from the remaining species within *Mycosphaerella,* however, further work is required to resolve this issue
- Novel tools for the identification of *Mycosphaerella* species pathogenic on eucalypts, including species-specific primers for *M. cryptica* and *M. nubilosa* have been developed
- The genotypic diversity of the south-western Australian population of *M. nubilosa* was significantly lower than that of south-eastern Australia,

indicating that this pathogen was recently introduced into south-western Australia

The implications of these research findings are discussed below.

8.2 Mycosphaerella *taxonomy*

The study of MLD must be underpinned by a reliable and definitive taxonomy of the species that comprise this genus. Epidemiological work may be weakened or lead to erroneous conclusions if it is based on incorrect taxonomic assumptions. The work in the current thesis has led to a clearer understanding of the taxonomy of *Mycosphaerella* on eucalypts. Species, which were previously thought to be separate, have been demonstrated to probably be conspecific on the basis of ITS rDNA sequence comparison. Rapid methods for the reliable differentiation of *Mycosphaerella* species have been developed that will enable forest pathologists working in different laboratories and on separate continents to compare species amongst each other, with confidence. Using and extending the techniques outlined within, the taxonomic mistakes that have been made in some previous epidemiological studies of *Mycosphaerella* (Beresford 1978, Cheah 1977) need not be repeated.

Ideally, the taxonomic placement of *Mycosphaerella* at all levels from genus through to class should reflect its natural phylogeny. The phylogenetic evidence from the current study is that *Mycosphaerella* is an assemblage of polyphyletic anamorph genera. However, although *Mycosphaerella* as a teleomorph genus is probably mostly monophyletic, this is not entirely clear from the current study, or from previous similar work. There is some evidence that the '*Dissoconium*' clade may be of a separate origin from other clades within *Mycosphaerella.* The *Dissoconium* clade may be phylogenetically closer to other genera within the Dothideales, such as *Botryosphaeria* or *Dothidea,* than to other lineages within *Mycosphaerella.* This conclusion is based on a tree that compared the ITS rDNA sequences of the sub-set of *Mycosphaerella* species occurring on eucalypts, and using more than one out group taxa (*Botryosphaeria* and *Dothidea*). Also, there are well-differentiated lineages within *Mycosphaerella* that may be phylogenetically

equivalent to genera within the Dothideales, in terms of the degree of ITS rDNA sequence divergence that they exhibit.

The separation of the *Dissoconium* anamorph clade of *Mycosphaerella* from the remaining *Mycosphaerella* species off eucalypts has also been discussed in earlier studies (Crous *et al.* 1999, Crous *et al.* 2000, Crous *et al.* 2001a). Their tree derived from the ITS rDNA regions indicated that the *Dissoconium* clade is distinctly separate from *Mycosphaerella sensu-stricto* (Crous *et al.* 2001a). Whereas, their cladogram inferred from the large sub-unit (LSU) of the rDNA, indicates that *Dissoconium* clusters within *Mycosphaerella sensu-stricto.* However, the latter of these cladograms, uses *Cladosporium* as an out-group. As this anamorph genus is known to have a *Mycosphaerella* teleomorph, it was inappropriate for the comparison of divergence within *Mycosphaerella*. Therefore, the topology of the LSU tree probably reflected a similarity between the *Colletogloeopsis* clade and the *Cladosporium* outgroup, not a similarity between the *Dissoconium* and the larger cercosporoid clade within *Mycosphaerella*. The advantage of the LSU tree however, is that this region is more conserved than the ITS rDNA, and the former is therefore more suited to resolving differences at higher taxonomic levels.

The remaining lineages outlined in the current study may represent phylogenetic sections within *Mycosphaerella*. Attempts so far to relate the molecular phylogeny of *Mycosphaerella* to its currently accepted taxonomic sections (Barr 1972, Crous *et al.* 2000) have been inconsistent. This is due to a number of reasons. Firstly, the criteria for differentiating these sections are artificial and based on characters that are not necessarily phylogenetically conserved. Secondly, many species of *Mycosphaerella* may not be readily or accurately ascribed to a particular section.

The criteria that are used to differentiate sections within *Mycosphaerella* include the arrangement and shape of asci and ascospores, ecology (for example parasitic v. saprobic), and anamorph affiliation (Barr 1972, Crous *et al.* 2000). There is no good evidence to suggest that these are phylogenetically conserved characters. The character states of parasitic versus saprobic ecology are correlated with climatic zone (Crous *et al.* 2000), and hence are homoplasious rather than homologous states. Anamorph states appear to be mostly polyphyletic, and did not correlate

strongly with the clades that emerge from molecular phylogenetic trees within *Mycosphaerella* in the current study and in previous work (Crous *et al.* 2001a, Crous, Kang & Braun 2001b, Goodwin & Zismann 2001, Stewart *et al.* 1999). Furthermore, molecular studies have not indicated whether the characters used to delineate *Mycosphaerella* from morphologically similar genera, are phylogenetically conserved. Characters such as ascal arrangement, ascospore pigmentation or number of septa, although convenient for classification, may not reflect phylogenetic differentiation of genera within the Dothideaceae. Although deeper level studies placing the Dothideales within the Dothidideomycetes are available (Guo, Hyde & Liew 2000, Liu *et al.* 1999, Reynolds 1998, Silva-Hanlin & Hanlin 1999, Winka, Eriksson & Bang 1998), there have been no comprehensive studies considering the molecular phylogeny within the family Dothideaceae that may help delimit *Mycosphaerella* and other closely related genera. Until such studies are conducted, the status of the *Dissoconium* clade and even other clades within *Mycosphaerella* will remain ambiguous. The need for such studies is emphasised by the observation that the Dothideaceae, and at least one other family within the Dothideales (Phaeotrichaceae), appear to be polyphyletic (Liu *et al.* 1999, Lumbsch, Lindemuth & Schmitt 2000). More genes need to be sequenced and a multi-gene based phylogeny worked out for *Mycosphaerella* and other genera within the Dothideales.

Mycosphaerella is a large genus comprising more than 1800 species (Corlett 1991). The present study supports the hypothesis, raised previously (Barr 1972, Corlett 1991, Crous *et al.* 2000), that this number may be artificially high, due to such factors as the separate naming of the same species on different hosts. An example of species synonymy revealed in the current study was that of *M. molleriana*. This species has been recorded on eucalypt hosts from a number of countries and has some reasonably distinctive morphological features, including an acervular anamorph state, *Colletogloeopsis.* Yet, on the basis of ITS rDNA sequence data a synonym for this species has recently been described as *M. vespa* (Carnegie & Keane 1998, Milgate *et al.* 2001), with an apparently different anamorph state (*Coniothyrium ovatum*). The anamorph state for this species is therefore variable, probably according to environmental conditions, although it could also be strain

related; with some strains expressing the acervular form and others the pycnidial form.

Variability in the expression of features such as conidiogenesis and mitospore pigmentation has clouded the distinction of some anamorph genera connected with *Mycosphaerella*. The above example from the current thesis is a case in point. *Colletogloeopsis* forms thick-walled conidia in an acervulus, not in a pycnidium (Crous 1998); the conidia of *Coniothyrium* are formed in a pycnidium (Carnegie & Keane 1998, Milgate *et al.* 2001). Therefore, the character of fruiting structure (acervular v. pycnidial) is influenced by strain or environmental conditions. As this character is not stable, it makes the taxonomy of these anamorph genera very difficult. Similar kinds of variability have been recorded in other anamorph genera, including *Septoria* (Verkley 1998, Verkley & Priest 2000). Further work is required in determining which characters are stable, under what conditions, for the delimitation of anamorph taxa.

Such a study is needed for *M. molleriana,* which along with *M. vespa* should also consider the recently described *M. ambiphylla* (Chapter 3) that shares a similar ITS rDNA sequence to *M. molleriana,* but has a different anamorph state. These taxa have different anamorphs: *Phaeophleospora ambiphylla* in the case of *M. ambiphylla; Colletogloeopsis* in the case of *M. molleriana;* and *Coniothyrium* in the case of *M. vespa.* In *P. ambiphylla* the conidia and the conidiogenous cells are pale brown and finely verruculose, and conidiogenesis is both percurrent and sympodial (Chapter 3). Whereas, the conidia of *Coniothyrium* are verruculose and the conidiogenous cells are hyaline and smooth-walled, conidiogenesis is by percurrent proliferation only (Carnegie & Keane 1998, Milgate *et al.* 2001). Therefore, numerous isolates of each of these three species should be grown on the same kind of substrate under the same conditions in order to compare conidiogenesis, conidia and fruiting structure. In the same study, the sequence from a range of neutral, appropriately variable non-coding areas of genes should be compared for each of these three species.

Molecular information has aided in the identification of synonymous taxa within *Mycosphaerella* in the current study. This has been possible through the comparison

of ITS rDNA sequences, which were identical or at least 99% similar between some species. Sequences obtained directly in the current study, indicated that intra-species variation of the ITS rDNA was in the order of 1–2 nt, a figure which agrees with that of Goodwin & Zismann (2001) for the 25 *Mycosphaerella* species that they considered. However, when sequences from the NCBI GenBank database are added to those from the current study, the degree of intra-specific variation increases somewhat. It is likely that most of this increase in intra-specific variation was due to incompletely edited sequences, or misidentified species lodged with GenBank. Considering these errors, it was argued in the current thesis, that intra-species variation within *Mycosphaerella* may be up to 5 nt within the ITS 1 and 2 regions of the rDNA (discounting large insertions). Taxa that vary by 1–2 nt are likely to be conspecific. Those that vary by 3–6 nt may be more difficult to circumscribe with certainty. Taxonomic conclusions for these species must be based on sequences from large collections of isolates (20–30) from throughout their host and geographic range. If the differences are conserved as two distinct genotypes, with no gradation between them, then it is likely that the taxa are different species. However, if there is a gradation between the genotypes then they are conspecific.

On the basis of sequence information and morphological evidence the current study proposes that *M. grandis* is probably synonymous with *M. parva* and *M. vespa* is probably synonymous with *M. molleriana.* Other closely related taxa, such as the *M. heimii* complex, remain cryptic and further work is required on these. One species considered in the current study, *M. lateralis*, has two distinct genotypes that are different at 4 nt sites. These genotypes appear to be geographically separate. However, as this species is polyphagous, occurring on several host genera including *Eucalyptus* (Crous *et al.* 1999, Hoog, Hijwegen & Batenburg-van der Vegte 1991), it is unlikely that populations would become geographically isolated on the same continent. Further work describing and sequencing more isolates of this species is required to ascertain if it is in fact two separate species, or whether they are simply two geographically isolated populations with limited gene flow.

8.3 Impact and Biogeography

The current study has determined that MLD is more severe and widespread in *E. globulus* plantations in south-western Australia, than in indigenous forest areas. This is in contrast with previous studies (Abbott *et al.* 1993, Carnegie *et al.* 1997), which found the disease to be low in plantations and generally higher at selected regrowth locations of endemic eucalypt forest of *E. diversicolor* or *E. marginata.* This shift in relative severity of the disease may be due to the increased area of plantation estate and the increased time that that estate has now been exposed to *Mycosphaerella* species. Close to 10 years has elapsed since those earlier studies, allowing for an increase in inoculum load within the *E. globulus* estate. Also, the studies of Abbott *et al.* (1993) may have been selective and only considered areas of indigenous forest where disease was known to occur, whereas the sampling in the current study was random.

The current thesis has shown that MLD of *E. globulus* in south-western Australia is a disease complex. The number of species associated with the disease there has increased from the three recorded in 1994 (Carnegie *et al.* 1997), to ten in 2001 (Maxwell *et al.* 2003, Maxwell *et al.* 2001, Maxwell *et al.* 2000) including two new species and five new records. Significantly, *M. nubilosa,* previously not recorded in south-western Australia, was found to be the most widespread and damaging foliar pathogen of *E. globulus* plantations there.

The increase in the number of records of *Mycosphaerella* species associated with MLD in south-western Australia is due to the rapidly expanding *E. globulus* estate in the region. The plantation estate in south-western Australia has grown from 5000 ha in 1988 (Bailey & Dunconson 1998, Loch & Floyd 2001) to over 150 000 ha by 2001 (Anonymous 2000). This rapid expansion has resulted in a large area of even aged, closely spaced *E. globulus* in the higher rainfall regions of the state. The juvenile foliage of this eucalypt species is particularly susceptible to *Mycosphaerella*. As the size of the plantation estate has grown, so too has the inoculum load. Thus the likelihood of finding more species has increased along with the impact of the disease. However, except for *M. nubilosa,* the origin of these *Mycosphaerella* species on the exotically planted *E. globulus* remains uncertain.

Apart from *M. cryptica* and *M. marksii,* the *Mycosphaerella* species present in south-western Australia have only been recorded on *E. globulus,* although recently more species have been recorded on *E. diversicolor* (Jackson *et al.* In prep.). These include *M. marksii* and *M. parva* along with new species not previously described. *Mycosphaerella cryptica* and *M. nubilosa* were the most severe and frequent causes of MLD on *E. globulus.* Similarly these two species are the major cause of MLD in south-eastern Australia (Carnegie *et al.* 1998, Park 1988a, Park *et al.* 2000). However, unlike south-eastern Australia, *M. marksii* was a widely recorded primary cause of disease at many plantations in south-western Australia. This pathogen frequently caused disease on adult foliage, and so like *M. cryptica* presents a threat for the entire life of the plantation. Consequently, more work needs to be conducted on the importance of this pathogen, including testing its pathogenicity.

The current study quantified the leaf phase and leaf surface occurrence of *Mycosphaerella* species on *E. globulus*. Although observations of this nature have been recorded previously, this is the first time that quantitative data have been assembled. It was found, for the first time, that *M. nubilosa* caused disease on adult as well as juvenile foliage. However, disease on adult foliage was rare, and the quantitative assessment of leaf phase preference supports previous work, in that *M. nubilosa* predominantly infects juvenile foliage. Over time, it will be interesting to observe whether the ability of *M. nubilosa* to infect adult foliage increases, a result of selection pressure. *Mycosphaerella nubilosa* was the most frequently occurring pathogen across the *E. globulus* estate. However, *M. cryptica* and *M. nubilosa* caused similar intensity of disease on juvenile foliage, and *M. cryptica* was the dominant cause of disease on adult leaves.

In the indigenous eucalypt forest of south-western Australia, MLD was present on *E. diversicolor, E. jacksonii* and *E. marginata* but absent on the more distantly related eucalypt, *Corymbia calophylla.* Disease severity was most pronounced on *E. diversicolor,* in contrast to earlier work which found disease on south-western Australian eucalypts to be greatest on *E. rudis* and *E. marginata* (Abbott *et al.* 1993, Carnegie *et al.* 1997). It was apparent from the current thesis that the major cause

of disease on *E. diversicolor, E. jacksonii* and *E. marginata* was *M. cryptica*. The reason for the absence of the remaining *Mycosphaerella* species on indigenous eucalypts, is unknown. Further work is required to determine the potential of host switching of *Mycosphaerella* species between indigenous eucalypts and the exotic *E. globulus*.

There is evidence that *M. nubilosa,* which has only been isolated from eucalypts within the series *Viminales*, is able to infect *E. diversicolor* but it is not yet clear if it can cause disease on this host (Jackson *et al.* 2001, Jackson *et al.* 2002). Infection studies and pathogenicity trials are required to determine the host range of *Mycosphaerella* species. Such studies have been hampered by the difficulty of obtaining sufficient quantities of pure ascospore suspensions, of the later sporulating species in particular. Obtaining pure ascospore suspensions from fresh material is compromised by the common occurrence of more that one *Mycosphaerella* species on a lesion and reliable methods for inducing ascospores in culture have not been developed for these pathogens.

8.4 Population genetics of M. nubilosa

The population of *M. nubilosa* in south-western Australia was found to be genetically uniform on the basis of RAPD analysis, in contrast to that of eastern Australia which was significantly more variable. Also, no variation in the rDNA was detected in the population in south-western Australia, as opposed to eastern Australia where there was some sequence variation at this locus. This pathogen was first detected in south-western Australia in 1998 (Chapter 3), twenty years after the first *E. globulus* plantations were established in the region. By 1998 *M. nubilosa* was present at every *E. globulus* plantation surveyed in south-western Australia (Chapter 4). In contrast, four years earlier it was not found at any of the plantations visited by Carnegie *et al.* (1997). The low population diversity of *M. nubilosa* suggests that it was recently introduced to south-western Australia and has spread rapidly from this limited introduction. The host range of *M. nubilosa* is thought to be narrow as it has only been isolated from eucalypts from the series *Viminales,* of the *Symphomyrtus* sub-genus. There are no representatives of this series in south-western Australia, therefore, it is unlikely that inoculum was

present on indigenous eucalypts and infected the introduced *E. globulus.* However, there are numerous eucalypts from the *Symphomyrtus* in this region that could possibly be hosts of *M. nubilosa.* Further surveys and pathogenicity testing of these hosts, such as *E. diversicolor,* needs to be conducted to be certain of the host range of *M. nubilosa* in south-western Australia.

