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# PCR-identification of *Mycosphaerella* species associated with leaf diseases of *Eucalyptus*

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A PCR-based technique based on the ITS1-5.8s-ITS2 domain of the rRNA gene for identifying five species associated with *Mycosphaerella* leaf disease (MLD) of eucalypts was developed. Primer pairs MC2F and MC2R; ML1F and ML1R; MM1F and MM1R; MN1F and MN1R; and MP1F and MP1R amplified a product for DNA extracted from their single target species, those being *M. cryptica*, *M. lateralis*, *M. marksii*, *M. nubilosa* and *M. parva*, respectively. The possibility of false positive amplification by each primer pair was tested in reactions with DNA extracts from 16 other *Mycosphaerella* species associated with eucalypts and against non-infected *Eucalyptus globulus* leaves. Under the PCR conditions used, there were no false positive amplifications of the 16 non-target *Mycosphaerella* species, or from non-symptomatic *E. globulus* leaves for the primer pairs ML1F and ML1R; MM1F and MM1R; MN1F and MN1R; and MP1F and MP1R. The primer pair MC2F and MC2R amplified a 402 nt product from both the target *M. cryptica* and non-target *M. nubilosa*. However, these two species were differentiated by digesting the product with the restriction enzyme *Sacc II* which resulted in a single 402 nt product for *M. cryptica*, and two products of 78 and 324 nt for *M. nubilosa*. All of the primers were able to detect their target *Mycosphaerella* species from *Eucalyptus globulus* lesions. PCR reactions with these primers on DNA extracted from *Mycosphaerella* lesions confirmed the presence of all five species from leaf material collected from three plantations in Western Australia.

## INTRODUCTION

*Mycosphaerella* leaf disease (MLD) is a widespread, economically important disease of *Eucalyptus* species worldwide. MLD is common in naturally occurring eucalypt forest areas (Park *et al.* 2000) and is the major foliar disease of eucalypt plantations in southern Australia (Park & Keane 1982a, Carnegie 1991, 2000, Park *et al.* 2000, Maxwell *et al.* 2003). Crous (1998) attributes the cessation of *E. globulus* forestry in South Africa in the 1930s to MLD.

*Mycosphaerella* species are generally thought to have a narrow host range in that the taxonomy of the genus is largely host related. *Mycosphaerella* pathogens of eucalypts vary in their host-range. For example, *M. cryptica* has a comparatively wide host range and is known to infect at least 14 and 24 species in the subgenera *Monocalyptus* and *Symphomyrtus*, respectively, whereas *M. nubilosa* has a comparatively narrow host range and infects only six species within subgenus *Symphomyrtus* (Park *et al.* 2000). There is also inter-and intra-host variation in the susceptibility to MLD (Carnegie *et al.* 1994). Because, MLD often occurs as a disease complex, it would be useful in epidemiological and host resistance studies to determine what species are present and in what proportion.

*Mycosphaerella* taxonomy based on morphological features is difficult, in that the 2000 species within this genus are discriminated on the basis of small differences in ascospore size, shape, germination pattern (Crous 1998), and host. These features often overlap between species and vary considerably within a single species. The formation of conidia and fruiting structures are also important in differentiating *Mycosphaerella* species. These structures may not form easily in culture, or it may require weeks or months of growth on defined media under specific conditions for conidia to develop. Thus, there is disagreement between *Mycosphaerella* taxonomists on the identification of some *Mycosphaerella* species; further, non-specialists are not able to confidently recognise species within this genus. In addition, epidemiological or host resistance studies may be hampered by difficulties in identifying the complex of *Mycosphaerella* species causing the disease.

There are important examples of misidentification of *Mycosphaerella* species from eucalypts, such as studies on what was thought to be *M. nubilosa* in New Zealand

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(Cheah 1977, Beresford 1978) that was later identified as *M. cryptica* (Park & Keane 1982b). *Colletogloeopsis nubilosum*, the anamorph of *M. cryptica*, was first incorrectly connected to the teleomorph *M. nubilosa* (Ganapathi & Corbin 1979). In addition, early records have attributed MLD outbreaks to *M. molleriana*, when other *Mycosphaerella* species were the cause (Crous 1998). Molecular-based methods provide an additional tool that can make species identification more certain.

Comparison of sequences of ITS 1 and 2 of the rRNA genes has proved useful in delimiting and differentiating species (Berbee *et al.* 1995, Crous *et al.* 1999, Guo, Hyde & Liew 2000). Sequence comparison of the ITS rDNA has enabled a more secure differentiation of *Mycosphaerella* species on eucalypts in south-western Australia (Maxwell *et al.* 2000, 2003). However, DNA sequencing is now a relatively expensive and time-consuming technique for identifying species. The data obtained from ITS sequences may be utilised to develop less expensive and more rapid molecular means for the recognition of *Mycosphaerella* species. These include PCR-based restriction fragment length polymorphic DNA (PCR-RFLPs) and species-specific primers for DNA amplification and visualisation *via* agarose gel electrophoresis.

PCR-RFLPs are utilised for the identification of eucalypt pathogens such as *Cryphonectria cubensis* and related species (Myburg, Wingfield & Wingfield 1999), and *Mycosphaerella* species on other hosts (Ueng *et al.* 1998). Species-specific primers are also used for the detection of *M. fijiensis* and *M. musicola* on banana (Johanson & Jeger 1993, Johanson *et al.* 1994, Johanson 1995).

The large and expanding size of the genus, the difficulty in identifying the species by conventional means, and the increasing impact of diseases caused by *Mycosphaerella*, highlight the need to develop fast molecular methods for the detection of *Mycosphaerella* species associated with eucalypt diseases. Worldwide, *M. cryptica* and *M. nubilosa* are the two most important causes of MLD. In south-western Australia, *M. lateralis*, *M. marksii* and *M. parva* are also commonly associated with this disease (Maxwell *et al.* 2003). Therefore, the aim of the present study was to develop species-specific primers for the identification and detection of *M. cryptica*, *M. lateralis*, *M. marksii*, *M. nubilosa* and *M. parva*, and to test the sensitivity and specificity of these primers against diseased *E. globulus* leaves.

## MATERIALS AND METHODS

### *Fungal isolates*

Single ascospore isolates of ten *Mycosphaerella* species (Table 1) were obtained from lesions of *E. globulus* from south-western Australia following a method adapted from Crous (1998). These were identified on morphological characters (Crous 1998, Maxwell *et al.* 2003) and maintained on 2% malt extract agar (MEA; Difco, Sparks, MD). DNA was extracted from these isolates, ITS rDNA sequenced for species-specific primer development, and then these primers were tested against the DNA extract. Also, the primer pairs were tested against *Mycosphaerella* cultures and DNA obtained from collections in eastern Australia and from Vietnam as indicated in Table 1. In all, 48 *Mycosphaerella* species from eucalypt and non-eucalypt hosts worldwide, including DNA sequences obtained in the current study, were aligned (An1085729532; EMBL database) and used to screen for unique primer binding sites in the development of species-specific primers.

### *DNA extraction and sequencing*

Axenic mycelia were obtained and DNA extracted. Briefly, microfuge tubes containing lyophilized mycelia were immersed in liquid nitrogen and the mycelia then ground into fine powder with an electric pellet mixer (Kontes, Vineland, NJ). Extraction buffer (300 µl; 200 mmol Tris HCl, pH 8.5, 250 mmol NaCl, 25 mmol EDTA and 0.5% SDS; Raeder & Broda 1985) was added to each microfuge tube containing up to 200 µl volume of ground mycelia, mixed and incubated for 1–2 h at 65 °C and then centrifuged at 13 200 ***g*** (Beckman Microfuge E, Fullerton, CA) for 10 min. DNA from the resulting supernatant was purified using the UltraClean (Mo Bio, Carlsbad, CA) silica binding kit according to the manufacturer's instructions. The DNA concentration was determined using a Hoefer DyNA Quant 200 fluorometer.

The extracted DNA was stored at −20 ° until required for PCR reactions and for the testing of species-specific primers. Initial PCR reactions for ITS sequencing were conducted using the primers ITS1f and ITS4 (White *et al.* 1990, Gardes & Bruns 1993) as described below. Amplification solutions (50 µl) were made aseptically in sterile 200 µl microfuge tubes, containing; 1–5 ng genomic DNA, 0.2 µM primer, 2.5 mM $MgCl_2$ (Fisher Biotech Australia, West Perth), 2.5 U *Tth* plus polymerase (Biotech International), 1 × polymerisation buffer (Biotech International) equivalent to 67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg $ml^{-1}$ gelatin, 0.2 mM dNTPs and sterile, deionised water to make up the reaction volume of 50 µl. These solutions were vortexed for 1–2 s, then centrifuged for 5 s at 13 200 ***g***. The PCR reactions were performed (Applied Biosystems, Foster City, CA; Gene Amp 9600 thermocycler) according to the following parameters: Initial denaturing step of 96 ° for 2 min; then 30 cycles of 94 ° (30 s) denaturing, 55 ° (30 s) annealing, 72 ° (2 min) extension; this was followed by a 7 min extension cycle at 72 °, then a hold cycle at 10 °. Products of the PCR reaction were stored at 4 ° prior to cleaning and sequencing. The isolates and species tested for species-specific primers are listed (Table 1).

