

Development of Nested Polymerase Chain Reaction Detection of *Mycosphaerella* spp. and Its Application to the Study of Leaf Disease in *Eucalyptus* Plantations

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ABSTRACT

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Mycosphaerella leaf disease (MLD) is a serious disease of two of the major eucalypt species grown in temperate regions worldwide, *Eucalyptus globulus* and *E. nitens*. More than 30 species of *Mycosphaerella* have been reported on eucalypts worldwide. Accurate, rapid, and early discrimination of *Mycosphaerella* spp. causing crown damage to *E. globulus* and *E. nitens* will assist the development of sustainable management strategies. This study describes the development, and incorporation in a nested polymerase chain reaction (PCR) approach, of specific primers for the detection and identification of *Mycosphaerella* spp. commonly reported from leaf lesions of *E. globulus* and *E. nitens* in Australia. Primer design was assisted by sequence alignment and phylogenetic analysis of 165 nonredundant sequences from the nuclear ribosomal DNA internal transcribed spacer regions of *Mycosphaerella* and related species. Phylogenetic analysis revealed very high sequence similarity for two taxon groups, *Mycosphaerella grandis* and *M. parva*, and *M. vespa*, *M. ambi-*

phylla, and *M. molleriana*, and primers were designed to differentiate each of the two groups. Three other species, *M. cryptica*, *M. nubilosa*, and *M. tasmaniensis*, were distinct and distinguished by species-specific primers. In double-blind trials, the detection test accurately and rapidly identified *Mycosphaerella* spp. in cultures and discriminated against other pathogens that co-occur in or on *Eucalyptus* leaves, thereby verifying its reliability. The detection test has an internal amplification control in the first-round PCR with fungal-specific primers to raise confidence in test results, particularly to highlight negative results due to PCR inhibition. When applied to DNA extracted from leaf or stem samples either as multiple or single lesions, it detected and identified up to five *Mycosphaerella* spp. or taxon groups in both positively identified and in young (putative) MLD lesions. The samples were 20 mm² or larger in surface area and were collected while undertaking disease rating assessments in an experimental investigation of *Eucalyptus* plantations and regrowth forest. Using nested PCR detection, *Mycosphaerella* spp. were positively identified in 2 days, 1 to 5 months earlier than by classical methods, demonstrating the potential application of this detection test to the early discrimination of MLD components in ecological, epidemiological, and genetic investigations.

Eucalyptus spp. are a major global hardwood pulp crop. *Mycosphaerella* spp. cause severe leaf diseases of temperate *Eucalyptus* plantations in wetter areas of Australia and serious disease in New Zealand, South Africa, Spain, and some regions of Chile (19). Many species appear to have spread to other temperate regions from Australia, and species that also occur in the Australian subtropics appear to have spread to Indonesia, Vietnam, China, and South America (21,58,62). Indigenous temperate Australian forests have mixed eucalypt species where *Mycosphaerella* leaf disease (MLD) generally is of minor significance, except in coppice regrowth in certain Western Australian forests (1,14). In southern Australia, MLD in plantations appears to be caused principally by *Mycosphaerella nubilosa* and *M. cryptica*, although *M. vespa*, *M. tasmaniensis*, *M. parva*, and *M. grandis* occur frequently. *M. marksii* and other species are encountered less often (13,49,51,52,60). The most damaging are reported to be *M. nubilosa* and *M. cryptica* (10–12,14,25,59–62), which cause the most severe leaf disease, resulting in significant crown damage of *E. globulus*, *E. nitens*, and their hybrids (30,39), the most widely

grown temperate plantation species. Crown damage in *E. globulus* in Australia has been reported to range from 10% leaf necrosis to complete defoliation and tree death in locations strongly conducive to disease development (22,23,50,52). MLD has restricted the use of commercially desirable eucalypt species in Australia, New Zealand, and South Africa (28).

Currently, MLD is detected visually by lesion characteristics that also are used in crown damage assessment of plantations (71). Many foliar diseases that have much lower impact also produce symptoms similar to MLD and are difficult to distinguish without experience or training. MLD identification may be confirmed using conventional methods. Conventional methods for positive identification of these and other leaf pathogens involve waiting until lesions produce “typical” reproductive structures or forms, isolating them, and identification of putative pathogens using taxonomic criteria. A challenge for those evaluating *Mycosphaerella* spp. affecting eucalypts is that reproductive structures may or may not form in leaves or cultures and, often, the most readily available identifying characteristics, such as germinating ascospores, are also taxonomically ambiguous (10,15,49,51,59–62). In addition, pseudothecia do not form in axenic cultures of most species and are found only in older lesions or, for a few species, on inoculated aseptic leaves in culture (59,60). Identification is further confounded by the fact that more than one

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Mycosphaerella spp. may be present in a single lesion (43,62). Other limitations of conventional methods include low isolation frequencies of all or even most *Mycosphaerella* spp. from lesions. Detailed sampling does not always deliver all of the *Mycosphaerella* spp. present in each lesion. Hence, crown damage assessment based on lesions is not necessarily attributable to a particular *Mycosphaerella* spp.

Molecular detection of plant pathogens is used in many agricultural spheres to assist decision support systems. These include immunological and DNA and polymerase chain reaction (PCR)-based methods. Immunological detection of *Mycosphaerella brassicicola* from spore traps has been developed to rationalize pesticide applications within crop protection programs (42). However, pathogen discrimination is still dependent on spore production, which may occur at a late stage in development in the disease cycle and by which stage prophylactic intervention may not be a management option. Furthermore, fungicide use may be financially or environmentally prohibitive in tree plantations. Detection at an earlier stage in the fungal life-cycle would be preferable and may permit development of silvicultural options to prevent disease or reduce disease severity (9,11,52). PCR-based methods exploit genetic sequence differences to provide early, rapid, specific, and sensitive detection and identification of pathogens of concern regardless of the presence of reproductive structures. PCR amplification using species-specific primers has been widely used for disease diagnosis in humans, other animals, and plants (2,67). Specific PCR tests have been developed for *M. graminicola* (*Septoria tritici*) in wheat (27) and to distinguish *M. fijiensis* and *M. musicola* from banana leaves (40). PCR detection may target specific genes or anonymous products, such as microsatellites, random amplified polymorphic DNA, or amplified fragment length polymorphism fragments (35,67,68,72). In order to provide simultaneous discrimination for several *Mycosphaerella* spp. of eucalypts with high reproducibility and reliability, a well-characterized DNA region which is likely to be highly stable must be targeted. There are more than 30 species of *Mycosphaerella* on *Eucalyptus* spp., and related anamorphs increase this number to over 50 (5,6,15,17,18,49). In wheat, the *M. graminicola*-specific PCR amplifies a fragment of the β -tubulin gene (27). However, many more species occur in eucalypts than on wheat, and few β -tubulin sequences are currently available for eucalypt species. The specific primers for *M. fijiensis* and *M. musicola* (banana pathogens) were designed to sequence divergence within internal transcribed spacer (ITS)1 (40). In addition, molecular phylogenetic studies in the families Mycosphaerellaceae on Myrtaceae have been based mainly on ITS1 and ITS2 sequences (19,20,33,62,64,70). These studies have provided reliable characters for the discrimination of species for which morphological characters such as spore dimensions are variable within species and overlap among species (62), leading to taxonomic confusion and frequent misidentifications. The value of ITS sequence data is attested to by the numerous *Mycosphaerella* sequences available on public databases. Multiple sequences are available for species causing serious diseases of industrial crops, allowing comparison of geographically diverse isolates from different host species. In addition to providing interspecific discrimination, the ribosomal RNA genes, including the ITS regions, are multicopy, assisting in sensitive detection from plant material. Sensitivity is essential as lesions may have mixtures of many fungal species in addition to several *Mycosphaerella* spp. A PCR test based on ITS sequences exists for some *Mycosphaerella* spp. on eucalypts; however, the published test requires a restriction digestion after PCR, with discrimination based on fragment size (43), adding considerably to the processing and analysis time. Analysis of restriction fragments also may be confounded when multiple species are present, as is often the case in MLD.