The introduction of *M. nubilosa* into south-western Australia indicates the need to improve quarantine measures to reduce gene flow between pathogen populations of south-eastern and south-western Australia. Previously, eucalypt seed and seedling material has been moved amongst Australian states (Young, pers. comm.). Seedlings are sprayed with fungicide, however, this may act to mask the presence of MLD rather than to exclude it. A better approach would be to more closely monitor for disease symptoms before and after the transport of seedling material. This highlights the importance of good hygiene and quarantine strategies in controlling the spread of disease in the plantation eucalypt industry. It provides a warning of the ease with which a potentially more serious disease such as the guava rust (*Puccinia psidii*) could be introduced and spread within Australia via the plantation eucalypt industry.

8.5 Future research

There are five major directions that research in MLD needs to take. Firstly, a better understanding of the phylogeny of this genus should be pursued. Secondly, the role of different species that make up the disease complex needs to be understood, particularly in terms of disease epidemiology at a regional level. Thirdly, the population genetics of the more important *Mycosphaerella* pathogens should be elucidated. Fourthly, the mode of disease resistance in the host must be investigated. Fifthly, the economic impact of MLD should be monitored.

Research into the phylogeny of *Mycosphaerella* should involve a multi-gene approach, such as that of Geiser *et al.* (1998) that utilises some slower and faster evolving loci, in order to get good resolution at the intra- and inter-genus level. The possibility that *Mycosphaerella* may be polyphyletic, and that some lineages

within *Mycosphaerella* may be aligned with lineages in other generic taxa of the Dothideales, should not be ignored.

The role of the different species that comprise the MLD complex in south-western Australia needs to be investigated. This is in relation to host-range, and disease epidemiology. Is the host range of species greater than that suggested by results from the current thesis? Importantly, the disease epidemiology can now be investigated with a greater understanding of the *Mycosphaerella* species present in the region. The species specific primers developed in the current study could be used to verify the identification of ascospores counted in spore capturing devices. Alternatively, polyclonal antibodies could be developed against species of *Mycosphaerella* to accurately measure ascospore release of particular species, using techniques developed for *M. brassicicola* (Kennedy, Wakeham & Cullington 1999, Wakeham 2000).

The population genetics of *M. cryptica, M. marksii* and *M. nubilosa* should be investigated. For *M. cryptica* the question of whether there are differences amongst populations on different hosts needs to be addressed, as does the degree of sexual recombination in this heterothallic species. For *M. nubilosa* the structure of populations in eastern Australia must be compared with that in south-western Australia. This is a homothallic fungus with a limited host range, and so more divergence between populations might be expected than that between populations of *M. cryptica.* These questions could be addressed using the RAPD markers tested in the current study, or with more powerful markers such as those targeting microsatellite DNA.

Disease resistance should be assessed in the field against multiple species of MLD. The species specific primers developed in the current study could be used to determine which species are responsible for the disease present in provenance trials. It is important in breeding resistance that it is known what taxa resistance is being selected against. The type of resistance that works for *M. nubilosa* may not correspond to that which works for *M. cryptica*. For example, a more rapid switch to the juvenile leaf phase may affect resistance to *M. nubilosa*, however, this may have little effect on *M. cryptica* which attacks adult foliage as readily as juvenile

foliage. Dungey *et al* (1997) found significant juvenile adult correlations in provenance susceptibility to MLD and Carnegie and Ades (2002) quantified the *Mycosphaerella* species involved. Although Carnegie (2000) found a correlation between provenances resistant to MLD in their juvenile foliage and those resistant in their adult foliage, a study by Maxwell, Hardy & Dell (1998) found that there was no correlation between provenances that were resistant in their juvenile foliage and those resistant in their adult foliage. These differing conclusions may be due to differing suites of *Mycosphaerella* species at the two study sites. Therefore, more work needs to be conducted comparing the correlation between provenances resistant in their juvenile phase foliage and those resistant in their adult phase foliage. This needs to be done with the knowledge of the specific *Mycosphaerella* species involved in causing the disease. Again, species-specific primers would facilitate these objectives.

In terms of the eucalypt plantation industry it is important that strategies for the early detection of new *Mycosphaerella* species are established. Such strategies need to ascertain, what these species are, where they occur and the degree of threat that they pose. Also, changes in the distribution and impact of currently described species, needs to be monitored. A coordinated approach of plantation managers throughout Australia is needed to facilitate this. Staff, with skill in identifying *Mycosphaerella* and other disease causing species in eucalypt plantations need to be employed to measure and monitor disease throughout the plantation growing regions. Currently, plantations in Western Australia are routinely assessed at regular intervals in order to measure increases in wood volume and thereby determine the optimal time for harvest (Young pers. comm.). The assessment of pest and disease problems should become part of this inventory practice, such that plantation managers have a measure of current and past disease levels in their plantations. Currently, formal forest health surveillance units conduct annual detailed pest and disease surveys of plantations in eastern Australia (Carnegie pers. comm.) but my research into plantation managers records in WA indicate that pest and disease assessment is conducted in an ad hoc and uncoordinated way in Western Australia. With good quantitative pest and disease data, changes in disease level across geographic locations and over time could be measured. This

would help in targeting important areas for future research and in assessing the effectiveness of silvicultural methods in controlling disease. This work needs to occur in addition to basic research aimed at understanding and reducing the impact of MLD.

Further areas of research, which are important for the plantation industry, include the determination of *Mycosphaerella* species present in native eucalypt forests. This is an area of real concern in Australia where plantations are established alongside diverse areas of closely related eucalypt forest. This presents the opportunity of host switching of disease causing organisms, or the exposure of plantation eucalypts to a pathogen pre-adapted to that species. Another aspect of the close proximity of diverse eucalypt habitats is the potentially greater degree of genetic diversity in the pathogen population that may lead to novel combinations of alleles and consequently increased pathogenicity. This is particularly a problem if the industry moves towards clonal forestry in order to increase wood volume, harvesting and processing efficiencies and wood quality, as has occurred elsewhere. Where plantations are clonal, there is the potential for catastrophic losses to disease, if that particular genotype is susceptible.

There are also potential negative environmental impacts of plantations in close proximity to indigenous eucalypt forests. This is because disease levels may reach epidemic proportions in the monoculture conditions of the plantation, and thereby become a massive inoculum load for nearby forest. Thus high levels of disease could result in native forest because of disease epidemics in plantations. Also, there is the potential for *Mycosphaerella* species to be transported to new areas with seed or seedling material as has occurred with *M. nubilosa* on *E. globulus* (Chapter 3, 4 & 7). If such disease causing species are moved to new areas and are able to infect a newly encountered host, then this could be a serious issue. Particularly, if the level of pathogenicity is high on the non-adapted host, as has occurred for some new encounter host pathogen interactions such as the well known example of potato and *Phytophthora infestans* (Fry & Goodwin 1997).

Related to the new encounter interaction, is the potential for host switching of pathogens that are not pre-adapted to the newly encountered host. *Mycosphaerella*

species that move with plantation material into new areas may not initially be able to cause disease on newly encountered hosts. However, if the inoculum loads from plantations remain high, and there is mutation or sexual recombination in the pathogen population, over time evolution may occur in the pathogen population such that it is able to switch hosts to indigenous eucalypt species.

The impact of MLD on growth rates of *E. globulus* was not considered in the current study. However, work by Carnegie *et al.* (1998) found that MLD leaf infection levels as low as 10 % resulted in a 17 % reduction in height of *E. globulus.* Similar impacts have been observed in chemical exclusion trials of pests and diseases of *E. globulus* in south-western Australia (Neumiester-Kemp *et al.* 2003). This work is on-going in south-western Australia and similar trials should be repeated elsewhere in order to gauge the economic impact of MLD on this industry. Particularly as this impact is likely to increase as inoculum levels rise over time and the industry plants a narrower genetic base of trees (possibly clonal), selected primarily for growth rate properties.

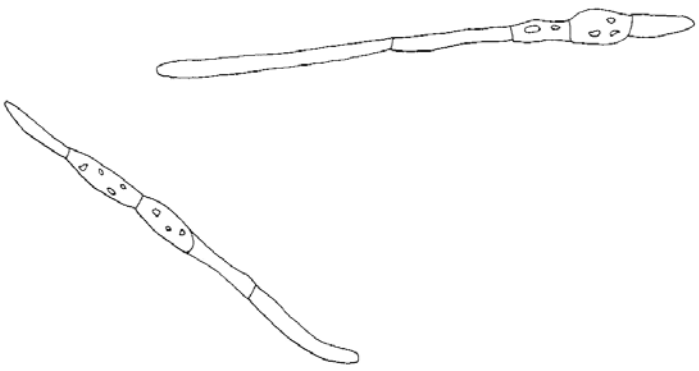
8.6 Conclusion

The differentiation of *Mycosphaerella* species on morphological characters is difficult and has led to erroneous placement of taxa in the past. The current study has shown that sequence variation of the ITS rDNA is suitable for the differentiation of *Mycosphaerella* species, but that more genes need to be sequenced to adequately answer phylogenetic questions pertaining to this and related genera. Molecular techniques were developed, that are more reliable than conventional means, for the identification of *Mycosphaerella* species occurring on eucalypts. These techniques include a PCR based method that will enable more a powerful resolution of important ecological and epidemiological questions regarding MLD. These are areas of research that must be pursued to effectively control MLD in eucalypt plantations into the future.

The increase in the level of MLD in plantations in south-western Australia has been marked over the past ten years. This is such that levels of disease were low

compared with native eucalypt forestry stands. Significantly, *M. nubilosa* the most important pathogen of juvenile *E. globulus,* which was absent from plantations in south-western Australia ten years ago, is now the most widespread cause of disease in this region. In addition, the number of species associated with this disease in south-western Australia has increased from 3 to at least 10 over this same time period. The presence of *M. nubilosa* in particular can be attributed to the movement of plantation material and its impact underscores the need for improved quarantine in this industry. It is likely that disease levels will continue to increase in plantation forestry due to inoculum build up, the movement of pathogen species and genotypes and the reduction in genetic diversity in eucalypts planted. It remains important for the eucalypt plantation industry that research continues into MLD.

Appendices



Appendix 2.1a Foliar analysis of Eucalyptus globulus *plantations assessed for pests and diseases in south-western Australia*

Sample	N mg/g	[P] mg/g	[K] mg/g	[S] mg/g	[Ca] mg/g	[Mg] mg/g	[Fe] mg/g	[Zn] mg/g	[Mn] mg/g	[Cu] mg/g	[B] mg/g
frankland R. plot 1	16.1	1.1	1.0	11.0	3.8	2.2	8.1	21.8	45.7	19.3	17.4
frankland R. plot 2	17.3	1.3	1.0	9.1	3.3	2.1	9.5	28.4	175	16.9	14.9
frankland R. plot 3	18.3	1.4	1.1	11.5	3.5	2.0	10.9	24.9	66.4	20.3	15.7
frankland R. plot 4	14.4	1.1	1.0	12.0	3.5	2.2	7.1	19.2	64.2	17.6	15.2
range Montana97 P1	32.8	2.4	2.0	15.0	3.4	2.3	10.1	37.4	39.7	27.6	22.2
range Montana97 P2	31.4	2.4	1.7	15.4	4.4	1.9	6.0	32.7	72.9	35.1	24.4
range Montana97 P3	17.2	1.2	1.1	12.8	4.6	1.8	6.5	23.2	75.1	25.1	18.2
range Montana97 P4	18.1	1.1	1.0	10.2	5.7	2.4	5.0	16.2	86.6	19.3	16.0
Kelora 8/2/00 P2 R1	18.8	1.7	12.0	1.5	3.7	2.0	12.6	23.4	37.5	9.5	16.8
Kelora 8/2/00 P2 R2	19.1	1.6	10.0	1.4	6.0	2.4	11.5	20.9	29.9	8.4	20.8
Kelora 8/2/00 P2 R3	18.2	1.7	10.9	1.3	4.5	2.4	14.9	21.2	25.7	8.4	19.2
Kelora 8/2/00 P3 R1	17.8	1.4	7.5	1.2	5.1	2.4	21.3	17.7	48.2	5.8	17.7
Kelora 8/2/00 P3 R2	20.0	1.6	11.2	1.5	4.3	2.4	13.3	24.6	28.0	7.9	20.2
Kelora 8/2/00 P3 R3	19.6	1.4	9.0	1.3	5.8	2.6	20.4	20.0	34.4	7.0	18.8
Kelora 8/2/00 P4 R1	20.6	1.6	10.3	1.5	5.6	2.5	20.4	24.1	78.2	7.3	22.7
Kelora 8/2/00 P4 R2	25.8	1.9	12.9	1.6	5.1	2.5	12.2	26.4	82.0	8.5	20.5
Kelora 8/2/00 P4 R3	23.2	1.6	10.4	1.6	6.0	2.6	23.2	20.9	74.2	6.7	20.3
deficient	17	0.9	7	1.2			15	11	19	2.6	10
adequate	25	1.3	9	1.3	3	0.8	33	15	100	5	12

Appendix 2.1b Foliar analysis of Eucalyptus globulus *plantations assessed for pests and diseases in south-western Australia*

Sample	[Cu]	[Fe]	[Zn]	[Mn]	[B]	[P]	[S]	[K]	[Mg]	[Ca]	[Na]	[N]
Chelgiup 1	2.9	44.3	10.6	158.2	35.2	1.0	0.9	6.2	1.7	10.2	2.8	1.7
Chelgiup 2	2.5	25.3	10.4	41.9	21.6	1.0	1.0	8.8	1.7	6.5	2.4	5.3
Chelgiup 3	4.2	26.4	9.3	38.1	25.7	0.9	0.9	8.8	1.5	9.6	2.9	1.4
Chelgiup 4	3.2	36.4	13.7	135.9	15.2	1.4	1.4	9.5	2.4	9.8	1.9	1.9
Cobertup 1	5.2	21.9	10.0	119.5	29.9	0.8	0.9	7.4	2.2	11.6	1.2	1.2
Cobertup 2	2.5	34.8	9.5	230.1	26.5	0.9	0.9	7.0	2.3	11.2	2.6	1.1
Cobertup 3	5.0	25.2	11.3	70.7	25.0	1.0	0.9	11.2	2.3	8.1	2.0	1.2
Frankland 1	6.7	43.5	15.6	39.3	17.5	1.1	1.2	10.0	3.4	7.7	1.5	1.5
Frankland 2	6.1	46.0	20.3	294.8	20.8	1.3	1.3	5.1	3.7	8.7	3.5	1.6
Frankland 3	8.4	43.6	17.5	99.2	14.7	1.4	1.6	10.1	3.4	8.3	1.5	1.6
Frankland 4	5.5	36.7	13.2	60.4	11.9	1.1	1.1	10.1	3.2	6.5	1.6	1.4
Kelora 2	3.4	37.2	12.4	46.7	16.4	1.6	1.3	8.4	2.6	10.3	1.9	1.8
Kelora 3	4.1	38.0	15.1	88.7	16.6	1.4	1.3	8.7	2.6	10.5	2.3	2.2
Range Montana 1	3.6	52.6	17.4	118.3	19.6	1.5	1.5	8.8	2.8	9.0	2.6	2.1
Range Montana A 1	4.5	31.4	18.5	62.7	18.3	1.1	1.2	8.8	2.5	7.4	2.4	1.7
Range Montana J2	2.9	43.1	21.7	331.3	16.9	1.7	1.5	7.7	2.2	9.7	3.8	2.0
Range Montana A2	4.3	33.8	21.3	150.0	15.2	1.1	1.2	7.8	2.4	8.8	1.8	1.5
Range Montana J3	1.1	41.5	14.8	132.2	12.9	1.3	1.0	8.1	2.5	8.6	2.0	1.2
Range Montana A3	3.5	32.5	17.1	182.4	21.4	1.1	1.0	8.7	2.2	8.7	2.9	1.1
range Montana J4	6.6	42.2	17.0	294.5	21.7	1.4	1.2	8.1	3.1	8.4	2.3	1.2
Range Montana A4	3.3	21.0	11.7	112.0	11.3	1.2	0.8	8.3	2.5	8.2	2.4	1.1
Range Montana A5	3.0	30.7	14.3	186.5	25.8	1.3	1.1	6.7	2.0	6.3	5.0	1.3

Appendix 2.1c Foliar analysis of Eucalyptus globulus *plantations assessed for pests and diseases in south-western Australia*

Sample	[N] mg/g	[P] mg/g	[S] mg/g	[K] mg/g	[Ca] mg/g	[Mg] mg/g	[Cu] mg/g	[Zn] mg/g	[Mn] mg/g	[Fe] mg/g	[B] mg/g
Kelora 97, C3 P2	1.8	1.2	1.2	5.8	9.8	2.3	5.0	25.1	167.9	62.7	25.1
Kelora 97, C2 P3	1.8	1.0	1.1	4.3	9.1	1.8	5.0	27.5	127.3	44.9	22.5
Kelora 97, C2 P4	2.1	1.1	1.3	4.2	8.8	2.1	5.0	17.7	254.7	25.2	22.7
Range Montana 97, C4 P1	1.8	1.3	1.4	6.6	7.4	1.5	4.9	17.3	315.7	46.9	22.2
Range Montana 97, C5 P2	2.1	1.5	1.3	6.4	10.1	2.1	2.5	22.7	1283.7	32.8	37.8
Range Montana 97, C6 P3	2.7	1.7	1.4	8.1	10.1	1.7	4.9	17.3	530.2	49.3	29.6
Range Montana 97, C14 P4	1.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Frankland River, C7 spl1	2.0	1.4	1.3	6.2	5.8	2.0	7.6	20.2	57.9	22.7	20.2
Frankland River, C1 spl2	2.5	2.0	1.6	6.6	5.9	1.6	10.0	32.3	947.8	14.9	19.9
Frankland River, C2 spl3	2.4	1.9	1.6	9.3	5.2	1.5	9.9	27.2	108.8	22.2	14.8
Frankland River, C3 spl4	2.0	1.5	1.2	9.6	6.1	1.9	7.5	17.5	147.1	10.0	17.5

Appendix 2.2 Procedures for rating pest and disease symptoms in **Eucalyptus globulus** *plantations*

Nutrient disorders. Crown decline and stem distortion are two symptoms associated with micronutrient deficiency. Stem distortion was given a rating from 1 to 3 with 1 being non-distorted and 3 being severely distorted. Trees were rated for crown decline according to the % loss of leaves in the upper 25% of the tree canopy. The rating scale was log-linear following the increments: 0—6, 7—12, 13—25, 26—50 and >51% loss of leaves to crown area.