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

Sequence products were separated by electrophoresis on 5 % acrylamide gels, which were then washed for 10 min with 1 litre of 20 % ethanol, stained and exposed to Kodak SB film. The DNA sequence data was read by an electronic digitiser and aligned by LaserGene (v. 1.60dz) using the CLUSTAR V method. The sequencing of the DNA template was conducted using the software package, Sequencase (v. 2.0).

### *Phylogenetic analysis*

The forward and reverse sequence data for each isolate were edited and aligned with SEQUED (v. 1.04; PE Applied Biosystems, Foster City, CA) or GeneTool (v. 1.0; Oakland, CA) with manual adjustments where necessary. Additional *Mycosphaerella* sequence data from all species recorded from eucalypts were downloaded from GenBank. *Botryosphaeria rhodina* and *Dothidea insculpta* were chosen as out-groups on the basis that they are sister taxa with differing degrees of affinity to *Mycosphaerella*, within the *Dothideales*.

Sequence data from all species were aligned in Clustal W, saved in 'gcg msf' and in 'Phylip' format and optimised manually. Any large insertions opposite contiguous gaps were assumed to be due to single or few insertion events. Therefore these insertions were reduced to the first five bases (*cfr* Greenwood *et al*. 1991). In order to optimise the alignment it was necessary to exclude a 180 bp insertion in the *M. gregaria* sequence. The sequence alignment (An1085715770) for Fig. 1 is available in the EMBL database.

Aligned sequence data were imported into PAUP (v. 4.1b; Swofford 1998) and the most parsimonious trees constructed using the heuristic search option. The initial set of trees was obtained by the 'simple' option for the stepwise addition of taxa and these trees evaluated *via* the tree bisection reconstruction (TBR) method of branch swapping. Bootstrap analysis (10 000 replications) was used to evaluate the confidence of the branch nodes of the trees (Felsenstein 1985). In order to determine the robustness of the tree topologies derived via parsimony analysis, phylogenetic trees were also obtained using a distance method (neighbour-joining; Satou & Nei 1987) program in PHYLIP (Felsenstein 1995). The phylogenetic structure of *Mycosphaerella* species on *Eucalyptus* was investigated through a comparison of all unique sequences of isolates from this study with other *Mycosphaerella* species occurring on *Eucalyptus* elsewhere.

### *Species-specific primer development*

The consensus sequences for the ITS1f/ ITS4 rDNA region for each of the ten species of *Mycosphaerella* sequenced in the current study were imported, along with all other available *Mycosphaerella* species on the National Centre for Biotechnology Information (NCBI) GenBank database, into Gene Tool (Table 1). This included species from diverse hosts and locations worldwide. Forward and reverse primers were designed in the 'sequence editor' module of Gene Tool for *M. cryptica*, *M. lateralis*, *M. marksii*, *M. nubilosa*, and *M. parva*. Primer sites were chosen from the variable (ITS-1 or ITS-2) regions of the rDNA that were within the size range 17–23 nt and with a Tm of 55–65 °, that were free of structural impediments to annealing, and that would amplify a product of 300–400 nt. These were screened against all available sequences of each species worldwide in order to ensure their activity against their known populations. All other *Mycosphaerella* species on the NCBI database, including isolates from many regions of the world, were downloaded, aligned (An1085729532; EMBL), and then searched for matches to those primer sites to ensure species specificity. The basic local alignment search tool (BLAST) software available on the NCBI database was used to ensure that the primer sites were not present on other fungal species associated with eucalypts, or in the host plant DNA. Forward and reverse primers specific to *M. cryptica* (MC2F and MC2R), *M. lateralis* (ML1F and ML1R), *M. marksii* (MM1F and MM1R), *M. nubilosa* (MN1F and MN1R) and *M. parva* (MP1F and MP1R) (Table 2) were chosen and tested against DNA extracts from each of the species listed in Table 1.

### *Testing of species-specific primers on fungal DNA*

PCR reactions were performed on DNA from *Mycosphaerella cryptica* (11 isolates), *M. lateralis* (8), *M. marksii* (22), *M. nubilosa* (14), and *M. parva* (11).

**Table 1.** *Mycosphaerella* isolates for which ITS rDNA sequences were screened and tested for species-specific primer development.

| *Mycosphaerella* species | Isolate no. (MURU)[a] | GenBank accession no. | Host (*Eucalyptus*) | Origin[b] | Isolates sequenced (S) and DNA primers tested (T) against |
|---|---|---|---|---|---|
| *M. ambiphylla* | 3 | | *E. globulus* | WA | S, T |
| *M. ambiphylla* | 211 | AY150675 | *E. globulus* | WA | S, T |
| *M. aurantia* | 1 | AY509743 | *E. globulus* | WA | S |
| *M. aurantia* | 2 | AY509744 | *E. globulus* | WA | S |
| *M. aurantia* | 151 | AY 150331 | *E. globulus* | WA | S, T |
| *M. aurantia* | 152 | AY509742 | *E. globulus* | WA | S |
| *M. aurantia* | 340 | AY509742 | *E. globulus* | WA | T |
| *M. citri* | 251 | | *E. camaldulensis* | VTN | T |
| *M. colombiensis* | 352 | | *E. camaldulensis* | VTN | T |
| *M. cruenta* | 353 | | *E. camaldulensis* | VTN | T |
| *M. cryptica* | 089 | AY509747 | *E. globulus* | WA | S, T |
| *M. cryptica* | 090 | AY509748 | *E. globulus* | WA | S, T |
| *M. cryptica* | 091 | AY509749 | *E. globulus* | WA | S, T |
| *M. cryptica* | 101 | AY509750 | *E. globulus* | WA | S, T |
| *M. cryptica* | 102 | | *E. globulus* | WA | S, T |
| *M. cryptica* | 110 | AY509751 | *E. globulus* | WA | S, T |
| *M. cryptica* | 114 | AY509752 | *E. diversicolor* | WA | S, T |
| *M. cryptica* | 115 | AY509753 | *E. globulus* | WA | S, T |
| *M. cryptica* | 117 | | *E. grandis* × *camaldulensis* | Qld | S, T |
| *M. cryptica* | 118 | AY509754 | *E. delegatensis* | Vic | S, T |
| *M. cryptica* | 120 | | *E. globulus* | Vic | S, T |
| *M. cryptica* | 145 | | *E. diversicolor* | WA | S |
| *M. fori* | 324 | | *E. globulus* | WA | T |
| *M. gregaria* | 237 | AY509755 | *E. globulus* | WA | S, T |
| *M. gregaria* | 240 | AY509757 | *E. globulus* | WA | S |
| *M. gregaria* | 246 | AY509756 | *E. globulus* | WA | S |
| *M. lateralis* | 252 | AY509758 | *E. maidenii* | Qld | S, T |
| *M. lateralis* | 253 | AY509761 | *E. maidenii* | Qld | S, T |
| *M. lateralis* | 254 | AY509760 | *E. globulus* | WA | S, T |
| *M. lateralis* | 255 | AY509759 | *E. globulus* | WA | S, T |
| *M. lateralis* | 256 | | *E. globulus* | WA | T |
| *M. lateralis* | 257 | AY509762 | *E. globulus* | WA | S, T |
| *M. lateralis* | 258 | AY509763 | *E. globulus* | WA | S, T |
| *M. lateralis* | 177 | | *E. globulus* | WA | T |
| *M. marksii* | 234 | AY509764 | *E. globulus* | WA | S, T |
| *M. marksii* | 242 | AY509767 | *E. globulus* | WA | S, T |
| *M. marksii* | 243 | AY509766 | *E. globulus* | WA | S, T |
| *M. marksii* | 247 | AY509765 | *E. globulus* | WA | S |
| *M. marksii* | 178 | | *E. globulus* | WA | T |
| *M. marksii* | 179 | | *E. globulus* | WA | T |
| *M. marksii* | 180 | | *E. globulus* | WA | T |
| *M. marksii* | 181 | | *E. grandis* × *camaldulensis* | WA | T |
| *M. marksii* | 182 | | *E. grandis* × *camaldulensis* | WA | T |
| *M. marksii* | 183 | | *E. grandis* × *camaldulensis* | WA | T |
| *M. marksii* | 184 | | *E. grandis* × *camaldulensis* | WA | T |
| *M. marksii* | 185 | | *E. grandis* × *camaldulensis* | WA | T |
| *M. marksii* | 186 | | *E. globulus* | Qld | T |
| *M. marksii* | 187 | | *E. globulus* | Qld | T |
| *M. marksii* | 188 | | *E. pellita* | Qld | T |
| *M. marksii* | 189 | | *E. grandis* | Qld | T |
| *M. marksii* | 190 | | *E. dunnii* | Qld | T |
| *M. marksii* | 191 | | *E. dunnii* | Qld | T |
| *M. marksii* | 192 | | *E. dunnii* | Qld | T |
| *M. marksii* | 193 | | *E. tereticornis* | Qld | T |
| *M. marksii* | 194 | | *E. diversicolor* | WA | T |
| *M. marksii* | 195 | | *E. tereticornis* | Qld | T |
| *M. marksii* | 196 | | *E. rudis* | WA | T |
| *M. mexicana* | 006 | AY509768 | *E. globulus* | WA | S, T |
| *M. mexicana* | 007 | AY509769 | *E. globulus* | WA | S, T |
| *M. mexicana* | 008 | AY509770 | *E. globulus* | WA | S |
| *M. mexicana* | 197 | AY509771 | *E. globulus* | WA | S |
| *M. nubilosa* | 301 | | *E. globulus* | WA | S, T |
| *M. nubilosa* | 302 | AY509775 | *E. globulus* | WA | S, T |
| *M. nubilosa* | 304 | AY509776 | *E. globulus* | WA | S, T |
| *M. nubilosa* | 051 | AY509777 | *E. globulus* | Vic | S, T |

