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Development of polymorphic microsatellite markers for the *Eucalyptus* leaf pathogen *Mycosphaerella nubilosa*

Abstract: *Mycosphaerella nubilosa* is one of the most important *Eucalyptus* leaf pathogens, causing premature defoliation and stunting of growth. The aim of this study was to develop polymorphic microsatellite markers for *M. nubilosa*. Fifteen primer sets were developed and evaluated for polymorphism. Two primers were monomorphic, three primers did not amplify the desired region and 10 primer pairs were polymorphic. These microsatellite markers will be applied to population biology studies of *M. nubilosa* collections from several countries. These studies will promote an understanding of the genetics and the global movement of *M. nubilosa* that is severely limiting plantation development.

Published as: Hunter GC, Cortinas MN, Wingfield BD, Crous PW, Wingfield, MJ (2006). Development of polymorphic microsatellite markers for the *Eucalyptus* leaf pathogen *Mycosphaerella nubilosa*. *Molecular Ecology Notes* **6**: 900–903.

INTRODUCTION

Species of the ascomycete fungal genus *Mycosphaerella* Johanson are amongst the most serious leaf pathogens of agricultural and forestry crops. More than 60 *Mycosphaerella* spp. are known from *Eucalyptus* spp. alone, and many of these result in serious diseases such as Mycosphaerella Leaf Disease (MLD) (Crous 1998, Crous *et al*. 2004, Hunter *et al*. 2004a). Infection results in leaf spots and twig cankers causing premature defoliation and stunting of tree growth (Crous 1998, Park *et al*. 2000).

Mycosphaerella nubilosa (Cooke) Hansf. is one of the most important *Mycosphaerella* spp. causing MLD in South Africa (Crous *et al*. 2004, Hunter *et al*. 2004b). *Eucalyptus nitens*, the most widely planted cold tolerant species of *Eucalyptus* in the country, is particularly susceptible to MLD. This is especially evident during the first two years of tree growth when juvenile leaves become severely infected resulting in defoliation and growth loss (Purnell & Lundquist 1986, Lundquist & Purnell 1987). Despite the importance of MLD, very little is known regarding the genetic structure, population dynamics or reproductive strategies of *M. nubilosa*. The aim of this study was, therefore, to develop polymorphic microsatellite markers for *M. nubilosa*.

Polymorphic microsatellite markers were developed using a single isolate of *M. nubilosa* (CBS 114708). This isolate was collected from *E. nitens* in the KwaZulu-Natal Province of South Africa during the course of a survey of *Mycosphaerella* spp. on *Eucalyptus* (Hunter *et al*. 2004b). DNA was collected from this isolate using the 1 : 1 phenol : chloroform isolation method as described by Hunter *et al*. (2004a, b). Isolates of *M. nubilosa* used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

The fast isolation by AFLP of sequences containing repeats (FIASCO) method for microsatellite isolation (Zane *et al*. 2002) with modifications (Cortinas *et al*. 2006) was used for microsatellite development. Briefly, DNA of *M. nubilosa* (1 µg) was digested with *Mse*I and ligated to an adaptor in the presence of a high concentration of ligase enzyme. This mixture was incubated overnight at 37 °C and the reaction was terminated by incubation at 65 °C for 20 min. A 1 : 10 dilution was prepared of the digestion-ligation mixture and 5 µL used for subsequent polymerase chain reaction (PCR) amplification following the methods of Zane *et al*. (2002). Following amplification, PCR products were hybridised to $(ATCC)_5$, $(GATA)_6$,

$(AG)_{10}$, $(GT)_{17}$, $(TC)_{15}$ and $(CA)_{15}$ biotinylated probes. DNA-probe complexes were subsequently isolated through magnetic bead capture. *Mycosphaerella nubilosa* DNA-containing repeats were cloned into the TOPO TA Cloning Kit (Invitrogen) following the manufacturer`s instructions and Nakabonge *et al*. (2005).

Bacterial clones were selected and diluted in 25 µL sterile water. This suspension was incubated at 96 °C for 7 min and 1 µL was removed for further colony PCR reactions. Colony PCR reactions were carried out in 50 µL reaction volumes containing 10 × PCR buffer, 1.5 mM $MgCl_2$, 300 mM each of TOPO M13 primers (5`–GTAAAACGACGGCCAG–3`/5`–CAGGAAACAGCTATGAC–3`), 5.0 µM dNTPs, 5.0 U Taq DNA polymerase (Roche Diagnostics, South Africa) and sterile distilled water to achieve a final volume of 50 µL (Cortinas *et al*. 2006). Colony PCR reactions included an initial denaturation at 96 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 53 °C for 30 s, elongation at 72 °C for 1 min and a final elongation to complete the reaction at 72 °C for 7 min. PCR products between 100 and 500 bp were subsequently selected and purified through Sephadex G-50 (Sigma Aldrich, St. Louis, MO) in Centri-sep spin columns (Princeton separations, Adelphia, NJ) following the manufacturer`s instructions. The purified PCR products were used as template DNA for cycle sequencing reactions using the ABI Prism Big Dye Terminator Cycle sequencing reaction kit v. 3.1 (Applied Biosystems, Foster City, CA) following the manufacturer`s instructions. The same primers used for the PCR reactions were also used for sequencing reactions. Precipitated PCR products were run on an ABI PRISM^{TM} 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA) and evaluated for the presence of microsatellites. Following these protocols, a total of 126 clones were sequenced and 15 potential microsatellite regions were identified from the selected *M. nubilosa* isolate.

Primers for the 15 potential microsatellite regions were developed using the primer development software PRIMER 3 (Rozen & Skaletsky 2000) and used to test for polymorphism in nine isolates of *M. nubilosa* from various locations including Spain (CMW 12569, CMW 12568), Tanzania (CMW 18616, CMW 18617), Australia (CMW 18619, CMW 18618, CMW 18620, CMW 18621) and South Africa (CBS 114708). DNA from these *M. nubilosa* isolates was used as a template for PCR reactions in an Eppendorf Mastercycler Personal PCR machine (Eppendorf AG, Germany) with the designed primers. PCR reactions included an initial denaturation step of 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C or 60 °C for 30 s, extension at 72 °C for 1 min and a final

extension step at 72 °C for 10 min. DNA was amplified in a 25 µL reaction volume containing PCR Buffer (5 mM Tris-HCl, 0.75 mM $MgCl_2$, 25 mM KCl, pH 8.3) (Roche Diagnostics, South Africa), 5.0 mM dNTP's 0.2 µM of each forward and reverse primer, 1.25 U Taq Polymerase (Roche Diagnostics, South Africa) and sterile water was added to achieve a final volume of 25 µL. Amplified DNA was visualised on 2 % agarose gels stained with ethidium bromide and viewed under ultra violet light. PCR products were purified using Sephadex G-50 (Sigma Aldrich, St. Louis, MO) in Centri-sep spin columns (Princeton separations, Adelphia, NJ). PCR products were sequenced as described earlier and evaluated for sequence polymorphism between the nine isolates of *M. nubilosa*. Following sequence evaluation using the 15 primer pairs, it was found that two primer pairs amplified a region monomorphic for all *M. nubilosa* isolates, three primer pairs did not amplify the desired region and 10 primer pairs were polymorphic for the *M. nubilosa* isolates.

Primer pairs that exhibited polymorphism were flourescently labelled (Applied Biosystems, South Africa) (Table 1) and used for further analysis on 18 *M. nubilosa* isolates (CMW 18616, CMW 18617, CBS 114708, CMW 12569, CMW 12568, CMW 12546, CMW 12562, CMW 12598, CMW 12600, CMW 12574, CMW 12551, CMW 12556, CMW 12557, CMW 12549, CMW 18619, CMW 18618, CMW 18620, CMW 18621). All forward primers were labelled with fluorescent dyes, except for locus MN-2 in which the reverse primer was flourescently labelled (Table 1). Allele sizes of amplified PCR products were determined by electrophoresis on an ABI PRISM™ 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA) and compared against a GeneScan 500-LIZ size standard (Applied Biosystems, Warrington, U.K.). Data analysis was conducted with GENESCAN and GENEMAPPER software (Applied Biosystems, Foster City, CA). A total of 32 alleles were obtained across the 10 loci for the 18 isolates of *M. nubilosa*. The most polymorphic locus was MN-8, which exhibited six alleles for the *M. nubilosa* isolates tested. Loci were tested for linkage disequilibrium using the program MULTILOCUS (Agapow & Burt 2001). From this test an observed $\bar{r}_s$ value of -0.02 (P = 0.728) was obtained, indicating that there is random association of alleles in the test population.

The polymorphic primer sets that were developed for *M. nubilosa* were also tested for cross amplification on six other *Mycosphaerella* spp. that are phylogenetically closely related to *M. nubilosa*. These were, *M. ohnowa* Crous & M.J. Wingf., *M. molleriana* (Thüm) Lindau, *M. vespa* Carnegie & Keane, *M. ambiphylla* A. Maxwell, *M. toledana* Crous & G. Bills and *M. cryptica* (Cooke) Hansf. PCR amplification with primers for locus MN-1 resulted in a

single band of the predicted size for *M. molleriana*, *M. vespa* and *M. ambiphylla* (data not shown). Amplification with all other polymorphic primer pairs resulted in multiple fragments or no amplification for the *Mycosphaerella* spp. tested.

The polymorphic microsatellite markers developed for *M. nubilosa* in this study will be applied to populations of this species from several countries. This will promote a better understanding of the genetic structure and the reproductive mechanisms of this important pathogen. This knowledge will contribute to improved breeding strategies and longer-term durability of resistance in trees chosen for plantation development.

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Table 1. Characteristics of 10 polymorphic microsatellite markers developed for *Mycosphaerella nubilosa* (MN). **H**: Gene Diversity (Nei 1973), calculated using MULTILOCUS (Agapow & Burt 2001).

Locus name	Fluorescent label	Primer Sequence (5`–3`)	Tm (°C)	No. of alleles	H	PCR product size (bp)	Core sequence	Individuals typed per locus	GenBank Accession no.
MN-1	NED	TCCTGAAATGAGTGCAGACG TCCTCATCCTCTGTGGAACC	60	2	0.20	257–271	$(AG)_{10}(TG)_{10}$	18	DQ096633
MN-2	6-FAM	CATTGCTTCGGCGGTTATAG ATGCACGAAGTCGTTGTTTG	60	3	0.20	182–266	$(ACT)_8$.59bp.$(AC)_{11}$	18	DQ096634
MN-3	VIC	GACTCAACCGTCGTCGAAAC CGAACTGAATCCGCTGTGTA	60	3	0.30	306–320	$(AC)_{13}$	18	DQ096635
MN-4	NED	TGTCACAAGACTTTGGATTGC CCACCACAATCTCCTCACAA	60	4	0.44	137–165	$(ATTGTGG)_{10}$	18	DQ096636
MN-7	6-FAM	CGCCTCACAGTTACACATGG CGAAAGGCTGAGGCTGAA	60	2	0.20	377–395	$(TGTA)_6$	18	DQ096637
MN-8	PET	TTCTATATACTATATTCTATTTAGG ATATACTATATCTAAAAGAGGTAG	53	6	0.51	202–322	$(CTCTCTATA)_{20}$	18	DQ096638
MN-9	NED	CGAATGGGCTATCAGAAACG ACAGGGCAAGGACCTCGTAT	60	4	0.38	211–221	$(CT)_{20}$	18	DQ096639
MN-10	PET	ACACCTCGAAATCGCTCATC TAGCTCTGTGCTGCCTTTGA	60	2	0.20	136–144	$(TC)_{11}$	18	DQ096640
MN-11	VIC	CTCACCAGTGCCGTCTAGGT GGAAATCCTGCCCTAACCTC	60	3	0.44	193–223	$(TTGGTG)_5$	18	DQ096641
MN-14	6-FAM	TCGACTACCGTAGGGGACTACT ATGCACGAAGTCGTTGTTTG	60	3	0.20	100–112	$(AC)_{13}$	18	DQ096642

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Global movement and population biology of the *Eucalyptus* leaf pathogen *Mycosphaerella nubilosa*

Abstract: Approximately 80 species of the ascomycete genus *Mycosphaerella* are responsible for leaf diseases on *Eucalyptus*. One of these species, *M. nubilosa*, is highly pathogenic to cold-tolerant *Eucalyptus* spp. which are commonly propagated for commercial forestry operations. Infection by *M. nubilosa* causes a disease known as Mycosphaerella Leaf Blotch (MLB) causing premature leaf abscission and in severe cases, stunting of tree growth. Although the taxonomy of *M. nubilosa* has been treated relatively closely, little is known regarding its population biology. Using 10 polymorphic DNA-based microsatellite markers, the genetic diversity of *M. nubilosa* populations from several different countries was considered. Results of these studies show that *M. nubilosa* from eastern Australia (New South Wales) has a higher gene and genotypic diversity than all introduced populations, supporting the view that this represents the origin of the pathogen. It was also evident that *M. nubilosa* populations from Europe and Tanzania are clonal, with the same multilocus haplotypes occurring in South Africa, but being absent in Australia. This suggests that *M. nubilosa* may have been introduced into Europe via Africa. Based on these results, we propose a pathway of gene flow of *M. nubilosa* from Australia to South Africa, into Africa and finally to Europe.

INTRODUCTION

The ascomycete fungal genus *Mycosphaerella* Johanson includes more than 80 species of fungi that have been associated with leaf diseases of *Eucalyptus* spp. (Crous 1998, Crous *et al*. 2004, 2006). Infection by *Mycosphaerella* spp. can result in a decreased capacity of young *Eucalyptus* leaves to undergo photosynthesis, resulting in premature leaf defoliation, leading to reduced growth of trees (Park & Keane 1982b, Lundquist & Purnell 1987, Carnegie & Ades 2003, Milgate *et al*. 2005a, Pinkard & Mohammed 2006). Recognition that there can be a large number of *Mycosphaerella* spp. known from *Eucalyptus* leaves has led to the understanding that *Eucalyptus* trees are susceptible to different *Mycosphaerella* spp. throughout their growth phase (Carnegie & Ades 2005).

Mycosphaerella nubilosa (Cooke) Hansf. is one of the most important *Mycosphaerella* spp. on *Eucalyptus* and it causes the disease commonly referred to as Mycosphaerella Leaf Blotch (MLB) particularly on cold-tolerant *Eucalyptus* spp. such as *E. globulus* and *E. nitens* (Park & Keane 1987, Carnegie *et al*. 1998, Carnegie & Ades 2002, Hunter *et al*. 2004a). This fungus typically infects juvenile leaves and susceptible trees can be severely defoliated (Park & Keane 1982b, Lundquist & Purnell 1987, Milgate *et al*. 2005a). In severe cases, trees do not enter the adult leaf stage and plantation establishment can fail (Lundquist & Purnell 1987).

Mycosphaerella nubilosa was first described from *Eucalyptus* leaves in Victoria, Australia (Cooke 1891). Subsequent to its description, the fungus has been recognized as a severe *Eucalyptus* leaf pathogen in Tasmania, Victoria, New South Wales, South and Western Australia (Hansford 1956, Park & Keane 1982 a, b, Park 1988, Maxwell *et al*. 2001, Milgate *et al*. 2001, 2005a). Furthermore, *M. nubilosa* is also known from the North Island of New Zealand (Dick 1982). In Africa, *M. nubilosa* has been identified from several countries including Ethiopia, Kenya, South Africa, Tanzania and Zambia (Crous *et al*. 2004, 2006, Hunter *et al*. 2004a, Alemu *et al*. 2006). Likewise, *M. nubilosa* has been accidentally introduced into commercial plantations of *E. globulus*, where it has caused serious damage in Spain and Portugal (Crous *et al*. 2004).

Mycosphaerella nubilosa infects several species of *Eucalyptus*, including *E. bridgesiana*, *E. cypellocarpa*, *E. grandis*, *E. globulus*, *E. nitens*, *E. quadrangulata* (Dick 1982, Crous 1998, Crous *et al*. 2004, Hunter *et al*. 2004 a, b, Milgate *et al*. 2005a). Due to their rapid growth, frost tolerance and favourable wood qualities, *E. nitens* and *E. globulus* are amongst the most popular *Eucalyptus* spp. propagated for commercial forestry in countries

such as Australia, Spain, Portugal and several African countries. These *Eucalyptus* spp. are particularly susceptible to *M. nubilosa*. Thus, MLB caused by *M. nubilosa* can be a serious impediment to the sustainability of *E. nitens* and *E. globulus* plantations.

Mycosphaerella nubilosa is a haploid ascomycete that in nature has been seen only in its sexual state. This is represented by many small black pseudothecia, containing eight-spored asci, that are amphigenous but predominantly hypophyllous on lesion surfaces (Park & Keane 1982a, Crous 1998, Crous *et al*. 2004). Upon sufficient wetting, ascospores are released from pseudothecia and act as primary inoculum that is predominantly wind-dispersed (Park & Keane 1982b, Park 1988). Ascospores germinate on *Eucalyptus* leaves where they infect via stomata and after successive developmental stages result in the formation of mature ascocarps on diseased leaf tissue (Park & Keane 1982b). Although the anamorph state *Uwebraunia juvenis* Crous & M.J. Wingf. has been linked to *M. nubilosa*, this connection has not been reconfirmed (Crous *et al*. 2004) and remains doubtful. Recognising the presence of an anamorph state is important because it represents a source of secondary inoculum in nature, responsible for short distance dispersal and infections of the same plant. This has for example been shown for *Septoria tritici* Desm. (teleomorph: *Mycosphaerella graminicola* (Fuckel) J. Schröt.), where conidia are splash-dispersed resulting in short distance dispersal and infection within the same plant or neighbouring plants (McDonald & Martinez 1990, Boeger *et al*. 1993, Linde *et al*. 2002).

The only research conducted on the population biology of *Mycosphaerella* spp. occurring on *Eucalyptus* has been by Milgate *et al.* (2005b), who considered the important pathogen *Mycosphaerella cryptica* (Cooke) Hansf. In contrast, extensive research has been conducted on other pathosystems involving *Mycosphaerella* spp. and their anamorphs on a wide range of important agronomic crops. These include *M. graminicola* (anamorph: *S. tritici*) that causes leaf blotch disease on wheat as well as *Mycosphaerella fijiensis* M. Morelet and *Mycosphaerella musicola* R. Leach ex Mulder, causing leaf diseases of banana (Carlier *et al*. 1996, Linde *et al*. 2002, Hayden *et al*. 2003a, b). These population biology studies have promoted an increased knowledge of population structure, distribution of genetic diversity, gene flow, centres of origin, reproductive modes and the contribution of mating strategy to population structure (Carlier *et al*. 1996, Linde *et al*. 2002, Hayden *et al*. 2003a, b, Zhan *et al.* 2003).