Therefore, the primary aim of this investigation was to develop PCR-based systems for direct detection and identification of several

Mycosphaerella spp. from lesions of all ages in eucalypt species as a research tool aimed at refining current knowledge of epidemiology, ecology, and host resistance and to provide the potential for certification of disease-free germ plasm. Although the tests developed here were primarily for use on *Eucalyptus* spp. leaves and stems, they also were intended to be applicable to a wider range of woody species that are potential hosts for *Mycosphaerella* spp. For example, other members of the family Myrtaceae, members of other Myrtales families, and species of the family Proteaceae are actual or potential hosts that co-occur naturally throughout the landscape in Australia with *Eucalyptus* spp. Plantations are grown in close proximity to natural vegetation harboring *Mycosphaerella* spp. in Australia, South Africa, most probably South America, which shares a Gondwanan origin, and Indonesia. In addition to detecting *Mycosphaerella* spp. directly from leaves, a detection system also could be used to verify whether samples collected or isolated during earlier surveys belong to the species or taxon group in which they had been identified according to the taxonomic criteria at the time.

PCR, although fast, sensitive, and capable of being automated, is not without problems. These include enzyme inhibition by substances present in the template DNA, which may cause false negative results. Plant material is particularly notorious for PCR inhibitor compounds, many of which often are co-precipitated during DNA extraction (45). Strategies to overcome PCR inhibition and increase confidence in negative results include using a range of DNA template dilutions (40), spiking of negative samples (40), buffer additions during the DNA extraction process (4,34,63) magnetic DNA capture (4,45,63), post-extraction purification (44), and the use of alternative polymerases (3). In the interests of speed, economy, and wide applicability of the tests, it is desirable to avoid repetitious assays or lengthy and involved DNA purification techniques. A rapid, simple DNA purification method based on binding of DNA to silica in the presence of chaotropic salts (31) avoids the use of toxic and volatile reagents and has been used extensively in our laboratory for obtaining amplifiable DNA from a wide range of material. Nested PCR also has been used to amplify DNA containing inhibitors from plant material (53,75). Because none of these strategies for overcoming PCR inhibition were completely successful, detection of PCR inhibition is important to raise confidence in negative results. This can be achieved by the use of internal amplification controls (IACs). Amplification of endogenous IACs using a different primer pair has been shown to be unreliable in detecting PCR inhibition (37); therefore, we constructed an exogenous IAC that can be added at a controlled concentration to each sample in the first round of a nested PCR. Specific primers were used in the second-round PCR to detect the presence of five of the most commonly reported *Mycosphaerella* spp. in southern Australian plantations. The results of a survey of *Mycosphaerella* spp. in an *E. globulus* plantation in northern Tasmania to establish a baseline of the species present prior to the setting up of experimental trials also are presented here. The increased coverage, spatial resolution, and accuracy provided by using the detection test as a survey tool for *Mycosphaerella* spp. are discussed.

MATERIALS AND METHODS

Fungal material. Cultures (Table 1) were maintained on 2% malt extract agar. Nonviable cultures up to 10 years old also were used for DNA extraction. Standard reference cultures for each of the target species were nominated based on the most reliably identified material available. These were *M. cryptica* (AA/1/3/10), morphologically identified by Milgate (51), ITS sequenced here, sequence consistent with sequences in GenBank for nine other *M. cryptica* isolates; *M. grandis* (Q/1/1/1), morphologically identified by Milgate in collaboration with A. J. Carnegie, and ITS

sequenced (51), sequence identical with that of AC165, neotype material from A. J. Carnegie; *M. nubilosa* (Z/1/1/11), morphologically identified by Milgate (51), ITS sequenced, and consistent with 13 other sequences in GenBank; *M. tasmaniensis* (STE-U1555), an ex-type culture from Tasmania (21); and

M. vespa (BS/3/2/1), morphologically identified by Milgate in collaboration with A. J. Carnegie, and ITS sequenced (51). These cultures have been deposited in the Department of Agriculture Western Australia Plant Pathogen Collection (WDCM77).

TABLE 1. Fungal cultures used to test species-specific primers

Species	Code ^a	Host	Origin
<i>Botryosphaeria</i> sp.	WA36 ^b	<i>Eucalyptus wandoo</i>	Western Australia
<i>Cryptosporiopsis eucalypti</i>	I00108a ^b	<i>E. camaldulensis</i>	Queensland
<i>Cylindrocladium retaudii</i>	I00150 ^b	<i>E. urophylla</i>	Queensland
<i>Mycosphaerella africana</i>	CBS680.95 ^{c,d}	<i>E. viminalis</i>	South Africa
<i>M. cryptica</i>	AA/1/3/10 ^e	<i>E. globulus</i>	Tasmania
	NZ301H ^e	<i>E. nitens</i>	New Zealand
	E7444a ^f	<i>E. pilularis</i>	New South Wales
	I00205 ^b	<i>E. globulus</i>	Victoria
	I00207 ^b	<i>E. globulus</i>	Victoria
	I00208 ^b	<i>E. globulus</i>	Victoria
	I00210 ^b	<i>E. globulus</i>	Victoria
	I00214 ^b	<i>E. globulus</i>	Victoria
	I00216 ^b	<i>E. globulus</i>	Victoria
	I00217 ^b	<i>E. globulus</i>	Victoria
	I00225 ^b	<i>E. obliqua</i>	Victoria
	I00226 ^b	<i>E. obliqua</i>	Victoria
	I00234 ^b	<i>E. globulus</i>	Victoria
	I00235 ^b	<i>E. cytellocarpa</i>	New South Wales
	I00236 ^b	<i>E. cytellocarpa</i>	New South Wales
<i>M. grandis</i>	AC165 ^{g,h}	<i>E. saligna</i>	Victoria
	I00206 ^b	<i>E. globulus</i>	Victoria
	I00219 ^b	<i>E. globulus</i>	Victoria
	S/1/2/3 ^c	<i>E. globulus/nitens</i>	Tasmania
	Q/1/1/1 ^e	<i>E. nitens</i>	Tasmania
	H/1/3/1 ^e	<i>E. nitens</i>	Tasmania
<i>M. nubilosa</i>	AC106 ^{g,h}	<i>E. globulus</i>	Victoria
	L/1/2 multispore ^e	Not recorded	Victoria
	StMarys/2/4 ^c	<i>E. globulus</i>	Tasmania
	StMarys/2/8 ^c	<i>E. globulus</i>	Tasmania
	Z/1/1/11 ^e	<i>E. globulus</i>	Tasmania
	E1 multispore ^e	<i>E. globulus</i>	Tasmania
	Z/1/1/13 ^c	<i>E. globulus</i>	Tasmania
	S/2/1/1 ^e	<i>E. globulus</i>	Tasmania
<i>M. parkii</i>	CBS 387.92 ^{c,d}	<i>E. grandis</i>	Brazil
<i>M. parva</i>	AC83 ^{g,h}	<i>E. globulus</i>	Victoria
	AC85 ^{g,h}	<i>E. globulus</i>	Victoria
	AC89 ^{g,h}	<i>E. globulus</i>	Victoria
	CBS110503 ^c	<i>E. globulus</i>	Western Australia
<i>M. pini</i>	DT3 ^b	<i>Pinus radiata</i>	New South Wales
<i>M. suberosa</i>	CBS 436.92 ^c	<i>E. dunnii</i>	Brazil
<i>Mycosphaerella</i> sp. 1 (received as <i>M. parkii</i>)	CBS 208.94 ^c	<i>E. grandis</i>	Indonesia
	CBS 209.94 ^c	<i>E. grandis</i>	Indonesia
<i>Mycosphaerella</i> sp. 2 (received as <i>M. parkii</i>)	CBS516.93 ^c	<i>E. globulus</i>	Brazil
<i>M. tasmaniensis</i>	I555 ^{d,e,h}	Not recorded	Tasmania
	BS10/2 ^{e,h}	<i>E. nitens</i>	Tasmania
	S/3/2/7 ^{e,h}	<i>E. globulus/nitens</i>	Tasmania
<i>M. vespa</i>	A/1/3 ^e	<i>E. globulus</i>	Tasmania
	B/3/2/1 ^e	<i>E. globulus</i>	Tasmania
	A/1/7 ^e	<i>E. globulus</i>	Tasmania
	B/3/2/4 ^e	<i>E. globulus</i>	Tasmania
	BrunyIs/1/1 ^e	<i>E. globulus</i>	Tasmania
<i>Phaeophleospora eucalypti</i>	NZFS85C/1 ⁱ	<i>E. nitens</i>	New Zealand
	NZFS85C/3 ⁱ	<i>E. nitens</i>	New Zealand
	NZFS85C/23 ⁱ	<i>E. nitens</i>	New Zealand
<i>Quambalaria cyanescens</i>	IMI178848 ^j	<i>E. pauciflora</i>	New South Wales
<i>Q. eucalypti</i>	S8 ^k	<i>E. globulus</i> (bark)	Uruguay
<i>Q. pitereka</i>	E7440 ^f	<i>Corymbia maculata</i>	Western Australia