**Table 1.** (*Cont.*)

| *Mycosphaerella* species | Isolate no. (MURU)[a] | GenBank accession no. | Host (*Eucalyptus*) | Origin[b] | Isolates sequenced (S) and DNA primers tested (T) against |
|---|---|---|---|---|---|
| *M. nubilosa* | 052 | | *E. globulus* | Vic | T |
| *M. nubilosa* | 055 | | *E. globulus* | Vic | T |
| *M. nubilosa* | 056 | | *E. globulus* | Vic | T |
| *M. nubilosa* | 057 | AY509778 | *E. globulus* | Vic | S, T |
| *M. nubilosa* | 025 | AY509772 | *E. globulus* | WA | S, T |
| *M. nubilosa* | 026 | AY509773 | *E. globulus* | WA | S, T |
| *M. nubilosa* | 328 | | *E. grandis* × *resinifera* | WA | T |
| *M. nubilosa* | 317 | | *E. grandis* × *resinifera* | WA | T |
| *M. nubilosa* | 329 | | *E. grandis* × *resinifera* | WA | T |
| *M. nubilosa* | 346 | | *E. globulus* × *urophylla* | WA | T |
| *M. parva* | 248 | AY509779 | *E. globulus* | WA | S, T |
| *M. parva* | 012 | AY509780 | *E. globulus* | WA | S |
| *M. parva* | 250 | AY509781 | *E. globulus* | WA | S |
| *M. parva* | 013 | AY509782 | *E. globulus* | WA | S |
| *M. parva*[c] | 204 | | *E. grandis* | Tas | T |
| *M. parva* | 170 | | *E. globulus* | WA | T |
| *M. parva* | 171 | | *E. globulus* | WA | T |
| *M. parva* | 172 | | *E. globulus* | WA | T |
| *M. parva* | 173 | | *E. grandis* × *camaldulensis* | WA | T |
| *M. parva* | 174 | | *E. grandis* × *camaldulensis* | WA | T |
| *M. parva* | 175 | | *E. grandis* × *camaldulensis* | WA | T |
| *M. parva* | 176 | | *E. dunnii* | Qld | T |
| *M. parva* | 213 | | *E. globulus* | WA | S, T |
| *M. parva* | 337 | | *E. grandis* × *urophylla* | WA | T |
| *M. suberosa* | 263 | | *E. globulus* | WA | S, T |
| *M. suberosa* | 245 | | *E. globulus* | WA | S, T |
| *M. suttoniae* | 327 | | *E. globulus* | WA | T |
| *M. tasmaniensis* | 323 | | *E. globulus* | WA | T |
| *M. vespa* | 200 | | *E. globulus* | Tas | T |

[a] MURU, culture collection of Murdoch University.
[b] Origin of isolates: Vietnam (VTN), Western Australia (WA), Queensland, Australia (Qld), Victoria, Australia (Vic) and Tasmania, Australia (Tas).
[c] As *M. grandis*.

The isolates of *M. cryptica*, *M. lateralis*, *M. marksii*, *M. nubilosa* and *M. parva* for which the species-specific PCR primers were tested were from geographically dispersed locations and from four, two, eight, three, and four different host species, respectively (Table 1). To ensure against 'false-positive' amplification, the primer pairs, were tested against isolates of each of an additional 16 closely related non-target *Mycosphaerella* species as listed in Table 1. Template DNA (1 ng) from each species was added to separate PCR reactions.

PCR reactions were performed as described previously for the ITS1f and ITS4 primers, with the exception that the primer pairs used were MC2F/MC2R, ML1F/ML1R, MM1F/MM1R, MN1F/MN1R and MP1F/MP1R (Table 2) and the annealing temperature was set in a 'touchdown' format as 68 ° for two cycles, 66 ° for four cycles, 64 ° for eight cycles, 63 ° for eight cycles, and 62 ° for eight cycles.

To confirm the presence and size of the PCR products, they were separated using electrophoresis on a 1% agarose gel in 40 mM Tris-HCl acetate pH 8.5, 1 mM EDTA buffer (TAE) at 90 V for 30–60 min. In scoring these reactions, 6 µl of product was loaded into individual lanes. However, for the purpose of visualisation on the agarose gels used, the products of the reactions of each of the non-target species were combined in a single lane for each of the primer pairs.

The size of the DNA bands, were determined against a 100 bp DNA marker (Geneworks, Adelaide) as molecular weight standard. DNA fragments were visualised under UV following gel staining with ethidium bromide (0.5 µg $ml^{-1}$) for 20 to 30 min and de-staining in 1 × TAE buffer for 10 min. Products from selected isolates were then sequenced to confirm that they matched the ITS ribosomal region for the target species. Sequencing was performed as described previously with the exception that each of the species-specific primers were used rather than the generic ITS primers.

Products generated by the primer pair MC2F/MC2R were further characterised through their digestion with the restriction enzyme *Sacc* II according to the manufacturer's instructions (Fermentas; Hanover, MD). Digested products were separated via electrophoresis on a 1.5% agarose gel at 90 V for 120 min, stained with ethidium bromide and visualised under UV light as previously described.

**Table 2.** List of species-specific primers developed for five *Mycosphaerella* species causing disease of eucalypt foliage.

| Species | Primer name | Direction | Sequence (5′–3′) | Length (nt) | Tm (° C) |
|---|---|---|---|---|---|
| *M. cryptica* | MC2F | Forward | cccgcccgacctccaacc | 18 | 58 |
| *M. cryptica* | MC2R | Reverse | cggtcccggaagcgaaacag | 20 | 58 |
| *M. lateralis* | ML1F | Forward | aaacgccggggccttcg | 17 | 54 |
| *M. lateralis* | ML1R | Reverse | cgacgtctccgccgatgttttcc | 23 | 61 |
| *M. marksii* | MM1F | Forward | cggcccgacctccaacc | 17 | 57 |
| *M. marksii* | MM1R | Reverse | gatgccacaacgctcggaga | 20 | 55 |
| *M. nubilosa* | MN1F | Forward | gcgccagcccgacctcc | 17 | 57 |
| *M. nubilosa* | MN1R | Reverse | ggtccccgtcagcgaaacagt | 21 | 56 |
| *M. parva* | MP1F | Forward | cctccgggctcgacctcca | 19 | 60 |
| *M. parva* | MP1R | Reverse | tctcgcaagcggatgattaaacc | 23 | 55 |

### *Species-specific primer sensitivity*

The sensitivity of the primer pairs was tested in PCR reactions against known amounts of DNA for each target species in a dilution series. PCR reactions were conducted with the following amounts of DNA template: 10 ng, 1 ng, 100 pg, 10 pg and 1 pg for three isolates of each species: *Mycosphaerella cryptica* (MURU 089–091) *M. lateralis* (MURU 253–255), *M. marksii* (MURU 234, 242, 243), *M. nubilosa* (MURU 301, 302, 304), and *M. parva* (MURU 012, 013, 250) in 25 μl reactions as described previously. Products were separated via electrophoresis, stained and visualised under UV as described in the specific primer protocol.

### *Testing of species-specific primers against leaf material*

Non-diseased leaves and leaves with lesions typical of *Mycosphaerella cryptica*, *M. marksii*, *M. nubilosa* and multiple species infection were collected from three *Eucalyptus globulus* plantations in Western Australia. Lesion pieces ranging from 2–50 mm² were dissected from leaves that had been washed in a solution of sodium hypochlorite (2.5 % w/v) for 30 s and rinsed three times in deionised water in order to remove surface spores. DNA was extracted and quantified and adjusted as described previously.