Most research conducted on *M. nubilosa* has focussed on its epidemiology, host susceptibility, taxonomy and phylogenetic placement with respect to other species of *Mycosphaerella*. However, little is known regarding the population biology of this important

Eucalyptus leaf pathogen. The aim of this study was, therefore, to investigate the population biology of *M. nubilosa* from several countries by employing allele size data for 10 polymorphic microsatellite loci. There were four primary objectives in this study that included a consideration of the genetic diversity of *M. nubilosa*, examination of variation in genetic diversity between different countries, determination of the likely origin and global movement of *M. nubilosa* and to determine the likely mating strategy of the pathogen.

MATERIALS AND METHODS

Isolation and isolates

Diseased *Eucalyptus* leaves infected with *M. nubilosa* were collected from plantations in five countries on three continents including, Australia, Portugal, South Africa, Spain and Tanzania (Table 1). A hierarchical sampling strategy was followed for South Africa where one diseased tree at the centre of a plantation was selected for sampling and many diseased leaves were collected from this single tree (–1T). One diseased leaf was then taken from 40–60 trees randomly sampled throughout the plantation around the central tree, extending outwards (–MT). In all other locations, only the second level of hierarchy was sampled, where individual leaves were taken from individual trees in a single plantation (–MT). Furthermore, a third level of hierarchy was incorporated where one lesion from one leaf off one tree was sampled and several isolates were collected from this lesion (–1L).

Two populations of *M. nubilosa* were collected in Australia. From eastern Australia, one leaf was randomly collected from 40 different *E. globulus* trees in one plantation near Bonalbo, north-eastern New South Wales (EA-MT). Similarly, in Western Australia one leaf was randomly collected from 32 different *E. globulus* trees in several different plantations in the south of the state. For the purposes of this study, the samples collected from Western Australia were grouped together into one population and analysed as at the plantation level of hierarchy (WA-MT). One plantation of *E. globulus* was sampled in northern Spain and one leaf was selected from 55 different trees (S-MT). Likewise, one plantation of *E. globulus* was sampled in central Portugal where 42 leaves were taken from an equal number of different *E. globulus* trees (P-MT).

Isolates of *M. nubilosa* were collected from two countries in Africa, namely South Africa and Tanzania. In Njombe, Tanzania, 56 different *E. globulus* trees showing MLB symptoms were randomly sampled, and one leaf was collected from each tree (T-MT). In

South Africa, three plantations of *E. nitens* were sampled in the Mpumalanga Province, namely the Issabelladale, Wynton and Rooihoogte plantations. From the Rooihoogte plantation, 48 leaves displaying spots were randomly collected from a single tree in the centre of the plantation (R-1T). At this plantation, a single lesion from a single leaf was sampled and 32 isolates were collected from this (R-1L). In addition, a single leaf was randomly collected from 56 different trees within the Roooihoogte plantation (R-MT). From the Wynton plantation, 64 leaves exhibiting MLB symptoms were collected from a single tree at the centre of the plantation (W-1T). Furthermore, 64 trees showing MLB symptoms in this plantation were randomly selected and one diseased leaf was taken from each tree (W-MT). From the Issabelladale plantation, 40 leaves showing MLB lesions were randomly collected from one tree at the centre of the plantation (I-1T). In addition, 60 trees within the same plantation were randomly selected and one leaf was collected from each of these trees (I-MT).

Isolation, culture growth and DNA extraction

Isolations of *Mycosphaerella nubilosa* from diseased *Eucalyptus* leaves followed the protocol of Crous (1998). One lesion containing pseudothecia of *M. nubilosa* was excised from each *Eucalyptus* leaf collected from the various locations and placed in water for approximately 2 h. Lesions were then dried and attached to the undersides of Petri dish lids with adhesive tape with pseudothecia facing downward over 2 % malt extract agar (MEA) (Biolab, South Africa). Petri dishes, containing lesions, were incubated in the dark at room temperature for 24 h to allow for ascospore discharge from pseudothecia and germination on agar medium. After 24 h, Petri dishes were evaluated for the presence of germinating ascospores. One ascospore per lesion, exhibiting the type F ascospore germination pattern (Crous 1998, Crous *et al*. 2004), was cut from the agar surface and transferred to fresh 2 % MEA and incubated at 25 °C for approximately 2–3 mo to ensure sufficient mycelial growth. All isolates of *M. nubilosa* used in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Mycelium from actively growing single-spore cultures of *M. nubilosa* was scraped from the surface of cultures, freeze dried for 24 h and ground to a fine powder using liquid nitrogen. DNA was isolated using the phenol : chloroform (1 : 1) extraction protocol as described in Hunter *et al*. (2004a, b). DNA was precipitated by the addition of absolute ethanol (98 % EtOH). Isolated DNA was cleaned by washing with 70 % ethanol and dried under vacuum. Triple distilled water was used to resuspend the isolated DNA. RNaseA (10 µg/µL) was added to the resuspended DNA and incubated at 37 °C for approximately 2 h to

digest any residual RNA. Isolated DNA was separated by electrophoresis in 1 % agarose gels (wt/v) (Roche Diagnostics, Mannheim), stained with ethidium bromide and visualised under ultra-violet light.

PCR amplification and allele size determination

DNA from isolates of *M. nubilosa* served as template DNA for polymerase chain reactions (PCR) in an Eppendorf Mastercycler PCR machine (Eppendorf AG, Germany). Flourescently labelled primers used for PCR reactions were the 10 polymorphic microsatellite primers developed by Hunter *et al*. (2006). The PCR reaction mixture and conditions were the same as those described previously (Hunter *et al*. 2006). Amplified DNA was visualised in 2 % agarose gels stained with ethidium bromide and viewed under ultra violet light. Sizes of PCR amplicons were estimated by comparison against a 100 bp molecular weight marker (O' RangeRuler^{TM} 100bp DNA ladder) (Fermentas Life Sciences, U.S.A.). PCR products were purified using Sephadex G-50 (Sigma Aldrich, St. Louis, MO) in Centri-sep spin columns (Princeton separations, Adelphia, NJ) as outlined by the manufacturer.

Allele sizes of amplified PCR products were determined by electrophoresis on an ABI PRISM^{TM} 3100 Automated DNA sequencer (Applied Biosystems, Foster City, CA) and compared against a GENESCAN –500 LIZ (Applied Biosystems, Warrington, U.K.) internal size standard. Allele sizes were analysed with GENESCAN and GENEMAPPER software (Applied Biosystems, Foster City, CA). Allele size data from GENESCAN and GENEMAPPER were collected in spreadsheets and the number of alleles per locus and the total number of multilocus haplotypes (MLH's) (hereafter referred to as haplotypes) across all the *M. nubilosa* populations were determined.

Gene and genotypic diversity

The frequency of alleles at each locus was calculated for every *M. nubilosa* population. Allele diversity was also determined using the software program POPGENE (Yeh *et al*. 1999) and the gene diversity (*H*) of Nei (1973) was calculated at each locus and also averaged over all loci ($H = 1 - \sum x_k^2$), where x_k is the frequency of the k^{th} allele. Chi square tests for differences in allele frequencies between the *M. nubilosa* populations were also calculated from clone corrected datasets (Workman & Niswander 1970).

Every *M. nubilosa* isolate was assigned a haplotype code according to the observed allele sizes across the 10 microsatellite loci. Genotypic diversity (*G*) was calculated using the

equation of Stoddard & Taylor (1988) ($G = 1 \Big/ \sum_{x=0}^{N} \left[f_X \left(X/N \right)^2 \right]$), where N is the sample size and f_x is the observed frequency of the x^{th} genotype. To compare genotypic diversities between the various *M. nubilosa* populations, the maximum percentage of genotypic diversity ($\hat{G}$) was calculated ($\hat{G} = G/N \times 100$), where G is the observed genotypic diversity and N is the sample size (Chen *et al.* 1994). Genotypic diversity values were also compared between *M. nubilosa* populations at the plantation level for significant differences using a *t*-test (Chen *et al*. 1994).

Population differentiation and gene flow

Clone corrected populations of *M. nubilosa* were used to calculate population differentiation (θ) between the South African and Australian *M. nubilosa* populations using the computer software program Multilocus (Agapow & Burt 2001), using 1000 randomised datasets. The population differentiation value (θ), which is Weir's formulation of Wrights' $F_{ST,}$ was calculated ($Q = \Sigma Q_2 \text{-} \Sigma Q_3 / \Sigma \left(1\text{-}Q_3 \right)$), where Q_2 is the probability that two alleles from the same population are the same and Q_3 is the probability that two alleles from differing populations are the same and for multiple loci Q_2 andQ_3 are summed across the evaluated loci (Weir 1996, Agapow & Burt 2001). Furthermore, gene flow (M) was calculated from theta (θ) using the equation of Cockerham & Weir (1993) ($M = \left[\left(1/\theta \right) - 1 \right] / 2$).

Mode of reproduction

Multilocus linkage disequilibrium between the 10 microsatellite loci was tested for each South African *M. nubilosa* population at the plantation level separately (R-MT, I-MT, W-MT) and the two Australian (WA-MT, EA-MT) populations and also the combined South African *M. nubilosa* populations (R-MT + I-MT + W-MT) at the plantation level by determining the Index of Association (I_A) using the program Multilocus (Maynard Smith *et al*. 1993, Agapow & Burt 2001). The I_A value was determined for both clone corrected and uncorrected datasets. In Multilocus, the expected I_A data for 1000 randomisations were calculated and compared to the observed I_A value. The null hypothesis for the Index of Association test is random association of alleles indicating sexual reproduction and heterothallism. For this to be true the observed I_A value will fall within the distribution of the randomised values. However, if the I_A value falls outside of the distribution of randomised values, it is an indication of non-random association of alleles and homothallism.

RESULTS

Isolates

A total of 497 isolates of *M. nubilosa* were collected from diseased *Eucalyptus* leaves for this study (Table 1). These represented a wide range of geographic locations and also the different hierarchical levels. From Europe, a total of 97 *M. nubilosa* isolates were collected and these included 55 isolates from Spain and 42 isolates from Portugal. Fifty-six isolates were retrieved from the Tanzanian collection and 293 isolates from the three *E. nitens* plantations in South Africa. Nineteen isolates were from New South Wales in eastern Australia and 32 isolates were from Western Australia.

PCR amplification and allele size determination

A total of 66 different alleles were observed across all 10 loci for all the *M. nubilosa* populations (Table 2) with an average of 6.6 alleles per locus. The Australian *M. nubilosa* populations, WA-MT and EA-MT, exhibited the greatest number of alleles across the 10 loci, namely 22 and 44 respectively. Many unique alleles were present in the eastern Australian population (EA-MT), with 32 unique alleles for this population across all 10 loci. The Western Australian population (WA-MT) had seven unique alleles within loci MN-2, MN-7, MN-8 and MN-11.

South African *M. nubilosa* populations exhibited much lower numbers of alleles across the 10 loci than those observed from Australia. In total, 25 different alleles were observed across all of the South African *M. nubilosa* populations at the various hierarchical levels and the majority of these alleles were shared between the South African *M. nubilosa* populations (Table 2). South African populations R-MT and I-1T exhibited the most alleles for any of the South African populations with 18 alleles across the 10 loci. Allele numbers were similar for all of the South African *M. nubilosa* populations at the single tree or plantation hierarchical level and ranged from 16 alleles (I-MT, W-1T), 17 alleles (R-1T, W-MT) and 18 alleles (R-MT, I-1T) (Table 2). Interestingly, one *M. nubilosa* population (R-1L) collected from a single lesion on an *E. nitens* leaf from the Rooihoogte plantation in South Africa yielded a total of 13 different alleles for the 10 microsatellite loci across 32 *M. nubilosa* isolates (Table 2). Only three alleles were unique for the South African populations, namely allele 209 (locus MN-8) for population R-MT, allele 340 (locus MN-8) for population

W-1T and allele 192 (locus MN-11) for population R-1T (Table 2). For the Spain, Portugal and Tanzanian *M. nubilosa* populations, all loci were monomorphic.

When considering all the *M. nubilosa* populations together, all of the 10 microsatellite loci were polymorphic. However, among the South African *M. nubilosa* populations, loci MN-1, MN-2, MN-7 and MN-14 were monomorphic, thus only 60 % of the loci were polymorphic for the South African populations. In contrast, 60 % of the loci were polymorphic for the Western Australian (WA-MT) population and 80 % were polymorphic for the New South Wales (EA-MT) population.

Gene and genotypic diversity

The mean gene diversity (*H*) values across all loci were moderate to low across all of the *M. nubilosa* populations (Table 2). The South African *M. nubilosa* populations had a relatively low gene diversity, ranging from 0.149–0.250. The lowest gene diversity observed for South Africa was for population R-1L that had a gene diversity of 0.149, while the highest level of gene diversity observed in South Africa was for the W-MT population with a value of 0.250. The gene diversity for the Western Australia population (WA-MT) was comparable to those observed for the South African *M. nubilosa* populations with a value of 0.242. In contrast, a substantially higher gene diversity value of 0.506 was observed for the New South Wales population (EA-MT).

A total of 68 different haplotypes were observed across all *M. nubilosa* populations from the various locations considered. Almost half (49 %) of the haplotypes were unique and were detected only once. The South African populations had a total of 35 different haplotypes. The majority of these haplotypes were distributed among the various South African *M. nubilosa* populations. However, seven haplotypes were unique to certain South African populations namely, MNG-38 and MNG-39 were unique to population R-1T, MNG-22 and MNG-23 were unique to population I-1T, MNG-16 was unique to I-MT, MNG-36 was unique to population W-1T and MNG-29 was unique to population W-MT. Therefore, 9.7 % of the haplotypes that were observed for the South African populations were unique. Haplotype MNG-2 was observed a total of 48 times across the South African *M. nubilosa* populations and occurred in all South African populations except population R-1L. None of the haplotypes that were observed for the South African populations occurred in either the Western Australian population (WA-MT) or the New South Wales population (EA-MT). However, the Spanish and Portuguese *M. nubilosa* populations both had only one haplotype, namely MNG-1, that was also observed in the R-MT, W-1T and W-MT *M. nubilosa*

populations of South Africa. Furthermore, the Tanzanian *M. nubilosa* population also had only one haplotype, namely MNG-2, that was also observed in the R-1T, R-MT, I-1T, I-MT, W-1T and W-MT South African *M. nubilosa* populations.

A total of 26 different haplotypes were observed across the two Australian *M. nubilosa* populations (WA-MT and EA-MT). Population WA-MT had 10 haplotypes that were unique to this population and did not occur in the EA-MT population. The most frequently observed haplotype from the WA-MT population was MNG-59 that occurred 10 times in this population. The EA-MT population had a total of 16 different haplotypes that were also unique to this population. The haplotypes of the EA-MT population either occurred only once, while only three haplotypes occurred twice namely, MNG-43, MNG-44 and MNG-45.

Genotypic diversity (G) varied between the various populations of *M. nubilosa* from the various locations (Table 3). The lowest maximum percentage of genotypic diversity ($\hat{G}$), 7.1 %, was observed for the South African *M. nubilosa* population from one lesion (R-1L). However, maximum percentage of genotypic diversity values for South African *M. nubilosa* populations at the plantation level were comparable with values of 30.14 %, 30.10 % and 30.20 % for R-MT, I-MT and W-MT respectively (Table 3). Interestingly, the Western Australian population (WA-MT) had the second lowest value of genotypic diversity of 15.20 % of the theoretical maximum value. This was lower than those for all the South African populations except for population R-1L. The highest genotypic diversity was observed for the New South Wales population (EA-MT) where a value of 76 % of the theoretical maximum was observed. No significant differences ($P < 0.05$) in genotypic diversities were observed between the *M. nubilosa* populations at the plantation level (R-MT, I-MT, W-MT, WA-MT, EA-MT) using a *t*-test (data not shown).

Population differentiation and gene flow

Based on contingency chi squared tests, there were no significant differences in allele frequencies at any of the loci when the three *M. nubilosa* sub-populations (R-1L, R-1T and R-MT) from the Rooihoogte *E. nitens* plantation in South Africa were considered (Table 4). Similarly, no significant differences between the allele frequencies at any of the loci from the Issabelladale *M. nubilosa* sub-populations (I-1T, I-MT) and the Wynton *M. nubilosa* sub-populations (W-1T, W-MT) were observed (Table 4). Three sub-populations at the plantation level from South Africa (R-MT, I-MT, W-MT) were also compared with each other and no significant differences were observed between the allele frequencies at any of the 10 loci

(Table 4). It was, therefore, decided to combine the three South African populations at the plantation level (R-MT, I-MT, W-MT) and to clone-correct this dataset, in order to compare it to the two Australian *M. nubilosa* populations from Western Australia and New South Wales, eastern Australia (WA-MT, EA-MT).

Based on contingency chi squared tests, significant differences ($P < 0.05$) in allele frequencies were observed at nine of the 10 polymorphic loci when the two Australian *M. nubilosa* populations (WA-MT, EA-MT) were compared against each other (Table 5). These differences were observed at loci MN-1, MN-4, MN-7, MN-8, MN-9, MN-10, MN-11 and MN-14. Significant differences ($P < 0.05$) in allele frequencies were also observed at five loci when the combined South African *M. nubilosa* population (RSA) was compared with the Western Australian population (WA-MT). These differences were observed at loci MN-3, MN-4, MN-7, MN-8 and MN-11. Significant differences in allele frequencies were detected for all 10 loci (MN-1 to MN-14) when the combined South African *M. nubilosa* population (RSA) was compared with the New South Wales *M. nubilosa* population (EA-MT) (Table 5).