MLD and LBSF rating procedure Leaf disease symptoms on juvenile and adult leaves were rated separately according to the log rating scale (Figure 2.2).

Firstly, the presence or absence of lesions due to *Mycosphaerella* on the entire tree was recorded. Then juvenile foliage was rated on a branch at 1.5m high for 1996 plantings and on a branch at 0.75m high for 1997 plantings. The branch most at right angles to the row of trees was selected. Juvenile foliage was rated as two separate categories: (a) recent flush; and (b) previous flush (season) of growth. Previous season's growth was assessed on a secondary branch occurring 1/3 of the branch distance from the trunk. The middle ten leaves from this secondary branch (Figure 2.3) were rated by comparing with diagrams representing a log-linear increase in leaf area affected (Figure 2.2). New season's growth was assessed on the final ten leaves of the above-mentioned primary branch. Where adult foliage was affected, damage was assessed according to the log-linear scale for the lower 25% of adult foliage.

Fungal canker rating Canker symptoms included cracking and darkening of the bark, formation of callus tissue, gummosis or bleeding from wounds, the formation of a distinct lesion front where healthy 'green' tissue met unhealthy brown tissue. Five categories were awarded to the canker status of a tree (Figure 2.3).

C1 if no canker greater than 20% of tree circumference in any direction.

C2 if greater than 20% of circumference but not encircling the entire trunk, pycnidia present

C3 if encircling the entire trunk and pycnidia present

A category of C5 was for trees that had canker symptoms of a C3 but no pycnidia.

Where signs were present but symptoms did not exceed those required for a C2 then a C6 category was assigned to the tree.

For the purpose of statistical analysis the C6 was combined with the C2 category and the C3 with the C5 category.

Fungi causing cankers were identified in the field with the aid of a 10x hand lens. *Cryphonectria* pycnidia were clearly visible as orange pycnidia on cankers. Pieces

of tissue from three cankers at each site were collected and plated out to confirm the presence of *Cryphonectria* or other canker causing species, *Botryosphaeria* , *Cryphonectria*. and *Pestalotiopsis*

Leaf chewing insect damage Leaf damage due to insects was rated according to a log scale (Figure 2.2) using the following increments: 0, 1—3, 4—6, 7—12, 13—25, 26—50, 51—75, 76+% damaged leaf area for entire canopy, unless otherwise stated. A single all encompassing chewing category was firstly rated according to the log-linear scale, followed by specific sub-categories.

Adult weevil damage was rated according to the percentage of canopy with unevenly chewed leaf margins (Figure 2.3) using the log-linear system described.

Chrysomelid beetles were assessed according to a scalloping symptom on leaves Figure 2.3), which was simply rated as present or absent on each tree.

Larval symptoms for weevils were irregularly grazed portions of the leaf (Figure 2.3).

Larval symptoms for beetles were evenly trimmed portions of the leaf (Figure 2.3).

Larval symptoms for weevils and chrysomelid beetles were simply rated as present or absent as were the following categories of damage: leaf miner ('shot-hole'), leaf and stem galls, small chlorotic lesions due to psyllids and other sap-sucking insects, leaf skeletalisation, AGM symptoms (Figure 2.3). Damage caused by leaf blister sawfly was rated in terms of the percentage of blistered leaf (Figure 2.3) area within the juvenile and adult portion of canopy separately, according to the log-linear scale described.

Tip damage caused by psyllids was rated by randomly selecting 10 leaves and assessing the number of tips that were damaged. This was expressed as a percentage along a linear scale: 0, 1—10, 11—20, 21—30, 31—40, 41—50, 51—60, 61—70, 71—80, 81—90, 91—100%.

Where insects were present on trees this was also recorded.

Appendix 5.1 Sequence alignment of Mycosphaerella nubilosa *and* M. cryptica

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                1       ……10          ……20            30          ……40          …..50
'98-125'        TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCGCCCG--C
AYO45496        ----------------------ACCTGCGGA GGNA ----------------------------------CCC----C
'98-191'        TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCGCCCG--C
R089            TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCGCCCG--C
AYO45494        TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCGCCCG--C
AYO45495        TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCGCCCG--C
R090            TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCGCCCG--C
AYO45498        TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCGCCCG--C
AF309623        TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCGCCCG--C
R091            TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCGCCCG--C
R101            TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCGCCCG--C
R110            TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCGCCCG--C
R114            TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCGCCCG--C
R115            TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCGCCCG--C
R118            TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCGCCCG--C
AF309622        TCCGTAGGTG AACCTGCGTA GGGATCATTA CCGAGTGAGG GCCTCCGGGT
AYO45507        TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
AYO45508        TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
AY045505        TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
AY045506        TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
AY045509        TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
R051            TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
R004            TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
R002            TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
R001            TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
'98-101'        TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
'98-099'        TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
R057            TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
AF449097        TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
AF449098        TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
AF449094        TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
AF449096        TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
AF449099        TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG GCGCCAG--C
AF309618        TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCGGCAG--C
AF449095        TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGCGG G--GCCAG--C
AF243401        TCCGTAGGTG AACCTGCGGA AGGATCATTA CCGAGTTCTCG GCTTCGGC
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                    ……60          …..70          .80          …90          ..100
'98-125'        CCGAC-CTCC AACCCCATGT TTTCCAACC- ATGTTGCCTC GGGGGCGACC
AYO45496        CCGAC-CTCC AACCCCATGC TTTCCAACC- ATGTTGCCTC GGGGGCGACC
'98-191'        CCGAC-CTCC AACCCCATGT TTTCCAACC- ATGTTGCCTC GGGGGCGACC
R089            CCGAC-CTCC AACCCCATGT TTTCCAACC- ATGTTGCCTC GGGGGCGACC
AYO45494        CCGAC-CTCC AACCCCATGT TTTCCAACC- ATGTTGCCTC GGGGGCGACC
AYO45495        CCGAC-CTCC AACCCCATGT TTTCCAACC- ATGTTGCCTC GGGGGCGACC
R090            CCGAC-CTCC AACCCCATGT TTTCCAACC- ATGTTGCCTC GGGGGCGACC
AYO45498        CCGAC-CTCC AACCCCATGT TTTCCAACC- ATGTTGCCTC GGGGGCGACC
AF309623        CCGAC-CTCC AACCCCATGT TTTCCAACC- ATGTTGCCTC GGGGGCGACC
R091            CCGAC-CTCC AACCCCATGT TTTCCAACC- ATGTTGCCTC GGGGGCGACC
R101            CCGAC-CTCC AACCCCATGT TTTCCAACC- ATGTTGCCTC GGGGGCGACC
R110            CCGAC-CTCC AACCCCATGT TTTCCAACC- ATGTTGCCTC GGGGGCGACC
R114            CCGAC-CTCC AACCCCATGT TTTCCAACC- ATGTTGCCTC GGGGGCGACC
R115            CCGAC-CTCC AACCCCATGT TTTCCAACC- ATGTTGCCTC GGGGGCGACC
R118            CCGAC-CTCC AACCCCATGT TTTCCAACC- ATGTTGCCTC GGGGGCGACC
AF309622        CCGAC-CTCC AACCCTTTGT GAACGCATC- CCGTTGCGTC GGGGCCGACC
AYO45507        CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
AYO45508        CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
AY045505        CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
AY045506        CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
AY045509        CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
R051            CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
R004            CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
R002            CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
R001            CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
'98-101'        CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
'98-099'        CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
R057            CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
AF449097        CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
AF449098        CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
AF449094        CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
AF449096        CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
AF449099        CCGAC-CTCC AACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
AF309618        CCGAC-CTCC TACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
AF449095        CCGAC-CTCC TACCCCATGT TTTCCCACC- ACGTTGCCTC GGGGGCGACC
AF243401        TCGACTCTCC CACCCTTTGT GAACGTACC TCTGTTGCTT TGGCGGC--TC
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                    ……110         …….120         …..130          140                150
'98-125'        CGGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
AYO45496        CGGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
'98-191'        CGGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
R089            CGGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
AYO45494        CGGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
AYO45495        CGGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
R090            CGGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
AYO45498        CGGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
AF309623        CGGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
R091            CGGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
R101            CGGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
R110            CGGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
R114            CGGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
R115            CGGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
R118            CGGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
AF309622        CTGCCGCCGT GCCGGGGCCC CCGGCGGACC CCTCAACT-C TGCATCTTTG
AYO45507        CGGCCACCGC GCCGGGGCCC TCGCAGGACG CCTCAACG-C TGCATCTGTG
AYO45508        CGGCCACCGC GCCGGGGCCC TCGCAGGACG CCTCAACG-C TGCATCTGTG
AY045505        CGGCCACCGC GCCGGGGCCC TCGCAGGACG CCTCAACG-C TGCATCTGTG
AY045506        CGGCCACCGC GCCGGGGCCC TCGCAGGACG CCTCAACG-C TGCATCTGTG
AY045509        CGGCCACCGC GCCGGGGCCC TCGCAGGACG CCTCAACG-C TGCATCTGTG
R051            CGGCCACCGC GCCGGGGCCC TCGCAGGACG CCTCAACG-C TGCATCTGTG
R004            CGGCCACCGC GCCGGGGCCC TCGCAGGACG CCTCAACG-C TGCATCTGTG
R002            CGGCCACCGC GCCGGGGCCC TCGCAGGACG CCTCAACG-C TGCATCTGTG
R001            CGGCCACCGC GCCGGGGCCC TCGCAGGACG CCTCAACG-C TGCATCTGTG
'98-101'        CGGCCACCGC GCCGGGGCCC TCGCAGGACG CCTCAACG-C TGCATCTGTG
'98-099'        CGGCCACCGC GCCGGGGCCC TCGCAGGACG CCTCAACG-C TGCATCTGTG
R057            CGGCCACCGC GCCGGGGCCC TCGCAGGACG CCTCAACG-C TGCATCTGTG
AF449097        CGGCCCCCGC GCCGGGGCCC TCGCAGGACC CCTCAACG-C TGCATCTGTG
AF449098        CGGCCCCCGC GCCGGGGCCC TCGCAGGACC CCTCAACG-C TGCATCTGTG
AF449094        CGGCCCCCGC GCCGGGGCCC TCGCAGGACC CCTCAACG-C TGCATCTGTG
AF449096        CGGCCCCCGC GCCGGGGCCC TCGCAGGACC CCTCAACG-C TGCATCTGTG
AF449099        CGGCCCCCGC GCCGGGGCCC TCGCAGGACC CCTCAACG-C TGCATCTGTG
AF309618        CGGCCCCCGC GCCGGGGCCC TCGCAGAACC CCTCAACGGC TGGATCTGTG
AF449095        CGGCCCCCGC GCCGGGGCCC TCGCAGGACC CCTCAACG-CT GCATCTGTG
AF243401        CGGCCGCCA--- -AAGGCCTTC AAA-----CTCCA GTCAGTAAAC GCAGA
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                    160        170        180        190        200
'98-125'      CGTCTGAGTG ATAACGAAAA ATCAATCAAA ACTTTCAACA ACGGATCTCT
AYO45496      CGTCTGAGTG ATAACGAAAA ATCAATCAAA ACTTTCAACA ACGGATCTCT
'98-191'      CGTCTGAGTG ATAACGAAAA ATCAATCAAA ACTTTCAACA ACGGATCTCT
R089          CGTCTGAGTG ATAACGAAAA ATCAATCAAA ACTTTCAACA ACGGATCTCT
AYO45494      CGTCTGAGTG ATAACGAAAA ATCAATCAAA ACTTTCAACA ACGGATCTCT
AYO45495      CGTCTGAGTG ATAACGAAAA ATCAATCAAA ACTTTCAACA ACGGATCTCT
R090          CGTCTGAGTG ATAACGAAAA ATCAATCAAA ACTTTCAACA ACGGATCTCT
AYO45498      CGTCTGAGTG ATAACGAAAA ATCAATCAAA ACTTTCAACA ACGGATCTCT
AF309623      CGTCTGAGTG ATAACGAAAA - TCAATCAAA ACTTTCAACA ACGGATCTCT
R091          CGTCTGAGTG ATAACGAAAA ATCAATCAAA ACTTTCAACA ACGGATCTCT
R101          CGTCTGAGTG ATAACGAAAA ATCAATCAAA ACTTTCAACA ACGGATCTCT
R110          CGTCTGAGTG ATAACGAAAA ATCAATCAAA ACTTTCAACA ACGGATCTCT
R114          CGTCTGAGTG ATAACGAAAA ATCAATCAAA ACTTTCAACA ACGGATCTCT
R115          CGTCTGAGTG ATAACGAAAA ATCAATCAAA ACTTTCAACA ACGGATCTCT
R118          CGTCTGAGTG ATAACGAAAA ATCAATCAAA ACTTTCAACA ACGGATCTCT
AF309622      CGTCTGAGTG ATAACGAAAA –TCAATCAAA ACTTTCAACA ACGGATCTCT
AYO45507      CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
AYO45508      CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
AY045505      CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
AY045506      CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
AY045509      CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
R051          CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
R004          CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
R002          CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
R001          CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
'98-101'      CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
'98-099'      CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
R057          CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
AF449097      CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
AF449098      CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
AF449094      CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
AF449096      CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
AF449099      CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
AF309618      CGT-GGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACA ACGGATCTCT
AF449095      CGTCGGAGTA ATA-CAACCA ATCAATTAAA ACTTTCAACAACGGATCTCT
AF243401      CGTCTGA-TA ACA-AGTTAAT AAACT- -AAA ACTTTCAACAACGGATCTCT
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                    210        220        230        240        250
'98-125'    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AYO45496    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
'98-191'    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
R089        TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AYO45494    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AYO45495    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
R090        TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AYO45498    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AF309623    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
R091        TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
R101        TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
R110        TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
R114        TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
R115        TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
R118        TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AF309622    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AYO45507    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AYO45508    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AY045505    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AY045506    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AY045509    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
R051        TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
R004        TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
R002        TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
R001        TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
'98-101'    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
'98-099'    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