Firstly the sensitivity of the PCR-based technique was tested using *M. nubilosa*-specific primer-pair (MN1F and MN1R) against DNA extracts from three replicate *E. globulus* leaves in each of the following four categories: non-infected unexpanded leaves at the growing tip of the branch; sub-symptomatic lesions characterised by a small circular shaped loss of the waxy bloom on the leaf surface; pale yellow-green to necrotic lesion without pseudothecia; necrotic lesion with abundant pseudothecia. PCR reactions (25 μl) were conducted using 10 ng, 1 ng, 100 pg, 10 pg and 1 pg of template DNA as described previously for the species-specific primer protocol.

Secondly, PCR reactions (25 μl with 1 ng template DNA) were conducted to test the effectiveness of the five *Mycosphaerella*-specific primers on DNA extracted from ten lesions from the following five categories: non-infected leaves at the growing tip of the branch; *M. cryptica* lesions characterised by ascomata densely arranged on both surfaces of young leaves with a waxy bloom; *M. marksii* lesions characterised by ascomata only on the adaxial leaf surface of young leaves; *M. nubilosa* lesions characterised by lesions only on the abaxial leaf surface of young leaves; mixed species lesions characterised by lesions on older leaves without a waxy bloom with pseudothecia densely arranged on the abaxial surface and more sparsely arranged on the adaxial surface. A 100 mm² piece of each lesion was dissected and single spore isolations made according to the method of Crous (1998) in order to identify the *Mycosphaerella* species associated with each lesion using conventional means.

The products from the *Mycosphaerella*-species specific PCR reactions using DNA extracted from leaves were separated on agarose gels and visualised as previously described. Products from these reactions were purified and sequenced in 10 μl reactions using their respective specific primers as described previously. Each sequence was used in a BLAST search to identify those sequences that were most homologous on the database.

## RESULTS

### *Phylogenetic analysis*

In the phylogenetic analysis of ITS sequences, the morphological species of *Mycosphaerella* isolated from *Eucalyptus* in the current study, grouped with their corresponding sequences, where they were available on GenBank (Fig. 1). The sequences for *M. cryptica* clustered in a distinct group (Fig. 1). The *M. cryptica* isolates sequenced in the current study clustered with the *M. cryptica* isolate from Chile (Fig. 1) despite the Chilean sequence being the most divergent sequence for this species on Genbank, and differs from the consensus of the remaining isolates at 28 nucleotide positions. Three of the four remaining fully resolved *M. cryptica* ITS sequences available on GenBank were identical with the sequences from the current study, and the other one differed at two of 539 nucleotide sites (data not shown).

The south-western Australian *M. marksii* isolates clustered with the GenBank sequence of *M. marksii*.

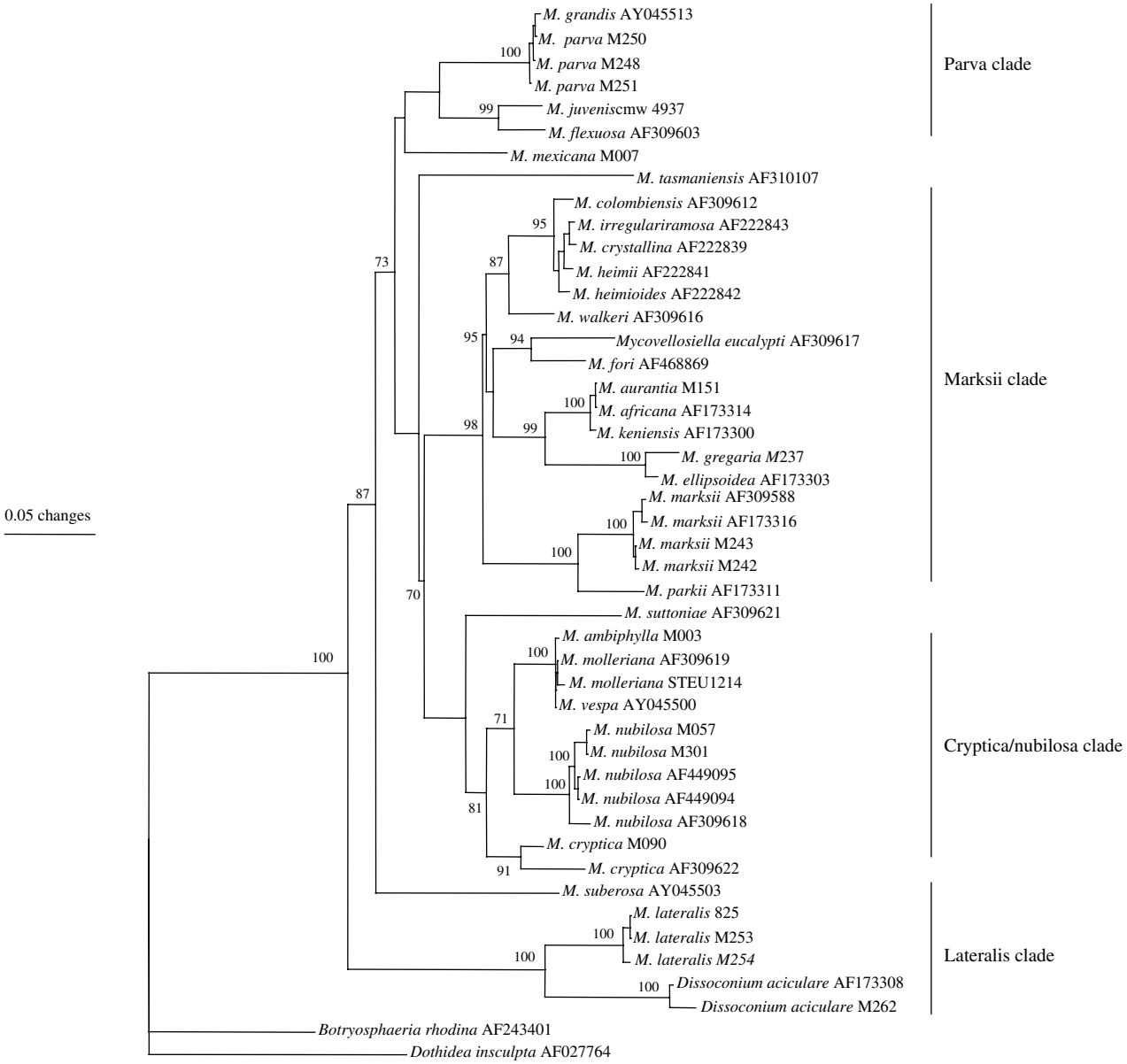


**Fig. 1.** Phylogenetic tree of *Mycosphaerella* species from *Eucalyptus* based on distance analysis (neighbour-joining; PHYLIP) of ITS rDNA sequences. Bootstrap (10 000 replicates) values over 70 indicated.

The *M. parva* isolates from the current study clustered with the published sequence for *M. grandis* (Fig. 1). There was greater than 99% similarity between all *M. parva* and *M. grandis* isolates (data not shown).

The target species for specific primer development in the current study mostly occurred in separate clades of related species (Fig. 1). The exceptions to this were *M. cryptica* and *M. nubilosa* which occurred in the same clade of related species that included *M. vespa* and *M. ambiphylla*.

The *M. lateralis* clade was the most distant clade from the remaining *Mycosphaerella* species. *M. lateralis* isolates from south-western Australia formed a separate group within the same clade as *M. lateralis* isolates from eastern Australia and elsewhere. The *M. parva* clade also contained *M. juvenis* and *M. mexicana* and was distinct from the remaining clades.

*M. marksii* was most similar to *M. parkii* and comprised the largest of the clades in this study. Other species in this clade include *M. aurantia*, *M. colombiensis*, *M. fori*, and *M. gregaria*.