^a Superscripts identify culture source.

^b CSIRO FFP Canberra.

^c Centraal Bureau voor Schimmelcultures.

^d Type strain.

^e University of Tasmania.

^f CSIRO FFP Perth.

^g Angus Carnegie, LaTrobe University.

^h DNA was extracted from an old, nongrowing culture.

ⁱ New Zealand Forest Research.

^j International Mycological Institute.

^k University Viçosa, Brazil.

Sampling of leaf material from plantations and forest re-growth sites. A survey of 2-year-old *E. globulus* was conducted at Smithton in northwestern Tasmania on 8 August 2003 (S40°55'21", E144°59'39"). The plantation of approximately 21 ha was divided into six sections and 10 leaf samples were taken from separate trees in each section. Juvenile leaves were sampled from branches at a height of 1.2 m from trees that were up to 4 m tall. These trees were selected using systematic sampling starting from the first two trees, moving over a row and sampling the next two trees, and so on, until the required number of samples was taken. Leaves were taken from the fourth to fifth leaf pair on each branch to minimize variation in leaf age, physiological development, height in the canopy, and age of the lesions. Lesions from lateral branch stems also were sampled from seven randomly selected *E. globulus* trees in the same trial.

Additional samples for confirmation that the test was applicable to other host species were collected at four different sites in north and northeastern of Tasmania. In all, 16 *E. nitens* samples were taken from plantations at Weldborough (S41°13'14", E147°51'60"), St. Helens (S41°20'41", E148°3'55"), and Scottsdale (S41°15'34", E147°25'25"). Leaf material was sampled from branches at a height of 1.2 m and sampled from the fourth to fifth pair on each branch from trees ≈5 to 6 m tall, selected using a systematic sampling regime as described above. A collection of four samples was made from natural forest regeneration of *E. regnans* at a roadside site near Bicheno (S41°48'19", E148°14'53"). Leaves were taken from the fourth pair on branches. Trees were ≈1.5 m tall and ≈12 months old.

Lesions were excised with a scalpel, using a fresh blade for each sample to avoid DNA cross-contamination. Each sample was 1 cm² and consisted of up to 15 lesions per leaf, pooled. The stem samples from *E. globulus* were obtained by scraping the surface of stem lesions with a scalpel to produce shavings and approximately 80 mm² of stem surface area was taken.

DNA extraction, PCR, and sequencing. Plant material and fungal mycelium were ground in 1.5-ml microfuge tubes with a pellet pestle mixer (in liquid nitrogen for leaf and stem samples), mixed with extraction buffer (65), and incubated for 1 h at 65°C. Tubes were centrifuged at 14,000 rpm for 15 min and the supernatant removed. Purification was achieved by binding to a silica matrix in the presence of NaI (31). PCR was carried out in an Applied Biosystems (Foster City, CA) GeneAmp PCR System 2700 thermocycler. Reactions of 25 µl contained 0.55 U of TTH+ Polymerase (Fisher Biotec, Subiaco, Western Australia) in 1x polymerization buffer (Fisher Biotec), 2.0 mM MgCl₂, bovine serum albumen at 0.2 mg/ml, 0.2 mM each dNTP, and 0.25 µM each primer. For sequencing, the entire ITS1, 5.8S, and ITS2 regions were amplified using primers ITS1-F (29) and ITS4 (76) and the following thermocycler program: 94°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; followed by an extension step of 72°C for 7 min. PCR products were electrophoresed on a 1% agarose (Fisher Biotec) gel at 4 V/cm for 1 h. Before sequencing, PCR products were purified with an UltraClean PCR Clean-up DNA purification kit (MoBio Laboratories, Inc., West Carlsbad, CA). Sequences were determined with an ABI Prism Dye Terminator Cycle Sequencing kit on a Bio-Rad 373 Sequencer (Hercules, CA) with stretch upgrade. Sequences were obtained in both directions and assembled with Lasergene software (DNASar, Madison, WI).

Phylogenetic analysis and primer design. Sequences generated in this project are listed in Table 2. Further sequences were retrieved from GenBank and EMBL. *Mycosphaerella* and anamorph species included in the analysis were selected on the basis of (i) occurrence on members of Myrtaceae or (ii) high sequence similarity to a species from Myrtaceae. All available sequence variants for each species were included with the exception of two related anamorph groups (*Cercospora* and *Trimmatostroma*), with sequence similarity of <85% to any of the

target species and which previously have been shown to form distinct clusters in phylogenetic analyses (20,33). *Hortaea werneckii* was included on the basis of high sequence similarity, but only one ITS sequence was selected from the 17 available at the time. Alignments were performed with CLUSTALW (73) and adjusted manually. Phylogenetic analyses were executed with DNAPars and DNAML of the PHYLIP package (26) on the Australian National Genome Information Service (ANGIS, Sydney, NSW, Australia). Initial analysis was by DNAPars, with *Phaeoramularia hachijoensis* (AY251086) set as the outgroup, jumbling 10 times, and all other options as default. The set of most parsimonious trees were subjected to maximum likelihood analysis by DNAML with *Phaeoramularia hachijoensis* (AY251086) set as the outgroup, global rearrangements on, and all other options as default. The sequence alignment and phylogenetic tree were deposited in TreeBase (accession number TBA). Primers were designed following the general concepts outlined by Dieffenbach (24), with two additional aims. To permit future multiplexing of the five specific PCRs, primer sites were selected to produce amplicons of different size for each species and primer lengths were modified to result in the same annealing temperature for all primers. BLAST searches of GenBank and EMBL also were carried out using the taxon-specific primer sequences.

Primer specificity testing. Primers were tested for specificity on a broad range of template DNA, including pure cultures of *Mycosphaerella* spp., other eucalypt leaf pathogens (Table 1), and clean eucalyptus leaves. To obtain optimum specificity and efficiency of amplification, several different annealing temperatures were tested. Primer lengths were adjusted by 1 or 2 bases until all primer pairs amplified the target species or group at the same annealing temperature, with no amplification from other species.

Construction of an IAC for first-round PCR. A plasmid containing primer-binding sites for the primers ITS1-F (29), ITS5, and ITS4 (76) was constructed by ligation of a histone gene fragment amplified from *Phaeophleospora eucalypti* DNA using composite primers (Table 3). The forward primer sequence is a composite of ITS1-F, ITS5, and H3-1a (32), with H3-1a at the 3' end, and the reverse a composite of ITS4 and H3-1b (32), with H3-1b at the 3' end. A product of ≈1,100 bp was amplified using conditions for the H3-1a/H3-1b primers (32). This product was purified with an UltraClean PCR Clean-up DNA purification kit (MoBio Laboratories, Inc.) and transformed into *Escherichia coli* using a pGEM-T cloning kit (Promega Corp. Madison, WI). Plasmid DNA was extracted using an Ultraclean plasmid extraction kit (MoBio Laboratories, Inc.).