Population differentiation values (θ) were not significantly different between any of the South African *M. nubilosa* populations at the plantation level (R-MT, I-MT, W-MT) (Table 6). Gene flow (M) between the three South African populations was high with the greatest number of migrants ($M = 82.47$) occurring between the W-MT and I-MT populations and the least number of migrants ($M = 9.63$) occurring between the I-MT and R-MT populations (Table 6). There were, however, significant differences ($P < 0.001$) between the three South African *M. nubilosa* populations at the plantation level (R-MT, I-MT, W-MT) and the two Australian *M. nubilosa* populations at the plantation level (WA-MT, EA-MT) based on the values of θ (Table 6). Similarly, θ values indicated a significant population differentiation ($P < 0.001$) between the two Australian (WA-MT, EA-MT) *M. nubilosa* populations (Table 6). Very little gene flow ($M = 0.52$) between the Western Australian and New South Wales *M. nubilosa* populations was observed. However, there was low gene flow ($M = 1.13–1.54$) between the Western Australian *M. nubilosa* population (WA-MT) and the three South African *M. nubilosa* populations (R-MT, I-MT, W-MT) and extremely limited gene flow ($M = 0.41–0.43$) between the New South Wales population and the three South African populations (Table 6).

Mode of reproduction

The Index of association values (I_A) differed for the clone corrected and clone uncorrected datasets. For clone corrected datasets, the observed I_A values did not differ significantly from the recombined datasets of *M. nubilosa* for all of the populations at the plantation level from South Africa and Western Australia and when the three South African populations were combined (Table 7). However, using the clone corrected data for the New South Wales *M. nubilosa* population resulted in an observed I_A value that did significantly differ ($P < 0.001$) from the recombined dataset.

When *M. nubilosa* populations from South Africa, Western Australia and New South Wales were used in an uncorrected dataset, significant differences in the observed I_A values from the recombined datasets were observed (Table 7). This was true for the three South African *M. nubilosa* populations at the plantation level (R-MT, I-MT, W-MT). However, the level of significance was not high (Table 7). When the three South African *M. nubilosa* populations from the plantation level were combined into a single population (RSA), there was a high significance value ($P < 0.001$) supporting the difference of the observed I_A from the recombined datasets. This was also true for the Western Australian (WA-MT) and New South Wales (EA-MT) *M. nubilosa* populations (Table 7, Fig. 1). As *M. nubilosa* is a sexually reproducing fungus, this is indicative of a homothallic (selfing) mating system.

DISCUSSION

Mycosphaerella nubilosa is the most important foliar pathogens of *Eucalyptus nitens* and *E. globulus* and in turn, these trees represent two of the most important sources of wood fibre derived from plantations. South Africa has the oldest plantation forestry programme based on non-native species in the world (Burgess & Wingfield 2001) and *M. nubilosa* was the first pathogen of *Eucalyptus* to be reported from this country (Doidge 1950). It has subsequently appeared in many areas of the world where *E. nitens* and *E. globulus* are grown. Although it has been assumed that the pathogen originated in Australia, and that it was accidentally introduced into other parts of the world, this hypothesis has never been tested experimentally. Results of this study thus provide the first evidence that *M. nubilosa* has moved from Australia to other parts of the world, where it has become one of the most important constraints to the propagation of *E. nitens* and *E. globulus* in plantations.

Mycosphaerella nubilosa was originally identified from Victoria in eastern Australia (Cooke 1891). Since this initial identification, it has been hypothesized that eastern Australia

would represent the centre of origin for *M. nubilosa*. Gene diversity and maximum percentage of genotypic diversity for the New South Wales *M. nubilosa* population was the highest of all populations evaluated during this study. Furthermore, the New South Wales *M. nubilosa* population had a greater number of alleles when compared to the other *M. nubilosa* populations. It is known that older pathogen populations representing centres of origin have higher gene diversity values due to the accumulation of mutations over time (McDonald 1997). Results of this study, therefore, support the view that *M. nubilosa* originated from eastern Australia.

An interesting observation in this study was the presence of shared alleles between Western Australia and South Africa. This finding suggests that *M. nubilosa* could have been introduced into South Africa from Western Australia and not from eastern Australia. However, *M. nubilosa* was only detected in Western Australia in 1999 (Maxwell *et al*. 2001), despite previous surveys (Carnegie *et al*. 1997) suggesting that it is relatively newly introduced into that area. There are also no known hosts of *M. nubilosa* that naturally occur in Western Australia. Furthermore, commercial forestry with *E. globulus* (a species native to south-eastern Australia) only began in Western Australia during the 1980's. It thus seems unlikely that *M. nubilosa* was introduced into South Africa from Western Australia. We thus support the alternative hypothesis that *M. nubilosa* populations from eastern Australia, not sampled in this study, acted as a source population for both Western Australia and South Africa. The fact that *E. globulus* seed from eastern Australia was the first *Eucalyptus* planting stock to be planted in South Africa would provide additional support, albeit anecdotal, for this view.

Gene diversities derived in this study for the various *M. nubilosa* populations from South Africa were comparable with each other. These similar gene diversity values and lack of significant population differentiation, combined with the high level of gene flow between the South African *M. nubilosa* populations, indicate that these populations are highly homogenous. Similar findings have emerged for the related pathogens *Cercospora zeae-maydis* Tehon & E.Y. Daniels and *M. graminicola*, where high levels of gene flow have served to homogenise populations of these pathogens (Okori *et al*. 2003, Zhan & McDonald 2004).

In South Africa, the gene diversity of isolates from a single tree was as great as the gene diversity of isolates from a single plantation and gene flow between plantations was very high. Similar findings of genetic diversity have been found for *M. fijiensis* on banana where the majority of genetic diversity for that pathogen exists at the plant and plantation levels

(Rivas *et al*. 2004). Our results are also consistent with those for other *Mycosphaerella* pathosystems. For example, the majority of diversity for *S. tritici* has been observed within a single plot of a wheat field (McDonald & Martinez 1990, Zhan *et al*. 2003). Likewise in *Septoria musiva* Peck from north-eastern America where more than 90 % of the genetic diversity was distributed within a single tree (Feau *et al*. 2005).

Results of this study have shown that haplotypes of *M. nubilosa* are shared between South Africa, Tanzania and Europe. Thus, the single haplotype (MNG-1) found in both the Spanish and Portuguese *M. nubilosa* populations was also found in the Rooihoogte and Wynton *M. nubilosa* populations of South Africa, but not in any of the other *M. nubilosa* populations considered. Likewise, the single haplotype (MNG-2) in the Tanzanian *M. nubilosa* population was present in the Rooihoogte, Issabelladale and Wynton *M. nubilosa* populations from South Africa. These results indicate that gene flow has occurred between South Africa, Tanzania and Europe, resulting in the establishment and persistence of specific haplotypes in Europe and in Tanzania. Based on gene flow data between Australia and South Africa and the same haplotypes that are shared between South Africa, Tanzania and Europe, it seems likely that *M. nubilosa* was first introduced into South Africa from Australia and that it was subsequently moved from South Africa into other parts of Africa and into Europe. This route of movement is also consistent with the pattern of establishment of *Eucalyptus* plantation development in Africa and Europe.

Populations of *M. nubilosa* from South Africa, Western Australia and eastern Australia were significantly differentiated. No haplotypes were shared between the South African, Western Australian or New South Wales *M. nubilosa* populations, also indicating significant population differentiation. Furthermore, gene flow between South Africa and the two Australian *M. nubilosa* populations was low. This is in contrast to the genetic similarity observed for *M. graminicola* populations from different continents (Linde *et al*. 2002). Based on the significant differentiation of the South African, Western Australian and New South Wales populations, it is possible these three populations may represent distinct species. However, DNA sequence data from the Internal Transcribed Spacer (ITS), Translation Elongation Factor 1-alpha (EF-1α), Beta tubulin (Bt) and Calmodulin (CAL) gene regions of *M. nubilosa* representatives from the South African, Western and New South Wales populations, no nucleotide differences were observed (data not shown). Furthermore, considering the extensive distances between *M. nubilosa* populations from South Africa, Western Australia and New South Wales, it is evident that these populations are isolated through distance.

Prior to this study, there has been no knowledge regarding the mode of reproduction of *M. nubilosa*. Park & Keane (1982b) hypothesised that *M. nubilosa* would most likely be homothallic due to the ability of this species to produce pseudothecia containing viable ascospores once grown in agar medium supplemented with *Eucalyptus* leaves. Linkage disequilibrium analyses in this study showed that there is non-random association of alleles within *M. nubilosa.* This would be consistent with a homothallic life history and supports the earlier view of Park & Keane (1982b). Likewise, Milgate *et al*. (2005b) showed that a *M. cryptica* population in Tasmania, Australia, exhibited significant linkage disequilibrium indicating that this population was not strictly heterothallic. The fact that two of the most important *Mycosphaerella* pathogens on *Eucalyptus* most likely have a homothallic mating strategy is intriguing because most *Mycosphaerella* spp. known from other hosts that have been studied are heterothallic (Carlier *et al*. 1996, Linde *et al*. 2002, McDonald & Linde 2002).

Despite the fact that *M. nubilosa* appears to be homothallic, a relatively high number of haplotypes exist in the South African and Australian populations of the pathogen. This is not necessarily unusual as relatively high levels of diversity have been found in various other homothallic fungi. This diversity is typically accounted for by rare mitotic crossing over or mutation events that generate recombinant genotypes (Taylor *et al*. 1999). Furthermore, the presence of sexual structures of *M. nubilosa* in nature is an indication that potential exists for sexual reproduction where chance outcrossing events could generate novel genotypes (Milgroom 1996). Considering that several haplotypes of *M. nubilosa* occupy the same lesion, it is likely that mycelium of different haplotypes will come into contact, leading to outcrossing events. These unique haplotypes also may be introductions from outside of South Africa most likely introduced through *Eucalyptus* plant material.

Numerous haplotypes were found in the South African and Australian *M. nubilosa* populations. Considering the number of different haplotypes and alleles observed in these *M. nubilosa* populations it would be difficult to effectively breed *Eucalyptus* trees that are tolerant to *M. nubilosa* infection. Genetically diverse and large pathogen populations have a greater evolutionary potential than small populations (McDonald & Linde 2002). Due to the large population sizes of *M. nubilosa* in South Africa and Australia, these populations would have greater evolutionary potential to overcome new *Eucalyptus* genotypes that are deployed in commercial forestry. However, considering that eastern Australia appears to be the centre of origin of *M. nubilosa*, resistance genes present in *Eucalyptus* hosts would most likely be found within this area and could be used for breeding tolerant *Eucalyptus* genotypes. It has for

instance, already been shown that wide variation exists within *E. globulus* at the subspecies, provenance and family level in susceptibility to MLD (Carnegie *et al*. 1994, Carnegie & Ades 2005, Milgate *et al*. 2005a).

A high number of haplotypes was observed in the Australian and South African *M. nubilosa* populations but not in those from other parts of the world. This presents a strong case for ensuring that movement of *Eucalyptus* germplasm does not occur. This knowledge should be incorporated into quarantine regulations and actionable lists in Australia and countries in Africa and Europe. It is known that seedborne infections can lead to epidemics of crop foliage (Milgroom & Peever 2003) and it has been suggested, but not proven, that *Mycosphaerella* spp. may be transferred on seed material. It has also been suggested that gene flow of *M. graminicola* genotypes may occur on a global scale due to the movement of infected wheat seed (McDonald *et al*. 1996). The movement of *Eucalyptus* seed should, therefore, be monitored and tested for the presence of *Mycosphaerella* propagules. This could be achieved through the use of *Mycosphaerella* species-specific primers or PCR-RFLP based techniques to identify *M. nubilosa* (Kularatne *et al*. 2004, Maxwell *et al*. 2005).

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Table 1. Origin of *Mycosphaerella nubilosa* isolates used for this study. Number of isolates per hierarchical sampling level. Population code (**P.C**) indicates the population code assigned to each population and sub-population.

Country	Area	Plantation	Hierarchical Level	P.C	Host	Collector	Date	No. Isolates
South Africa	Mpumalanga	Rooihoogte	1 Lesion	R-1L	*E. nitens*	G.C. Hunter	2005	32
			1 Tree	R-1T	*E. nitens*	G.C. Hunter	2005	48
			Plantation	R-MT	*E. nitens*	G.C. Hunter	2005	56
		Issabelladale	1 Tree	I-1T	*E. nitens*	G.C. Hunter	2005	30
			Plantation	I-MT	*E. nitens*	G.C. Hunter	2005	29
		Wynton	1 Tree	W-1T	*E. nitens*	G.C. Hunter	2005	49
			Plantation	W-MT	*E. nitens*	G.C. Hunter	2005	49
Australia	Western Australia	Albany	Plantation	WA-MT	*E. globulus*	A. Maxwell	2002	32
	New South Wales	Bonalbo	Plantation	EA-MT	*E. globulus*	A.J. Carnegie	2004	19
Spain			Plantation	S-MT	*E. globulus*	J.P.M. Vasquez	2002	55
Portugal			Plantation	P-MT	*E. globulus*	M.J. Wingfield	2005	42
Tanzania			Plantation	T-MT	*E. globulus*	J. Roux	2004	56
							Total	497

Table 2. Allele size (bp) and allele frequency at 10 microsatellite loci (MN-1 – MN-14) determined using the software program POPGENE for populations of *Mycosphaerella nubilosa* collected from South Africa (Rooihoogte, Issabelladale and Wynton) and Australia (Western Australia and New South Wales).

			South Africa						Australia	
			Rooihoogte		Issabelladale		Wynton		Western	NSW
Locus	**Allele**	**R-1L**	**R-1T**	**R-MT**	**I-1T**	**I-MT**	**W-1T**	**W-MT**	**WA-MT**	**EA-MT**
MN-1	**267**	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-
	253	-	-	-	-	-	-	-	-	1.000
MN-2	**183**	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.906	0.053
	185	-	-	-	-	-	-	-	0.094	-
	192	-	-	-	-	-	-	-	-	0.053
	194	-	-	-	-	-	-	-	-	0.053
	214	-	-	-	-	-	-	-	-	0.158
	222	-	-	-	-	-	-	-	-	0.158
	234	-	-	-	-	-	-	-	-	0.105
	262	-	-	-	-	-	-	-	-	0.053
	264	-	-	-	-	-	-	-	-	0.053
	265	-	-	-	-	-	-	-	-	0.158
	272	-	-	-	-	-	-	-	-	0.158
MN-3	**307**	-	-	-	-	-	-	-	-	0.211
	309	1.000	0.500	0.446	0.300	0.241	0.469	0.469	1.000	0.800
	315	-	0.500	0.554	0.700	0.759	0.531	0.531	-	-

		South Africa							Australia	
			Rooihoogte		Issabelladale		Wynton		Western	NSW
Locus	**Allele**	**R-1L**	**R-1T**	**R-MT**	**I-1T**	**I-MT**	**W-1T**	**W-MT**	**WA-MT**	**EA-MT**
MN-4	**141**	-	-	-	-	-	-	-	-	0.421
	148	-	-	-	-	-	-	-	-	0.263
	155	0.500	0.458	0.518	0.433	0.483	0.388	0.571	1.000	0.053
	162	0.500	0.452	0.482	0.533	0.517	0.612	0.429	-	0.158
	169	-	-	-	0.033	-	-	-	-	0.105
MN-7	**376**	-	-	-	-	-	-	-	0.344	-
	380	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.656	-
	396	-	-	-	-	-	-	-	-	0.684
	400	-	-	-	-	-	-	-	-	0.316
MN-8	**193**	-	-	-	-	-	-	-	-	0.053
	202	-	-	-	-	-	-	-	0.031	-
	209		-	0.018	-	-	-	-	-	-
	229	-	-	-	-	-	-	-	-	0.053
	238	0.563	0.625	0.554	0.633	0.724	0.653	0.429	-	0.580
	242	-	-	0.018	-	-	-	-	-	-
	247	-	-	-	-	-	-	-	0.344	0.158
	265	-	-	-	0.633	-	-	-	0.156	-
	274	-	-	-	-	-	-	-	-	0.053
	283	-	-	-	-	-	-	-	0.031	-
	292	-	-	-	-	-	-	-	-	0.105
	301	-	-	-	-	-	-	-	0.375	-
	319	0.438	0.375	0.411	0.333	0.276	0.327	0.571	0.031	-

		South Africa							Australia	
			Rooihoogte		Issabelladale		Wynton		Western	NSW
Locus	**Allele**	**R-1L**	**R-1T**	**R-MT**	**I-1T**	**I-MT**	**W-1T**	**W-MT**	**WA-MT**	**EA-MT**
	340	-	-	-	-	-	0.020	-	-	-
	355	-	-	-	-	-	-	-	0.031	-
MN-9	**202**	-	-	-	-	-	-	-	-	0.053
	210	-	-	-	-	-	-	-	-	0.474
	214	-	-	-	-	-	-	-	-	0.105
	216	-	0.250	0.375	0.133	0.207	0.286	0.408	0.844	-
	218	1.000	0.750	0.607	0.833	0.793	0.714	0.571	0.156	-
	220	-	-	0.018	-	-	-	-	-	0.053
	222	-	-	-	0.033	-	-	0.020	-	0.053
	226	-	-	-	-	-	-	-	-	0.053
	230	-	-	-	-	-	-	-	-	0.158
	234	-	-	-	-	-	-	-	-	0.053
MN-10	**135**	-	0.021	-	-	-	-	0.020	-	-
	137	1.000	0.979	1.000	1.000	1.000	1.000	0.980	1.000	0.158
	141	-	-	-	-	-	-	-	-	0.053
	145	-	-	-	-	-	-	-	-	0.158
	147	-	-	-	-	-	-	-	-	0.527
	153	-	-	-	-	-	-	-	-	0.105
MN-11	**192**	-	0.021	-	-	-	-	-	-	-
	194	0.469	0.458	0.375	0.467	0.379	0.367	0.367	0.500	1.000
	200	-	-	-	-	-	-	-	0.156	-
	206	-	-	-	-	0.035	-	-	0.344	-

		South Africa							Australia	
			Rooihoogte		Issabelladale		Wynton		Western	NSW
Locus	**Allele**	**R-1L**	**R-1T**	**R-MT**	**I-1T**	**I-MT**	**W-1T**	**W-MT**	**WA-MT**	**EA-MT**
	224	0.531	0.521	0.625	0.533	0.586	0.633	0.633	-	-
MN-14	**98**	-	-	-	-	-	-		-	0.158
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.875	-
	102	-	-	-	-	-	-	-	0.125	0.421
	104	-	-	-	-	-	-	-	-	0.263
	106	-	-	-	-	-	-	-	-	0.158
N^A		32	48	56	30	29	49	49	32	19
N^B		4	24	24	16	17	22	23	10	16
Alleles		13	17	18	18	16	16	17	22	44
P		3	6	5	5	5	5	6	6	8
P (%)		30	60	50	50	50	50	60	60	80
H		0.149	0.240	0.248	0.222	0.211	0.231	0.250	0.242	0.506
H*		0.125	0.258	0.255	0.248	0.242	0.247	0.255	0.246	0.506

N^A: Number of isolates used in the uncorrected dataset.