### *Species-specific primer development and testing*

A BLAST search for the sequence sites for each of the primer pairs MC2F and MC2R; ML1F and ML1R; MM1F and MM1R; and MN1F and MN1R found no 100% matches of both sets of primers in a primer pair with non-target fungal or plant DNA. The primer pair MP1F and MP1R did not match any DNA sequences from the non-target *M. parva*, except for *M. grandis*, which have identical or almost identical ITS sequences to *M. parva* and is believed to be conspecific with *M. parva* based on

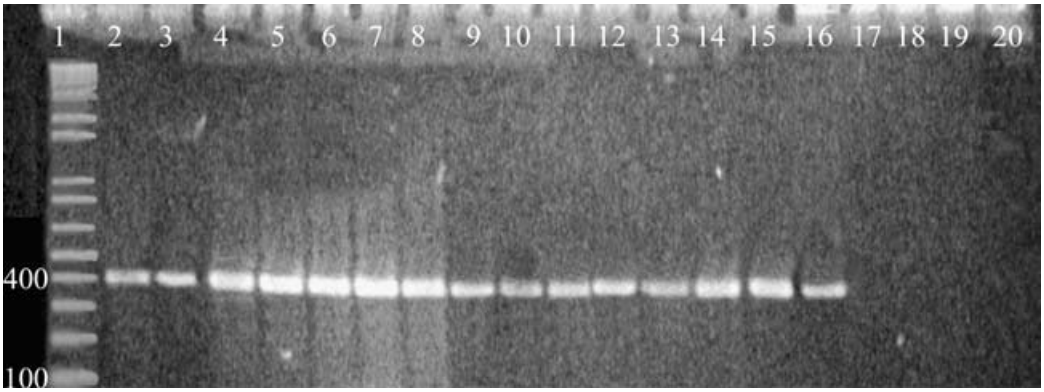


**Fig. 2.** Agarose gel (1%) of PCR product from *Mycosphaerella* DNA using primers specific for either *M. cryptica* or *M. nubilosa*. Lane 1, 1 Kb+DNA ladder, 100 and 400 bp fragments indicated. Lanes 2–9 *M. nubilosa* (MURU301, MURU302, MURU304, MURU051, MURU055–057) amplified with MN1F & MN1R primers. Lanes 10–16 *M. cryptica* (MURU089–90, MURU102, MURU114, MURU115, MURU118, MURU120) amplified with MC2F & MC2R primers; L17 Negative controls *M. ambiphylla*, *M. aurantia*, *M. citri*, *M. colombiensis*, *M. cruenta*, *M. gregaria*, *M. lateralis*, *M. marksii*, *M. mexicana*, *M. parva*, *M. suberosa*, *M. suttoniae*, *M. tasmaniensis*, and *M. vespa* with MC2F & MC2R; L18 *M. ambiphylla*, *M. aurantia*, *M. citri*, *M. colombiensis*, *M. cruenta*, *M. cryptica*, *M. gregaria*, *M. lateralis*, *M. marksii*, *M. mexicana*, *M. parva*, *M. suberosa*, *M. suttoniae*, *M. tasmaniensis*, and *M. vespa* combined with MN1F, MN1R primers. Lanes 19–20 are blank. Negative control isolates listed (T) in Table 1.

morphological and molecular data (Crous 1998, Maxwell 2004).

PCR reactions resulted in products of 402, 432, 306, 404 and 407 bp by the MC2 ML1, MM1, MN1and MP1 primer pairs respectively (Figs 2–4). Primer pairs ML1F and ML1R; MM1F and MM1R; MN1F and MN1R; and MP1F and MP1R amplified a product for DNA extracted from all target species isolates, those being *M. lateralis*, *M. marksii*, *M. nubilosa* and *M. parva* respectively. There were no false positive amplifications of the 16 non-target *Mycosphaerella* species that these four primer pairs were tested against (Figs 2–3). The primer pair MC2F and MC2R only amplified DNA from the target species *M. cryptica* and the non-target species *M. nubilosa*. However, the products from these two species were differentiated on the basis of their restriction profiles using the enzyme *Sacc* II (Fig. 4). There was no false positive amplification of the remaining 15 non-target *Mycosphaerella* species for the primer pair MC2F and MC2R (Fig. 2).

### *Species-specific primer sensitivity against DNA extract*

In sensitivity tests against target fungal DNA extract, MC2F/MC2R was able to detect DNA at the upper limit of 1 ng and the remaining primer pairs were able to detect DNA at the highest range tested of 10 ng per 25 µl reaction. Primer pairs MC2F/MC2R and MM1F/MM1R detected DNA at the lowest concentration tested of 1 pg per reaction. The lowest detection limit of the remaining primer pairs was 10 pg of DNA per 25 µl reaction. This result was consistent across the three isolates tested for each species. The intensity of banding pattern for each of the primers is illustrated for the DNA template amounts 1 pg to 1 ng in Fig. 5. It is evident from Fig. 5 that the intensity of the product at 1 ng is lower than that at 100 pg for MC2F/MC2R, whereas for the remaining primers the intensity of the band from greatest DNA template amount was equal to or higher than all other amounts. The PCR product band intensity generally decreased with decreasing amount of DNA template.

### *Detection of target species in leaf material*

The sensitivity of the MN1F/MN1R primer pair to the target *Mycosphaerella nubilosa* DNA in lesions varied according to the type of lesion (Table 3). PCR products resulted from 10 pg of template DNA from highly diseased lesions, whereas a mean of 40 pg and 1 ng of DNA was required for moderate and sub-chlorotic diseased lesions, respectively. No product resulted from amplification of DNA from non-diseased, unexpanded *Eucalyptus globulus* leaf tips by the primer pair MN1F/MN1R.

All five primer pairs were able to detect each of their target species from DNA extract of lesions (Fig. 6, Table 4). Sequencing of the PCR products from each of the primer pairs confirmed their identity as belonging to each of their respective target species on the basis that they shared more than 99% sequence homology (Table 4). Frequently more than one species was associated with a particular lesion type. For example, specimen MURU031 gave products for *M. lateralis* and *M. marksii*. Conventional isolation techniques from these 20 specimens only yielded three species, *M. marksii*, *M. nubilosa* and *M. cryptica*, for specimens MURU031, 037 and 094 respectively. In this study only a single species was isolated from each of these lesions. Unlike the conventional isolations in this study, *Mycosphaerella* species were identified according to PCR products generated from all of the lesions tested. PCR products were not generated from any non-infected leaf tips by any of the five species-specific primer pairs.

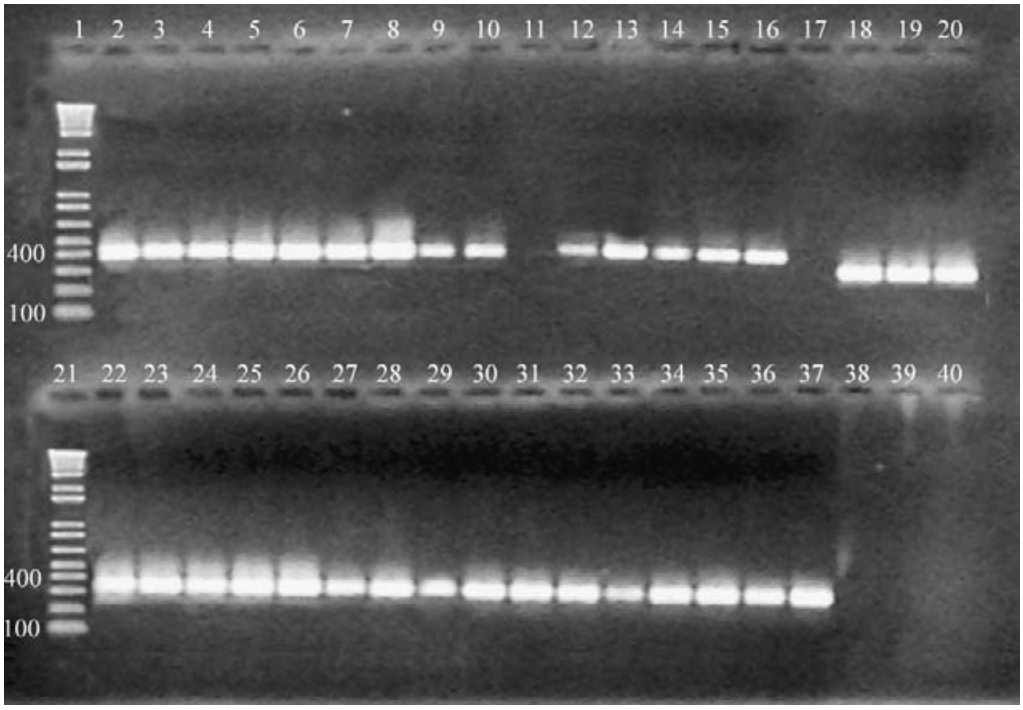


**Fig. 3.** Agarose gel (1 %) of purified PCR product from *Mycosphaerella* DNA using primers specific for *M. parva*, *M. lateralis* or *M. marksii*. Lanes 1 and 21, 1 Kb + DNA ladder, bands at 100 and 400 bp indicated. Lanes 2–10 *M. parva* (MURU170–176, MURU204, MURU213) amplified with MP1F & MP1R. Lane 11 negative control of *M. ambiphylla*, *M. aurantia*, *M. citri*, *M. colombiensis*, *M. cruenta*, *M. cryptica*, *M. gregaria*, *M. lateralis*, *M. marksii*, *M. mexicana*, *M. nubilosa*, *M. suberosa*, *M. suttoniae*, *M. tasmaniensis*, and *M. vespa* amplified with MP1F & MP1R; Lanes 12–16 *M. lateralis* (MURU177, MURU253, MURU256–258) amplified with ML1F & ML1R. Lane 17 negative control of *M. ambiphylla*, *M. aurantia*, *M. citri*, *M. colombiensis*, *M. cruenta*, *M. cryptica*, *M. gregaria*, *M. marksii*, *M. mexicana*, *M. nubilosa*, *M. parva*, *M. suberosa*, *M. suttoniae*, *M. tasmaniensis*, and *M. vespa* amplified with ML1F & ML1R. Lanes 18–37 *M. marksii* (MURU178–196) amplified with MM1F & MM1R. Lane 38 negative control of *M. ambiphylla*, *M. aurantia*, *M. citri*, *M. colombiensis*, *M. cruenta*, *M. cryptica*, *M. gregaria*, *M. lateralis*, *M. mexicana*, *M. nubilosa*, *M. parva*, *M. suberosa*, *M. suttoniae*, *M. tasmaniensis*, and *M. vespa* amplified with MM1F & MM1R. Lanes 39–40 are blank. Isolates used as negative controls are listed as tested (T) in Table 1.