Nested PCR. All reactions were set up in UV-irradiated laminar flow hoods with positive (DNA from pure culture of target species) and negative (DNA from pure cultures of nontarget species and no added template) controls included in each set of reactions. Aerosol barrier tips were used routinely and gloves

TABLE 2. Sequences generated in this project for primer design and species verification

Species	Accession numbers
<i>Mycosphaerella africana</i>	AY626981
<i>M. cryptica</i>	AY667576, AY626989, DQ471928
<i>M. grandis</i>	AY626986
<i>M. nubilosa</i>	AY667577, DQ471926
<i>M. parkii</i>	AY626979 (type isolate)
<i>M. parva</i>	AY626980
<i>Mycosphaerella</i> sp. 1	AY66982, AY626983
<i>Mycosphaerella</i> sp. 2	AY626984
<i>M. suberosa</i>	AY626985
<i>M. tasmaniensis</i>	AY667578 (type isolate)
<i>M. vespa</i>	DQ471925, DQ471927
<i>Phaeophleospora eucalypti</i>	AY626987, AY626988

were always worn and changed frequently to minimize cross-contamination. First-round PCR used primers ITS1-F and ITS4 as outlined above, with the addition of 25 fg of IAC in each 25- μ l reaction. Failure of first-round amplification (as occurred in a few leaf samples) demonstrated the need to repeat the first-round PCR using a more dilute sample. First-round product was diluted 1/10 and a 5- μ l aliquot was used as template in a 25- μ l nested PCR reaction with species-specific primers. Concentrations were 0.25 μ M for each primer, 2 mM MgCl₂, and 0.2 mM each dNTP in 1 \times polymerization buffer (Fisher Biotec), with 0.55 U of TTH+ polymerase (Fisher Biotec) per 25- μ l reaction.

Cloning and sequencing of PCR products from multiple fungal species in leaf samples. Independent confirmation of PCR test results by isolation of corresponding species from the same leaf samples was not possible because not all samples had pseudothecia and not all samples with pseudothecia had mature spores. Therefore, confirmation was sought by amplification, cloning, and sequencing of fungal PCR products from selected leaf samples. Fungal ITS products were amplified with ascomycete-specific primer combination ITS1-F (29) and ITS4-A (46) using the same reagent concentrations and thermocycler program as given above for ITS1-F/ITS4. Products were purified with an UltraClean PCR Clean-up DNA purification kit (MoBio Laboratories, Inc.) and cloned using a pGEM-T cloning kit (Promega Corp.). These primers had not been used previously in the laboratory and amplified a larger fragment than any primers used previously, so that the possibility of chance contamination with PCR product was eliminated. Individual clones were tested with the species-specific primers, and a representative of each species from each leaf sample was sequenced using primers ITS1-F and ITS4 as described previously.

Plantation survey. Every set of PCR reactions included five samples containing DNA from cultures of each of the five species or taxa groups and one sample with no template DNA. The four nontarget species in each of the species-specific tests were treated as additional negative controls. The test was repeated on \approx 30% of samples to demonstrate the consistency of the analysis. Although the ITS primers for *M. vespa* also are able to detect *M. ambiphylla* and *M. molleriana*, these species previously have not been recorded in Tasmania, whereas *M. vespa* has been recorded over the majority of the state (51). For simplicity, the positive results for this group are referred to as *M. vespa*. The possibility of *M. molleriana* and *M. ambiphylla* occurring in Tasmania has not been eliminated.

RESULTS

Phylogenetic analysis and primer design. A phylogenetic analysis of ITS sequences was carried out after sequence alignment to accommodate the following points: (i) intraspecific variation in ITS sequences of some *Mycosphaerella* spp., (ii) a degree

of taxonomic uncertainty for some species, and (iii) quality control for sequences obtained from public databases (the accuracy and reliability of which was beyond our control). Phylogenetic analysis showed species with ITS sequences most similar to the target species to facilitate the selection of interspecific divergent regions most suitable for species-specific primer design. Phylogenetic analysis also assisted in the detection of misidentified species from public sequence databases or culture collections.

In all, 165 nonredundant ITS sequences from 95 *Mycosphaerella* and related anamorph species that were obtained from GenBank or produced here were included in the phylogenetic analysis (Fig. 1). All available sequences of *Mycosphaerella* spp. that occur on *Eucalyptus* spp. were included in the analysis, as were several species from other hosts. Species known from eucalypts for which ITS sequences were not available include *M. delegatensis*, *M. endophytica*, *M. gracilis*, *M. longibasalis*, and *M. swartii*. Sequences AY045519 (*M. suttoniae*) and AY626984 (*Mycosphaerella* sp.) had segments of 157 and 181 bp, respectively, removed from ITS1 after the initial alignment revealed that these were most probably insertions. The insertions were at different sites and did not appear to be homologous. Analysis by DNAPARS produced 428 equally most parsimonious trees of 3,170 steps. DNAML analysis of this set produced an ML tree with an Ln likelihood = -13,649.6.

For each of the five target species (*M. cryptica*, *M. nubilosa*, *M. vespa*, *M. grandis*, and *M. tasmaniensis*), variable regions were visually selected from the alignment of 95 *Mycosphaerella* spp. Particular attention was paid to discriminating species that had high overall sequence similarity and that clustered closely in the ML tree. The variable regions anchored the 3' end of species-specific primers and primer length was varied at the 5' end to give similar annealing temperatures. Species-specific primers were designed for *M. cryptica*, *M. nubilosa*, and *M. tasmaniensis*. High interspecific sequence similarity combined with intraspecific variation prevented the design of species-specific primers for *M. vespa* and *M. grandis*. Therefore, primers were designed for two taxa groups: one primer pair for *M. vespa*, *M. ambiphylla*, and *M. molleriana*, and one primer pair for *M. grandis* and *M. parva*. Regions were chosen to give a different product size for each species with a view to future multiplexing of the tests. Primer sequences were checked for self-annealing and BLAST search results were checked to ensure that only the target species matched both primers. Primer sequences and expected product sizes are given in Table 3.

Arrows on branches indicate groups that have an exact match to primer sequences (Fig. 1). Each of these branches is supported by 99% confidence limits. This includes all available sequences for each target taxon, with two exceptions: AF173307 was very different from all other *M. tasmaniensis* sequences, grouping instead with *Mycovellosiella eucalypti* and *Phaeoramularia saururi*, and AY534227 grouped with the other *M. vespa* sequences but dif-

TABLE 3. Species-specific primers designed from *Mycosphaerella* spp. internal transcribed spacer (ITS) sequences and composite primers used to construct an internal amplification control for ITS1-F/ITS4 and ITS5/ITS4 PCR

Species	Primer name	Primer sequence	Product size (bp)
<i>Mycosphaerella cryptica</i>	McrypF	5' CATCTTTGCGTCTGAGTGATAACG	331
	McrypR	5' GGGGGTTGACGGCGCGAC	
<i>M. grandis/parva</i>	MgpF	5' CCCATTGTATTCCGACCTCTTG	359
	MgpR	5' CGCTTAGAGACAGTTGGCTCAG	
<i>M. nubilosa</i>	MnubF	5' CAACCCCATGTTTTCCACCACG	395
	MnubR	5' CGCCAGACCGGTCCCCGTC	
<i>M. tasmaniensis</i>	MtasF	5' GTCACGCGGCCGACCGC	298
	MtasR	5' CATTAGGGCACGCGGGCTG	
<i>M. vespa/ambiphylla/molleriana</i>	MvamF	5' GCATCTCTGCGTCTGAGTCAC	264
	MvamR	5' GCTCGCCGGAGACTTCG	
<i>Phaeophleospora eucalypti</i>	ITSPCF	5' CTTGGTCATTAGAGGAAAGTAAAAGTCGT AACAAGGACTAAGCAGACCCGCCAGG	1,100
	ITSPCR	5' TCCTCCGCTTATTGATATGCGCGGGCG AGCTGGATGTCCTT	