N^B: Number of isolates used in the clone corrected dataset.

P: Number of polymorphic loci.

P (%): Percentage of polymorphic loci.

H: Gene diversity of the population (Nei 1973).

H**: Gene diversity of the clone corrected population.

Table 3. Multi Locus Haplotypes (MLH's) of *Mycosphaerella nubilosa* isolates compiled from 10 polymorphic microsatellite markers. Haplotypes from Spain (S-MT), Portugal (P-MT), Tanzania (T-MT) all at the plantation level. From three plantations in South Africa at different hierarchical levels namely, Rooihoogte plantation at the plantation level (R-MT), within one tree (R-1T), within one lesion (R-1L), Issabelladale plantation at the plantation level (I-MT), within one tree (I-1T) and the Wynton plantation at the plantation level (W-MT), within one tree (W-1T). Also two *M. nubilosa* populations both at the plantation level from Australia, Western Australia (WA-MT) and New South Wales (EA-MT).

				South Africa							Australia	
	Spain	Portugal	Tanzania	Rooihoogte			Issabelladale		Wynton		Western	NSW
MLH	S-MT	P-MT	T-MT	R-1L	R-1T	R-MT	I-1T	I-MT	W-1T	W-MT	WA-MT	EA-MT
MNG-1	55	42				1			1	3		
MNG-2			56		7	8	10	7	11	5		
MNG-3					2	2	1	5	3			
MNG-4					1	3	2	2	1	4		
MNG-5					1	2		1				
MNG-6				14	3	3	4	1	8	6		
MNG-7					1		1	1				
MNG-8				1	2	1	1	2		1		
MNG-9							1	1				
MNG-10							1	1	1			
MNG-11					3	4		1	1	1		
MNG-12				16	5	1		1	3	1		
MNG-13						1		1				
MNG-14						4		1	5	1		

				South Africa							Australia	
	Spain	Portugal	Tanzania	Rooihoogte			Issabelladale		Wynton		Western	NSW
MLH	S-MT	P-MT	T-MT	R-1L	R-1T	R-MT	I-1T	I-MT	W-1T	W-MT	WA-MT	EA-MT
MNG-15					1	3	1	1		6		
MNG-16								1				
MNG-17						1	1	1		1		
MNG-18					2	2	1	1	1			
MNG-19							1			1		
MNG-20					3	2	1		2			
MNG-21				1	2	3	2		1	2		
MNG-22							1					
MNG-23							1					
MNG-24					1					3		
MNG-25					2				1			
MNG-26					1				2	1		
MNG-27					3				1	2		
MNG-28						3				1		
MNG-29										1		
MNG-30					1	1			1	1		
MNG-31						3				2		
MNG-32					2				1	1		
MNG-33					1				1	1		
MNG-34						3			1	2		

					South Africa						Australia	
	Spain	Portugal	Tanzania		Rooihoogte		Issabelladale		Wynton		Western	NSW
MLH	S-MT	P-MT	T-MT	R-1L	R-1T	R-MT	I-1T	I-MT	W-1T	W-MT	WA-MT	EA-MT
MNG-35					1	2			1	2		
MNG-36									1			
MNG-37					1				1			
MNG-38					1							
MNG-39					1							
MNG-40						1						
MNG-41						1						
MNG-42						1						
MNG-43												2
MNG-44												2
MNG-45												2
MNG-46												1
MNG-47												1
MNG-48												1
MNG-49												1
MNG-50												1
MNG-51												1
MNG-52												1
MNG-53												1
MNG-54												1

				South Africa							Australia	
	Spain	Portugal	Tanzania	Rooihoogte			Issabelladale		Wynton		Western	NSW
MLH	**S-MT**	**P-MT**	**T-MT**	**R-1L**	**R-1T**	**R-MT**	**I-1T**	**I-MT**	**W-1T**	**W-MT**	**WA-MT**	**EA-MT**
MNG-55												**1**
MNG-56												**1**
MNG-57												**1**
MNG-58												**1**
MNG-59											**10**	
MNG-60											**4**	
MNG-61											**1**	
MNG-62											**9**	
MNG-63											**1**	
MNG-64											**2**	
MNG-65											**1**	
MNG-66											**2**	
MNG-67											**1**	
MNG-68											**1**	
N	55	42	56	32	48	56	30	29	49	49	32	19
N (g)	1	1	1	4	24	24	16	17	22	23	10	16
G	N/A	N/A	N/A	2.26	15.78	16.88	6.63	8.73	9.61	14.82	4.86	14.49
$\hat{G}$	N/A	N/A	N/A	7.1	32.9	30.14	22.1	30.1	19.6	30.2	15.2	76.3

N: Number of *M. nubilosa* isolates.

N (g): Number of Multi Locus Haplotypes (MLH's).

G: Genotypic diversity (Stoddard & Taylor 1988).

$\hat{\boldsymbol{G}}$: Maximum percentage of genotypic diversity.

Table 4. Gene Diversity (*H*) and contingency χ^2 tests for differences in allele frequencies for the 10 microsatellite loci across the clone corrected populations of *M. nubilosa* from different hierarchical levels. **(A)** Within one lesion (R-1L), within one tree (R-1T), within one plantation (R-MT) of the Rooihoogte plantation. **(B)** Within one tree (I-1T), within one plantation (I-MT) of the Issabelladale plantation. **(C)** Within one tree (W-1T), within one plantation (W-MT) of the Wynton plantation. **(D)** Comparison between three South African plantations at the plantation level (R-MT, I-MT, W-MT). Determined χ^2 values not significant. **N** = Number of *M. nubilosa* isolates from the clone corrected dataset.

	Gene Diversity (*H*)							A		B		C		D	
	R-1L	**R-1T**	**R-MT**	**I-1T**	**I-MT**	**W-1T**	**W-MT**	χ^2	**d.f.**	χ^2	**d.f.**	χ^2	**d.f.**	χ^2	**d.f.**
MN-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-	-	-
MN-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-	-	-
MN-3	0.00	0.49	0.49	0.43	0.46	0.48	0.50	3.0	2	0.06	1	0.22	1	2.2	2
MN-4	0.38	0.50	0.49	0.55	0.50	0.48	0.50	0.9	2	1.22	2	0.22	1	0.7	2
MN-7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-	-	-
MN-8	0.38	0.50	0.57	0.54	0.48	0.52	0.48	3.3	6	1.10	2	1.07	2	3.6	6
MN-9	0.00	0.47	0.52	0.46	0.46	0.48	0.54	3.6	4	1.37	2	1.07	2	3.9	6
MN-10	0.00	0.08	0.00	0.00	0.00	0.00	0.08	1.2	2	-	-	0.98	1	1.8	2
MN-11	0.50	0.53	0.49	0.49	0.53	0.50	0.45	2.2	4	1.10	2	1.07	1	5.8	4
MN-14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-	-	-
N	4	24	24	16	17	22	23								
Mean	0.13	0.23	0.25	0.25	0.24	0.25	0.25								

Table 5. Gene Diversity (*H*) and contingency χ^2 tests for differences in allele frequencies for the 10 polymorphic microsatellite loci across the clone corrected *M. nubilosa* populations from (**A**) South Africa [RSA = (R-MT + I-MT + W-MT)] and Western Australia (WA-MT), (**B**) South Africa [RSA = (R-MT + I-MT + W-MT)] and New South Wales, eastern Australia (EA-MT), (**C**) Western Australia (WA-MT) and New South Wales (EA-MT).

	Gene Diversity (*H*)			A		B		C	
Locus	**RSA**	**WA-MT**	**EA-MT**	**χ^2**	**d.f.**	**χ^2**	**d.f.**	**χ^2**	**d.f.**
MN-1	0.00	0.00	0.00	-	-	51.0*	1	26.0*	1
MN-2	0.00	0.18	0.88	3.6	1	46.5*	9	22.2*	10
MN-3	0.50	0.00	0.30	7.8*	1	15.9*	2	2.1	1
MN-4	0.50	0.00	0.69	9.4*	1	38.2*	4	22.2*	4
MN-7	0.00	0.32	0.43	7.3*	1	51.0*	2	26.0*	3
MN-8	0.54	0.84	0.63	39.6*	9	22.6*	8	20.4*	10
MN-9	0.54	0.32	0.76	5.1	3	46.4*	9	26.0*	9
MN-10	0.06	0.00	0.63	0.3	1	42.2*	5	19.0*	4
MN-11	0.52	0.62	0.00	19.0*	3	15.0*	2	9.9*	2
MN-14	0.00	0.18	0.73	3.6	1	51.0*	4	22.4*	4
N	35	10	16						
Mean	0.27	0.25	0.51						

* Indicates significant χ^2 values ($p < 0.05$).

Table 6. Population differentiation values (θ), above the diagonal, calculated after 1000 randomisations using the program Multilocus. Gene Flow (Number of migrants) (M) indicated below the diagonal.

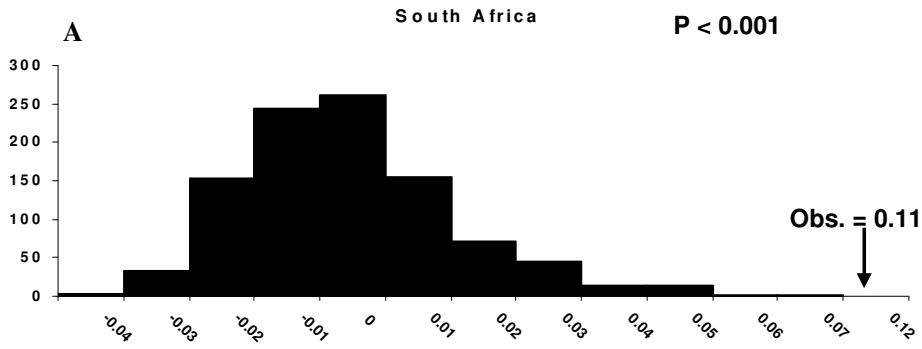
	R-MT	I-MT	W-MT	WA-MT	EA-MT
R-MT		-0.0054	-0.03034	0.25*	0.55*
I-MT	9.63		-0.0061	0.31*	0.54*
W-MT	16.93	82.47		0.29*	0.55*
WA-MT	1.54	1.13	1.24		0.49*
EA-MT	0.41	0.43	0.41	0.52	

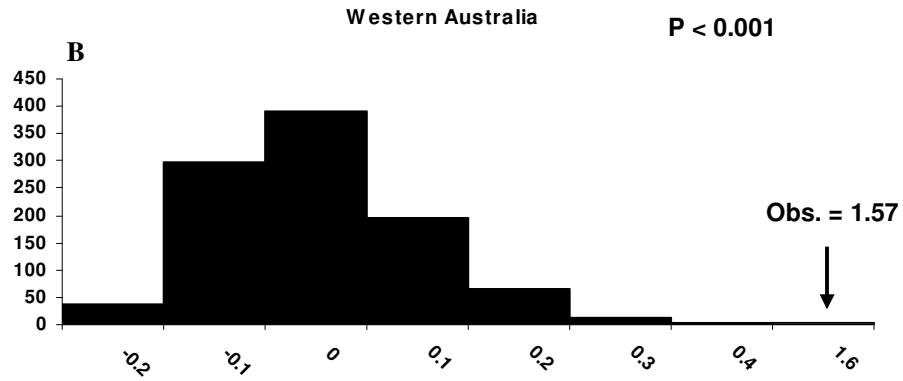
*: Indicates significant values ($p < 0.001$).

Table 7. Linkage disequilibrium (I_A) values for populations of *M. nubilosa* from South Africa at the plantation level (R-MT, I-MT, W-MT) and for the combined South African population (RSA) and for the two Australian *M. nubilosa* populations (WA-MT, EA-MT). Linkage disequilibrium values were determined for both (**A**) clone corrected and (**B**) uncorrected datasets using the program Multilocus with 1000 randomisations. N = Number of isolates, P = p-value.

	A			B		
	N	I_A	**P**	**N**	I_A	**P**
R-MT	24	-0.10	0.951	56	0.14	0.002
I-MT	17	-0.10	0.773	29	0.30	0.004
W-MT	23	-0.10	0.916	49	0.06	0.071
RSA	42	-0.12	1.0	134	0.11	< 0.001
WA-MT	10	0.66	0.031	32	1.57	< 0.001
EA-MT	16	1.30	< 0.001	19	1.38	< 0.001

Figure 1. Histograms of frequency distributions of Index of Association (I_A) values following 1000 randomisations in Multilocus of clone uncorrected *M. nubilosa* populations. (**A**) South African *M. nubilosa* populations combined at the plantation level (R-MT, I-MT, W-MT), (**B**) Western Australian *M. nubilosa* population (WA-MT), (**C**) New South Wales *M. nubilosa* population (EA-MT). Frequency indicated on Y-axis and Index of Association (I_A) indicated on X-axis.


A
South Africa
P < 0.001
300
250
200
150
100
50
0
Obs. = 0.11
-0.04
-0.03
-0.02
-0.01
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.12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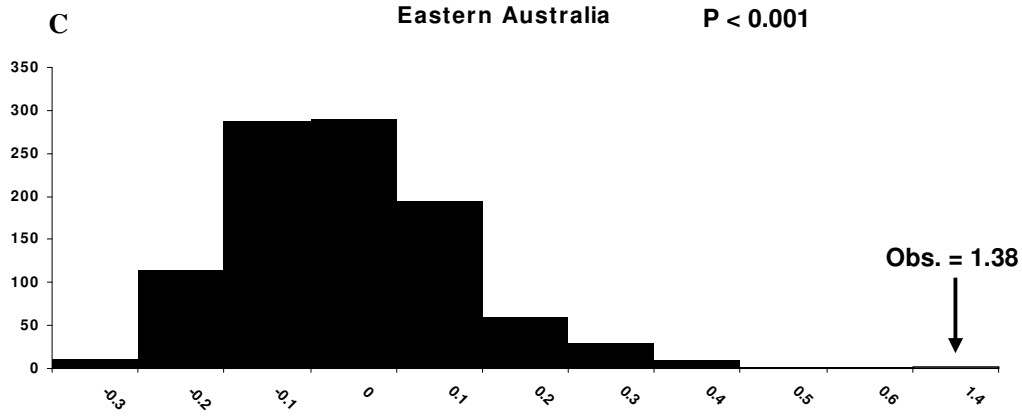
0.4
1.6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0.2
0.3
0.4
0.5
0.6
1.4

6

Intra-specific variation in *Mycosphaerella nubilosa sensu lato*

Abstract: *Mycosphaerella nubilosa* is one of the most important pathogens of *Eucalyptus* leaves and causes premature defoliation and stunting of tree growth. Recent surveys of diseased *Eucalyptus* plantations have resulted in extensive collections of *M. nubilosa*. From comparisons of DNA sequences for the Internal Transcribed Spacer (ITS) region of the rRNA operon, it has become apparent that variation exists within this species. The aim of this study was to critically compare a large collection of isolates of *M. nubilosa* from different hosts and regions of the world. This was achieved by comparing isolates based on DNA sequence data from three nuclear gene regions, as well as analysis of microsatellite marker data. Results of this study indicate that intra-specific variation exists within the fungus known as *M. nubilosa*, which can be linked to host association and culture morphology. From DNA sequence data we designate two *M. nubilosa* ITS lineages indicative of host associations and geographic distribution. Although these may represent discrete taxa, current data are insufficient to describe the first lineage as a new species.

INTRODUCTION

Species of *Eucalyptus* are native to Australia, Papua New Guinea, Indonesia and the Philippines where they occur in large natural forests (Turnbull 2000). Many of these *Eucalyptus* spp. have been removed from their native ranges and propagated in commercially important plantations in various tropical and sub-tropical countries (Poynton 1979). In many non-native environments, *Eucalyptus* spp. have become second only to *Pinus* spp. in their use as a commercial forestry crop (Old *et al*. 2003). This is due to their favourable growth properties, relatively short rotation periods, climate adaptability and favoured wood properties (Poynton 1979, Turnbull 2000).

Mycosphaerella nubilosa (Cooke) Hansf. is one of the many species of *Mycosphaerella* Johanson that infect *Eucalyptus* leaves (Crous 1998, Crous *et al*. 2004, Crous *et al*. 2006). This species was originally identified from *Eucalyptus* leaves in Victoria, eastern Australia (Cooke 1891). Currently *M. nubilosa* is known from Australia, New Zealand and several countries from Africa and Europe (Dick 1982, Park & Keane 1982, Maxwell *et al*. 2001, Crous *et al*. 2004, Alemu *et al*. 2006). In these countries, *M. nubilosa* has become a serious impediment to the continued propagation of *E. globulus* and *E. nitens* (Park *et al*. 2000, Crous *et al*. 2004, Hunter *et al*. 2004). *M. nubilosa* infects juvenile *Eucalyptus* leaves causing reduced photosynthetic capability and premature defoliation of *Eucalyptus* trees (Park & Keane 1982, Pinkard & Mohammed 2006). Recently, however, *M. nubilosa* has also been identified from mature *Eucalyptus* foliage and in this regard it poses an increased threat to commercial *Eucalyptus* forestry.

DNA sequencing has become the definitive tool used for identification of *Mycosphaerella* species (Crous *et al*. 2006). The Internal Transcribed Spacer (ITS) region of the rRNA operon has been the preferred gene region used for phylogenetic analyses and identification of *Mycosphaerella* spp. (Crous *et al*. 2004, Hunter *et al*. 2004). Recently, however, DNA Multi Locus Sequence Typing (MLST) has resulted in a mutli-gene phylogeny for species of *Mycosphaerella* occurring on *Eucalyptus* leaves (Hunter *et al*. 2006b). Thus, existing DNA sequence data have clarified species concepts in *Mycosphaerella* and more particularly, have resulted in the identification of several species complexes (Crous *et al*. 2004, 2006). Analysis of sequences of the ITS region of the rRNA operon for more than 120 *Mycosphaerella* isolates from *Eucalyptus* enabled Crous *et al*. (2006) to identify 9 *Mycosphaerella* species complexes including several novel cryptic species of *Mycosphaerella*.