R057        TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AF449097    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AF449098    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AF449094    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AF449096    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AF449099    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AF309618    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AF449095    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
AF243401    TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
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                260        270        280        290        300
'98-125'        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AYO45496        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
'98-191'        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
R089            TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AYO45494        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AYO45495        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
R090            TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AYO45498        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AF309623        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
R091            TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
R101            TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
R110            TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
R114            TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
R115            TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
R118            TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AF309622        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCCCT
AYO45507        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AYO45508        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AY045505        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AY045506        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AY045509        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
R051            TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
R004            TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
R002            TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
R001            TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
'98-101'        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
'98-099'        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
R057            TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AF449097        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AF449098        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AF449094        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AF449096        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AF449099        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AF309618        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AF449095        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCTCT
AF243401        TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCCTT
```

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                    310        320        330        340        350
'98-125'      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTACACC CCTCCAGCCT
AYO45496      GGTATTCCNG AGGGCATGCC TGTTCGAGCG TCATTACACC CCTCCAGCCT
'98-191'      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTACACC CCTCCAGCCT
R089          GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTACACC CCTCCAGCCT
AYO45494      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTACACC CCTCCAGCCT
AYO45495      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTACACC CCTCCAGCCT
R090          GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTACACC CCTCCAGCCT
AYO45498      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTACACC CCTCCAGCCT
AF309623      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTACACC CCTCCAGCCT
R091          GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTACACC CCTCCAGCCT
R101          GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTACACC CCTCCAGCCT
R110          GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTACACC CCTCCAGCCT
R114          GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTACACC CCTCCAGCCT
R115          GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTACACC CCTCCAGCCT
R118          GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTACACC CCTCCAGCCT
AF309622      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTACACC ACTCCAGCCT
AYO45507      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCT
AYO45508      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCT
AY045505      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCT
AY045506      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCT
AY045509      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCT
R051          GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCT
R004          GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCT
R002          GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCT
R001          GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCT
'98-101'      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCT
'98-099'      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCT
R057          GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCT
AF449097      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCC
AF449098      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCC
AF449094      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCC
AF449096      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCC
AF449099      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCC
AF309618      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCC
AF449095      GGTATTCCGG AGGGCATGCC TGTTCGAGCG TCATTTCACC ACTCCAGCCC
AF243401      GGTATTCCGG GGGGCATGCC TGTTCGAGCG TCATTACAAC CCTCAAGCTC
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                    360        370        380        390        400
'98-125'      CGCTGGGTGT TGGGCATCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AYO45496      CGCTGGGTGT TGGGCATCGC GGTCTCCGCG ----CGCCTC AATGTCTCCG
'98-191'      CGCTGGGTGT TGGGCATCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
R089          CGCTGGGTGT TGGGCATCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AYO45494      CGCTGGGTGT TGGGCATCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AYO45495      CGCTGGGTGT TGGGCATCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
R090          CGCTGGGTGT TGGGCATCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AYO45498      CGCTGGGTGT TGGGCATCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AF309623      CGCTGGGTGT TGGGCATCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
R091          CGCTGGGTGT TGGGCATCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
R101          CGCTGGGTGT TGGGCATCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
R110          CGCTGGGTGT TGGGCATCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
R114          CGCTGGGTGT TGGGCATCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
R115          CGCTGGGTGT TGGGCATCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
R118          CGCTGGGTGT TGGGCATCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AF309622      CGCTGGGTAT TGGGCGTCTC GG-  CTCCGCG ----CGCCTC AATGTCTCCG
AYO45507      CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AYO45508      CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AY045505      CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AY045506      CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AY045509      CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
R051          CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
R004          CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
R002          CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
R001          CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
'98-101'      CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
'98-099'      CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
R057          TGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AF449097      CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AF449098      CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AF449094      CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AF449096      CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AF449099      CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AF309618      CGCTTGGTAT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AF449095      CGCTGGGTCT TGGGCGCCGC GGCCTCCGCG ----CGCCTC AATGTCTCCG
AF243401      TGCTTGGAAT TGGGCACCGT CCTCACTGCG GACGCGCCTC AAAGACCTCG
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                  410        420        430        440        450
'98-125'          GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCT-TCC
AYO45496          GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCTATCC
'98-191'          GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCT-TCC
R089              GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCT-TCC
AYO45494          GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCT-TCC
AYO45495          GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCT-TCC
R090              GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCT-TCC
AYO45498          GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCT-TCC
AF309623          GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCT-TCC
R091              GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCT-TCC
R101              GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCT-TCC
R110              GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCT-TCC
R114              GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCT-TCC
R115              GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCT-TCC
R118              GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCT-TCC
AF309622          GCCG-AGCCG ACCGTCTCT- AAGCGTTGTG GCACAACTGT TTCGCT-TCC
AYO45507          GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACCACTGT TTCGCTGACG
AYO45508          GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACCACTGT TTCGCTGACG
AY045505          GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACCACTGT TTCGCTGACG
AY045506          GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACCACTGT TTCGCTGACG
AY045509          GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACCACTGT TTCGCTGACG
R051              GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACCACTGT TTCGCTGACG
R004              GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACCACTGT TTCGCTGACG
R002              GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACCACTGT TTCGCTGACG
R001              GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACCACTGT TTCGCTGACG
'98-101'          GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACCACTGT TTCGCTGACG
'98-099'          GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACCACTGT TTCGCTGACG
R057              GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACCACTGT TTCGCTGACG
AF449097          GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACTACTGT TTCGCTGACG
AF449098          GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACTACTGT TTCGCTGACG
AF449094          GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACTACTGT TTCGCTGACG
AF449096          GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACTACTGT TTCGCTGACG
AF449099          GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACTACTGT TTCGCTGACG
AF309618          GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACTACTGT TTCGCTGACG
AF449095          GCCG-AGCCG ACCGTCTCT- CAGCGTTGTG GCACTACTGT TTCGCTGACG
AF243401          GCGGTGGCTG TTCAGCCCT CAAGCGTAGT AGAATA-CAC CTCGCT---T
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                  460        470        480        490        500
'98-125'    GGGACCGGTC CGGCGTCGC- -GCCG--TCA ACCCCCT--- --CTCTCACA
AYO45496    GGGACCGGTC CGGCGTCGC- -GCCG--TCA ACCCCCT-----CTCTCACA
'98-191'    GGGACCGGTC CGGCGTCGC- -GCCG--TCA ACCCCCT-----CTCTCACA
R089        GGGACCGGTC CGGCGTCGC- -GCCG--TCAA CCCCCT-----CTCTCACA
AYO45494    GGGACCGGTC CGGCGTCGC- -GCCG--TCA ACCCCCT-----CTCTCACA
AYO45495    GGGACCGGTC CGGCGTCGC- -GCCG--TCA ACCCCCT-----CTCTCACA
R090        GGGACCGGTC CGGCGTCGC- -GCCG--TCA ACCCCCT-----CTCTCACA
AYO45498    GGGACCGGTC CGGCGTCGC- -GCCG--TCA ACCCCCT-----CTCTCACA
AF309623    GGGACCGGTC TGGCGTCGC- -GCCG--TCA ACCCCCT-----CTCTCACA
R091        GGGACCGGTC CGGCGTCGC- -GCCG--TCA ACCCCCT-----CTCTCACA
R101        GGGACCGGTC CGGCGTCGC- -GCCG--TCA ACCCCCT-----CTCTCACA
R110        GGGACCGGTC CGGCGTCGC- -GCCG--TCA ACCCCCT-----CTCTCACA
R114        GGGACCGGTC CGGCGTCGC- -GCCG--TCA ACCCCCT-----CTCTCACA
R115        GGGACCGGTC CGGCGTCGC- -GCCG--TCA ACCCCCT-----CTCTCACA
R118        GGGACCGGTC CGGCGTCGC- -GCCG--TCA ACCCCCT-----CTCTCACA
AF309622    GGGACCGGTC TGGCGTCGC- -GCCG--TCA ACCCCCT-----CTCTCACA
AYO45507    GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
AYO45508    GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
AY045505    GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
AY045506    GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
AY045509    GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
R051        GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
R004        GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
R002        GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
R001        GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
'98-101'    GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
'98-099'    GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
R057        GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
AF449097    GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
AF449098    GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
AF449094    GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
AF449096    GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
AF449099    GGGACCGGTC TGGCGGCGC- -GCCG--TTA AACCCTT-----TCACCAAA
AF309618    GGGACCGGTC TGGCG-CGC- -GCCG--TTA AACCCTT-----TCACCAAA
AF449095    GGGACCGGTC TGGCGGCGC- -GCCG--TT AAACCCTT-----TCACCAAA
AF243401    TGGAGCGGT- TGGCGTCGCC CGCCGGAC GAACCTTCTGAACTTTTCTCAA
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                510                 520                 530                 540                 550
'98-125'        GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
AYO45496        GGTTAACCTC NGATCAGTAG GGGAT-ACCC CCTGAACTTA AGCATATCAA
'98-191'        GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
R089            GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
AYO45494        GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
AYO45495        GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
R090            GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
AYO45498        GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
AF309623        GGTTGACCTC GGATCAGGTA GGGA---------- ----------
R091            GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
R101            GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
R110            GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
R114            GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
R115            GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
R118            GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
AF309622        GGTTGACCTC GGATCAGGTA GGGAT-A------- ----------
AYO45507        GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
AYO45508        GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
AY045505        GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
AY045506        GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
AY045509        GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
R051            GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
R004            GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
R002            GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
R001            GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
'98-101'        GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
'98-099'        GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
R057            GGTTGACCTC GGATCAGGTA GGGAT-ACCC GCTGAACTTA AGCATATCAA
AF449097        GGTTGACCTC GGATCAGGTA GGGAT-A------- ----------
AF449098        GGTTGACCTC GGATCAGGTA GGGAT-A-------- ----------
AF449094        GGTTGACCTC GGATCAGGTA GGGAT-A-------- ----------
AF449096        GGTTGACCTC GGATCAGGTA GGGAT-A-------- ----------
AF449099        GGTTGACCTC GGATCAGGTA GGGAT-A-------- ----------
AF309618        GGTTGACCTC GGATCAGGTA GGGAT-A-------- ----------
AF449095        GGTTGACCTC GGATCAGGTA GGGAT-A-------- ----------
AF243401        GGTTGACCTC GGATCAGGTA CGGATTACCC GCTGAACTTA AGCATATCAA
```