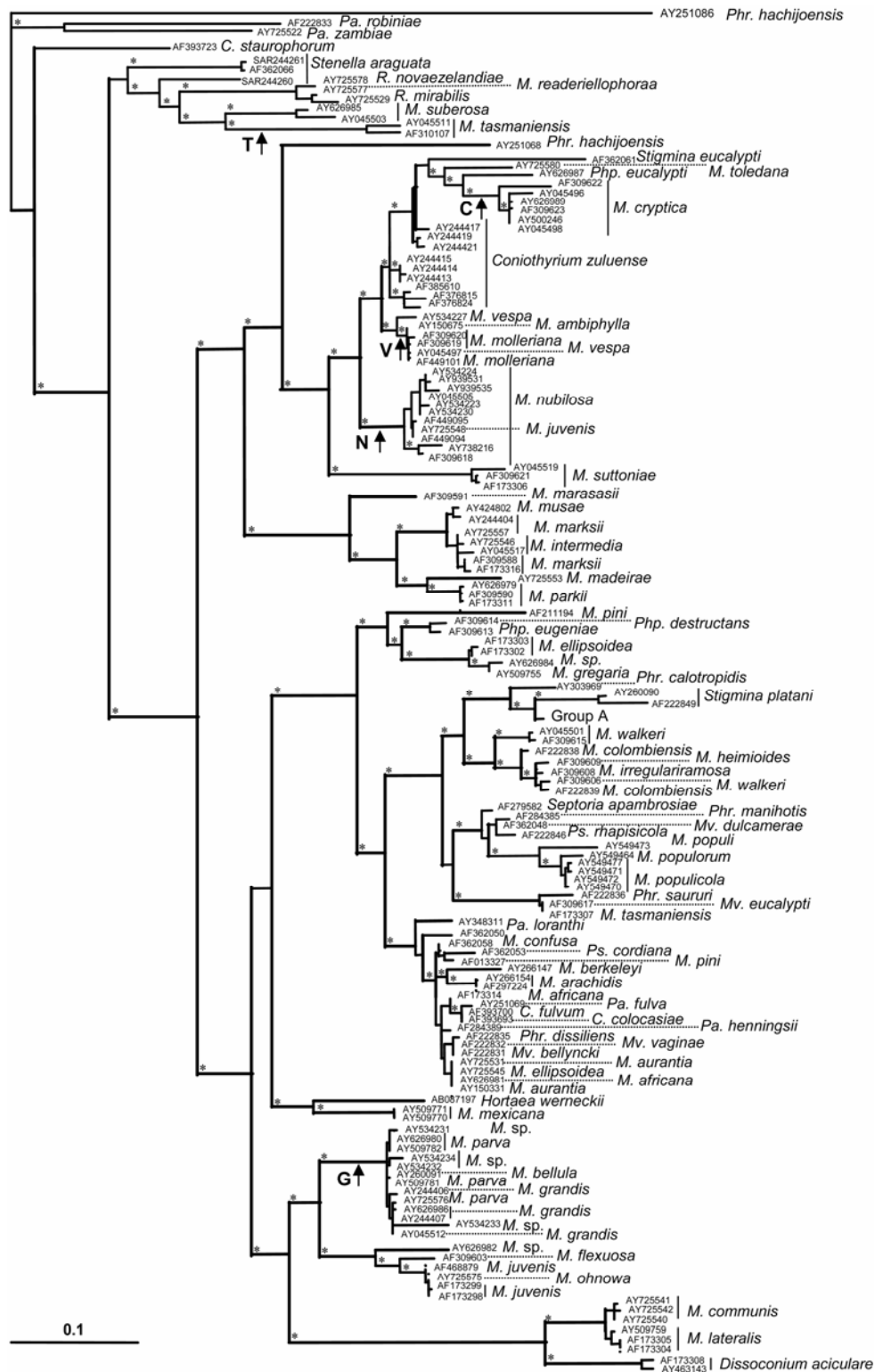


Fig. 1. Maximum likelihood tree produced by phylogenetic analysis of 165 internal transcribed spacer sequences from *Mycosphaerella* and related anamorph species, Ln likelihood = -13,649.6. The outgroup is *Phaeoramularia hachijoensis* and the scale bar represents expected nucleotide variation of 10%. Clades that are supported with 99% confidence are denoted by an asterisk above the branch. Arrows on branches represent the groups that have 100% similarity to the taxon-specific primer sequences as follows: G, *Mycosphaerella grandis/parva* group; V, *M. vespa/ambiphyllo/molleriana* group; N, *M. nubilosa*; C, *M. cryptica*; and T, *M. tasmaniensis*. Abbreviations for genera are: *M.*, *Mycosphaerella* and the anamorph genera *C.*, *Cladosporium*; *Mv.*, *Mycovellosiella*; *Pa.*, *Passalora*; *Php.*, *Phaeophleopora*; *Phr.*, *Phaeoramularia*; *Ps.*, *Pseudocercospora*; and *R.*, *Readeriella*. The anamorph genera are those given in the GenBank accessions, although many of them recently have been recombined (5,6,15), appropriate formal name changes have not been made by the sequence submitters. In addition, some sequences listed in GenBank as belonging to *M. juvenis* and *M. ellipsoidea* are included as belonging to those species, though the isolates that were sequenced probably represent different taxa (P. W. Crous, *personal communication*). To improve readability of the tree, one branch, distant from the target taxa, has been pruned. Group A represents two *Mycosphaerella* spp., *M. fori* and *M. musicola*, and 14 *Pseudocercospora* spp.: *P. basiramifera*, *P. cordiana*, *P. cruenta*, *P. eriodendri*, *P. eucalyptorum*, *P. hibbertiaeasperae*, *P. luzardi*, *P. macrospora*, *P. natalensis*, *P. paraguayensis*, *P. platyloba*, *P. pseudoecalyptorum*, *P. robusta*, and *P. syzygicola*. These are included in the tree submitted to Treebase.

ferred by 2.3% and had two mismatches to the forward primer for *Mycosphaerella vespa/ambiphylla/molleriana*.

By contrast, *M. ambiphylla* and *M. molleriana* sequences grouped closely with the remaining *M. vespa* sequences with 99% confidence and had a 100% match to the *M. vespa* primers. Percent difference among this group was only 0.2 to 1.0%. In addition, sequences for *M. grandis* and *M. parva* were very similar to each other (99.2%) and form a single clade with high support. Furthermore *M. bellula*, derived from a Proteaceae family host, and other unnamed isolates from eucalypts also fall within this clade and all have a 100% match to the *M. grandis/parva* primers.

IAC for first-round PCR. Plasmid p35.12 was selected for use as an IAC because it had an insert of the correct size ($\approx 1,100$ bp) that was amplifiable with the ITS1-F and ITS4 primers. A PCR product was visible when 2.5 fg of IAC was the only DNA template added to a 25- μ l reaction (Fig. 2); however, addition of leaf or fungal DNA required a higher concentration of IAC (Fig. 3). Therefore 25 fg was added to each 25- μ l PCR, which equates to 6×10^4 copies per reaction. Successful first-round amplification with ITS1-F and ITS4 primers verified that the template DNA from plant material or fungal cultures was amplifiable, even if fungal DNA was at too low a concentration to be detected after one round of PCR. This increased confidence that subsequent negative results for taxon-specific primers were not false negatives caused by PCR inhibition. If fungal DNA was present at a much higher concentration, amplification of IAC was prevented by competition. Thus, a visible product from either the IAC or fungal DNA or, in some cases, both, indicated successful first-round PCR.