Crous *et al*. (2004, 2006) showed that *M. nubilosa* could be divided into two well-supported phylogenetic clades based on ITS sequence data. Recent surveys of diseased *Eucalyptus* plantations have resulted in many collections of *M. nubilosa* from several different countries where this pathogen is known to occur. These have made it possible to consider the phylogenetic relationships between *M. nubilosa* isolates more closely. The aim of this study was thus to use DNA sequence data to determine whether *M. nubilosa* represents a single monophyletic species, or a polyphyletic assemblage of more than one phylogenetic lineage. This was achieved by sequencing the ITS, Translation Elongation Factor 1-alpha (EF-1α) and Beta tubulin-2 (Bt-2) gene regions of several isolates of *M. nubilosa* from different geographical areas and *Eucalyptus* hosts. Furthermore, these sequence data were combined with culture studies and microsatellite amplification experiments of *M. nubilosa* isolates to determine if, and to what extent, variation exists within *M. nubilosa*.

MATERIALS AND METHODS

Isolation and isolates

Isolates of *M. nubilosa* were collected from several different geographic locations. In Australia, several areas were sampled including Western Australia, Tasmania, Victoria and New South Wales, constituting the major areas where *Eucalyptus* spp. are grown commercially. *Mycosphaerella nubilosa* isolates were also collected from New Zealand, Spain, Portugal, South Africa and Tanzania (Table 1).

Isolations of *M. nubilosa* from diseased *Eucalyptus* leaves followed the protocols of Crous (1998). Lesions bearing pseudothecia of *M. nubilosa* were excised from symptomatic *Eucalyptus* leaves and placed in water for 2 h. Lesions were then dried and attached to the undersides of Petri dish lids with adhesive tape having the pseudothecia facing downward over 2 % Malt Extract Agar (MEA) (Biolab, South Africa). Petri dishes were then incubated in the dark at room temperature for 24 h to allow for ascospore discharge, and germination on the agar medium. Petri dishes were then evaluated for the presence of germinating ascospores. Ascospores exhibiting the type F ascospore germination pattern (Crous 1998, Crous *et al.* 2004), were cut from the agar surface and transferred to fresh 2 % MEA agar plates and incubated at 25 °C for the establishment of pure cultures.

All *M. nubilosa* isolates used in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (CMW), University of Pretoria, Pretoria, South Africa and the herbarium collection of Murdoch University (MURU), Perth,

Western Australia, Australia (Table 1). Duplicates of the New South Wales collection are kept at the fungal collection of forestry New South Wales (NSWF), Sydney, Australia.

DNA extraction and PCR

Mycelium from actively growing single-ascospore cultures was scraped from the surface of agar plates, freeze dried for 24 h and ground to a fine powder using liquid nitrogen. DNA was isolated using a phenol : chloroform (1 : 1) extraction protocol as described in Hunter *et al.* (2004) and precipitated by the addition of absolute ethanol (98 % EtOH). Isolated DNA was cleaned by washing with 70 % ethanol and dried under vacuum. Triple distilled water was used to resuspend the isolated DNA. RNase A (10 µg/µL) was added to the resuspended DNA and incubated at 37 °C for approximately 2 h to digest residual RNA. Isolated DNA was separated by electrophoresis in 1 % agarose gels (wt/v) (Roche Diagnostics, Mannheim), stained with ethidium bromide and visualised under ultra-violet light.

DNA from *M. nubilosa* isolates was used as template DNA for amplification using the polymerase chain reaction (PCR). All PCR reactions were mixed in a total volume of 25 µL containing 10 × PCR Buffer (5 mM Tris-HCl, 0.75 mM $MgCl_2$, 25 mM KCl, pH 8.3) (Roche Diagnostics, South Africa), 2.5 mM of each dNTP (dATP, dTTP, dCTP, dGTP) (Roche Diagnostics, South Africa), 0.2 µM of forward and reverse primers (Inqaba Biotech, South Africa) and 1.25 U Taq DNA Polymerase (Roche Diagnostics, South Africa) and DNA (20 ng/µL). Sterilised distilled water was added to obtain a final volume of 25 µL.

The ITS-1, ITS-2 and the 5.8 S gene regions of the ITS region of the rRNA operon were amplified using primers ITS-1 (5`–TCC GTA GGT GAA CCT GCG G–3`) and LR-1 (5`–GGT TGG TTT CTT TTC CT–3`) (White *et al.* 1990, Vilgalys & Hester 1990). A portion of the translation elongation factor 1-alpha (EF-1α) gene region was amplified using the primers EF1-728F (5'–CAT CGA GAA GTT CGA GAA GG–3') and EF1-986R (5'–TAC TTG AAG GAA CCC TTA CC–3') (Carbone & Kohn 1999). A portion of the Bt-2 gene region was amplified using primers Bt-2a (5'–GGT AAC CAA ATC GGT GCT GCT TTC–3') and Bt-2b (5'–ACC CTC AGT GTA GTG ACC CTT GGC–3') (Glass & Donaldson 1995). Reaction conditions for the ITS gene region followed those of Crous *et al.* (2004) and Hunter *et al.* (2004). Reaction conditions for the EF-1α gene region were the same as those used by Hunter *et al.* (2006b). The reaction conditions for the Bt-2 gene region were the same as those described for the ITS region (Hunter *et al.* 2004), however, the annealing temperature for the Bt-2 gene region was set to 55 °C.

All PCR products were visualised in 1.5 % agarose gels (wt/v) stained with ethidium bromide and viewed under ultra-violet light. Sizes of PCR amplicons were estimated by comparison against a 100 bp molecular weight marker (O' RangeRuler™ 100 bp DNA ladder) (Fermentas Life Sciences, U.S.A.). Prior to DNA sequencing, PCR products were purified through Centri-sep spin columns (Princeton Separations, Adelphia, NJ) containing Sephadex G-50 (Sigma Aldrich, St. Louis, MO) as outlined by the manufacturer.

DNA sequencing and phylogenetic analyses

Purified ITS, EF-1α and Bt-2 PCR products of *M. nubilosa* isolates were used as template DNA for sequencing reactions on an ABI PRISM™ 3100 Automated DNA sequencer (Applied Biosystems, Foster City, CA). The ABI Prism Big Dye Terminator Cycle sequencing reaction kit v. 3.1 (Applied Biosystems, Foster City, CA) was used for sequencing reactions following the manufacturer's instructions. Most sequencing reactions were performed with the same primers used for PCR reactions. Exceptions were in the case of the ITS region where two internal primers ITS-2 (5'–GCT GCG TTC TTC ATC GAT GC–3') and ITS-3 (5'–GCA TCG ATG AAG AAC GCA GC–3') (White *et al.* 1990) were included for the sequencing reactions.

ITS, EF-1α and Bt-2 sequences of *M. nubilosa* were also downloaded from GenBank and incorporated into phylogenetic analyses. All resulting sequences were analysed with Sequence Navigator v. 1.0.1 (Applied Biosystems, Foster City, CA). Sequence alignments were done using MAFFT (Multiple alignment program for amino acid or nucleotide sequences) v. 5.667 (Katoh *et al.* 2005) and manually adjusted where necessary. Phylogenetic distance and parsimony analyses of individual DNA sequence datasets and combined sequence datasets were conducted in PAUP v. 4.0b10 (Swofford 2002). For all distance and parsimony analyses that were conducted, isolates of *Mycosphaerella molleriana* (Thüm) Lindau and *Mycosphaerella cryptica* (Cooke) Hansf. were used to root phylogenetic trees.

For distance analyses, Modeltest v. 3.04 (Posada & Crandall 1998) was used to determine the best evolutionary model to fit the individual DNA sequence datasets and the combined DNA sequence alignment. A neighbour-joining analysis with the chosen evolutionary models was conducted in PAUP. For the ITS sequence dataset, the distance measure employed was the Hasegawa-Kishino-Yano (HKY) model. Here, the number of substitution types were two, transition/transversion ratio = 1.1144, base frequencies A = 0.21300, C = 0.3108, G = 0.25270 and T = 0.22350, assumed proportion of invariable sites (I)

= 0.8567, the distribution of rates at variable sites was equal. Where they were encountered, ties were broken randomly.

For the Bt-2 DNA sequence dataset a General Time Reversible model (GTR + I) was selected from the Modeltest program. Here, the number of substitution types = 6, substitution rate matrix [A-C] =5.500, [A-G] = 2.7405, [A-T] = 0.000, [C-G] = 1.2594, [C-T] = 9.6068, [G-T] = 1.000, base frequencies A = 0.21220, C = 0.30270, G = 0.28620 and T = 0.19890, assumed proportion of invariable sites (I) = 0.6625, Distribution of rates at variable sites were equal and ties were broken randomly when they were encountered.

The Tamura-Nei (TrNef + I) evolutionary model was selected as the distance measure to be applied to the EF-1α DNA sequence alignment. Here, the number of substitution types = 6, substitution rate matrix [A-C] = 1.0000, [A-G] = 1.2098, [A-T] = 1.000, [C-G] = 1.000, [C-T] = 2.7318 and [G-T] = 1.000, base frequencies were equal, assumed proportion of invariable sites (I) = 0.4033, distribution of rates at variable sites were equal. Ties were broken randomly when they were encountered.

A Partition Homogeneity Test (PHT) (Farris *et al*. 1994), of all possible combinations, consisting of 1000 replicates on all informative characters was conducted in PAUP to determine if the ITS, Bt-2 and EF-1α sequence datasets could be combined. From the PHT, a P-value of 1.000 was obtained and therefore the ITS, Bt-2 and EF-1α sequence datasets were combined.

A modeltest of the combined DNA sequence dataset selected a transition model (TIM) to be applied to the combined DNA sequence dataset. For this distance analysis, the number of substitution types = 6, substitution rate matrix [A-C] = 1.0000, [A-G] = 1.0293, [A-T] = 0.5885, [C-G] = 0.5885, [C-T] = 2.5721, [G-T] = 1.0000, base frequencies A = 0.22090, C = 0.29600, G = 0.27040, T = 0.21270, assumed proportion of invariable sites (I) = 0.6478, distribution of rates at variable sites were equal. Where ties were encountered, they were broken randomly.

Parsimony analysis was also conducted on the combined ITS, Bt-2 and EF-1α sequence datasets. Here, heuristic searches were conducted in PAUP with starting trees obtained through stepwise addition with simple addition sequence and with the MULPAR function enabled. Tree Bisection Reconnection (TBR) was employed as the swapping algorithm. All gaps were coded as missing data and characters were assigned equal weight. Branch support for nodes was obtained by performing 1000 bootstrap replicates of the aligned sequences. For parsimony analyses, measures that were calculated included tree length (TL),

retention index (RI), consistency index (CI), rescaled consistency index (RC) and homoplasy index (HI). All sequences of *M. nubilosa* isolates used in this study have been deposited in GenBank (Table. 1).

Growth in culture

Growth characteristics of representative *M. nubilosa* isolates that grouped in different ITS clades were determined on MEA. Here, the ex-epitype isolate of *M. nubilosa* (CMW 3282, CBS 116005), that resided in ITS lineage 2 and *M. nubilosa* isolate CMW 6518, that was accommodated in ITS linage 1 were used for growth studies. Actively growing cultures were used, and mycelial plugs (3 mm diam) were cut from the margins of cultures and transferred to the centres of 90 mm MEA agar plates. Agar plates were incubated in the dark at temperatures between 0 °C and 35 °C at 5° intervals. Colony diameters were measured every 7 d for 1 mo. Two plates were used per isolate at each temperature, and the entire experiment was repeated once. Colony colours (Rayner 1970) and morphology was assessed after 1 mo of growth in culture.

Microsatellite amplification

DNA from three *M. nubilosa* isolates that resided in different ITS lineages were used for microsatellite amplification experiments. These isolates included the *M. nubilosa* ex-epitype isolate (CMW 3282, CBS 116005) which resided in ITS lineage 2 and two isolates of *M. nubilosa* (CMW 6518 and CMW 23911) from ITS lineage 1. DNA from these *M. nubilosa* isolates was used as template DNA for the amplification of 10 microsatellite regions.

Microsatellite primers developed by Hunter *et al*. (2006a) were used to amplify the desired microsatellite regions. PCR reactions included an initial denaturation step of 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C (MN-8) or 60 °C for 30 s, extension at 72 °C for 1 min and a final extension step at 72 °C for 10 min. DNA was amplified in 25 µL reaction volumes containing PCR Buffer (5 mM Tris-HCl, 0.75 mM $MgCl_2$, 25 mM KCl, pH 8.3) (Roche Diagnostics, South Africa), 5.0 mM dNTP's 0.2 µM of each forward and reverse primer, 1.25 U Taq Polymerase (Roche Diagnostics, South Africa) and sterile water was added to achieve a final volume of 25 µL. Amplified DNA was visualised in 1.5 % agarose gels stained with ethidium bromide and viewed under ultra violet light

RESULTS

DNA sequencing and phylogenetic analyses

ITS phylogeny: The total alignment of the ITS sequence dataset consisted of 341 characters and 90 taxa. Following neighbour-joining analyses of the aligned ITS dataset, two distinct ITS lineages were resolved, designated *M. nubilosa* ITS lineage 1 and *M. nubilosa* ITS lineage 2 (Fig. 1). *Mycosphaerella nubilosa* ITS lineage 1 was well supported with a bootstrap value of 76 % and included isolates of *M. nubilosa* from Victoria (eastern Australia, west of Melbourne), New Zealand, Tasmania and western Australia. *Mycosphaerella nubilosa* ITS lineage 2 was also well supported with a bootstrap value of 75 % and accommodated *M. nubilosa* isolates from Ethiopia, Kenya, Portugal, South Africa, Spain, Tanzania, western Australia, New South Wales and Victoria (eastern Australia, east of Melbourne). Lineage 2 also included the ex-epitype strain of *M. nubilosa* (CMW 3282) and isolates of *Mycosphaerella juvenis* Crous & M.J. Wingf. Lineage 1 could be distinguished from lineage 2 by four fixed base-pair polymorphisms, two transitions and two transversions, at nucleotide positions 13 (A/C), 37 (G/C), 255 (T/C) and 334 (C/T) (Table 2, Fig. 1).

Bt-2 phylogeny: The Bt-2 dataset consisted of a total of 348 characters and 56 taxa. Neighbour-joining analyses of the Bt-2 sequence data set resolved isolates of *M. nubilosa* into one main clade (Clade 1) supported with a bootstrap value of 99 %. However, isolates in clade 1 could be further separated into two sub-clades (Fig 2). *Mycosphaerella nubilosa* isolates accommodated in sub-clade 1 originated from New Zealand, Tasmania, western Australia, New South Wales and Victoria (eastern Australia). Sub-clade 1 was well supported with a bootstrap value of 84 % and the isolates residing in it could be differentiated from the *M. nubilosa* isolates in sub-clade 2 by a 3 base pair insertion at nucleotide position 191 (ACA/XXX) and a single base pair transition at position 215 (A/G) (Table 2, Fig. 2).

Interestingly, isolates of *M. nubilosa* from New South Wales (eastern Australia) (CMW 20469, CMW 23913, CMW 20468, CMW 32910, CMW 20467, CMW 23915) that were accommodated in *M. nubilosa* ITS lineage 2 of the ITS phylogeny, were accommodated in sub-clade 1 of the Bt-2 phylogeny along with isolates of *M. nubilosa* that were accommodated in *M. nubilosa* ITS lineage 1 of the ITS phylogeny (Fig. 1).

EF-1α phylogeny: A total of 291 characters and 55 taxa of the EF-1α sequence alignment were considered in the phylogenetic analysis. Following neighbour-joining analyses, the *M. nubilosa* isolates were not well resolved. All the *M. nubilosa* taxa were

accommodated in a single clade with a bootstrap value of 100 % (Fig. 3). All *M. nubilosa* isolates exhibited identical EF-1α sequences with no base pair polymorphisms (Table 2).

Combined phylogeny: Results from a Partition Homogeneity Tests (PHT), to determine if the ITS, Bt-2 and EF-1α datasets could be combined, gave a P-value of 1.000. Therefore the ITS, Bt-2 and EF-1α datasets were combined and a total of 980 characters and 53 taxa were considered for the phylogenetic analysis of this combined dataset. Following neighbour-joining analysis, the combined sequence data set could be resolved into two main clades (Fig. 4). Clade 1, supported with a bootstrap value of 93 % included *M. nubilosa* isolates from New Zealand, Tasmania, western Australia and Victoria (eastern Australia, west of Melbourne). Clade 2 was weakly supported with a bootstrap value of 59 % and contained *M. nubilosa* isolates from Spain, Tanzania, South Africa, Kenya, Portugal, western Australia, New South Wales and Victoria (eastern Australia, east of Melbourne) (Fig. 4).

For Parsimony analyses of the combined DNA sequence data set, 769 characters were constant, 159 characters were variable and parsimony uninformative and 52 characters were parsimony informative. Following heuristic searches of the combined DNA sequence dataset one most parsimonious tree was retained (Length = 53, CI = 0.981, RI = 0.990, RC = 0.972, HI = 0.019) (Fig. 5). This tree could be resolved into two main clades. Following 1000 bootstrap replicates, both clade 1 and clade 2 of the parsimony analyses were well supported with clade 1 having a support value of 75 % and contained *M. nubilosa* isolates from western Australia, New Zealand, Tasmania and Victoria (eastern Australia). Clade 2 was well supported with a bootstrap value of 83 % and contained the same *M. nubilosa* isolates as that for the neighbour-joining analyses. Both the neighbour-joining and parsimony phylogenetic trees had the same topologies which were also consistent with the topology of the ITS phylogram.

Growth in culture

Following 1 mo growth on MEA, isolates of *M. nubilosa* representing the two distinct ITS clades, ITS lineage 1 (*M. nubilosa* ex-epitype CMW 3282) and ITS lineage 2 (CMW 6518) had the same optimal growth temperature (Fig. 6). Isolate CMW 3282 had a colony diameter of 12 mm at 25 °C, which was the same as that of isolate CMW 6518. Isolate CMW 3282, did, however, exhibit more rapid growth at 15 and 20 °C than that of CMW 6518 (Fig. 6). At 15 and 20 °C, CMW 3282 had colony diameters of 8 mm and 11 mm respectively, while CMW 6518 had colonies of 6 and 9 mm at 15 and 20 °C degrees respectively.

Colonies of the *M. nubilosa* ex-epitype (CMW 3282) residing in ITS lineage 2 were circular with even to irregular margins; slight folding occurred with sectors of white mycelial growth, while aerial mycelium was sparse. Colonies pale greenish grey (surface) and olivaceous grey (reverse) (Rayner 1970). Colonies reaching a diameter of 12 mm after 1 mo growth on MEA (Fig. 7A).