'98-125'	TAAGCGGAGG A
AYO45496	TAAGCG-----
'98-191'	TAAGCGGAGG A
R089	TAAGCGGAGG A
AYO45494	TAAGC------
AYO45495	TAAGC------
R090	TAAGCGGAGG A
AYO45498	TAAGC------
AF309623	----------
R091	TAAGCGGAGG A
R101	TAAGCGGAGG A
R110	TAAGCGGAGG A
R114	TAAGCGGAGG A
R115	TAAGCGGAGG A
R118	TAAGCGGAGG A
AF309622	----------
AYO45507	TAAGCG-----
AYO45508	TAAGCG-----
AY045505	TAAGCG-----
AY045506	TAAGCG-----
AY045509	TAAGCG-----
R051	TAAGCGGAGG A
R004	TAAGCGGAGG A
R002	TAAGCGGAGG A
R001	TAAGCGGAGG A
'98-101'	TAAGCGGAGG A
'98-099'	TAAGCGGAGG A
R057	TAAGCGGAGG A
AF449097	----------
AF449098	----------
AF449094	----------
AF449096	----------
AF449099	----------
AF309618	----------
AF449095	----------
AF243401	TAAGCGGAGG A

Appendix 5.2: Sequence alignment all **Mycosphaerella** *species on eucalypts*

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                1……      10        20        30        40        50
R115    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCGCC
R118            TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCGCC
R114    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCGCC
R110    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCGCC
R101    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCGCC
R091    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCGCC
R090    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCGCC
R089    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCGCC
'98-191' TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCGCC
'98-125' TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCGCC
AF309622        TCCGTAGGT GAACCTGCGT AGGGATCATT A-----CCGA GTGAGGGCCTC
R210    TCCGTAGGT GAACCTGCGG AGGGATCATA -----CTGAG TGAGGGCGCA
R211    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGGCGCA
AY045497        TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGGCGCA
784             TCCGTAGGT GAACCTGCGG AGGGATCATA -----CTGAG TGAGGGCGCA
'98-099' TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGCGGGCGCC
'98-101' TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGCGGGCGCC
R001    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGCGGGCGCC
R002    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGCGGGCGCC
R004    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGCGGGCGCC
R051    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGCGGGCGCC
AY045505        TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGCGGGCGCC
R057    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGCGGGCGCC
AF449097        TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGCGGGCGCC
sutton1346      TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA  GCGAGGGCGTC
R215    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCCCC
SJ5             TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCCCC
R216    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCCCC
R216Y           TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCCCC
AY045503        TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CAGA GTTCTGGGTCC
AF310107        TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCCTT
'98-133' TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCAG AAGACGCCTCG
'98-163' TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCAG AAGACGCCTCG
dekk            TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCAG AAGACGCCTCG
'98-148' TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCAG AAGACGCCTCG
'98-149' TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCAG AAGACGCCTCG
R257    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCAG AAGACGCCTCG
R258    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCAG AAGACGCCTCG
R262    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCAG AAGCCGCGCCG
AF243401        TCCGTAGGT GAACCTGCGG AAGGATCATT A-----CC---- GAGTTCTCG
R234    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGTT--
R243    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGTT--
R247    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGTT--
R242    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGTT--
AY045517        ----------  ----------  ----------  ----------  ----------
AF173316        TCCGTAGGT GAACCTGCGG  AGGGATCATT A-----CCGA GTGAGGGT---
parkii353       TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGGTT--
R151    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGG----
R152    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGG----
R221    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGG----
R222    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGG----
AF173314        TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGG----
ken             TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGG----
R237    TCCGTAGGT GAACCTGCGG AGGGATCATT AAGAACCAAT ATGGGGATGTC
R246    TCCGTAGGT GAACCTGCGG AGGGATCATT AAGAACCAAT ATGGGGATGTC
R240    TCCGTAGGT GAACCTGCGG AGGGATCATT AAGAACCAAT ATGGGGATGTC
AF173303        TCCGTAGGT GAACCTGAGG AGGGATCATT A-----CTGA GTGAGGG----
AF468869        TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGG----
AF222839        TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGG----
AF222843        TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGGC---
AF222841        TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGG----
AF222842        TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGGC---
colomb          TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGGCC--
AF309616        TCGGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGC---
AF309603        TCGGTAGGT GAACCTGCGG AGGGATCATT A-----CTGA GTGAGGGCTCC
cmw4937         TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCTCC
R248    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCTCC
R251    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCCTC
R250    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCCTC
R249    TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCCTC
AY045516        TCCGTAGGT GAACCTGCGG AGGGATCATT A-----CCGA GTGAGGGCCTC
Dothidea        TCCGTAGGT GAACCTGCGG AAGGATCATT AA----AAG  AATTGGAGTGAC
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                    ……   60          70                  80              90   100
R115        C--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CAAC
R118        C--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CAAC
R114        C--------- ---G—CCC GACCTCCAAC CCCAT—GTT TTC---CAAC
R110        C--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CAAC
R101        C--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CAAC
R091        C--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CAAC
R090        C--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CAAC
R089        C--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CAAC
'98-191'    C--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CAAC
'98-125'    C--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CAAC
AF309622             C--------- ----GGGTC CGACCTCCAAC CCTTT—GTG AAC---GCAT
R210        A--------- ----G—CCC GACCTCCAAC CCCAT—GTT TCC---AAAC
R211        A--------- ----G—CCC GACCTCCAAC CCCAT—GTT TCC---AAAC
AY045497             A--------- ----G—CCC GACCTCCAAC CCCAT—GTT TCC---AAAC
784                  A--------- ----G—CCC GACCTCCAAC CCCAT—GTT TCC---AAAC
'98-099'    A--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CCAC
'98-101'    A--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CCAC
R001        A--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CCAC
R002        A--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CCAC
R004        A--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CCAC
R051        A--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CCAC
AY045505             A--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CCAC
R057        A--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CCAC
AF449097             A--------- ----G—CCC GACCTCCAAC CCCAT—GTT TTC---CCAC
sutton1346           A--------- ----GG-CCC GACCTCCGAC CCTTTT-GTG TCCT--ACAC
R215        C--------- -----GGCCC GACCTCCTAC CCCAT—GTG ACC---TCAC
SJ5                  C--------- -----GGCCC GACCTCCTAC CCCAT—GTG ACC---TCAC
R216        C--------- -----GGCCC GACCTCCTAC CCCAT—GTG ACC---TCAC
R216Y                C--------- -----GGCCC GACCTCCTAC CCCAT—GTG ACC---TCAC
AY045503             TT-------- --CGGGGCCC GTCCTCCAAC CCCTT—GT ATAC---CAAC
AF310107             C---------- ---GGGCTCG ACCTCCAACC CCATGTTTGTG TC--GAAC
'98-133'    GCGGAA---A CGCCGGGGCC TTCGTCCAAC CCTTT—GTGA ACG--TATC
'98-163'    GCGGAA---A CGCCGGGGCC TTCGTCCAAC CCTTT—GTGA ACG--TATC
dekk                 GCGGAA---A CGCCGGGGCC TTCGTCCAAC CCTTT—GTGA ACG--TATC
'98-148'    GCGGAA---A CGCCGGGGCC TTCGTCCAAC CCTTT—GTGA ACG--TATC
'98-149'    GCGGAA---A CGCCGGGGCC TTCGTCCAAC CCTTT—GTGA ACG--TATC
R257        GCGGAA---A CGCCGGGGCC TTCGTCCAAC CCTTT—GTGA ACG--TATC
R258        GCGGAA---A CGCCGGGGCC TTCGTCCAAC CCTTT—GTGA ACG--TATC
R262        GCCGCA---A CGCCGGCGCC TTCGCCCAAC CCTTT—GTGA ATT--ACGC
AF243401             G--------G CTTCGGCTCG ACTCTCCCAC CCTTT—GTGA ACG--TACC
R234        ---------- -TCG—GCCCG ACCTCCAACC CTTT—GTGA AT--CA-AA
R243                  ---------- -TCG—GCCCG ACCTCCAAC CCTTT—GTGA AT--CA-AA
R247                  ---------- TCG—GCCCG ACCTCCAAC CCTTT—GTGA AT--CA-AA
R242        ---------- TCG—GCCCG ACCTCCAAC CCTTT—GTGA AT--CA-AA
AY045517             ---------- TCG—GCCCG ACCTCCAAC CCTTT—GTGA AT--CA-AA
AF173316             ---------- TCG—GGCCG ACCTCCAAC CCTTT—GTGA AT--CA-AA
parkii353             ---------- -TCACCGCCC GACCTCCAAC CCTTT—GTGA AC--CACAA
R151        ---------- CTC ACGCCC GACCTCCAAC CCTTT—GTGA AC--CA-AC
R152        ---------- CTC ACGCCC GACCTCCAAC CCTTT—GTGA AC--CA-AC
R221        ---------- CTCACGCCC GACCTCCAAC CCTTT—GTGA AC--CA-AC
R222        ---------- CTCACGCCC GACCTCCAAC CCTTT—GTGA AC--CA-AC
AF173314             ---------- CTCACGCCC GACCTCCAAC CCTTT—GTGA AC--CA-AC
ken                   ---------- -CGCAAGCC CGACCTCCAAC CCTTT—GTGA AC--CA-AC
R237        TCCGCCTGGC ACTGTTGCCC A—TTCTAAC CCTTT—GTG AACTACA-AC
R246        TCCGCCTGGC ACTGTTGCCC A—TTCTAAC CCTTT—GTG AACTACA-AC
R240        TCCGCCTGGC ACTGTTGCCC A—TTCTAAC CCTTT—GTG AACTACA-AC
AF173303             ---------- -CTCACGCCC A—TTCTAAC CCTTT—GTG AACTACA-AC
AF468869             ---------- -CTCACGCCC GACCTCCAAC CCTTT—GTG AAC-ACA-TC
AF222839             ---------- --TTCGGTCC GACCTCCAAC CCTTT—GTG AAC--CA-AA
AF222843             ---------- --TTCGGTCC GACCTCCAAC CCTTT—GTG AAC--CA-AA
AF222841             ---------- --CTAGGTCC GACCTCCAAC CCTTT—GTG AAC--CA-AA
AF222842             ---------- --TTCGGTCC GACCTCCAAC CCTTT—GTG AAC--CA-AA
colomb               ---------- --TCCGGTCC GACCTCCAAC CCTTT—GTG AAC--CA-AT
AF309616             ---------- --CCCGGCCC GACCTCCAAC CCTTT—GTG GAC--CC-AA
AF309603             G--------- ------GCCC GACCTCCAAC CCTTT—GTG AATT-CGACC
cmw4937              G--------- ------GCCC GACCTCCAAC CCCAT—GTG AATC-TCACC
R248        G--------- -----GGCTC GACCTCCAAC CCCATT-GTA TTCC-GACCT
R251        C--------- ----GGGCTC GACCTCCAAC CCCAT---TG TATCCGACCT
R250        C--------- ----GGGCTC GACCTCCAAC CCCATT-GTA TTCC-GACCT
R249        C--------- ----GGGCTC GACCTCCAAC CCCATT-GTA TTCC-GACCT
AY045516             C--------- ----GGGCTC GACCTCCAAC CCCATT-GTA TTCC-GACCT
Dothidea             T-------- AACCGTCC TCCGACTTCC AACCCTCTG-TTG TTATAACTAC
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	…… 110 120 130 140 150
R115	CATGTTGCCTCGG--GGGCGACCCGGCCG-CCGTGCCGGG----------
R118	CATGTTGCCTCGG--GGGCGACCCGGCCG-CCGTGCCGGG----------
R114	CATGTTGCCTCGG--GGGCGACCCGGCCG-CCGTGCCGGG----------
R110	CATGTTGCCTCGG--GGGCGACCCGGCCG-CCGTGCCGGG----------
R101	CATGTTGCCTCGG--GGGCGACCCGGCCG-CCGTGCCGGG----------
R091	CATGTTGCCTCGG--GGGCGACCCGGCCG-CCGTGCCGGG----------
R090	CATGTTGCCTCGG--GGGCGACCCGGCCG-CCGTGCCGGG----------
R089	CATGTTGCCTCGG--GGGCGACCCGGCCG-CCGTGCCGGG----------
'98-191'	CATGTTGCCTCGG--GGGCGACCCGGCCG-CCGTGCCGGG----------
'98-125'	CATGTTGCCTCGG--GGGCGACCCGGCCG-CCGTGCCGGG----------
AF309622	CCCGTTGCGTCGG--GGCCGACCCTGCCG-CCGTGCCGGG----------
R210	CACGTTGCCTCGG--GGGCGACCCGGCCG-CCGCGCCGGG----------
R211	CACGTTGCCTCGG--GGGCGACCCGGCCG-CCGCGCCGGG----------
AY045497	CACGTTGCCTCGG--GGGCGACCCGGCCG-CCGCGCCGGG----------
784	CACGTTGCCTCGG--GGGCGACCCGGCCG-CCGCGCCGGG----------
'98-099'	CACGTTGCCTCGG--GGGCGACCCGGCCA-CCGCGCCGGG----------
'98-101'	CACGTTGCCTCGG--GGGCGACCCGGCCA-CCGCGCCGGG----------
R001	CACGTTGCCTCGG--GGGCGACCCGGCCA-CCGCGCCGGG----------
R002	CACGTTGCCTCGG--GGGCGACCCGGCCA-CCGCGCCGGG----------
R004	CACGTTGCCTCGG--GGGCGACCCGGCCA-CCGCGCCGGG----------
R051	CACGTTGCCTCGG--GGGCGACCCGGCCA-CCGCGCCGGG----------
AY045505	CACGTTGCCTCGG--GGGCGACCCGGCCA-CCGCGCCGGG----------
R057	CACGTTGCCTCGG--GGGCGACCCGGCCA-CCGCGCCGGG----------
AF449097	CACGTTGCCTCGG--GGGCGACCCGGCCC-CCGCGCCGGG----------
sutton1346	CCTGTTGCCTCGG--GGGCGACCCGGCCG-CCGCGTCGGG----------
R215	TATGTTGCCTCGG--GGGCGACCCGGCCT-TCGGGCTGTTT---------
SJ5	TATGTTGCCTCGG--GGGCGACCCGGCCT-TCGGGCTGTTT---------
R216	TATGTTGCCTCGG--GGGCGACCCGGCCT-TCGGGCTGTTT---------
R216Y	TATGTTGCCTCGG--GGGCGACCCGGCCT-TCGGGCTGTTT---------
AY045503	CATGTTGCCTCGG--GGGCGACCCGGCCG-TCCGGCCGATC---------
AF310107	ATTGTTGCTTCGG--GGGCGACCCGGCCGTCCGGGCCGCCG---------
'98-133'	TCTATTGCCCCGG--GGGAACCCCGCCTGTCATGGGCGTGG---------
'98-163'	TCTATTGCCCCGG--GGGAACCCCGCCTGTCATGGGCGTGG---------
dekk	TCTATTGCCCCGG--GGGAACCCCGCCTGTCATGGGCGTGG---------
'98-148'	TCTATTGCCCCGG--GGGAACCCCGCCTGTCACGGGCGTGG---------
'98-149'	TCTATTGCCCCGG--GGGAACCCCGCCTGTCACGGGCGTGG---------
R257	TCTATTGCCCCGG--GGGAACCCCGCCTGTCACGGGCGTGG---------
R258	TCTATTGCCCCGG--GGGAACCCCGCCTGTCACGGGCGTGG---------
R262	CCGATTTCCCCGG--GGGGACCGCCTGCCCTGCGCGCGCGG---------
AF243401	TCTGTTGCTTTGG--CGGCTCCGGCCGCCAAAGGCCTTCAA---------
R234	CCTGTTGCTTCGG--GGGCGACCCTGCCGTTC--GCGGCGC---------
R243	CCTGTTGCTTCGG--GGGCGACCCTGCCGTTC--GCGGCGC---------
R247	CCTGTTGCTTCGG--GGGCGACCCTGCCGTTC--GCGGCGC---------
R242	CCTGTTGCTTCGG--GGGCGACCCTGCCGTTC--GCGGCGC---------
AY045517	CCTGTTGCTTCGG--GGGCGACCCTGCCGTTC--GCGGCGC---------
AF173316	CCTGTTGCTTCGG--GGGCGACCCTGCCGTTC--GGCGCGC---------
parkii353	CTTGTTGCTTCGG--GGGCGACCCTGCCGTTC--GCGGCAT---------
R151	TCTGTTGCTTCGG--GGGCGACCCCGCCGTTTCGGCGACGG---------
R152	TCTGTTGCTTCGG--GGGCGACCCCGCCGTTTCGGCGACGG---------
R221	TCTGTTGCTTCGG--GGGCGACCCCGCCGTTTCGGCGACGG---------
R222	TCTGTTGCTTCGG--GGGCGACCCCGCCGTTTCGGCGACGG---------
AF173314	TCTGTTGCTTCGG--GGGCGACCCCGCCGTTTCGGCGACGG---------
ken	TCTGTTGCTTCGG--GGGCGACCCCGCCGTTTCGGCGACGGG--------
R237	TCTGTTGCTTCGG--GGGCGACCCCGCCGTCTCGGCGGTGG---------
R246	TCTGTTGCTTCGG--GGGCGACCCCGCCGTCTCGGCGGTGG---------
R240	TCTGTTGCTTCGG--GGGCGACCCCGCCGTCTCGGCGGTGG---------
AF173303	TCTGTTGCTTCGG--GGGCGACCCCGCCGTCTCGGCGGTGG---------
AF468869	T-TGTTGCTTCGG--GGGCGACCCTGCCGGCCCTGCGTCGCC--------
AF222839	CTTGTTGCTTCGG--GGGCGACCCTGCCGCTTTGGCGGTGC---------
AF222843	CTTGTTGCTTCGG--GGGCGACCCTGCCGCTTCGGCGGTGC---------
AF222841	CTTGTTGCTTCGG--GGGCGACCCTGCCGCTTGGGCGGTGC---------
AF222842	CTTGTTGCTTCGG--GGGCGACCCTGCCGCTTCGGCGGTGC---------
colomb	CTTGTTGCTTCGG--GGGCGACCCTGCCGCTTCGGCGGTGC---------
AF309616	CTTGTTGCTTCGG--GGGCGACCCTGCCGTCTCGGCGGCGC---------
AF309603	TCTGTTGCCTCGG--GGGCGACCCGGCCCTCTGGGTGCCGG---------
cmw4937	TCTGTTGCCTCGG--GGGTGACCCGGCCCTCTGGGTGCCGG---------
R248	CTTGTTGCCTCGG--GGGCGACCCGGCCT-TCGGGCGTCGG---------
R251	CTTGTTGCCTCGG--GGGCGACCCGGCCT-TCGGGCGTCGG---------
R250	CTTGTTGCCTCGG--GGGCGACCCGGCCT-TCGGGCGTCGG---------
R249	CTTGTTGCCTCGG--GGGCGACCCGGCCT-TCGGGCGTCGG---------
AY045516	CTTGTTGCCTCGG--GGGCGACCCGGCCT-TCGGGCGTCGG---------
Dothidea	CTTGTTGCTTTGGCGGGACCGTTCGGTCCTCCGAGCGCACCAGTCTTCGG