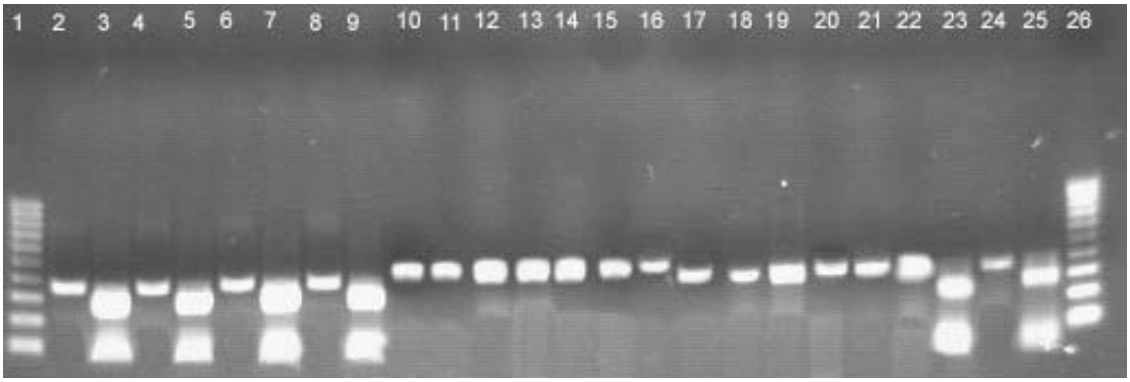


**Fig. 4.** Agarose gel (1·5 %) of restriction digest (*Sacc II*) profile of PCR products of *Mycosphaerella cryptica* and *Mycosphaerella nubilosa* DNA amplified with the primer pair MC2F & MC2R. Lanes 1 and 26 show a 100 bp DNA ladder. Lanes 2–9 *M. nubilosa* amplified with MC2F & MC2R; Lanes 2, 4, 6 and 8 are non-digested PCR products, Lanes 3, 5, 7 and 9 are digested PCR products of isolates MURU051, MURU055–057 respectively. Lanes 10–19 *M. cryptica* amplified with MC2F and MC2R. Lanes 10, 12, 14, 16 and 18 are undigested PCR products and Lanes 11, 13, 15, 17, and 19 are digested PCR products of isolates MURU089–90, MURU102, MURU114, MURU115 respectively. Lanes 20–21 are non-digested (L20) and digested (L21) PCR products of DNA extracted from *M. cryptica* infected *Eucalyptus globulus* leaves (MURU094). Lanes 22–25 are non-digested (L22, L24) and digested (L23, L25) PCR products of DNA extracted from *M. nubilosa* infected *E. globulus* leaves (MURU037, MURU039). Isolates are listed in Table 1.

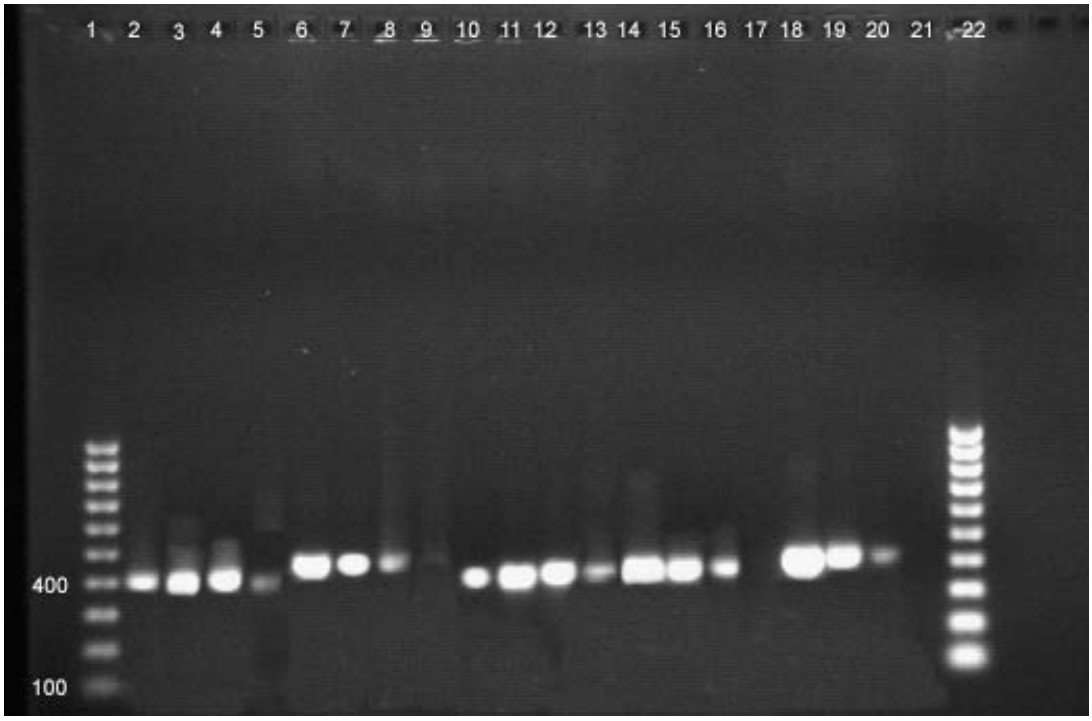


**Fig. 5.** Agarose gel (1 %) indicating the sensitivity of PCR primer pairs MC2F/MC2R, ML1/ML1R, MM1/MM1R, MN1/MN1R, MP1/MP1R specific for *Mycosphaerella cryptica* (L2–5), *M. lateralis* (L6–9), *M. marksii* (L10–13), *M. nubilosa* (L14–17) and *M. parva* (L18–21) respectively. DNA template amounts were 1ng (L2, L6, L10, L14 and L18), 100 pg (L3, L7, L11, L15, L19), 10 pg (L4, L8, L12, L16 and L20) and 1 pg (L5, L9, L13, L17 and L21). Lanes 1 and 22 a 100 bp DNA ladder with the 400 and 100 bp fragments indicated on the left of the gel. Lanes 1 and 22 100 bp ladder.

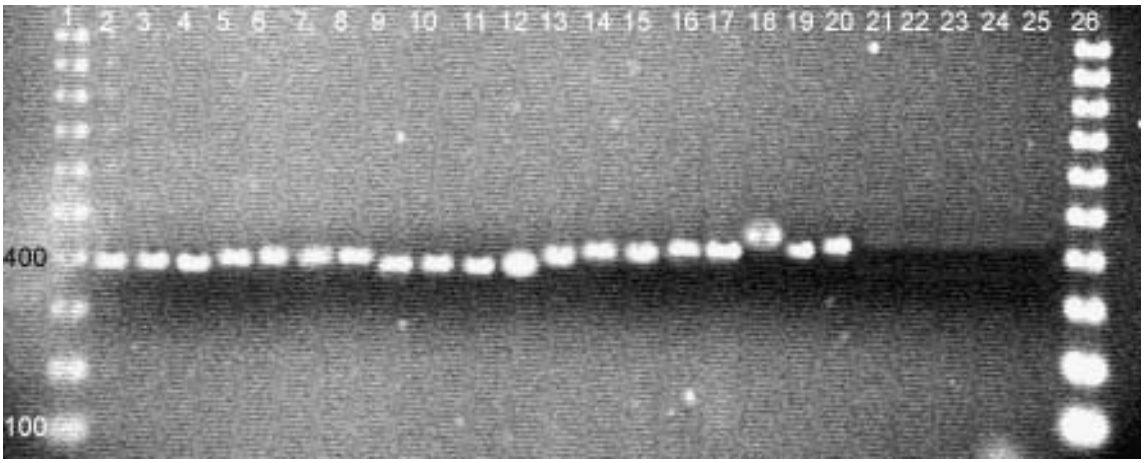


**Fig. 6.** Agarose gel (1 %) indicating the activity of PCR primer pairs MC2F/MC2R, ML1F/ML1R, MM1F/MM1R, MN1F/MN1R, MP1F/MP1R on DNA extracted from diseased *Eucalyptus globulus* leaves. Primer pairs MC2F/MC2R products amplified from sample leaves MURU 035, 037, 039, 094, 100, 149, 168 (L2–8); MM1F/MM1R amplified products from sample leaves MURU 031, 033, 035, 100 (L9–12); MN1F/MN1R amplified products from sample leaves MURU 035, 037, 039, 097 and 168 (L13–17); ML1F/ML1R amplified products from sample leaf MURU 031 (L18); MP1F/MP1R amplified products from sample leaf MURU 033 and 035 (L19–20); MC2F/MC2R, ML1F/ML1R, MM1F/MM1R, MN1F/MN1R, MP1F/MP1R on DNA extracted from non-diseased *E. globulus* leaves, respectively (L21–25). Lanes 1 and 26 100 bp ladder.