In contrast, *M. nubilosa* isolate CMW 6518 residing in ITS lineage 1 had irregular colonies with uneven and irregular margins with folding and sectoring with predominant white mycelial tufts occurring as extensive aerial mycelium. Colonies pale greenish grey (surface) with prominent white mycelial tufts and pale olivaceous grey (reverse) (Rayner 1970). Colonies reaching a diameter of 12 mm after 1 mo growth on MEA (Fig. 7B). Older cultures of CMW 6518 do however change in their culture morphology and exhibit irregular margins with sparse aerial mycelium, colony centres are pale olivaceous grey (surface) and colony borders are darker than colony centres being olivaceous grey (surface) while being olivaceous grey (reverse) (Fig. 7C).

Another isolate (CMW 23911) residing in *M. nubilosa* ITS lineage 1, exhibited a slightly different culture morphology to that of CMW 6518 and CMW 3282. Here, CMW 23911 grew extremely slowly and only reaching a diameter of 2 mm on MEA at 25 °C after 1 mo. Older colonies of CMW 23911 had irregular margins, extensive folding and convolutions with sparse to no aerial mycelium, submerged mycelium. Colonies, olivaceous grey (surface) and iron grey (reverse) (Fig. 7D).

Microsatellite amplification

Amplification of the DNA for *M. nubilosa* ex-epitype isolate CMW 3282 (ITS lineage 2), with 10 microsatellite primers resulted in amplification products of the required size for all of the microsatellite regions (Fig. 8). However, amplification of DNA in *M. nubilosa* isolates CMW 6518 and CMW 23911 residing in ITS lineage 1, with the same primers resulted in the amplification of only eight (MN-1, MN-2, MN-3, MN-7, MN-9, MN-10, MN-11, MN-14) of the 10 microsatellite primers. Microsatellite primers MN-4 and MN-8 did not result in amplification for *M. nubilosa* isolates CMW 6518 and CMW 23911 from ITS lineage 1 (Fig. 8).

DISCUSSION

Results of this study have provided clear evidence that there is considerable variation amongst isolates of *M. nubilosa* from different parts of the world and from different hosts. This variation is best expressed in terms of two clear ITS lineages for the fungus. These lineages have definitive *Eucalyptus* host associations and geographic distributions. *Mycosphaerella nubilosa* ITS lineage 2 includes the ex-epitype isolate of the species, and by default should be treated as *M. nubilosa sensu stricto*. In contrast, isolates residing in the ITS lineage 1 might represent a discrete species but there are insufficient data presently available to describe it as a new taxon.

Isolates of *M. nubilosa* used in this study and representing the two ITS lineages exhibited slight differences in culture growth and morphology. Park & Keane (1982) described two types of culture morphology for *M. nubilosa*, one characterised by black, tightly packed, submerged mycelium and dense dark-green aerial hyphae and another characterised by diffuse submerged mycelium and white to olive-green aerial hyphae. Both of these culture morphologies were observed in the present study. The majority of *M. nubilosa* isolates in this study, however, exhibited white to olive green aerial hyphae. An isolate of *M. nubilosa* (CMW 23911) collected from Victoria, eastern Australia exhibited a different culture morphology, and produced dense dark-green cultures with sparse aerial mycelium, a morphology similar to that described by Park & Keane (1982).

Further evidence for the presence of intra-specific variation in *M. nubilosa* was shown through the amplification of microsatellite regions using primers developed for *M. nubilosa* (Hunter *et al*. 2006a). Results of this study indicate that the majority of the microsatellite primers developed for *M. nubilosa*, can also amplify their specific microsatellite region for isolates of *M. nubilosa* in ITS lineage 1. However, two microsatellite primers, MN-4 and MN-8, did not result in amplification products for *M. nubilosa* isolates from this ITS lineage. Furthermore, there appears to be size polymorphisms between the ex-epitype of *M. nubilosa* from ITS lineage 2 and two other *M. nubilosa* isolates from ITS lineage 1 in their amplification products with microsatellite primers MN-2, MN-7, MN-9. These microsatellite primers could, therefore, be used as a diagnostic tool to distinguish between *M. nubilosa* isolates residing in the two lineages defined in this study.

Phylogenies of *M. nubilosa* generated from the three gene regions used in this study were generally discordant with respect to each other. The ITS gene region contained the highest level of sequence polymorphisms for *M. nubilosa* isolates. The Bt-2 gene region had

fewer polymorphisms and, the EF-1α gene region exhibited no sequence polymorphisms in isolates of this fungus. The ITS phylogeny was consistent with results of Crous *et al.* (2004), who also showed the presence of two *M. nubilosa* clades. The present study, however, included a large number of isolates and thus provides additional support for two *M. nubilosa* lineages. From the combined phylogeny, it may seem that *M. nubilosa* contains two phylogenetic species. But, for this to be true, phylogenies from different loci would need to be concordant in tree topologies as suggested by Geiser *et al*. (1998) for species of *Fusarium* Link. Future studies should thus incorporate DNA sequence data from several more unlinked loci to determine if *M. nubilosa* ITS lineage 1 represents a cryptic taxon.

An important outcome of this study is the knowledge that the two *M. nubilosa* lineages defined in the ITS and combined phylogenies have unique hosts associations (Fig. 4). Isolates of *M. nubilosa* accommodated in ITS lineage 1 are known only to infect *E. globulus*. In contrast, *M. nubilosa* isolates from ITS Lineage 2 are known from *E. dunnii*, *E. globulus*, *E. maidenii*, *E. nitens* and several other *Eucalyptus* hosts. Such host specificity has been shown previously. For example *M. nubilosa* was isolated from *E. globulus* in Tasmania and not *E. nitens* (Dungey *et al*. 1997, Carnegie *et al*. 1998, Milgate *et al*. 2001, Carnegie & Ades 2002). This has also been observed in New Zealand where *M. nubilosa* was most commonly found on sub-species of *E. globulus* but not on *E. nitens* (Hood *et al*. 2002). Results of the present study, showing that isolates of *M. nubilosa* in ITS lineage 1 occur only on *E. globulus* and only in Tasmania and New Zealand, provide support for previous studies that suggested this association.

The distribution of the *M. nubilosa* ITS lineages observed in this study and their host associations may be explained in several ways. *Eucalyptus globulus* ssp. *globulus* is native to Tasmania, islands of the Bass Strait and isolated pockets in Victoria (Kirkpatrick 1974, Poynton 1979, Jordan *et al*. 1993). *M. nubilosa* ITS lineage 1 may originate in Tasmania on *E. globulus* ssp. *globulus*. This lineage may have been spread to New Zealand and Victoria, where it currently occurs on other *E. globulus* sub-species such as *E. globulus* ssp. *pseudoglobulus*, *E. globulus* ssp. *maidenii* and *E. globulus* ssp. *bicostata*. Isolates belonging to ITS lineage 1 may have further adapted to infect various other *Eucalyptus* spp such as *E. nitens*. As such, it could have evolved to form ITS lineage 2 that has now become common in Australia, Africa and Europe. Alternatively, two initial populations of *M. nubilosa* occurred, one that is native on *E. globulus* ssp. *globulus* in Tasmania and another that is able to infect several *Eucalyptus* spp. and native to Victoria and New South Wales. Over time, with the movement of *Eucalyptus* plant material from Tasmania to Victoria, sexual outcrossing

between these two populations could have occurred, yet retaining their close relationship. These hypotheses could be tested by population level studies employing microsatellite markers developed by Hunter *et al*. (2006a).

It is interesting that some isolates of *M. nubilosa* from New South Wales (eastern Australia) (CMW 20469, CMW 23913, CMW 20468, CMW 23910, CMW 20467, CMW 23915) that grouped in lineage 2 of the ITS phylogeny, grouped in sub-clade 1 of the Bt-2 phylogeny. This suggests that these isolates may represent a hybrid between isolates representing the *M. nubilosa* lineages. This would not be unusual as *M. nubilosa* ITS lineage 1 and ITS lineage 2 both infect *E. globulus*, which would provide an opportunity for mating and hybridisation to occur between the different groups.

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Table 1. Isolates of *Mycosphaerella nubilosa* used during this study for DNA sequencing analyses.

Isolate	Species	Host	Area	Collector	GenBank Accession No.		
					ITS	Bt-2	EF-1α
MURU 40	*M. nubilosa*	*E. globulus*	Tasmania, Australia	A. Maxwell	DQ923540	DQ992034	DQ974117
MURU 42	*M. nubilosa*	*E. globulus*	Tasmania, Australia	A. Maxwell	DQ923541	DQ992035	DQ974118
MURU 46	*M. nubilosa*	*E. globulus*	Tasmania, Australia	A. Maxwell	DQ923542	DQ992036	DQ974119
MURU 52	*M. nubilosa*	*E. globulus*	Victoria, eastern Australia	A. Maxwell	DQ923543	DQ992037	DQ974120
MURU 64	*M. nubilosa*	*E. globulus*	Victoria, eastern Australia	A. Maxwell	DQ923544	DQ992039	DQ974122
CMW 23911	*M. nubilosa*	*E. globulus*	Victoria, eastern Australia	A.J. Carnegie	DQ923552	DQ992049	DQ974151
CMW 3282	*M. nubilosa*	*E. globulus*	Victoria, eastern Australia	A.J. Carnegie	AF309618	DQ992068	DQ235111
CMW 4042	*M. nubilosa*	*E. globulus*	Western Australia	A. Maxwell	DQ923546	DQ992041	DQ974124
CMW 4053	*M. nubilosa*	*Eucalyptus* sp.	Tasmania, Australia	A.W. Milgate	DQ923545	DQ992040	DQ974123
CMW 6518	*M. nubilosa*	*E. globulus*	Dartmoon, Victoria, eastern Australia	P. Barber	DQ923551	DQ992051	DQ974145
CMW 14928	*M. nubilosa*	*Eucalyptus* sp.	New Zealand	M.J. Wingfield	DQ923548	DQ992043	DQ974126
CMW 14930	*M. nubilosa*	*Eucalyptus* sp.	New Zealand	M.J. Wingfield	DQ923547	DQ992042	DQ974125
CMW 12548	*M. nubilosa*	*E. globulus*	Spain	J.P.M. Vasquez	DQ923570	DQ992067	DQ974127
CMW 12594	*M. nubilosa*	*E. globulus*	Spain	J.P.M. Vasquez	DQ923568	DQ992058	DQ974128
CMW 12600	*M. nubilosa*	*E. globulus*	Spain	J.P.M. Vasquez	DQ923569	DQ992065	DQ974129
CMW 18702	*M. nubilosa*	*E. nitens*	South Africa	G.C. Hunter	DQ923572	DQ992060	DQ974130
CMW 18796	*M. nubilosa*	*E. globulus*	Portugal	M.J. Wingfield	DQ923565	DQ992055	DQ974131
CMW 18805	*M. nubilosa*	*E. globulus*	Portugal	M.J. Wingfield	DQ923567	DQ992053	DQ974132
CMW 18828	*M. nubilosa*	*E. globulus*	Portugal	M.J. Wingfield	DQ923566	DQ992061	DQ974133
CMW 20207	*M. nubilosa*	*E. nitens*	South Africa	G.C. Hunter	DQ923571	DQ992056	DQ974134

Isolate	Species	Host	Area	Collector	GenBank Accession No.		
					ITS	Bt-2	EF-1α
CMW 20467	*M. nubilosa*	*E. globulus*	New South Wales, eastern Australia	A.J. Carnegie	DQ923549	DQ992045	DQ974139
CMW 20468	*M. nubilosa*	*E. globulus*	New South Wales, eastern Australia	A.J. Carnegie	DQ923550	DQ992047	DQ974140
CMW 20469	*M. nubilosa*	*E. globulus*	New South Wales, eastern Australia	A.J. Carnegie	DQ923577	DQ992044	DQ974141
CMW 21518	*M. nubilosa*	*E. nitens*	South Africa	G.C. Hunter	DQ923576	DQ992064	DQ974135
CMW 21603	*M. nubilosa*	*E. globulus*	Tanzania	J. Roux	DQ923563	DQ992070	DQ974136
CMW 21619	*M. nubilosa*	*E. globulus*	Tanzania	J. Roux	DQ923564	DQ992062	DQ974137
CMW 21633	*M. nubilosa*	*E. globulus*	Tanzania	J. Roux	DQ923562	DQ992066	DQ974138
CMW 22371	*M. nubilosa*	*E. globulus*	Western Australia	A. Maxwell	DQ923573	DQ992054	DQ974142
CMW 22381	*M. nubilosa*	*E. globulus*	Western Australia	A. Maxwell	DQ923574	DQ992057	DQ974143
CMW 22398	*M. nubilosa*	*E. globulus*	Western Australia	A. Maxwell	DQ923575	DQ992059	DQ974152
CMW 23908	*M. nubilosa*	*E. globulus*	New South Wales, eastern Australia	A.J. Carnegie	DQ923556	DQ992063	N/A
CMW 23909	*M. nubilosa*	*E. globulus*	New South Wales, eastern Australia	A.J. Carnegie	DQ923555	DQ992071	DQ974148
CMW 23910	*M. nubilosa*	*E. dunnii*	New South Wales, eastern Australia	A.J. Carnegie	DQ923558	DQ992046	DQ974154
CMW 23913	*M. nubilosa*	*E. dunnii*	New South Wales, eastern Australia	A.J. Carnegie	DQ923553	DQ992050	DQ974149
CMW 23912	*M. nubilosa*	*E. dunnii*	New South Wales, eastern Australia	A.J. Carnegie	DQ923554	N/A	N/A
CMW 23914	*M. nubilosa*	*E. globulus*	New South Wales, eastern Australia	A.J. Carnegie	DQ923559	DQ992052	DQ974155
CMW 23915	*M. nubilosa*	*E. dunnii*	New South Wales, eastern Australia	A.J. Carnegie	DQ923560	DQ992048	DQ974146
CMW 23916	*M. nubilosa*	*E. nitens*	New South Wales, eastern Australia	A.J. Carnegie	DQ923561	DQ992069	DQ974147
CMW 23907	*M. nubilosa*	*E. globulus*	New South Wales, eastern Australia	A.J. Carnegie	DQ923557	N/A	DQ974150

Table 2. Fixed polymorphisms observed between *Mycosphaerella nubilosa* isolates for the Internal Transcribed Spacer (ITS) and Beta-tubulin (Bt-2) gene regions. No polymorphisms were observed between *M. nubilosa* for the Translation Elongation Factor (EF-1α). X = deletion.

Locus	**Base pair fixed polymorphisms**			
ITS	**13**	**37**	**255**	**334**
	A/C	G/C	T/C	C/T
Bt-2	**191–201**	**215**	-	-
	ACA/XXX	A/G		
EF-1α	-	-	-	-

Figure 1. Neighbour-joining phylogram obtained from a distance analysis using the HKY substitution model on ITS sequence data of *M. nubilosa* isolates. Bootstrap values after 1000 replicates are shown above branches. *Mycosphaerella molleriana* and *M. cryptica* were used as outgroups. Fixed sequence polymorphisms, separating clade 1 from clade 2, and their base pair positions are indicated at dotted line.