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                    …… 160             170             180             190200
R115        --------GCCCCCGGCGGACCCC---TCAACTCT-GCATCTT----TGC
R118        --------GCCCCCGGCGGACCCC---TCAACTCT-GCATCTT----TGC
R114        --------GCCCCCGGCGGACCCC---TCAACTCT-GCATCTT----TGC
R110        --------GCCCCCGGCGGACCCC---TCAACTCT-GCATCTT----TGC
R101        --------GCCCCCGGCGGACCCC---TCAACTCT-GCATCTT----TGC
R091                --------GCCCCCGGCGGACCCC---TCAACTCT-GCATCTT----TGC
R090        --------GCCCCCGGCGGACCCC---TCAACTCT-GCATCTT----TGC
R089        --------GCCCCCGGCGGACCCC---TCAACTCT-GCATCTT----TGC
'98-191'    --------GCCCCCGGCGGACCCC---TCAACTCT-GCATCTT----TGC
'98-125'    --------GCCCCCGGCGGACCCC---TCAACTCT-GCATCTT----TGC
AF309622            --------GCCCCCGGCGGACCCC---TCAACTCT-GCATCTT----TGC
R210        --------GCCCCCGGTGGACCCC---TCAACTCT-GCATCTC----TGC
R211        --------GCCCCCGGTGGACCCC---TCAACTCT-GCATCTC----TGC
AY045497            --------GCCCCCGGTGGACCCC---TCAACTCT-GCATCTC----TGC
784                 --------GCCCCCGGTGGACCC----TCAACTCT-GCATCTC----TGC
'98-099'            --------GCCCTCGCAGGACGCC---TCAACGCT-GCATCTG----TGC
'98-101'    --------GCCCTCGCAGGACGCC---TCAACGCT-GCATCTG----TGC
R001        --------GCCCTCGCAGGACGCC---TCAACGCT-GCATCTG----TGC
R002        --------GCCCTCGCAGGACGCC---TCAACGCT-GCATCTG----TGC
R004        --------GCCCTCGCAGGACGCC---TCAACGCT-GCATCTG----TGC
R051        --------GCCCTCGCAGGACGCC---TCAACGCT-GCATCTG----TGC
AY045505            --------GCCCTCGCAGGACGCC---TCAACGCT-GCATCTG----TGC
R057        --------GCCCTCGCAGGACGCC---TCAACGCT-GCATCTG----TGC
AF449097            --------GCCCTCGCAGGACCCC---TCAACGCT-GCATCTG----TGC
sutton1346          --------CCCCCCTGAGGACCCT---CTAACCCT-GCGTCCTC--TTGC
R215        --------GCCCCCGGCGGACACC---TCAACTCT-GCATCTT----TGC
SJ5                 --------GCCCCCGGCGGACACC---TCAACTCT-GCATCTT----TGC
R216        --------GCCCCCGGCGGACACC---TCAACTCT-GCATCTT----TGC
R216Y               -------GCCCCCGGCGGACACC---TCAACTCT-GCATCTT----TGC
AY045503            --------GCCCCCGGTGGACCCC---TCAACTCT-GCATCTT----TGC
AF310107            --------CCCCCCGGTGGACCCCCTCTCAACTCTCGCGTCCC----GCC
'98-133'    --------GCCCCCGGTGGCCAAC--TCAAACTC-TGTTTTTAT--TGCC
'98-163'    --------GCCCCCGGTGGCCAAC--TCAAACTC-TGTTTTTAT--TGCC
dekk                --------GCCCCCGGTGGCCAAC--TCAAACTC-TGTTTTTAT--TGCC
'98-148'    --------GCCCCCGGCGGCCAAC--TCAAACTC-TGTTTTTAT--TGCC
'98-149'    --------GCCCCCGGCGGCCAAC--TCAAACTC-TGTTTTTAT--TGCC
R257        --------GCCCCCGGCGGCCAAC--TCAAACTC-TGTTTTTAT--TGCC
R258        --------GCCCCCGGCGGCCAAC--TCAAACTC-TGTTTTTAT--TGCC
R262        --------CCCCCCGGCGGACCCC--TCCAACTCGTGTTGTACC--TGCA
AF243401            --------ACTCCAGTCAGTAAAC--GCAGAC-----------------
R234        ----GGCG-CCCCCGGGGGAAA----TCAAACACT-GCGTCAAT--TTGT
R243                ----GGCG-CCCCCGGGGGAAA----TCAAACACT-GCGTCAAT--TTGT
R247        ----GGCG-CCCCCGGGGGAAA----TCAAACACT-GCGTCAAT--TTGT
R242        ----GGCG-CCCCCGGGGGAAA----TCAAACACT-GCGTCAAT--TTGT
AY045517            ----GGCG-CCCCCGGGGGAAA----TCAAACACT-GCGTCAAT--TTGT
AF173316            ----GGCG-CCCCCGGGGGAAA----TCAAACACT-GCGTCAAT--TTGT
parkii353           ----CGCG-CCCCCGGAGGATA----CTTAACCCT-GCATCA----TTGC
R151        ----CG---CCCCCGGAGGTCA----TCAAACACT-GCATCT----TTGC
R152        ----CG---CCCCCGGAGGTCA----TCAAACACT-GCATCT----TTGC
R221        ----CG---CCCCCGGAGGTCA----TCAAACACT-GCATCT----TTGC
R222        ----CG---CCCCCGGAGGTCA----TCAAACACT-GCATCT----TTGC
AF173314            ----CGG--CCCCCGGAGGTCA----TCAAACACT-GCATCT----TTGC
ken                 ----CGG--CCCCCGGAGGTCA----TCAAACACT-GCATCT----TTGC
R237        ----CG---CTCCCGGTGGCCAATTATTAAACTCT-GCATCTC---TTGC
R246        ----CG---CTCCCGGTGGCCAATTATTAAACTCT-GCATCTC---TTGC
R240        ----CG---CTCCCGGTGGCCAATTATTAAACTCT-GCATCTC---TTGC
AF173303            ----CG---CTCCCGGTGGCCAATTATTAAACTCT-GCATCTC---CTGC
AF468869            ----GGGCGCCCCCGAAGGTCT----CCAAACACT-GCATCT----TTGC
AF222839            ----GGCG-CCCCCGGAGGCCA----TTAAACACT-GCATCA----TTGC
AF222843            ----GGCGGCCCCCGGAGGCCA----TTAAACACT-GCATCA----TTGC
AF222841            ----GGCG-CCCCCGGAGGCCA----TTAAACACT-GCATCA----TTGC
AF222842            ----GGCG-CCCCCGGAGGCCA----T-AAACACT-GCATCA----TTGC
colomb              ----GGCG-CCCCCGGAGGCCA----TCAAACACT-GCATCA----TTGC
AF309616            ----GGCG-CCCCCGGAGGCCC----TCAAACACT-GCATCC----TCGC
AF309603            -------GGCCCCCGGCGGACACC---TCAACTCT-GCATCTT----TGC
cmw4937             -------GGCCCCCGGCGGACCAC---TCAACTAT-GCATCTG----TGC
R248        -------GGCCCCCGGTGGACCAT---CAAACTCT-GCATCTT---TGAC
R251                -------GGCCCCCGGTGGACCAT---CAAACTCT-GCATCTT---TGAC
R250        -------GGCCCCCGGTGGACCAT---CAAACTCT-GCATCTT---TGAC
R249        -------GGCCCCCGGTGGACCAT---CAAACTCT-GCATCTT---TGAC
AY045516            -------GGCCCCCGGTGGACCAT---CAAACTCT-GCATCTT---TGAC
Dothidea            ACAGGTGAGTGCCCGCCAGAGTCCAACCAAACTCTTGTTTTTAACCAGTC
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                  ……      160          170 180          190          200
R115      GTCTGAGTGATAA-CGAA-AAATCAATCAAAACTTTCAACAACGGATCTC
R118      GTCTGAGTGATAA-CGAA-AAATCAATCAAAACTTTCAACAACGGATCTC
R114      GTCTGAGTGATAA-CGAA-AAATCAATCAAAACTTTCAACAACGGATCTC
R110      GTCTGAGTGATAA-CGAA-AAATCAATCAAAACTTTCAACAACGGATCTC
R101      GTCTGAGTGATAA-CGAA-AAATCAATCAAAACTTTCAACAACGGATCTC
R091      GTCTGAGTGATAA-CGAA-AAATCAATCAAAACTTTCAACAACGGATCTC
R090      GTCTGAGTGATAA-CGAA-AAATCAATCAAAACTTTCAACAACGGATCTC
R089      GTCTGAGTGATAA-CGAA-AAATCAATCAAAACTTTCAACAACGGATCTC
'98-191'  GTCTGAGTGATAA-CGAA-AAATCAATCAAAACTTTCAACAACGGATCTC
'98-125'  GTCTGAGTGATAA-CGAA-AAATCAATCAAAACTTTCAACAACGGATCTC
AF309622          GTCTGAGTGATAA-CGAA-AA-TCAATCAAAACTTTCAACAACGGATCTC
R210      GTCTGAGTCACA--AAAT-AAATCAATCAAAACTTTCAACAACGGATCTC
R211      GTCTGAGTCACA--AAAT-AAATCAATCAAAACTTTCAACAACGGATCTC
AY045497          GTCTGAGTCACA--AAAT-CAATCAATCAAAACTTTCAACAACGGATCTC
784               GTCTGAGTCACA--AAAT-CAATCAATCAAAACTTTCAACAACGGATCTC
'98-099'  GTCGGAGTAATA--CAAC-CAATCAATTAAAACTTTCAACAACGGATCTC
'98-101'  GTCGGAGTAATA--CAAC-CAATCAATTAAAACTTTCAACAACGGATCTC
R001      GTCGGAGTAATA--CAAC-CAATCAATTAAAACTTTCAACAACGGATCTC
R002      GTCGGAGTAATA--CAAC-CAATCAATTAAAACTTTCAACAACGGATCTC
R004      GTCGGAGTAATA--CAAC-CAATCAATTAAAACTTTCAACAACGGATCTC
R051      GTCGGAGTAATA--CAAC-CAATCAATTAAAACTTTCAACAACGGATCTC
AY045505          GTCGGAGTAATA--CAAC-CAATCAATTAAAACTTTCAACAACGGATCTC
R057      GTCGGAGTAATA--CAAC-CAATCAATTAAAACTTTCAACAACGGATCTC
AF449097          GTCGGAGTAATA--CAAC-CAATCAATTAAAACTTTCAACAACGGATCTC
sutton1346        GTCTGAGTCGTGAGTAGA-AATTGAATCAAAACTTTCAACAATGGATCTC
R215      GTCGGAGTCTTA--TGAT-AAATCAATCAAAACTTTCAACAACGGATCTC
SJ5               GTCGGAGTCTTA--TGAT-AAATCAATCAAAACTTTCAACAACGGATCTC
R216      GTCGGAGTCTTA--TGAT-AAATCAATCAAAACTTTCAACAACGGATCTC
R216Y             GTCGGAGTCTTA--TGAT-AAATCAATCAAAACTTTCAACAACGGATCTC
AY045503          GTCGGAGTCTAA--TGAT-AAATCAATCAAAACTTTCAACAACGGATCTC
AF310107          GTCTAGTCTTTGATTATTGAATTGAAACAAAACTTTCAACAACGGATCTC
'98-133'  GTCTGAGTAACA---AACAAATCAAAACAAAACTTTCAACAACGGATCTC
'98-163'  GTCTGAGTAACA---AACAAATCAAAACAAAACTTTCAACAACGGATCTC
dekk              GTCTGAGTAACA---AACAAATCAAAACAAAACTTTCAACAACGGATCTC
'98-148'  GTCTGAGTAACA---AACAAATCAAAACAAAACTTTCAACAACGGATCTC
'98-149'  GTCTGAGTAACA---AACAAATCAAAACAAAACTTTCAACAACGGATCTC
R257      GTCTGAGTAACA---AACAAATCAAAACAAAACTTTCAACAACGGATCTC
R258      GTCTGAGTAACA---AACAAATCAAAACAAAACTTTCAACAACGGATCTC
R262      GTCCGAGTCTTAT--GAGAAATCAAACAAAAACTTTCAACAACGGATCTC
AF243401          GTCTGA-TAACA----AGTTAATAAACTAAAACTTTCAACAACGGATCTC
R234      GTCGGAGTA--C--TTGTTAATA-A-ACAAAACTTTCAACAACGGATCTC
R243      GTCGGAGTA--C--TTGTTAATA-A-ACAAAACTTTCAACAACGGATCTC
R247      GTCGGAGTA--C--TTGTTAATA-A-ACAAAACTTTCAACAACGGATCTC
R242      GTCGGAGTA--C--TTGTTAATA-A-ACAAAACTTTCAACAACGGATCTC
AY045517          GTCGGAGTA--C--TTGTTAATA-A-ACAAAACTTTCAACAACGGATCTC
AF173316          GTCGGAGTA--C--TTGTTAATA-A-ACAAAACTTTCAACAACGGATCTC
parkii353         GTCGGAGTAATT--TTATTAATA-ACATAAAACTTTCAACAACGGATCTC
R151      GTCGGAGTCTTA--AAGTAAATTTAAACAAAACTTTCAACAACGGATCTC
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R221      GTCGGAGTCTTA--AAGTAAATTTAAACAAAACTTTCAACAACGGATCTC
R222      GTCGGAGTCTTA--AAGTAAATTTAAACAAAACTTTCAACAACGGATCTC
AF173314          GTCGGAGTCTTA--AAGTAAATT-AAACAAAACTTTCAACAACGGATCTC
ken               GTCGGAGTCTTA--AAGTAAATT-AAACAAAACTTTCAACAACGGATCTC
R237      GTCGGAGTCTTA--AAG-AAATTTAAACAAAACTTTCAACAACGGATCTC
R246      GTCGGAGTCTTA--AAG-AAATTTAAACAAAACTTTCAACAACGGATCTC
R240      GTCGGAGTCTTA--AAG-AAATTTAAACAAAACTTTCAACAACGGATCTC
AF173303          GTCGGAGTCTTA--AAG-AAATTTAAACAAAACTTTCAACAACGGATCTC
AF468869          GTCGGAGTTT----AAACAAATT-AAACAAAACTTTCAACAACGGATCTC
AF222839          GTCGGAGTTA----AAGTAAATT-AAACAAAACTTTCAACAACGGATCTC
AF222843          GTCGGAGTTA----AAGTAAATT-AAACAAAACTTTCAACAACGGATCTC
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colomb            GTCGGAGTAA----AAGTAAATG-AAACAAAACTTTCAACAACGGATCTC
AF309616          GTCGGAGTCT----CAGTAAATG-AAACAAAACTTTCAACAACGGATCTC
AF309603          GTCTGAGTATGAT-ATTTGAATCAA-TCAAAACTTTCAACAACGGATCTC
cmw4937           GTCTGAGTA-AAT-ATTTGAATCAAATCAAAACTTTCAACAACGGATCTC
R248      GTCTGAGTAAAT----ATTGAATCAATCAAAACTTTTAACAACGGATCTC
R251      GTCTGAGTAAAT----ATTGAATCAATCAAAACTTTTAACAACGGATCTC
R250      GTCTGAGTAAAT----ATTGAATCAATCAAAACTTTTAACAACGGATCTC
R249      GTCTGAGTAAAT----ATTGAATCAATCAAAACTTTTAACAACGGATCTC
AY045516          GTCTGAGTAAAT----ATTGAATCAATCAAAACTTTTAACAACGGATCTC
Dothidea          GTCTGAGTATAAA-ATTTTAATTAAATTAAAACTTTCAACAACGGATCTC
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                  ……      210             220  230             240  250
R115      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R118      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R114      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R110      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R101      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R091      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R090      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R089      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
'98-191'  TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
'98-125'  TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AF309622          TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R210      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R211      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AY045497          TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
784               TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
'98-099'  TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
'98-101'  TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R001      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R002      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R004      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R051      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AY045505          TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R057      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AF449097          TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
sutton1346        TTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R215      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
SJ5               TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R216      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R216Y             TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AY045503          TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AF310107          TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
'98-133'  TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
'98-163'  TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
dekk              TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
'98-148'  TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
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R234      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
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R242      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AY045517          TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AF173316          TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
parkii353          TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R151      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
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ken               TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
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R240      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AF173303          TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AF468869          TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AF222839          TTGGTTCCAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AF222843          TTGGTTCCAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AF222841          TTGGTTCCAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AF222842          TTGGTTCCAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
colomb            TTGGTTCCAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AF309616          TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AF309603          TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
cmw4937           TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
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R250      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
R249      TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
AY045516          TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
Dothidea          TTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
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	…… 260 270 280 290300
R115	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
R118	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
R114	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
R110	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
R101	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
R091	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
R090	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
R089	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
'98-191'	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
'98-125'	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
AF309622	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
R210	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
R211	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
AY045497	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
784	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
'98-099'	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
'98-101'	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
R001	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
R002	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
R004	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
R051	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
AY045505	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
R057	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
AF449097	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
sutton1346	ATTGCAGAATTCCGTGAATAATCGAATCTTTGAACGCACATTGCGCCCT-
R215	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
SJ5	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
R216	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
R216Y	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
AY045503	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
AF310107	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
'98-133'	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
'98-163'	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
dekk	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
'98-148'	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
'98-149'	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
R257	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
R258	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
R262	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
AF243401	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
R234	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
R243	ATTGCAGGATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
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R242	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
AY045517	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGNGCCCCC
AF173316	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
parkii353	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
R151	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
R152	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
R221	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
R222	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
AF173314	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
ken	ATTGCAAAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
R237	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
R246	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
R240	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
AF173303	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
AF468869	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
AF222839	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
AF222843	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
AF222841	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
AF222842	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
colomb	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
AF309616	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT-
AF309603	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
cmw4937	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
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R250	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
R249	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
AY045516	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-
Dothidea	ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCC-