Specificity, sensitivity, and reliability testing of primer sets. Species-specific primers (0.25 μ M) were used in the second-round amplification of a nested PCR after first-round amplification with ITS1-F and ITS4. After specificity testing of all primer pairs at several annealing temperatures, the optimum thermocycler program to run the species-specific primers simul-

taneously was 94°C for 3 min; 20 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s; followed by 72°C for 10 min.

Each individual primer pair, designed to detect *Mycosphaerella* spp. or closely related species groups from plantation *Eucalyptus* spp., were highly specific for their respective targets (Fig. 4B to F). The specific primer pairs verified the taxa of 3 to 15 isolates of each of the target species from culture collections (Table 4). These tests were done in a series of experiments with replication of randomly chosen standard isolates and had identical results, showing the reliability of the test. In double-blind trials, the primers unequivocally discriminated among the five species or taxon groups and did not detect cultures of nontarget species, including possible co-occurring fungal pathogens, verifying their reliability (Table 4; Fig. 4B to F). With nested PCR, each primer pair was sensitive enough to detect pathogen DNA when present at concentrations of 10 to 100 fg per 25- μ l reaction (Fig. 5). Amplification at these low levels of template DNA was not reliable in repeated trials and this may be attributed to stochastic events involving target sequences at such low concentrations. Amplification from 10 fg of *M. grandis* DNA with no amplification from 100 fg (Fig. 5B and C) supports this hypothesis. The addition of plant DNA to the first-round PCR, equivalent to that in a commonly used dilution of the average-sized leaf sample, appeared to reduce final sensitivity by a factor of ≈ 10 (Fig. 5B). Addition of IAC appeared to have the reverse effect, though this also may have been a result of stochastic effects.

In 2 days in a double-blind trial, one person using the detection test successfully differentiated the appropriate species (*M. cryptica*, *M. grandis/parva*, *M. nubilosa*, and *Mycosphaerella* sp. nov.) (Table 1; data not shown) from 30 cultures from disease surveys in *Eucalyptus* plantations that had been identified at the time of isolation according to the taxonomic criteria available at that time. The PCR detection test results were verified by subsequent sequencing of the complete ITS regions of these isolates (Table 2; data not shown).

Testing of primers on plant material. The primers successfully detected *Mycosphaerella* spp. directly from infected leaves and stems (Fig. 6). At least one, and up to four, *Mycosphaerella* spp. were detected from each leaf or stem sample. The robustness of the detection systems to test for *Mycosphaerella* spp. in a wide range of tissue types was further indicated by amplification of specific products from dead, dried, herbarium collections. The nested PCR reactions enabled detection and discrimination of the five *Mycosphaerella* taxa in 30 samples with lesions at any developmental stage by one person within 2 days.

Verification of infection with multiple *Mycosphaerella* spp. by cloning of PCR products. Isolation of *Mycosphaerella* spp. from leaf lesions does not produce cultures of nonsporulating species; therefore, infection by multiple *Mycosphaerella* spp. was verified by cloning PCR products from selected leaf samples, then testing individual clones by species-specific PCR and sequencing. A comparison of species detected by taxon-specific nested PCR with those detected by cloning and sequencing (Table 5) shows that, although fewer species were detected by the latter method, the presence of multiple species in individual leaves or even individual lesions was confirmed. One leaf sample that had given a negative result for all the taxon-specific nested PCRs produced a clone containing an *M. grandis* sequence (Table 5, sample 3). Other species of possible leaf pathogens also were detected by the cloning method (data not shown). No clones containing *M. vespa* or *M. tasmaniensis* sequences were detected, even though five and two samples, respectively, had tested positive to these species by nested PCR. To verify that the nested PCR product was amplified from these two species and not from an untested fungus present in the leaf sample, the taxon-specific amplicons were sequenced. The sequences were 99 to 100% identical to published sequences from *M. vespa* and *M. tasmaniensis*, though single nucleotide polymorphisms occurred among the *M. vespa* sequences from different geographic areas (Table 5).

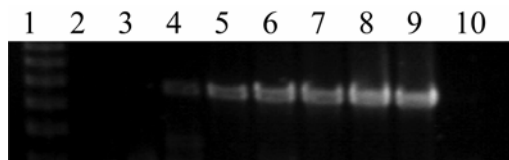


Fig. 2. Evaluation of sensitivity of amplification of the internal amplification control plasmid (IAC) using primers ITS1-F/ITS4. Lane 1 contains a 200-bp ladder showing fragments of 600 to 1,600 bp and lane 10 is no template control. Template concentrations are lane 2, 10^{-18} g/ μ l or 60 copies per 25- μ l reaction; lane 3, 10^{-17} g/ μ l or 600 copies; lane 4, 10^{-16} g/ μ l or 6,000 copies; lane 5, 1 fg/ μ l or 6×10^4 copies; lane 6, 10 fg/ μ l or 6×10^5 copies; lane 7, 100 fg/ μ l or 6×10^6 copies; lane 8, 1 pg/ μ l or 6×10^7 copies; and lane 9, 10 pg or 6×10^8 copies.

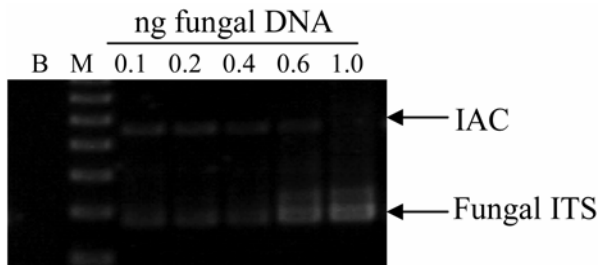


Fig. 3. Sensitivity of amplification of fungal DNA with primers ITS1-F and ITS4 with the addition of 25 fg of internal amplification control plasmid (IAC) to each 25- μ l polymerase chain reaction. Lane M contains a 200-bp ladder showing fragments of 400 to 1,400 bp. Amplification of the IAC is competitively inhibited at the higher fungal DNA concentrations.

Plantation survey. Most leaves contained more than one lesion and, therefore, most 1-cm² samples consisted of subsamples from more than one lesion. No relationship was observed between the number of lesions sampled per leaf and the number of species detected. Leaves from both *E. nitens* and *E. globulus* with only one lesion contained two to three *Mycosphaerella* spp. (Fig. 6).

The *Mycosphaerella* spp. most frequently detected on *E. globulus* were *M. cryptica*, *M. nubilosa*, and *M. grandis/parva*

(Table 6). All *E. nitens*, *E. globulus*, and *E. regnans* leaves from the plantation survey contained multiple *Mycosphaerella* spp., with three or more species occurring on most leaves (Table 7). The analysis for 30% of samples was repeated independently and produced consistent results. All leaf and stem lesions were analyzed at an early developmental stage. *E. globulus* leaves contained *M. cryptica*, *M. grandis/parva*, and either *M. vespa*, *M. tasmaniensis*, or *M. nubilosa* (Table 7), including several