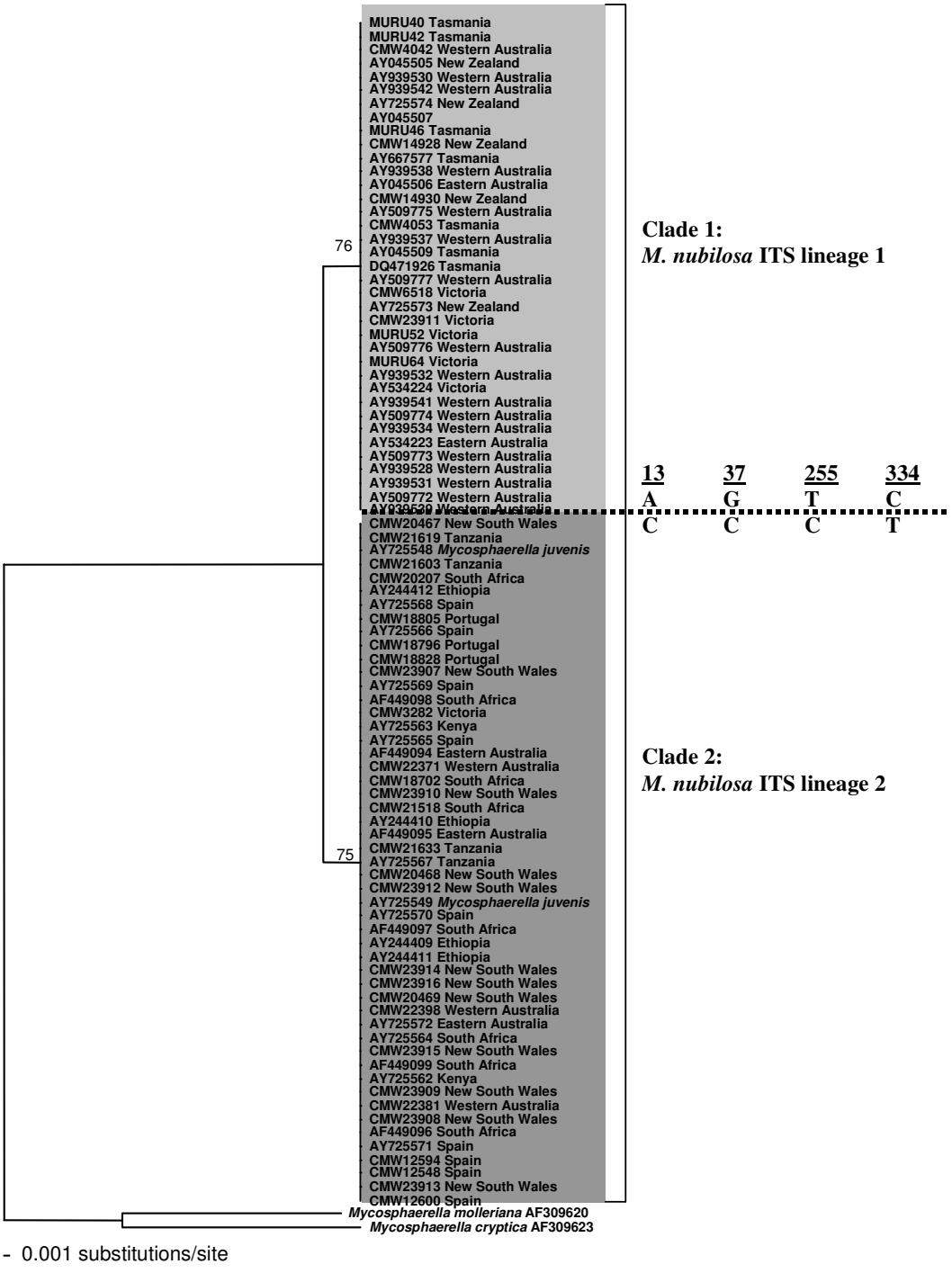

MURU40 Tasmania
MURU42 Tasmania
CMW4042 Western Australia
AY045505 New Zealand
AY939530 Western Australia
AY939542 Western Australia
AY725574 New Zealand
AY045507
MURU46 Tasmania
CMW14928 New Zealand
AY667577 Tasmania
AY939538 Western Australia
AY045506 Eastern Australia
CMW14930 New Zealand
AY509775 Western Australia
CMW4053 Tasmania
AY939537 Western Australia
76
AY045509 Tasmania
DQ471926 Tasmania
AY509777 Western Australia
CMW6518 Victoria
AY725573 New Zealand
CMW23911 Victoria
MURU52 Victoria
AY509776 Western Australia
MURU64 Victoria
AY939532 Western Australia
AY534224 Victoria
AY939541 Western Australia
AY509774 Western Australia
AY939534 Western Australia
AY534223 Eastern Australia
AY509773 Western Australia
AY939528 Western Australia
AY939531 Western Australia
AY509772 Western Australia
CMW20467 New South Wales
CMW21619 Tanzania
AY725548 Mycosphaerella juvenis
CMW21603 Tanzania
CMW20207 South Africa
AY244412 Ethiopia
AY725568 Spain
CMW18805 Portugal
AY725566 Spain
CMW18796 Portugal
CMW18828 Portugal
CMW23907 New South Wales
AY725569 Spain
AF449098 South Africa
CMW3282 Victoria
AY725563 Kenya
AY725565 Spain
AF449094 Eastern Australia
CMW22371 Western Australia
CMW18702 South Africa
CMW23910 New South Wales
CMW21518 South Africa
AY244410 Ethiopia
AF449095 Eastern Australia
75
CMW21633 Tanzania
AY725567 Tanzania
CMW20468 New South Wales
CMW23912 New South Wales
AY725549 Mycosphaerella juvenis
AY725570 Spain
AF449097 South Africa
AY244409 Ethiopia
AY244411 Ethiopia
CMW23914 New South Wales
CMW23916 New South Wales
CMW20469 New South Wales
CMW22398 Western Australia
AY725572 Eastern Australia
AY725564 South Africa
CMW23915 New South Wales
AF449099 South Africa
AY725562 Kenya
CMW23909 New South Wales
CMW22381 Western Australia
CMW23908 New South Wales
AF449096 South Africa
AY725571 Spain
CMW12594 Spain
CMW12548 Spain
CMW23913 New South Wales
CMW12600 Spain
Mycosphaerella molleriana AF309620
Mycosphaerella cryptica AF309623
- 0.001 substitutions/site
Clade 1:
M. nubilosa ITS lineage 1
13 37 255 334
A G T C
C C C T
Clade 2:
M. nubilosa ITS lineage 2

Figure 2. Neighbour-joining phylogram obtained from a distance analysis using the GTR + I substitution model on Bt-2 sequence data of *M. nubilosa* isolates. Bootstrap values after 1000 replicates are shown above branches. *Mycosphaerella molleriana* and *M. cryptica* were used as outgroups. Fixed sequence polymorphisms, separating sub-clade 1 from clade 1, and their base pair positions are indicated at dotted line. **X** = deletion.

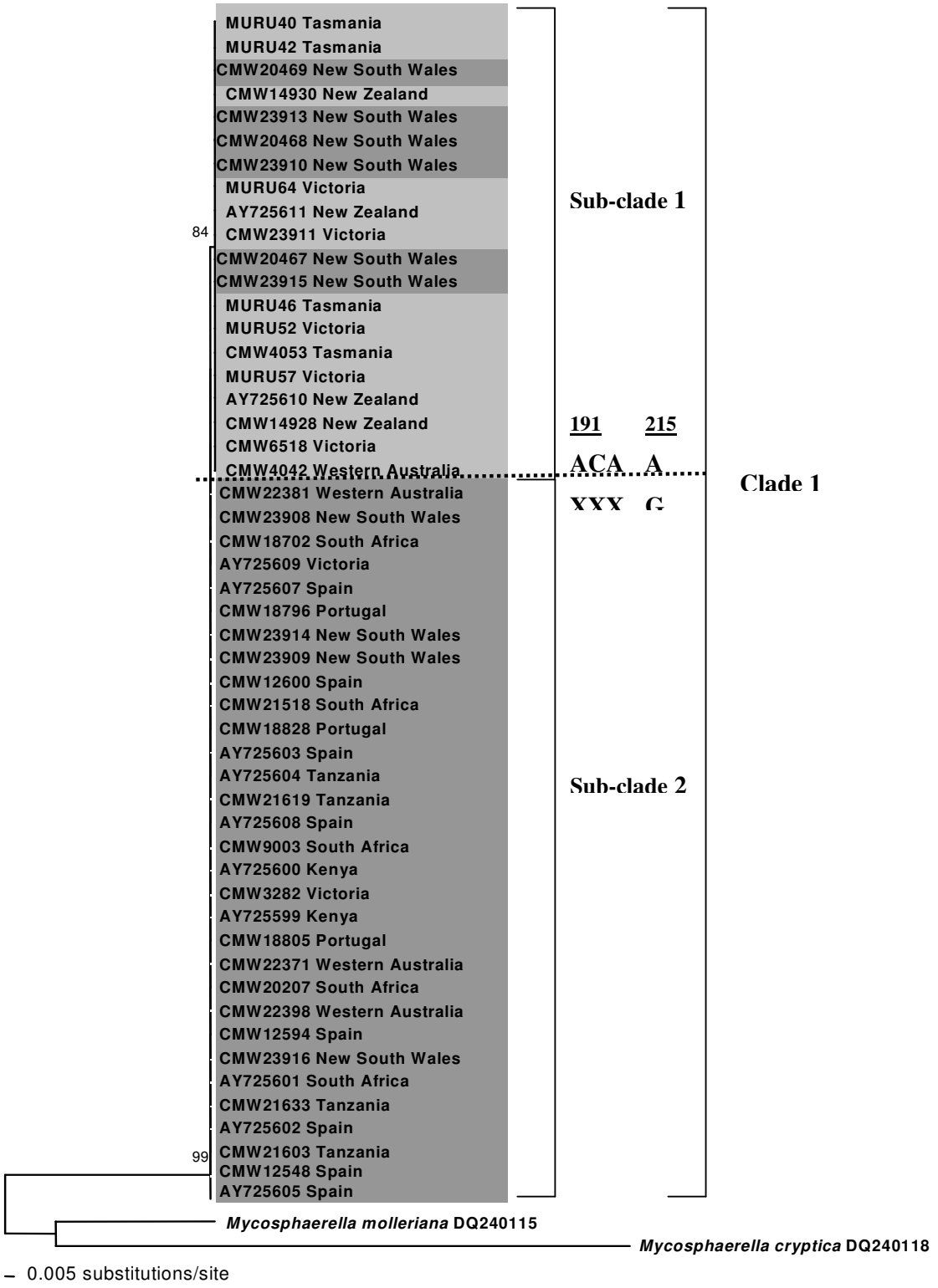

MURU40 Tasmania
MURU42 Tasmania
CMW20469 New South Wales
CMW14930 New Zealand
CMW23913 New South Wales
CMW20468 New South Wales
CMW23910 New South Wales
MURU64 Victoria
AY725611 New Zealand
84
CMW23911 Victoria
CMW20467 New South Wales
CMW23915 New South Wales
MURU46 Tasmania
MURU52 Victoria
CMW4053 Tasmania
MURU57 Victoria
AY725610 New Zealand
CMW14928 New Zealand
CMW6518 Victoria
CMW4042 Western Australia
CMW22381 Western Australia
CMW23908 New South Wales
CMW18702 South Africa
AY725609 Victoria
AY725607 Spain
CMW18796 Portugal
CMW23914 New South Wales
CMW23909 New South Wales
CMW12600 Spain
CMW21518 South Africa
CMW18828 Portugal
AY725603 Spain
AY725604 Tanzania
CMW21619 Tanzania
AY725608 Spain
CMW9003 South Africa
AY725600 Kenya
CMW3282 Victoria
AY725599 Kenya
CMW18805 Portugal
CMW22371 Western Australia
CMW20207 South Africa
CMW22398 Western Australia
CMW12594 Spain
CMW23916 New South Wales
AY725601 South Africa
CMW21633 Tanzania
AY725602 Spain
99
CMW21603 Tanzania
CMW12548 Spain
AY725605 Spain
Sub-clade 1
191
215
ACA
A
XXX
G
Clade 1
Sub-clade 2
Mycosphaerella molleriana DQ240115
Mycosphaerella cryptica DQ240118
0.005 substitutions/site

Figure 3. Neighbour-joining phylogram obtained from a distance analysis using the TrNef + I substitution model on EF-1α sequence data of *M. nubilosa* isolates. Bootstrap values after 1000 replicates are shown above branches. *Mycosphaerella molleriana* and *M. cryptica* were used as outgroups.

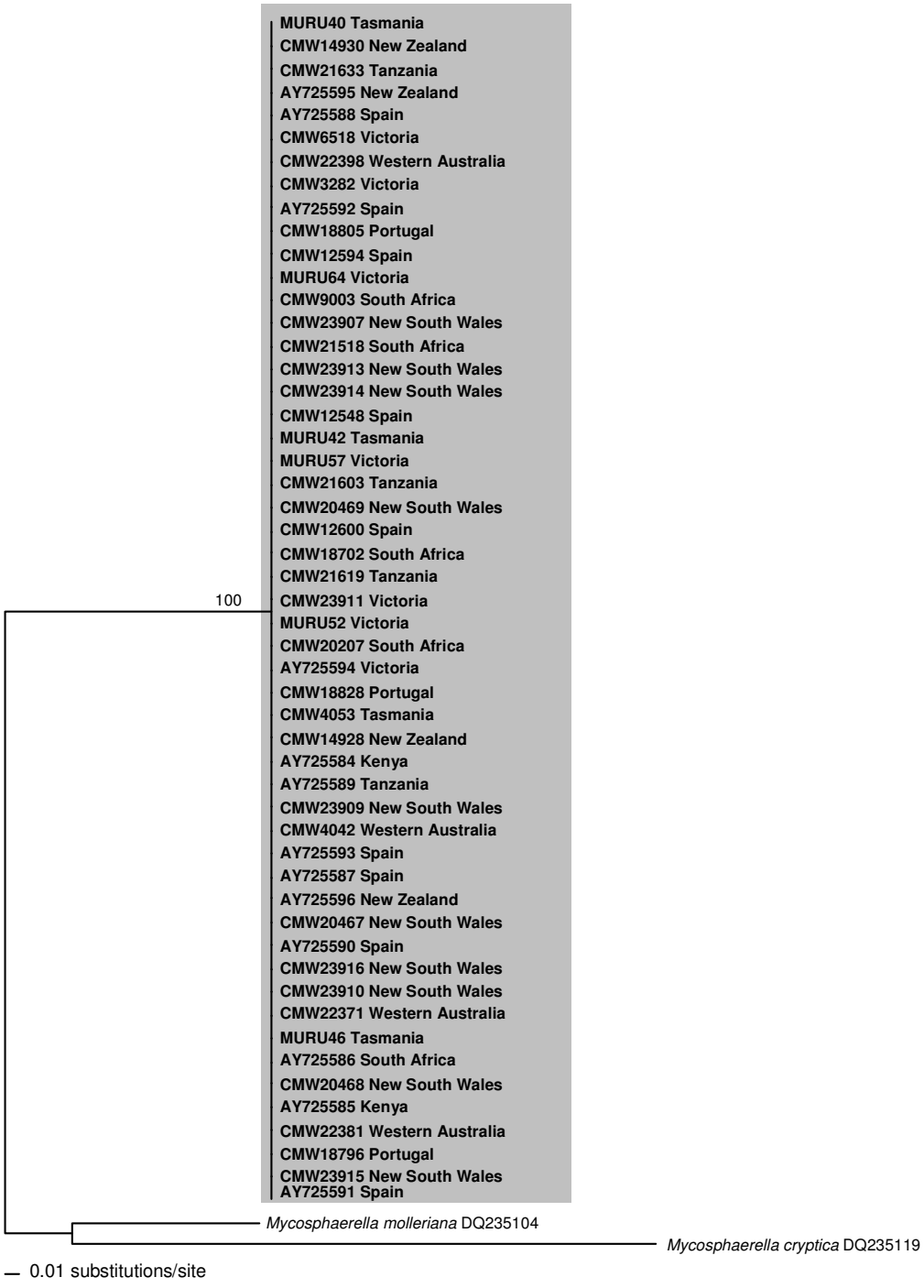

MURU40 Tasmania
CMW14930 New Zealand
CMW21633 Tanzania
AY725595 New Zealand
AY725588 Spain
CMW6518 Victoria
CMW22398 Western Australia
CMW3282 Victoria
AY725592 Spain
CMW18805 Portugal
CMW12594 Spain
MURU64 Victoria
CMW9003 South Africa
CMW23907 New South Wales
CMW21518 South Africa
CMW23913 New South Wales
CMW23914 New South Wales
CMW12548 Spain
MURU42 Tasmania
MURU57 Victoria
CMW21603 Tanzania
CMW20469 New South Wales
CMW12600 Spain
CMW18702 South Africa
CMW21619 Tanzania
100
CMW23911 Victoria
MURU52 Victoria
CMW20207 South Africa
AY725594 Victoria
CMW18828 Portugal
CMW4053 Tasmania
CMW14928 New Zealand
AY725584 Kenya
AY725589 Tanzania
CMW23909 New South Wales
CMW4042 Western Australia
AY725593 Spain
AY725587 Spain
AY725596 New Zealand
CMW20467 New South Wales
AY725590 Spain
CMW23916 New South Wales
CMW23910 New South Wales
CMW22371 Western Australia
MURU46 Tasmania
AY725586 South Africa
CMW20468 New South Wales
AY725585 Kenya
CMW22381 Western Australia
CMW18796 Portugal
CMW23915 New South Wales
AY725591 Spain
Mycosphaerella molleriana DQ235104
Mycosphaerella cryptica DQ235119
0.01 substitutions/site

Figure 4. Neighbour-joining phylogram obtained through distance analysis using the TIM substitution model on the combined ITS, Bt-2 and EF-1α DNA sequence datasets of *M. nubilosa* isolates. Bootstrap values after 1000 replicates are indicated above branches or by arrows. *Mycosphaerella molleriana* and *M. cryptica* were used as outgroups. *Eucalyptus* host associations of the *M. nubilosa* isolates are indicated on the right of the phylogram.

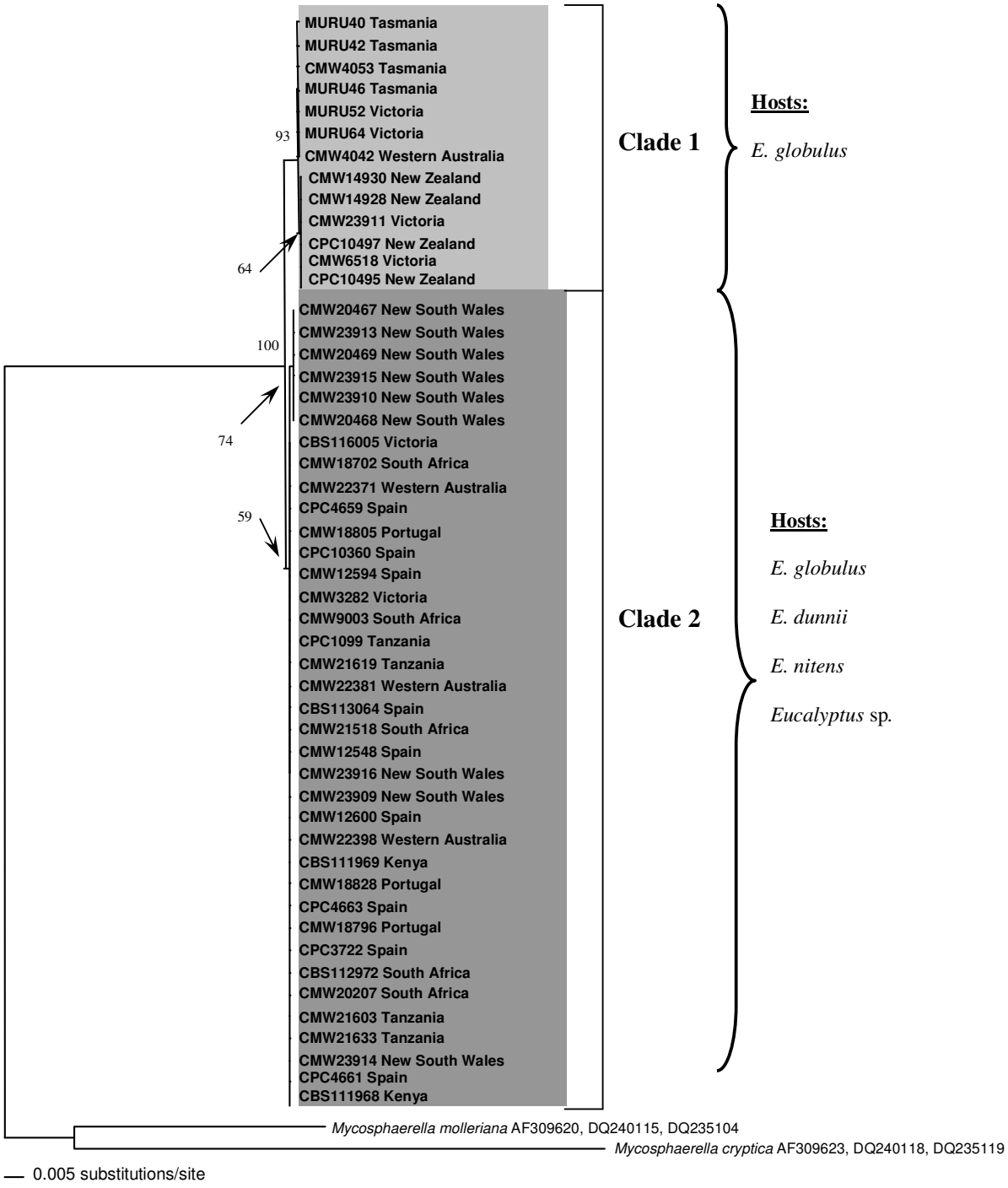

MURU40 Tasmania
MURU42 Tasmania
CMW4053 Tasmania
MURU46 Tasmania
MURU52 Victoria
MURU64 Victoria
CMW4042 Western Australia
CMW14930 New Zealand
CMW14928 New Zealand
CMW23911 Victoria
CPC10497 New Zealand
CMW6518 Victoria
CPC10495 New Zealand
CMW20467 New South Wales
CMW23913 New South Wales
CMW20469 New South Wales
CMW23915 New South Wales
CMW23910 New South Wales
CMW20468 New South Wales
CBS116005 Victoria
CMW18702 South Africa
CMW22371 Western Australia
CPC4659 Spain
CMW18805 Portugal
CPC10360 Spain
CMW12594 Spain
CMW3282 Victoria
CMW9003 South Africa
CPC1099 Tanzania
CMW21619 Tanzania
CMW22381 Western Australia
CBS113064 Spain
CMW21518 South Africa
CMW12548 Spain
CMW23916 New South Wales
CMW23909 New South Wales
CMW12600 Spain
CMW22398 Western Australia
CBS111969 Kenya
CMW18828 Portugal
CPC4663 Spain
CMW18796 Portugal
CPC3722 Spain
CBS112972 South Africa
CMW20207 South Africa
CMW21603 Tanzania
CMW21633 Tanzania
CMW23914 New South Wales
CPC4661 Spain
CBS111968 Kenya
93
64
100
74
59
Clade 1
Clade 2
Hosts:
E. globulus
Hosts:
E. globulus
E. dunnii
E. nitens
Eucalyptus sp.
Mycosphaerella molleriana AF309620, DQ240115, DQ235104
Mycosphaerella cryptica AF309623, DQ240118, DQ235119
0.005 substitutions/site

Figure 5. Parsimony phylogram of the combined ITS, Bt-2 and EF-1α sequence datasets of *M. nubilosa* isolates. Bootstrap values after 1000 replicates are indicated below branches and branch lengths are indicated above branches. *Mycosphaerella molleriana* and *M. cryptica* were used as outgroups.