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                  ……      310         320         330         340         350
R115      CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTACACC-C
R118      CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTACACC-C
R114      CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTACACC-C
R110      CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTACACC-C
R101      CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTACACC-C
R091      CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTACACC-C
R090      CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTACACC-C
R089      CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTACACC-C
'98-191'  CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTACACC-C
'98-125'  CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTACACC-C
AF309622          CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTACACC-A
R210      CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTACACC-A
R211      CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTACACC-A
AY045497          CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTACACC-A
784               CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTACACC-A
'98-099'  CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
'98-101'  CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R001      CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R002      CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R004      CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R051      CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
AY045505          CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R057      CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
AF449097          CTGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
sutton1346        CCGG--TATTCC---GGAGGGCATGCCTGTTCGAGCG-TCATCAACCC-A
R215      CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
SJ5               CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R216      CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R216Y             CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
AY045503          TTGG--TATTCC---GAGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
AF310107          CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
'98-133'  CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTACAAC-C
'98-163'  CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTACAAC-C
dekk              CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTACAAC-C
'98-148'  CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTGCAAC-C
'98-149'  CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTGCAAC-C
R257      CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTGCAAC-C
R258      CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTGCAAC-C
R262      CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTTCAAC-C
AF243401          TTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTACAAC-C
R234      GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R243      GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R247      GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R242      GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
AY045517          GTGGGTTATTCCCGCGGGGGGCATGCCTGGTCGAGCGGTCATTTCCCCCA
AF173316          GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
parkii353         GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R151      GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R152      GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R221      GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R222      GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
AF173314          GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
ken               GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R237      GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R246      GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R240      GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
AF173303          GTGG--TATTCC---GCGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
AF468869          TTGG--TATTCC---GAAGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
AF222839          CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
AF222843          CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
AF222841          CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
AF222842          CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
colomb            CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
AF309616          CTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
AF309603          TTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
cmw4937           TTGG--TATTCC---GGGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R248      TTGG--TATTCC---GAGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R251      TTGG--TATTCC---GAGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R250      TTGG--TATTCC---GAGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
R249      TTGG--TATTCC---GAGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
AY045516          TCGG--TATTCC---GAGGGGCATGCCTGTTCGAGCG-TCATTTCACC-A
Dothidea          TTGG--TATTCC---GAGGGGCATGCCTGTTCGAGCG-TCATTACACC-A
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                  ……      360         370         380         390         400
R115      CTCCA-GCCTCGCTGGGTGTTGGGCATCGCGG-C-----CTCC-GCGCGC
R118      CTCCA-GCCTCGCTGGGTGTTGGGCATCGCGG-C-----CTCC-GCGCGC
R114      CTCCA-GCCTCGCTGGGTGTTGGGCATCGCGG-C-----CTCC-GCGCGC
R110      CTCCA-GCCTCGCTGGGTGTTGGGCATCGCGG-C-----CTCC-GCGCGC
R101      CTCCA-GCCTCGCTGGGTGTTGGGCATCGCGG-C-----CTCC-GCGCGC
R091      CTCCA-GCCTCGCTGGGTGTTGGGCATCGCGG-C-----CTCC-GCGCGC
R090      CTCCA-GCCTCGCTGGGTGTTGGGCATCGCGG-C-----CTCC-GCGCGC
R089      CTCCA-GCCTCGCTGGGTGTTGGGCATCGCGG-C-----CTCC-GCGCGC
'98-191'  CTCCA-GCCTCGCTGGGTGTTGGGCATCGCGG-C-----CTCC-GCGCGC
'98-125'  CTCCA-GCCTCGCTGGGTGTTGGGCATCGCGG-C-----CTCC-GCGCGC
AF309622            CTCCA-GCCTCGCTGGGTATTGGGCGTCTCGG-C------TCC-GCGCGC
R210      CTCCG-GCCTCGCTGGGTATTGGGCGCCGCGG-C-----CTCC-GCGCGC
R211      CTCCG-GCCTCGCTGGGTATTGGGCGCCGCGG-C-----CTCC-GCGCGC
AY045497            CTCCG-GCCTCGCTGGGTATTGGGCGCCGCGG-C-----CTCC-GCGCGC
784                 CTCCG-GCCTCGCTGGGTATTGGGCGCCGCGG-C-----CTCC-GCGCGC
'98-099'  CTCCA-GCCTCGCTGGGTCTTGGGCGCCGCGG-C-----CTCC-GCGCGC
'98-101'  CTCCA-GCCTCGCTGGGTCTTGGGCGCCGCGG-C-----CTCC-GCGCGC
R001      CTCCA-GCCTCGCTGGGTCTTGGGCGCCGCGG-C-----CTCC-GCGCGC
R002      CTCCA-GCCTCGCTGGGTCTTGGGCGCCGCGG-C-----CTCC-GCGCGC
R004      CTCCA-GCCTCGCTGGGTCTTGGGCGCCGCGG-C-----CTCC-GCGCGC
R051      CTCCA-GCCTCGCTGGGTCTTGGGCGCCGCGG-C-----CTCC-GCGCGC
AY045505            CTCCA-GCCTCGCTGGGTCTTGGGCGCCGCGG-C-----CTCC-GCGCGC
R057      CTCCA-GCCTTGCTGGGTCTTGGGCGCCGCGG-C-----CTCC-GCGCGC
AF449097            CTCCA-GCCCCGCTGGGTCTTGGGCGCCGCGG-C-----CTCC-GCGCGC
sutton1346          CCTCAAGCCTCGCTTGGTGTTGGGCGTCGCGG-C-----CGCC-GCGCGC
R215      CTCAA-GCCTCGCTTGGTATTGGGCGCCGCGGCC-----TTCC-GCGCGC
SJ5                 CTCAA-GCCTCGCTTGGTATTGGGCGCCGCGGCC-----TTCC-GCGCGC
R216      CTCAA-GCCTCGCTTGGTATTGGGCGCCGCGGCC-----TTCC-GCGCGC
R216Y               CTCAA-GCCTCGCTTGGTATTGGGCGCCGCGGCC-----TTCC-GCGCGC
AY045503            CTCAA-GCCTCGCTTGGTATTGGGCGCCGCGGTC-----CGCC-GCGCGC
AF310107            CTCAA-GCCCGGCTTGGTATTGGGCCTCGCGGCC-----AGCCCGCGTGC
'98-133'  AATCCAGCCCCGCTGGGTATTGGGCGTCGCGGCC-----TGCCG-CGCGC
'98-163'  AATCCAGCCCCGCTGGGTATTGGGCGTCGCGGCC-----TGCCG-CGCGC
dekk                AATCCAGCCCCGCTGGGTATTGGGCGTCGCGGCC-----TGCCG-CGCGC
'98-148'  AATCCAGCCCCGCTGGGTATTGGGCGTCGCGGCC-----TGCCG-CGCGC
'98-149'  AATCCAGCCCCGCTGGGTATTGGGCGTCGCGGCC-----TGCCG-CGCGC
R257      AATCCAGCCCCGCTGGGTATTGGGCGTCGCGGCC-----TGCCG-CGCGC
R258      AATCCAGCCCCGCTGGGTATTGGGCGTCGCGGCC-----TGCCG-CGCGC
R262      AAACCAGCCCCGCTGGGTGTTGGGCGTCGCGGTC-----CGCCG-CGCGC
AF243401            C-TCAAGCTCTGCTTGGAATTGGGCACCGTCCTCAC---TGCGGACGCGC
R234      CTC-GAGTCTGACTCGGTATTGGGCGTCG-CGTTT----CGAT-GCGCGC
R243      CTC-GAGTCTGACTCGGTATTGGGCGTCG-CGTTT----CGAT-GCGCGC
R247      CTC-GAGTCTGACTCGGTATTGGGCGTCG-CGTTT----CGAT-GCGCGC
R242      CTC-GAGTCTGACTCGGTATTGGGCGTCG-CGTTT----CGAT-GCGCGC
AY045517            CTCAGAGTCTGACTCGGTATTGGCCGTCGGCGTTTT---CNAT-GCGCGC
AF173316            CTC-GAGTCTGACTCGGTATTGGGCGTCG-CGTTT----CGAT-GCGCGC
parkii353           CTC-GAGTCTGACTCGGTATTGGGCGTCGCGGCTTC---CGCC-GCGCGC
R151      CTC-AAGCCTAGCTTGGTATTGGGCGTCGCGGTTCC---G-----CGCGC
R152      CTC-AAGCCTAGCTTGGTATTGGGCGTCGCGGTTCC---G-----CGCGC
R221      CTC-AAGCCTAGCTTGGTATTGGGCGTCGCGGTTCC---G-----CGCGC
R222      CTC-AAGCCTAGCTTGGTATTGGGCGTCGCGGTTCC---G-----CGCGC
AF173314            CTC-AAGCCTAGCTTGGTATTGGGCGTCGCGGTTCC---G-----CGCGC
ken                 CTC-AAGCCTAGCTTGGTATTGGGCGTCGCGGTTCC---G-----CGCGC
R237      CTC-AAGCCTAGCTTGGTATTGGGCGTCGCGACTTC---GGTC-GCGCGC
R246      CTC-AAGCCTAGCTTGGTATTGGGCGTCGCGACTTC---GGTC-GCGCGC
R240      CTC-AAGCCTAGCTTGGTATTGGGCGTCGCGACTTC---GGTC-GCGCGC
AF173303            CTC-AAGCCTAGCTTGGTATTGGGCGTCGCGACTTC---GGTC-GCGCGC
AF468869            CTC-AAGCCTGGCTTGGTATTGGGCGTCGCGGCTCC---G-----CGCGC
AF222839            CTC-AAGCCTGGCTTGGTATTGGGCGTCGCGGCTCC---G-----CGCGC
AF222843            CTC-AAGCCTGGCTTGGTATTGGGCGTCGCGGCTCC---G-----CGCGC
AF222841            CTC-AAGCCTGGCTTGGTATTGGGCGTCGCGGCTCC---G-----CGCGC
AF222842            CTC-AAGCCTGGCTTGGTATTGGGCGTCGCGGCTTC---G-----CGCGC
colomb              CTC-AAGCCTGGCTTGGTATTGGGCGTCGCGGTGCC---G-----CGCGC
AF309616            CTC-AAGCCTGGCTTGGTATTGGGCGTCGCGGTGCC---G-----CGCGC
AF309603            CTC-AAGCCTGGCTTGGTATTGGGCGAGGCGGCTTC---CGGCCGCCCGC
cmw4937             CTC-AAGCCTGGCTTGGTATTGGGCGAGGCGGCTTCG--CGGCCGCCCGC
R248      CTC-AAGCCTGGCTTGGTATTGGGCGCCGCGGTT-----TGCC-GCGCGC
R251      CTC-AAGCCTGGCTTGGTATTGGGCGCCGCGGTT-----TGCC-GCGCGC
R250      CTC-AAGCCTGGCTTGGTATTGGGCGCCGCGGTT-----TGCC-GCGCGC
R249      CTC-AAGCCTGGCTTGGTATTGGGCGCCGCGGTT-----TGCC-GCGCGC
AY045516            CTC-AAGCCTGGCTTGGTATTGGGCGCCGCGGTT-----TGCC-GCGCGC
Dothidea            CTC-AAGCACTGCTTGGTATTGGGCATCGTCCGTCGAAAGGCGGGCGTGC
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          ……        410        420        430        440        450
R115      CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTAA-GCGTTGTGGCA-CA
R118      CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTAA-GCGTTGTGGCA-CA
R114      CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTAA-GCGTTGTGGCA-CA
R110      CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTAA-GCGTTGTGGCA-CA
R101      CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTAA-GCGTTGTGGCA-CA
R091      CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTAA-GCGTTGTGGCA-CA
R090      CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTAA-GCGTTGTGGCA-CA
R089      CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTAA-GCGTTGTGGCA-CA
'98-191'  CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTAA-GCGTTGTGGCA-CA
'98-125'  CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTAA-GCGTTGTGGCA-CA
AF309622  CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTAA-GCGTTGTGGCA-CA
R210      CTCGAAGTCT-CCGGCCGAGCC-GACCGTCTCCAA-GCGTTGTGGCA-CA
R211      CTCGAAGTCT-CCGGCCGAGCC-GACCGTCTCCAA-GCGTTGTGGCA-CA
AY045497  CTCGAAGTCT-CCGGCCGAGCC-GACCGTCTCCAA-GCGTTGTGGCA-CA
784       CTCGAAGTCT-CCGGCCGAGCC-GACCGTCTCCAA-GCGTTGTGGCA-CA
'98-099'  CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTCA-GCGTTGTGGCA-CC
'98-101'  CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTCA-GCGTTGTGGCA-CC
R001      CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTCA-GCGTTGTGGCA-CC
R002      CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTCA-GCGTTGTGGCA-CC
R004      CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTCA-GCGTTGTGGCA-CC
R051      CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTCA-GCGTTGTGGCA-CC
AY045505  CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTCA-GCGTTGTGGCA-CC
R057      CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTCA-GCGTTGTGGCA-CC
AF449097  CTCAATGTCT-CCGGCCGAGCC-GACCGTCTCTCA-GCGTTGTGGCA-CT
sutton1346 CCTAATGTCC-CCGGCCGAGCC-GGCCGTCCCGAA-GCGTTGTGGCGTCT
R215      CCCAATGTCT-CCGGCTGAGCC-ATCTATCTCAGA-GCGTTGTGGTA---
SJ5       CCCAATGTCT-CCGGCTGAGCC-ATCTATCTCAGA-GCGTTGTGGTA---
R216      CCCAATGTCT-CCGGCTGAGCC-ATCTATCTCAGA-GCGTTGTGGTA---
R216Y     CCCAATGTCT-CCGGCTGAGCC-ATCTATCTCAGA-GCGTTGTGGTA---
AY045503  CCCAATGTCT-CCGGCTCAGCCCAACGTCCTCGAA-GCGATTTGATT---
AF310107  CCTAATGTCT-C-GGCCGCGCC-GTCCGTCTCCCC-GCGTTGTGGCA---
'98-133'  CTCAAAGTCT-TCGGCGGAAGCCGCCCGTTCCTCT-GCGTGATGACACAT
'98-163'  CTCAAAGTCT-TCGGCGGAAGCCGCCCGTTCCTCT-GCGTGATGACACAT
dekk      CTCAAAGTCT-TCGGCGGAAGCCGCCCGTTCCTCT-GCGTGATGACACAT
'98-148'  CTCAAAGTCT-ACGGCGGAAGCCGCCCGTTCCTCT-GCGTGATGACACAT
'98-149'  CTCAAAGTCT-ACGGCGGAAGCCGCCCGTTCCTCT-GCGTGATGACACAT
R257      CTCAAAGTCT-ACGGCGGAAGCCGCCCGTTCCTCT-GCGTGATGACACAT
R258      CTCAAAGTCT-ACGGCGGAAGCCGCCCGTTCCTCT-GCGTGATGACACAT
R262      CTCAAAGTCT-TCGGCGGAAGCCGCCCGTTCCTCT-GCGTGATGCATCGT
AF243401  CTCAAAGACC-TCGGCGGTGGCTGTTCAGCCCTCAAGCGTAGTAGAATAC
R234      CTTAAAGTTT-CCGGCTGGACC-GTCCGTCTCCGA-GCGTTGTGG--CAT
R243      CTTAAAGTTT-CCGGCTGGACC-GTCCGTCTCCGA-GCGTTGTGG--CAT
R247      CTTAAAGTTT-CCGGCTGGACC-GTCCGTCTCCGA-GCGTTGTGG--CAT
R242      CTTAAAGTTT-CCGGCTGGACC-GTCCGTCTCCGA-GCGTTGTGG--CAT
AY045517  CTTAAAGTTT-CCGGCTGAACC-GTCCGTCTCCGA-GCGTTGTGG--CAT
AF173316  CTTAAAGTTT-CCGGCTGGACC-GTCCGTCTCCGA-GCGTTGTGG--CAT
parkii353 CTCAAAGTCT-CCGGCTGGGCA-GCCCGTCTCCGA-GCGTTGTGG--CAT
R151      CTTAAAGTCT-CCGGCTGAGCA-GTTCGTCTCTAA-GCGTTGTGG--CAT
R152      CTTAAAGTCT-CCGGCTGAGCA-GTTCGTCTCTAA-GCGTTGTGG--CAT
R221      CTTAAAGTCT-CCGGCTGAGCA-GTTCGTCTCTAA-GCGTTGTGG--CAT
R222      CTTAAAGTCT-CCGGCTGAGCA-GTTCGTCTCTAA-GCGTTGTGG--CAT
AF173314  CTTAAAGTCT-CCGGCTGAGCA-GTTCGTCTCTAA-GCGTTGTGG--CAT
ken       CTTAAAGTCT-CCGGCTGAGCA-GTTCGTCTCTAA-GCGTTGTGG--CAT
R237      CTTAAAGTCT-CCGGCTGAGCA-GTCTGTCTCCGA-GCGTTGTGA--TAC
R246      CTTAAAGTCT-CCGGCTGAGCA-GTCTGTCTCCGA-GCGTTGTGA--TAC
R240      CTTAAAGTCT-CCGGCTGAGCA-GTCTGTCTCCGA-GCGTTGTGA--TAC
AF173303  CTTAAAGTCT-CCGGCTGAGCA-GTGTGTCTCCGA-GCGTTGTGA--TAC
AF468869  CTTAAAGTCT-CCGGCTGAGCC-ATTCGTCTCTAA-GCGTTGTGGATTTT
AF222839  CTTAAAGTCTTCCGGCTGAGCT-GTCCGTCTCTAA-GCGTTGTGG--CAA
AF222843  CTTAAAGTCTTCCGGCTGAGCT-GTCCGTCTCTAA-GCGTTGTGG--CAA
AF222841  CTTAAAGTCTTCCGGCTGAGCT-GTCCGTCTCTAA-GCGTTGTGG--CAA