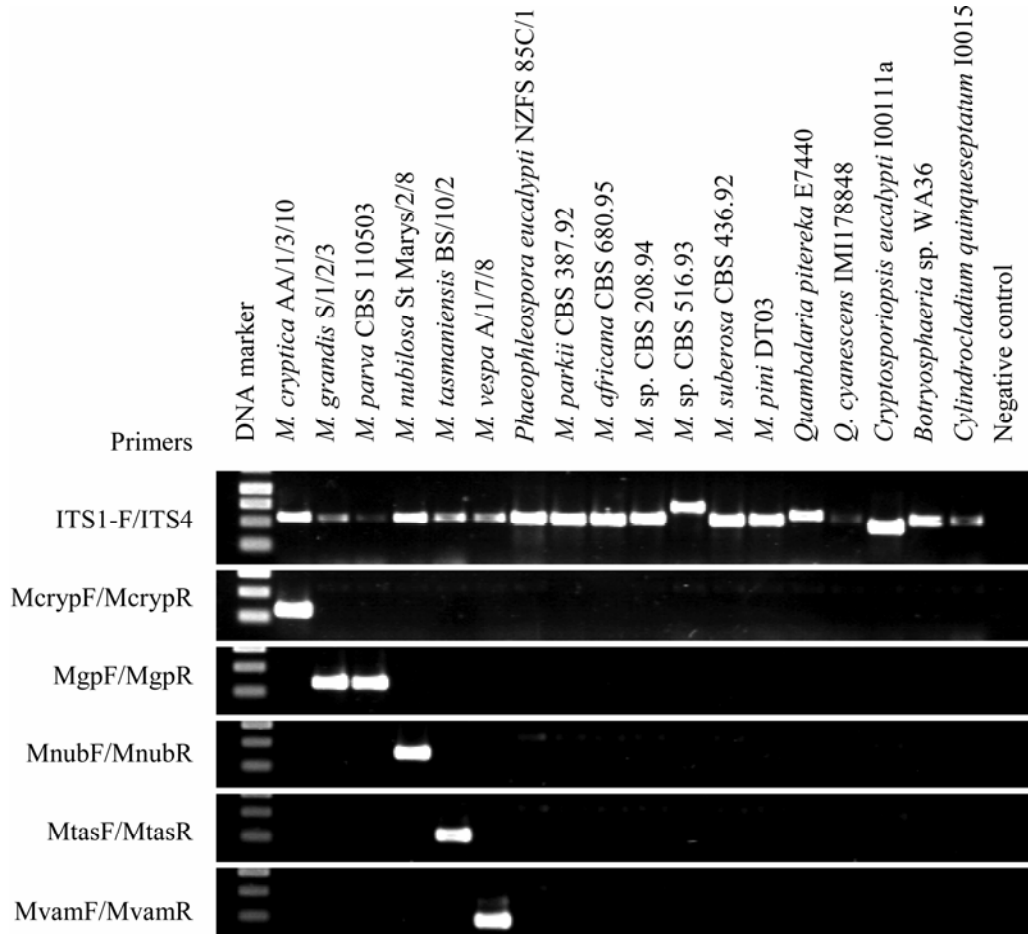


Fig. 4. Specificity of each pair of species-specific primers: polymerase chain reaction products from first-round amplification with primers ITS1-F/ITS4 and second-round amplification with specific primers.

TABLE 4. Results of taxon-specific nested polymerase chain reaction detection tests for the five *Mycosphaerella* taxa^a

Species (no. of isolates tested)	C	G	N	T	V
<i>Botryosphaeria</i> sp. (1)	-	-	-	-	-
<i>Cryptosporiopsis eucalypti</i> (1)	-	-	-	-	-
<i>Cylindrocladium retaudii</i> (1)	-	-	-	-	-
<i>Mycosphaerella africana</i> (1)	-	-	-	-	-
<i>M. cryptica</i> (15)	+	-	-	-	-
<i>M. grandis</i> (7)	-	+	-	-	-
<i>M. nubilosa</i> (8)	-	-	+	-	-
<i>M. parkii</i> (1)	-	-	-	-	-
<i>M. parva</i> (4)	-	+	-	-	-
<i>M. pini</i> (1)	-	-	-	-	-
<i>Mycosphaerella</i> sp. 1 (3)	-	-	-	-	-
<i>Mycosphaerella suberosa</i> (1)	-	-	-	-	-
<i>M. tasmaniensis</i> (3)	-	-	-	+	-
<i>M. vespa</i> (5)	-	-	-	-	+
<i>Phaeophleospora eucalypti</i> (3)	-	-	-	-	-
<i>Quambalaria cyanescens</i> (1)	-	-	-	-	-
<i>Q. eucalypti</i> (2)	-	-	-	-	-
<i>Q. pitereka</i> (1)	-	-	-	-	-

^a Columns C, G, N, T, and V represent results for taxon-specific tests for *M. cryptica*, the *M. grandis/parva* group, *M. nubilosa*, *M. tasmaniensis*, and the *M. vespa/ambiphyllo/molleriana* group, respectively.

leaves with a single lesion. All stem samples contained *M. grandis/parva*, with two testing positive for only *M. grandis/parva*. Five stem samples contained *M. tasmaniensis* and one had *M. cryptica*.

M. grandis/parva occurred on every leaf tested; however, it always occurred with either *M. cryptica* or *M. nubilosa* and often with *M. tasmaniensis*, *M. vespa*, or both. *M. nubilosa* was detected on one *E. nitens* sample. *M. tasmaniensis* was detected in 35% of *E. globulus* samples and 77% of *E. nitens* samples but was the only *Mycosphaerella* sp. absent from our samples of regrowth *E. regnans*. *M. vespa* occurred in 62% of *E. globulus*, 23% of *E. nitens*, and in samples from regrowth *E. regnans*.

The absence of false negatives due to PCR inhibition was demonstrated by amplification in first-round PCR, either of IAC or fungal ITS. Four samples that initially were negative for all species and the first-round amplification, gave positive results upon additional dilution of the DNA template (i.e., from 10- to 100-fold) for first-round PCR. Every positive was confirmed by lack of amplification in all of the negative controls. Samples from the various treatments and controls were processed randomly, and there was no evidence of cross contamination.

DISCUSSION

Five nested PCR detection tests were developed and their specificity verified against classically identified cultures of *Mycosphaerella* spp. and other genera pathogenic on *Eucalyptus* spp. The test identified cultures which had been given only a tentative identification during disease surveys and whose species relationship was confirmed by subsequent ITS sequence analysis. The sensitivity of all the nested PCR tests, at 10 to 100 fg using DNA from pure cultures, with or without the addition of plant DNA (Fig. 5), approximates to an ascospore or hyphal cell with two to three nuclei, assuming a genome size of 10 to 100 Mbp. This is an important attribute, potentially enabling the detection tests to discriminate a few *Mycosphaerella* cells in leaf or stem samples or to identify cultures nondestructively sampled 7 to 14 days after they are initiated from germinated ascospores. The specificity of the primers is high under the stringent conditions of the tests because they have not amplified DNA from a wide range of related organisms. The probability of 18 to 24 nucleotide primers

amplifying the DNA of an unrelated organism is $\approx 4^{-40}$ (equivalent to the probability of random occurrence of a 40-bp sequence, assuming that ITS sequences develop over a long period of time through a random process of unconstrained mutation and subsequent fixation), which is $< 10^{-24}$.

The detection system distinguished among pathogens in culture and produced accurate, rapid diagnosis at an early stage of infection by five *Mycosphaerella* taxon groups, which is impossible