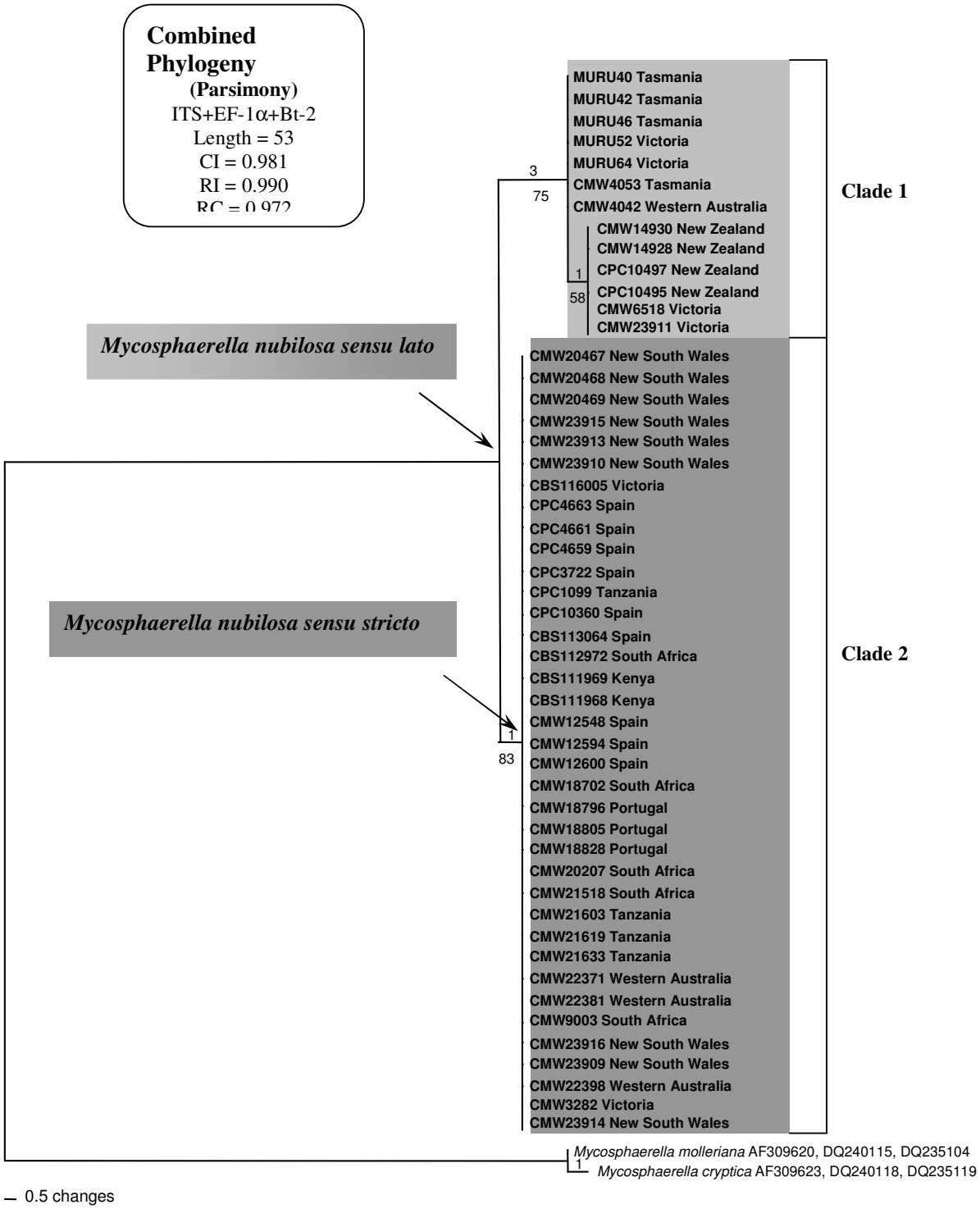
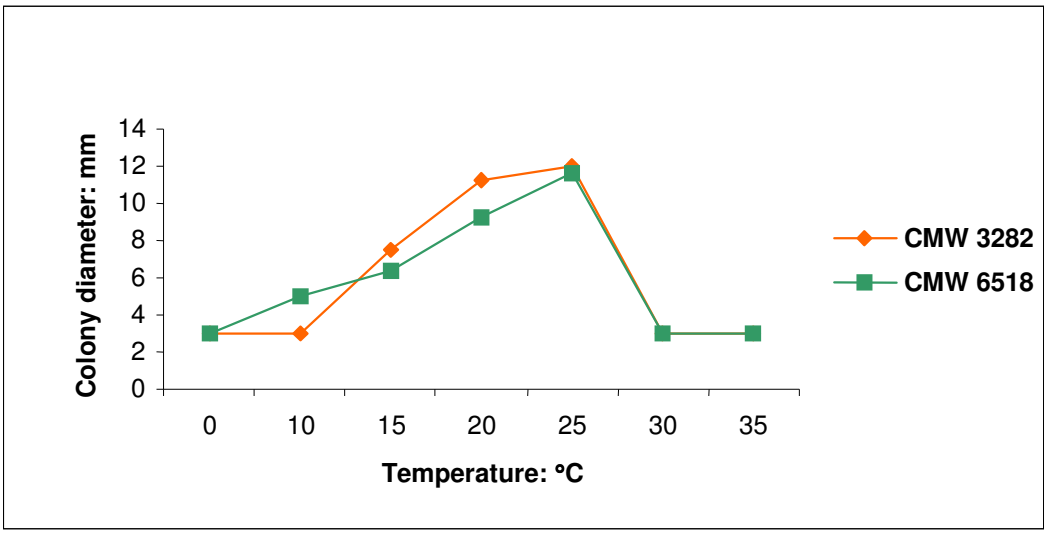

Combined Phylogeny
(Parsimony)
ITS+EF-1α+Bt-2
Length = 53
CI = 0.981
RI = 0.990
RC = 0.972
MURU40 Tasmania
MURU42 Tasmania
MURU46 Tasmania
MURU52 Victoria
MURU64 Victoria
CMW4053 Tasmania
CMW4042 Western Australia
CMW14930 New Zealand
CMW14928 New Zealand
CPC10497 New Zealand
CPC10495 New Zealand
CMW6518 Victoria
CMW23911 Victoria
3
75
1
58
Clade 1
Mycosphaerella nubilosa sensu lato
CMW20467 New South Wales
CMW20468 New South Wales
CMW20469 New South Wales
CMW23915 New South Wales
CMW23913 New South Wales
CMW23910 New South Wales
CBS116005 Victoria
CPC4663 Spain
CPC4661 Spain
CPC4659 Spain
CPC3722 Spain
CPC1099 Tanzania
CPC10360 Spain
CBS113064 Spain
CBS112972 South Africa
CBS111969 Kenya
CBS111968 Kenya
CMW12548 Spain
CMW12594 Spain
CMW12600 Spain
CMW18702 South Africa
CMW18796 Portugal
CMW18805 Portugal
CMW18828 Portugal
CMW20207 South Africa
CMW21518 South Africa
CMW21603 Tanzania
CMW21619 Tanzania
CMW21633 Tanzania
CMW22371 Western Australia
CMW22381 Western Australia
CMW9003 South Africa
CMW23916 New South Wales
CMW23909 New South Wales
CMW22398 Western Australia
CMW3282 Victoria
CMW23914 New South Wales
1
83
Clade 2
Mycosphaerella nubilosa sensu stricto
Mycosphaerella molleriana AF309620, DQ240115, DQ235104
1
Mycosphaerella cryptica AF309623, DQ240118, DQ235119
0.5 changes

Figure 6. Results of growth studies on *Mycosphaerella nubilosa* isolates (CMW 3282 ex-epitype, CMW 6518) after 1 mo growth on 2 % MEA at temperatures from 0 °C to 35 °C. CMW 3282 = ITS Lineage 2 and *M. nubilosa* ex-epitype, CMW 6518 = ITS Lineage 1.

Figure 7. Culture morphology of *Mycosphaerella nubilosa* isolates on 2 % MEA. (**A**) CMW 3282 following 1 mo growth on 2 % MEA, (**B**) CMW 6518 following 1 mo growth on 2 % MEA, (**C**) Older culture growth of CMW 6518 following 3 mo growth on 2 % MEA. (**D**) CMW 23911 following 3 mo growth on 2 % MEA.


Colony diameter: mm
14
12
10
8
6
4
2
0
0
10
15
20
25
30
35
Temperature: °C
CMW 3282
CMW 6518

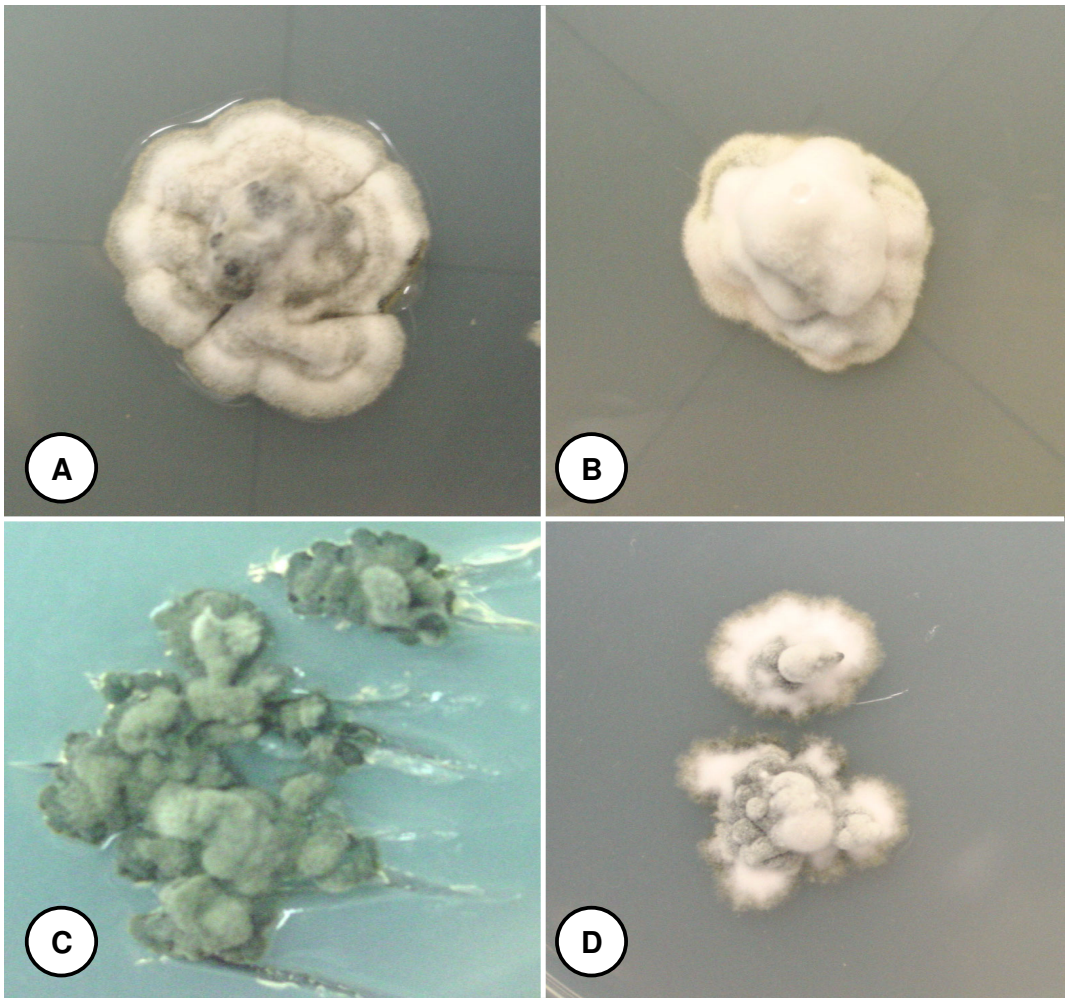
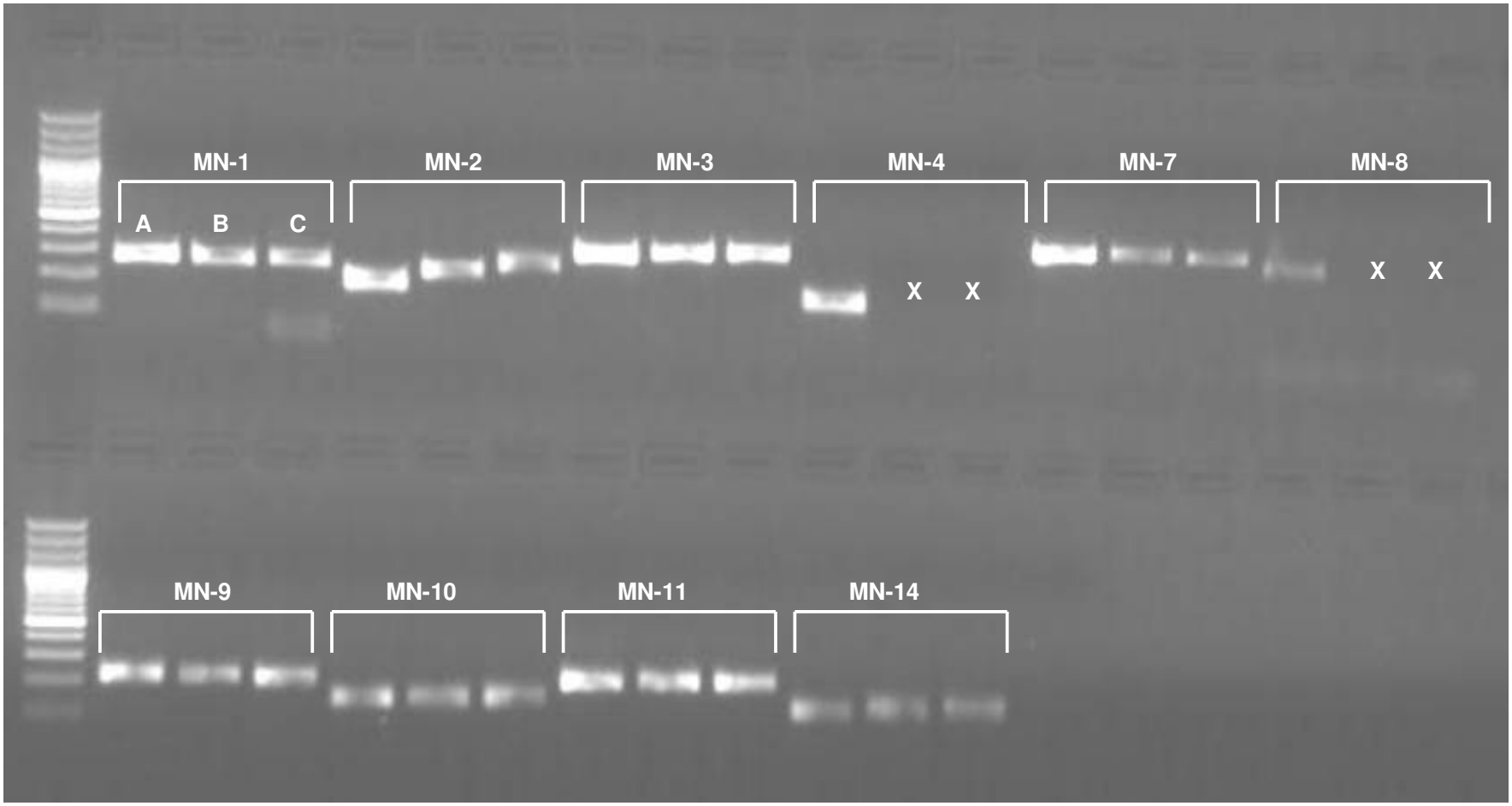

A
B
C
D

Figure 8. 1.5 % Agarose gel indicating the amplification of microsatellite regions for isolates of *Mycosphaerella nubilosa* using microsatellite primers developed by Hunter *et al*. (2006a). Lane **A** = CMW 3282, ITS Lineage 2 and *M. nubilosa* ex-epitype, Lane **B** = CMW 6518, ITS Lineage 1, Lane **C** = CMW 23911, ITS Lineage 1.


MN-1
A
B
C
MN-2
MN-3
MN-4
X
X
MN-7
MN-8
X
X
MN-9
MN-10
MN-11
MN-14