## DISCUSSION

Primers that selectively amplify DNA from *Mycosphaerella cryptica*, *M. lateralis*, *M. marksii*, *M. nubilosa*, and *M. parva*, important fungi associated with the *Mycosphaerella* disease complex of eucalypts in Australia were successfully developed in the current study. This will allow the identification of these species by non-specialists in *Mycosphaerella* taxonomy. This specific PCR technique offers advantages over

**Table 3.** Sensitivity of specific primers in detecting *Mycosphaerella nubilosa* in DNA extracted from highly-diseased (HD), moderately-diseased (MD), subchlorotic diseased (S-CD) and non-diseased (ND) *Eucalyptus globulus* leaf lesions.

| Symptom class[a] | Minimum DNA template (mean; $n=3$) |
|---|---|
| HD | 10 pg |
| MD | 40 pg |
| S-CD | 1 ng |
| ND | No product |

[a] HD, Necrotic with abundant pseudothecia; MD, chlorotic or necrotic without ascomata apparent; S-CD, translucent circular loss of wax-cuticle, not yet chlorotic; and ND, no disease symptoms.

**Table 4.** Identification of *Mycosphaerella* species by amplification and sequencing with species-specific primers from DNA extracts of *Eucalyptus globulus* leaves.

| Voucher specimen (MURU) | Symptom class[a] | Specific primers that generated products | NCBI Sequence reference | *Mycosphaerella* sequence homology[b] |
|---|---|---|---|---|
| 031 | marksii | ML1 | AY939544 | *lateralis* |
| 031 | | MM1 | AY939529 | *marksii* |
| 033 | marksii | MM1 | AY939526 | *marksii* |
| 033 | | MP1 | AY939527 | *parva* |
| 035 | marksii | MC2[c] | AY939528 | *nubilosa* |
| 035 | | MM1 | AY939525 | *marksii* |
| 035 | | MN1 | AY939530 | *nubilosa* |
| 035 | marksii | MP1 | NS[e] | NS |
| 037 | nubilosa | MC2[c] | AY939531 | *nubilosa* |
| 037 | | MN1 | AY939532 | *nubilosa* |
| 037 | | MP1 | AY939533 | *parva* |
| 039 | nubilosa | MC2[c] | AY939534 | *nubilosa* |
| 039 | | MN1 | AY939535 | *nubilosa* |
| 094 | cryptica | MC2[d] | AY939536 | *cryptica* |
| 097 | nubilosa | MC2[c] | AY939537 | *nubilosa* |
| 097 | | MN1 | AY939538 | *nubilosa* |
| 100 | mixed | MC2[c] | AY939539 | *nubilosa* |
| 100 | | MM1 | AY939540 | *marksii* |
| | | MP1 | NS | NS |
| 149 | mixed | MC2[c] | AY939541 | *nubilosa* |
| 149 | | MN1 | NS | *nubilosa* |
| 168 | mixed | MC2[c] | AY939542 | *nubilosa* |
| 168 | | MN1 | AY939543 | *nubilosa* |

[a] The symptom class 'cryptica' were lesions characterised by pseudothecia densely arranged on both surfaces of young leaves with a waxy bloom; 'marksii' by pseudothecia only on the adaxial leaf surface of young leaves; 'nubilosa' by lesions only on the abaxial leaf surface of young leaves; mixed species by lesions on older leaves without a waxy bloom with pseudothecia densely arranged on the abaxial surface and more sparsely arranged on the adaxial nubilosa.

[b] *Mycosphaerella* identities based on greater than 99% sequence homology.

[c] Restriction digest of PCR product with *Sacc* II generated 2 bands characteristic of *Mycophaerella nubilosa*.

[d] Restriction digest of PCR product with *Sacc* II generated one band consistent with *Mycosphaerella cryptica*.

[e] NS, Not sequenced.

randomly amplified polymorphic DNA (RAPDs), the previously published molecular method for the identification of *Mycosphaerella* species from eucalypts (Carnegie *et al*. 2001). The use of species-specific primers is more reliable and robust than RAPDs, which are often not reproducible between different laboratories (McDonald & McDermott 1993, Brown 1996).

In addition, these primers have been used in the detection of each of these species through the direct amplification of DNA from infected host plant tissue. The primers from the current study will enable early diagnosis of the causal organisms of MLD in a plantation or forest. These primers will also facilitate studies into *Mycosphaerella* disease development in that the presence of the pathogen may be detected prior to the appearance of symptoms. Studies may be conducted to determine the length of a hemi-biotrophic phase and the extent of tissue colonisation, both spatially and temporally, beyond the necrotic lesion in these *Mycosphaerella* species. Previously, such studies have been hampered by the slow growth rate of these fungi in culture and the lack of media that would allow their selective isolation and detection by directly plating diseased and non-diseased host tissue.

The primers were sensitive to target DNA amounts as low as 1 pg. The activity of the primers against *M. cryptica* DNA at 10 ng or more appeared to be inhibited. This may be due to the pigments formed by *M. cryptica* that are extracted along with the DNA, inhibiting the PCR process. Therefore in using these primers it is necessary to dilute the DNA to the point where the dark pigment is not apparent ($<1$–$5$ ng $\mu l^{-1}$). The primers were also able to detect the target species in lesion DNA extract. In highly diseased leaves that were heavily covered in ascomata the primers were able to detect target *Mycosphaerella* species from 10 pg of template DNA. However in moderate and low levels of disease the primers required a minimum of 40 and 1000 pg of DNA respectively. This is most likely because there is less target-DNA as a proportion of the total DNA extracted from the leaf in the early symptom lesions than in developed lesions. It is significant that *M. nubilosa* was detected in lesions that were barely visible or recognisable as such. These very early symptoms were expressed as a circular-shaped loss of the waxy bloom on the lower leaf surface.

It is possible that some *Mycosphaerella* parasites of *Eucalyptus* occur as endophytes and switch to a pathogenic phase after a period in the leaf or in response to some environmental cue. It is also possible that *Mycosphaerella* species may occur as endophytes and then become saprophytic as leaves die. For example *Mycosphaerella* species are thought to occur as endophytes in the palm *Livistona chinensis* grown in sub-tropical Hong Kong (Guo *et al*. 2000) and in *Fagus sylvatica* throughout Europe (Danti *et al*. 2002). Other than *M. endophytica* (Crous 1998), it is not known what endophytic *Mycosphaerella* species may occur on eucalypts. There are two implications for this. First, there may be undescribed *Mycosphaerella*

endophytes of eucalypts that could be amplified by these primers and give a false positive identification. This can be tested by sequencing the PCR products from leaf material. Second, these primers could be used to detect the presence of the target *Mycosphaerella* species prior to symptoms appearing. Thus they could be used to measure presence and the duration of an endophytic phase.

A comparison of primer specificity with the phylogenetic analysis indicated that, except for *M. cryptica* and *M. nubilosa*, each of the primer target species occurred in separate clades. Each primer was tested against DNA from at least one *Mycosphaerella* species from within the same clade as its target species. As these closely related, non-target species were not amplified this shows that these primers are specific at the species level. The only exception to this was MC2F/MC2R which amplified the non-target *M. nubilosa*. Three other primers specific for *M. cryptica* at the rRNA ITS locus were tested, but they also amplified *M. nubilosa* (data not shown). *M. cryptica* and *M. nubilosa* were only able to be differentiated by an additional restriction digest with *Sacc* II. Because of the close similarity of these two species at this locus it is unlikely that specific primers will be developed for *M. cryptica*. Sequencing of other more variable genes may yield primers that are able to unambiguously identify *M. cryptica* in a single step without the need for restriction digest.