AF222842  CTTAAAGTCTTCCGGCTGAGCT-GTCCGTCTCTAA-GCGATGTGG--CAA
colomb    CTTAAAGTCTTCCGGCTGAGCT-GTCCGTCTCTAA-GCGTTGTGG--CAA
AF309616  CTCAAAGTCTTCCGGCTGAGCT-GCCCGTCTCCAA-GCGTTGTGG--CGA
AF309603  -TCAAAGTCT-CCG-CTGGACC-GACCGTCTCTAA-GCGTTGTGAC---T
cmw4937   CTCAAAGTCT-CCGGCTGGACG-GATCGTCTCTAA-GCGTTGTGAC---T
R248      CTCAAAGTCT-CCGGCTGAGCC-AACTGTCTCTAA-GCGTTGTGGTTTAA
R251      CTCAAAGTCT-CCGGCTGAGCC-AACTGTCTCTAA-GCGTTGTGGTTCAA
R250      CTCAAAGTCT-CCGGCTGAGCC-AACTGTCTCTAA-GCGTTGTGGTTTAA
R249      CTCAAAGTCT-CCGGCTGAGCC-AACTGTCTCTAA-GCGTTGTGGTTTAA
AY045516  CTCAAAGTCT-CCGGCTGAGCC-AACTGTCTCTAA-GCGTTGTGGTTTAA
Dothidea  CTCGAAGACC-TCGGCGGGGTTTCTCCAACTTCGG-GCGTAGTAGA--GT
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              ……        460         470         480         490         500
R115        --ACTGTTTCGCTTCCGGG-ACCGGT-CCGGCGTCGCGCCGTCAACCCC-
R118        --ACTGTTTCGCTTCCGGG-ACCGGT-CCGGCGTCGCGCCGTCAACCCC-
R114        --ACTGTTTCGCTTCCGGG-ACCGGT-CCGGCGTCGCGCCGTCAACCCC-
R110        --ACTGTTTCGCTTCCGGG-ACCGGT-CCGGCGTCGCGCCGTCAACCCC-
R101        --ACTGTTTCGCTTCCGGG-ACCGGT-CCGGCGTCGCGCCGTCAACCCC-
R091        --ACTGTTTCGCTTCCGGG-ACCGGT-CCGGCGTCGCGCCGTCAACCCC-
R090        --ACTGTTTCGCTTCCGGG-ACCGGT-CCGGCGTCGCGCCGTCAACCCC-
R089        --ACTGTTTCGCTTCCGGG-ACCGGT-CCGGCGTCGCGCCGTCAACCCC-
'98-191'    --ACTGTTTCGCTTCCGGG-ACCGGT-CCGGCGTCGCGCCGTCAACCCC-
'98-125'    --ACTGTTTCGCTTCCGGG-ACCGGT-CCGGCGTCGCGCCGTCAACCCC-
AF309622    --ACTGTTTCGCTTCCGGG-ACCGGT-CTGGCGTCGCGCCGTCAACCCC-
R210        --ACTGTTTCGCTTTCGGG-ACCGGT-CTGGCGGCGCGCCGTTAAACCC-
R211        --ACTGTTTCGCTTTCGGG-ACCGGT-CTGGCGGCGCGCCGTTAAACCC-
AY045497    --ACTGTTTCGCTTTCGGG-ACCGGT-CTGGCGGCGCGCCGTTAAACCC-
784         --ACTGTTTCGCTTTCGGG-ACCGGT-CTGGCGGCGCGCCGTTAAACCC-
'98-099'    --ACTGTTTCGCTGACGGGGACCGGT-CTGGCGGCGCGCCGTTAAACCC-
'98-101'    --ACTGTTTCGCTGACGGGGACCGGT-CTGGCGGCGCGCCGTTAAACCC-
R001        --ACTGTTTCGCTGACGGGGACCGGT-CTGGCGGCGCGCCGTTAAACCC-
R002        --ACTGTTTCGCTGACGGGGACCGGT-CTGGCGGCGCGCCGTTAAACCC-
R004        --ACTGTTTCGCTGACGGGGACCGGT-CTGGCGGCGCGCCGTTAAACCC-
R051        --ACTGTTTCGCTGACGGGGACCGGT-CTGGCGGCGCGCCGTTAAACCC-
AY045505    --ACTGTTTCGCTGACGGGGACCGGT-CTGGCGGCGCGCCGTTAAACCC-
R057        --ACTGTTTCGCTGACGGGGACCGGT-CTGGCGGCGCGCCGTTAAACCC-
AF449097    --ACTGTTTCGCTGACGGGGACCGGT-CTGGCGGCGCGCCGTTAAACCC-
sutton1346  --ACTGTGCCGCTTCCGGG-ACCGGT-CTGGCGGTGTGCCGTCAAACCC-
R215        --AACGTTCCGCTTGCGAGTGC--GA-TGGCTGTG-CGCCGTTAAACCC-
SJ5         --AACGTTCCGCTTGCGAGTGC--GA-TGGCTGTG-CGCCGTTAAACCC-
R216        --AACGTTCCGCTTGCGAGTGC--GA-TGGCTGTG-CGCCGTTAAACCCC
R216Y       --AACGTTCCGCTTGCGAGTGC--GA-TGGCTGTG-CGCCGTTAAACCCC
AY045503    --ACAATCCCGCTAGCAGGCAC--GG-AGGGCGCGACGCCGCTAAACAC-
AF310107    --ATCATGTTCGCGACGGA-GCCGGC-CCGGCGTGG-GCCGTCAACGAC-
'98-133'    -CGTCGCTTGGGACACGGGGGTGAGCGCCCGGAAAACATCGGCGGAGAC-
'98-163'    -CGTCGCTTGGGACACGGGGGTGAGCGCCCGGAAAACATCGGCGGAGAC-
dekk        -CGTCGCTTGGGACACGGGGGTGAGCGCCCGGAAAACATCGGCGGAGAC-
'98-148'    -CGTCGCTTGGGACACGGGGGTGAGCGCCCGGAAAACATCGGCGGAGAC-
'98-149'    -CGTCGCTTGGGACACGGGGGTGAGCGCCCGGAAAACATCGGCGGAGAC-
R257        -CGTCGCTTGGGACACGGGGGTGAGCGCCCGGAAAACATCGGCGGAGAC-
R258        -CGTCGCTTGGGACACGGGGGTGAGCGCCCGGAAAACATCGGCGGAGAC-
R262        -CGTCGCTTGGGACACGGGGGTGAGCGCCCGGAAATCGTCGGCGGAGAC-
AF243401    ACCTCGCTTTGGA-GCGGTTGGCGTCGCCCGCCGGACGAACCTTCTGA--
R234        CTG---TCTCGCTAGGGAGCCGCGG---AGGGCGTTGGCCGTTAAACAC-
R243        CTG---TCTCGCTAGGGAGCCGCGG---AGGGCGTTGGCCGTTAAACAC-
R247        CTG---TCTCGCTAGGGAGCCGCGG---AGGGCGTTGGCCGTTAAACAC-
R242        CTG---TCTCGCTAGGGAGCCGCGG---AGGGCGTTGGCCGTTAAACAC-
AY045517    CTG---TCTCGCTAGGGANCCGCGG---AGGGCGTTGGCCGTTAAACAC-
AF173316    CTG---TCTCGCT-GAAAGCCGCGG---AGGGCGTTGGCCGTTAAACAC-
parkii353   CACAGTTCTCGCTAGGGAGTCGCGG---ACGGCGTCGGCCGTTAAATAC-
R151        ATA---TTTCGCTGAA-GAGTTCGG--ACGGCTTTTGGCCGTTAAATCT-
R152        ATA---TTTCGCTGAA-GAGTTCGG--ACGGCTTTTGGCCGTTAAATCT-
R221        ATA---TTTCGCTGAA-GAGTTCGG--ACGGCTTTTGGCCGTTAAATCT-
R222        ATA---TTTCGCTGAA-GAGTTCGG--ACGGCTTTTGGCCGTTAAATCT-
AF173314    ATA---TTTCGCTGAAAGAGTTCGGG-ACGGCTTTTGGCCGTTAAATCT-
ken         ATA---TTTCGCTGAAAGAGTTCGG--ACGGCTTTTGGCCGTTAAATCT-
R237        ATA---TT-CGCTAGG-GATGACAGG-TCTG-TCGCGGCCGTTAAATCT-
R246        ATA---TT-CGCTAGG-GATGACAGG-TCTG-TCGCGGCCGTTAAATCT-
R240        ATA---TT-CGCTAGG-GATGACAGG-TCTG-TCGCGGCCGTTAAATCT-
AF173303    ATA---TT-CGCTAGG-GATGACAGG-TCTG-TCGCGGCCGTTAAATCT-
AF468869    TCA---ATTCGCTTCG-GAGTGCGG---GTGGCCGCGGCCGTTAAATCT-
AF222839    CTA---TT-CGCTTCG-GAGGTCGG---GTGGCCGCGGCCGTTAAATCT-
AF222843    CTA---TT-CGCTTCG-GAGGCCGG---GTGGCCGCGGCCGTTAAATCT-
AF222841    CTA---TT-CGCTTCG-GAGGTCGG---GTGGCCGCGGCCGTTAAATCT-
AF222842    CTA---TC-CGCTTTG-GAGG-CGG---GTGGCC--GGCCGTTAAATCT-
colomb      CTA---TT-CGCTTCG-GAGGCCGG---GCGGCCGCGGCCGTTAAATCT-
AF309616    CTA---TT-CGCTTCG-GGGCGCGG---GCGGCCGCGGCCGTTAAATCT-
AF309603    AAATTGGACCGCTTGTGAGTATGGGA---CGTCCTCGGCCGTTAAACT--
cmw4937     TCGTTGGACCGCTTGCGAGTACGGGA---CGTCCTCGGCCGTTAAACCC-
R248        TCA----TCCGCTTGCGAG-ATCGAA----GGCGACGGCCGTTAAAC---
R251        TCA----TCCGCTTGCGAG-ATCGAA----GGCGACGGCCGTTAAAC---
R250        TCA----TCCGCTTGCGAG-ATCGAA----GGCGACGGCCGTTAAAC---
R249        TCA----TCCGCTTGCGAG-ATCGAA----GGCGACGGCCGTTAAAC---
AY045516    TCA----TCCGCTTGTGAG-ATCGAA----GGCGACGGCCGTTAAAC---
Dothidea    TAAATCGAACGTCTTATAAGCTTGGTGGGACTCCATTGCCGTTAAACCT-
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            ……      510         520         530         540         550
R115        --CTCTCTCACAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R118        --CTCTCTCACAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R114        --CTCTCTCACAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R110        --CTCTCTCACAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R101        --CTCTCTCACAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R091        --CTCTCTCACAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R090        --CTCTCTCACAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R089        --CTCTCTCACAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
'98-191'    --CTCTCTCACAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
'98-125'    --CTCTCTCACAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
AF309622    --CTCTCTCACAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R210        --TTTCACCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R211        --TTTCACCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
AY045497    --TTTCACCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
784         --TTTCAC-AAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
'98-099'    --TTTCACCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
'98-101'    --TTTCACCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R001        --TTTCACCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R002        --TTTCACCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R004        --TTTCACCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R051        --TTTCACCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
AY045505    --TTTCACCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R057        --TTTCACCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
AF449097    --TTTCACCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
sutton1346  --CTTCATCAAAG-GTTGACCTCGGATCA-GGCAGGG-AT-ACCCGCTGA
R215        TTTTCTATCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
SJ5         TTTTCTATCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R216        TTTTCTATCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R216Y       TTTTCTATCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
AY045503    ---CCCATCACAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
AF310107    --CCCATCTTCAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
'98-133'    -GTCGATTTCAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
'98-163'    -GTCGATTTCAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
dekk        -GTCGATTTCAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
'98-148'    -GTCGATTTCAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
'98-149'    -GTCGATTTCAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R257        -GTCGATTTCAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R258        -GTCGATTTCAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R262        -GCCGACTTCAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
AF243401    -ACTTTTCTCAAG-GTTGACCTCGGATCA-GGTACGG-ATTACCCGCTGA
R234        ---CCCATCAAAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
R243        ---CCCATCAAAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
R247        ---CCCATCAAAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
R242        ---CCCATCAAAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
AY045517    ---CCCATCAAAGAGTTGACCTCGGATCAAGGTAGGGGATACCCCGCTGN
AF173316    ---CCCATCAAAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
parkii353   ---CCCATCAAAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
R151        ---TTCTT-AA-G-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
R152        ---TTCTT-AA-G-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
R221        ---TTCTT-AA-G-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
R222        ---TTCTT-AA-G-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
AF173314    ---TTCTT-AAAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
ken         ---TTC---AAAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
R237        ---TTATA-ACAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
R246        ---TTATA-ACAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
R240        ---TTATA-ACAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
AF173303    ---TTATA-ACAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
AF468869    ---TTATTCAAAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
AF222839    ---TTCAC--AAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
AF222843    ---TTCAC--AAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
AF222841    ---TTCAC--AAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
AF222842    ---TTCAC--AAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
colomb      ---TTCAC--AAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
AF309616    ---TTCAC--AAG-GTTGACCTCGGATCA-GGTAGGG-ATACCC-GCTGA
AF309603    --TATTACACAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
cmw4937     --TTTTATCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R248        ---TTATTCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R251        ---TTATTCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R250        ---TTATTCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
R249        ---TTATTCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
AY045516    ---TTATTCAAAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
Dothidea    -TTTATTTTCTAG-GTTGACCTCGGATCA-GGTAGGG-AT-ACCCGCTGA
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                  ……      560         570         578
R115        ACTTAA-GCATAT-CAATAAGCGGAGGA
R118        ACTTAA-GCATAT-CAATAAGCGGAGGA
R114        ACTTAA-GCATAT-CAATAAGCGGAGGA
R110        ACTTAA-GCATAT-CAATAAGCGGAGGA
R101        ACTTAA-GCATAT-CAATAAGCGGAGGA
R091        ACTTAA-GCATAT-CAATAAGCGGAGGA
R090        ACTTAA-GCATAT-CAATAAGCGGAGGA
R089        ACTTAA-GCATAT-CAATAAGCGGAGGA
'98-191'    ACTTAA-GCATAT-CAATAAGCGGAGGA
'98-125'    ACTTAA-GCATAT-CAATAAGCGGAGGA
AF309622            ACTTAA-GCATAT-CAATAAGCGGAGGA
R210        ACTTAA-GCATAT-CAATAAGCGGAGGA
R211        ACTTAA-GCATAT-CAATAAGCGGAGGA
AY045497            ACTTAA-GCATAT-CAATAAGCGGAGGA
784                 ACTTAA-GCATAT-CAATAAGCGGAGGA
'98-099'    ACTTAA-GCATAT-CAATAAGCGGAGGA
'98-101'    ACTTAA-GCATAT-CAATAAGCGGAGGA
R001        ACTTAA-GCATAT-CAATAAGCGGAGGA
R002        ACTTAA-GCATAT-CAATAAGCGGAGGA
R004        ACTTAA-GCATAT-CAATAAGCGGAGGA
R051        ACTTAA-GCATAT-CAATAAGCGGAGGA
AY045505            ACTTAA-GCATAT-CAATAAGCGGAGGA
R057        ACTTAA-GCATAT-CAATAAGCGGAGGA
AF449097            ACTTAA-GCATAT-CAATAAGCGGAGGA
sutton1346          ACTTAA-GCATAT-CAATAAGCGGAGGA
R215        ACTTAA-GCATAT-CAATAAGCGGAGGA
SJ5                 ACTTAA-GCATAT-CAATAAGCGGAGGA
R216        ACTTAA-GCATAT-CAATAAGCGGAGGA
R216Y               ACTTAA-GCATAT-CAATAAGCGGAGGA
AY045503            ACTTAA-GCATAT-CAATAAGCGGAGGA
AF310107            ACTTAA-GCATAT-CAATAAGCGGAGGA
'98-133'    ACTTAA-GCATAT-CAATAAGCGGAGGA
'98-163'    ACTTAA-GCATAT-CAATAAGCGGAGGA
dekk                ACTTAA-GCATAT-CAATAAGCGGAGGA
'98-148'    ACTTAA-GCATAT-CAATAAGCGGAGGA
'98-149'    ACTTAA-GCATAT-CAATAAGCGGAGGA
R257        ACTTAA-GCATAT-CAATAAGCGGAGGA
R258                ACTTAA-GCATAT-CAATAAGCGGAGGA
R262        ACTTAA-GCATAT-CAATAAGCGGAGGA
AF243401            ACTTAA-GCATAT-CAATAAGCGGAGGA
R234        ACTTAA-GCATAT-CAATAAGCGGAGGA
R243        ACTTAA-GCATAT-CAATAAGCGGAGGA
R247        ACTTAA-GCATAT-CAATAAGCGGAGGA
R242        ACTTAA-GCATAT-CAATAAGCGGAGGA
AY045517            ACTTAAAGCATATTCAATAAGCGGAGGA
AF173316            ACTTAA-GCATAT-CAATAAGCGGAGGA
parkii353           ACTTAA-GCATAT-CAATAAGCGGAGGA
R151        ACTTAA-GCATAT-CAATAAGCGGAGGA
R152        ACTTAA-GCATAT-CAATAAGCGGAGGA
R221        ACTTAA-GCATAT-CAATAAGCGGAGGA
R222        ACTTAA-GCATAT-CAATAAGCGGAGGA
AF173314            ACTTAA-GCATAT-CAATAAGCGGAGGA
ken                 ACTTAA-GCATAT-CAATAAGCGGAGGA
R237        ACTTAA-GCATAT-CAATAAGCGGAGGA
R246        ACTTAA-GCATAT-CAATAAGCGGAGGA
R240        ACTTAA-GCATAT-CAATAAGCGGAGGA
AF173303            ACTTAA-GCATAT-CAATAAGCGGAGGA
AF468869            ACTTAA-GCATAT-CAATAAGCGGAGGA
AF222839            ACTTAA-GCATAT-CAATAAGCGGAGGA
AF222843            ACTTAA-GCATAT-CAATAAGCGGAGGA
AF222841            ACTTAA-GCATAT-CAATAAGCGGAGGA
AF222842            ACTTAA-GCATAT-CAATAAGCGGAGGA
colomb              ACTTAA-GCATAT-CAATAAGCGGAGGA
AF309616            ACTTAA-GCATAT-CAATAAGCGGAGGA
AF309603            ACTTAA-GCATAT-CAATAAGCGGAGGA
cmw4937             ACTTAA-GCATAT-CAATAAGCGGAGGA
R248        ACTTAA-GCATAT-CAATAAGCGGAGGA
R251        ACTTAA-GCATAT-CAATAAGCGGAGGA
R250        ACTTAA-GCATAT-CAATAAGCGGAGGA
R249        ACTTAA-GCATAT-CAATAAGCGGAGGA
AY045516            ACTTAA-GCATAT-CAATAAGCGGAGGA
Dothidea            ACTTAA-GCATAT-CAATAAGCGGAGGA
```

Appendix 5.3: Sequence alignment all **Mycosphaerella** *species irrespective of host*

This alignment is available on GenBank at:

http://www.ncbi.nlm.nih.gov/Genbank/index.html

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