The PCR-based system developed in this current study is useful for detecting *Mycosphaerella* species occurring on eucalypts. The primers were screened against sequences from a range of isolates off diverse hosts sourced from locations worldwide and tested against DNA extracts from isolates collected off a range of host species. Therefore, they are useful against the known diversity of their target species. However the primers are based on available sequence information which includes 31 of the 35 currently described species from eucalypts and additional sequences of 17 species from non-eucalypt hosts. This is from a total of 2000 named *Mycosphaerella* species. Thus there are many taxa for which the specificity of these primers is not known. However, most *Mycosphaerella* species have a narrow host range and therefore these non-sequenced taxa are not likely to occur on eucalypts. Nevertheless, it remains possible that these primers will amplify an unknown non-target species. Therefore, it is desirable where possible to confirm the species identification of products from leaf DNA extract by sequencing the PCR product.

The theoretical specificity of these primers across all fungal and plant taxa was tested through a BLAST query. Matches to non-target species were rare and where they occurred it was to another *Mycosphaerella* species. In no case did both primers in a primer pair match a non-target species. However, theoretical and actual specificity do not always correspond. In the current study the MC2F/MC2R primer pair amplified *M. nubilosa* for which they were not designed and products from this primer pair required a restriction digest to confirm whether *M. cryptica* or *M. nubilosa* was present. Thus despite differences between a primer and the target site, amplification can occur. This is a situation that is not unique to the primers designed in the current study and, as suggested above, identification should be confirmed by sequencing products where possible.

Although *M. cryptica* and *M. nubilosa* are well-studied, the role of other species in the MLD disease complex is not well understood. For example, *M. marksii* and *M. parva* are often isolated from diseased eucalypts, but their ability to cause disease has not been proven (Park & Keane 1982b, Park 1984, Park *et al.* 2000). This is because these fungi are often isolated from lesions that are also colonised by other *Mycosphaerella* species, and hence it is difficult to obtain pure ascospore suspensions of *M. parva* and *M. marksii* for pathogenicity studies. The development of species-specific primers will enable the order and pattern of species colonisation of a leaf to be established. It may be that some of the less commonly isolated species, or those that sporulate on older senescing leaves, infect and cause disease on healthy young leaves, but are slower than other *Mycosphaerella* species to sporulate. These primers could be used to test this hypothesis. They could also be used to confirm the presence of species involved in the disease complex in epidemiological or host resistance studies. Currently, studies into the interaction and succession process of the species involved in the *Mycosphaerella* disease complex in south-western Australia are underway.

## ACKNOWLEDGEMENTS

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## REFERENCES

Berbee, M. L., Yoshimura, A., Sugiyama, J. & Taylor, J. W. (1995) Is *Penicillium* monophyletic? An evaluation of phylogeny in the family *Trichocomaceae* from 18S, 5.8S and ITS ribosomal DNA sequence data. *Mycologia* **87**: 210–222.

Beresford, R. M. (1978) *Mycosphaerella nubilosa* (*Cooke*) *Hansf. on Eucalyptus delegatensis R.T. Baker: further studies of epidemiology in the North Island of New Zealand.* MSc thesis, University of Auckland.

Brown, J. K. M. (1996) The choice of molecular marker methods for population genetic studies of plant pathogens. *New Phytologist* **133**: 183–195.

Carnegie, A. J. (1991) *The susceptibility of certain Eucalyptus species and provenances to infection by Mycosphaerella species and other leaf parasites.* Honours thesis, La Trobe University, Melbourne.

Carnegie, A. J. (2000) *A study of species of Mycosphaerella on eucalypts in Australia and the impact of Mycosphaerella leaf disease on Eucalyptus globulus Labill*. PhD thesis, University of Melbourne.

Carnegie, A. J., Ades, P. K. & Ford, R. (2001) The use of RAPD-PCR analysis for the differentiation of *Mycosphaerella* species from *Eucalyptus* in Australia. *Mycological Research* **105**: 1313–1320.

Carnegie, A. J., Keane, P. J., Ades, P. K. & Smith, I. W. (1994) Variation in susceptibility of *Eucalyptus globulus* provenances to Mycosphaerella leaf disease. *Canadian Journal of Forest Research* **24**: 1751–1757.

Cheah, L. H. (1977) *Aerobiology and epidemiology of Mycosphaerella nubilosa* (*CKE*) *Hansf. in Eucalyptus* spp. MSc thesis, University of Auckland.

Crous, P. W. (1998) *Mycosphaerella* spp. and their anamorphs associated with leaf spot diseases of *Eucalyptus*. *Mycologia Memoir* **21**: 1–170.

Crous, P. W., Hong, L., Wingfield, M. J., Wingfield, B. D. & Kang, J. C. (1999) *Uwebraunia* and *Dissoconium*, two morphologically similar anamorph genera with different teleomorph affinity. *Sydowia* **51**: 155–166.

Danti, R., Seber, T. N., Sanguineti, G., Raddi, P. & Di Lonardo, V. (2002) Decline in diversity and abundance of endophytic fungi in twigs of *Fagus sylvatica* L. after experimental long-term exposure to sodium dodecylbenzene sulphonate (SDBS) aerosol. *Environmental Microbiology* **4**: 696–702.

Felsenstein, J. (1985) Confidence intervals on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Felsenstein, J. (1995) *PHYLIP* (*Phylogeny Inference Package*). Version 3.57c. University of Washington, Seattle.

Ganapathi, A. & Corbin, J. B. (1979) *Colletogloeum nubilosum* sp. nov., the imperfect state of *Mycosphaerella nubilosa* on *Eucalyptus* in New Zealand. *Transactions of the British Mycological Society* **72**: 237–244.

Gardes, M. & Bruns, T. D. (1993) ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhiza and rusts. *Molecular Ecology* **2**: 113–118.

Greenwood, S. J., Schlegel, M., Sogin, M. L. & Lynn, D. H. (1991) Phylogenetic relationships of Blepharissma americanum and Colpoda inflata within the phylum Ciliophora inferred from complete small subunit rRNA gene sequences. *Journal of Protozoology* **38**: 1–6.

Guo, L. D., Hyde, K. D. & Liew, E. C. Y. (2000) Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. *New Phytologist* **147**: 617–630.

Johanson, A. (1995) Detection of banana leaf spot pathogens by PCR. *EPPO Conference on New Methods of Diagnosis in Plant Protection, Wageningen* **25**: 99–107.

Johanson, A., Crowhurst, R. N., Rikkerink, E. H. A., Fullerton, R. A. & Templeton, M. D. (1994) The use of species-specific DNA probes for the identification of *Mycosphaerella fijiensis* and *M. musicola*, the causal agents of Sigatoka disease of banana. *Plant Pathology* **43**: 701–707.

Johanson, A. & Jeger, M. J. (1993) Use of PCR for detection of *Mycosphaerella fijiensis* and *M. musicola*, the causal agents of Sigatoka leaf spots in banana and plantain. *Mycological Research* **97**: 670–674.

McDonald, B. A. & McDermott, J. (1993) Population genetics of plant pathogenetic fungi. *Bioscience* **43**: 311–319.

Maxwell, A. (2004) *The taxonomy, phylogeny and impact of Mycosphaerella species on eucalypts in South-Western Australia*. PhD thesis, Murdoch University, Perth, WA.

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

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

Myburg, H., Wingfield, B. D. & Wingfield, M. J. (1999) Phylogeny of *Cryphonectria cubensis* and allied species inferred from DNA analysis. *Mycologia* **91**: 243–250.

Park, R. F. (1984) *The taxonomy, pathology and epidemiology of Mycosphaerella species associated with leaf diseases of Eucalyptus in Australia*. PhD thesis, La Trobe University, Melbourne.

Park, R. F. & Keane, P. J. (1982a) Three *Mycosphaerella species* from leaf diseases of *Eucalyptus*. *Transactions of the British Mycological Society* **79**: 95–100.

Park, R. F. & Keane, P. J. (1982b) Leaf diseases of *Eucalyptus* associated with *Mycosphaerella* species. *Transactions of the British Mycological Society* **79**: 101–115.

Park, R. F., Keane, P. J., Wingfield, M. J. & Crous, P. W. (2000) Fungal diseases of eucalypt foliage. In *Diseases and Pathogens of Eucalypts* (P. J. Keane, G. A. Kile, F. D. Podger & B. N. Brown, eds): 153–239. CSIRO Publishing, Collingwood.

Raeder, U. & Broda, P. (1985) Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**: 17–20.

Saitou, N. & Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology & Evolution* **4**: 406–425.

Swofford, D. L. (1998) *PAUP: Phylogenetic analysis using parsimony*. Version 4.01. Sinauer Associates, Sunderland, MA.

Ueng, P. P., Subramaniam, K., Chen, W., Arseniuk, E., Wang, L., Cheung, A. M., Hoffmann, G. M., Bergstrom, G. C. & Wang, L. X. (1998) Intra-specific genetic variation of *Stagonospora avenae* and its differentiation from *S. nodorum*. *Mycological Research* **102**: 607–614.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a guide to methods and applications* (M. A. Innis, D. H. Gelfand, J. J. Sninski & T. J. White, eds): 315–322. Academic Press, San Diego.

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