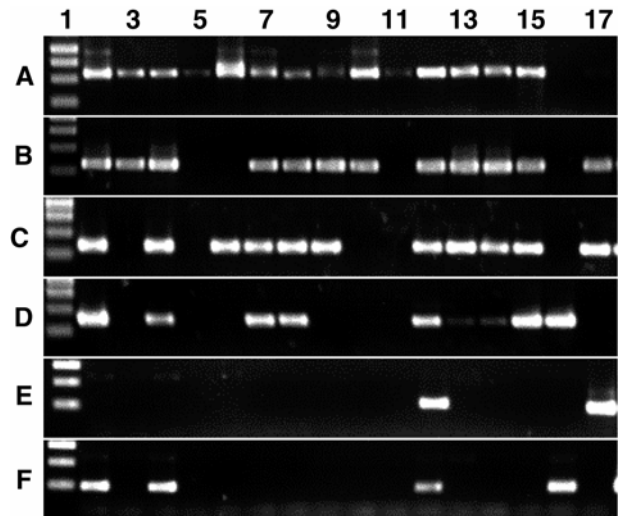


Fig. 6. Detection of *Mycosphaerella* spp. from leaf lesions. Polymerase chain reaction products from **A**, first-round amplification with primers ITS1-F/ITS4 and **B to F**, second-round amplification with specific primers. **B**, McrypF/McrypR; **C**, MgpF/MgpR; **D**, MnubF/MnubR; **E**, MtasF/MtasR; and **F**, MvamF/MvamR. Lane 1, Promega 1-kb ladder showing fragments of 1,000 (A, C, and D only), 750, 500, and 250 bp; lane 2, E7417 *Eucalyptus globulus*, all lesions on leaf; lane 3, E7418 *E. nitens*, all lesions on leaf; lane 4, E7445, *E. regnans*, all lesions on leaf; lane 5, *E. nitens*, random sample, no disease evident; lanes 6 to 10, *E. nitens*, single leaf lesions at a range of developmental stages, from small purplish spots with no reproductive structures (lane 6) to large necrotic lesions with abundant pycnidia (lane 10); lane 11, *E. globulus*, random sample, no disease evident; lanes 12 to 16, *E. globulus*, single leaf lesion at a range of developmental stages, from small purplish spots with no reproductive structures (lane 12) to large necrotic lesions with abundant pycnidia (lane 16); lane 17, *E. globulus*, single stem lesion.

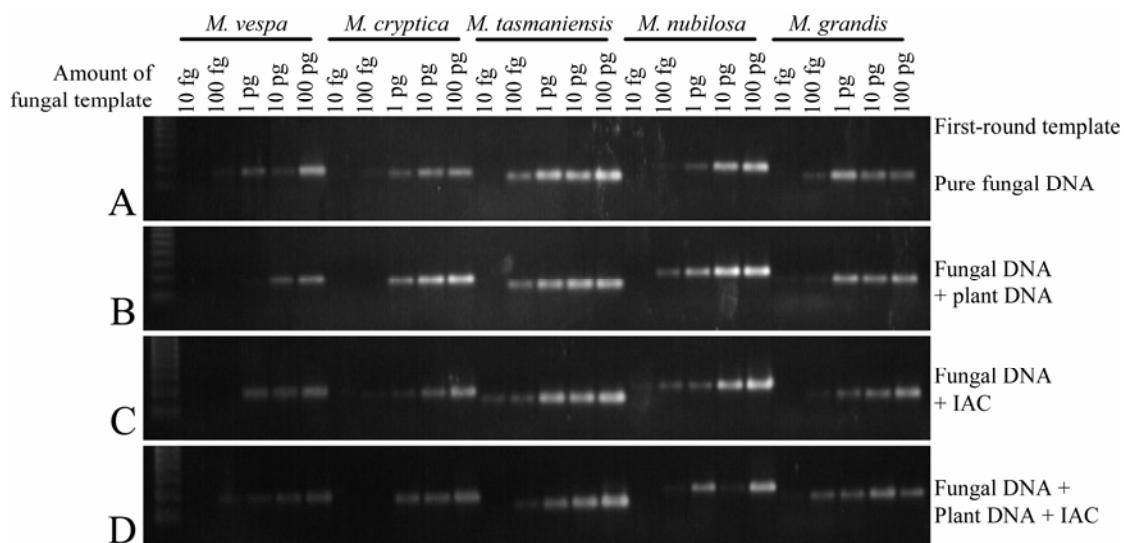


Fig. 5. Sensitivity of detection from cultures with and without internal amplification control (IAC) and plant DNA in the first-round amplification. Polymerase chain reaction (PCR) products from second-round amplification with specific primers are shown. Lane 1 contains a 200-bp DNA ladder showing fragments from 200 to 1,200 bp. **A**, Pure fungal DNA as template for round 1 PCR; **B**, equal volume of *Eucalyptus globulus* (lanes 2 to 16) or *E. grandis* (lanes 17 to 26) DNA, from a sample size and dilution equivalent to that used in testing diseased leaves; **C**, the IAC in round 1; and **D**, both plant DNA as in B and IAC as in C. Isolates details are: *Mycosphaerella cryptica* AA/1/3/10 amplified with McrypF/McrypR, *M. grandis* S/1/2/3 amplified with MgpF/MgpR, *M. nubilosa* St Marys/2/8 amplified with MnubF/MnubR, *M. tasmaniensis* BS/10/2 amplified with MtasF/MtasR, and *M. vespa* A/1/7 amplified with MvamF/MvamR.

using current methodology (52,62). Data in this article also raise concerns about the usefulness of lesion appearance as a diagnostic tool for identifying leaf pathogens. Different necrosis symptoms occur on *Phaseolus vulgaris* when co-inoculated with both common bacterial blight (*Xanthomonas campestris* pv. *phaseoli*) and rust (*Uromyces appendiculatus* var. *appendiculatus*) than those observed with single inoculations (78). Even within eucalypts there is considerable host variation with necrosis symptoms for the same *Mycosphaerella* spp. (8), and *M. cryptica* and *M. nubilosa* lesions often coalesce (59). Therefore, the identification of the full complement of *Mycosphaerella* spp. using lesion appearance, shape, and position of pseudothecia may not be accurate due to the presence of several species in lesions once identified as having only one species.

The first-round amplification with general fungal-specific primers increases sensitivity and avoids time-consuming procedures such as incubation (48). A high degree of species specificity is not essential at this stage, though preferential amplification of fungal DNA improves sensitivity in the nested PCR. Incorporation of an internal amplification control provides additional confidence that negative results are not due to PCR inhibition by some component of the DNA extract, a common problem with plant, particularly eucalypt, DNA (7). The first-round PCR is more critical for enzyme inhibition, because plant components will be considerably diluted by the second round of PCR. The larger size of the IAC product was planned to ensure the IAC amplified less efficiently than the fungal DNA target.

The inability to obtain cloned PCR products of *M. tasmaniensis* and *M. vespa* from leaf samples that had tested positive for these species is puzzling. *M. grandis* also was lacking in cloned products from five of the samples. This discrepancy may have been caused by preferential amplification of other fungal species with the primers used, though amplification of these three species with ITS1-F/ITS4A was verified from pure fungal DNA. All possible precautions were taken to prevent cross-contamination

with PCR product. The variation among the *M. vespa* sequences (Table 5), corresponding with geographic location of the leaf samples, is evidence against cross-contamination. A more likely explanation is that these three species were present in much smaller amounts and testing of a larger number of clones may have been necessary to locate clones containing DNA from these underrepresented species. Development of species-specific real-time PCR may provide further information.

The phylogenetic analysis revealed discrepancies among isolates within species indicative of possible misidentifications. Three isolates received as *M. parkii* (Table 1) were shown to be two new species quite distinct from *M. parkii* (Fig. 1), although they had produced a *Stenella* anamorph in culture (P. W. Crous, personal communication). Several GenBank accessions also appeared to come from misnamed isolates (AF173298, AF173299, AF173307, AF468879, AY251086, AY725545, and SAR244260) (Fig. 1). These difficulties are compounded when inaccurately identified cultures are used as reference strains; therefore, culture verification is vital for any studies involving molecular identification.

Phylogenetic analysis showed the close relationships for *M. grandis* and *M. parva*, and *M. vespa*, *M. ambiphylla*, and *M. molleriana*, which are all species from *Eucalyptus*. The high degree of interspecific ITS sequence similarity and lack of alternative well-characterized genomic loci preclude differentiation of each currently recognized species in these groups. Additional studies based on collections from many hosts and geographic regions are needed to confirm that these species are distinct taxa and not merely subpopulations of a single species (22). The ecological niche occupied or pathogenicity factors are secondary taxonomic characters for *Mycosphaerella* spp. from eucalypts (62). They have been used as part of the rationale for discriminating between *M. grandis* and *M. parva* (12), with *M. grandis* regarded as pathogenic and *M. parva* as endophytic, though some workers regard these as a single species (18). The

TABLE 5. Recombinant clones and sequence accession numbers of fungal ribosomal DNA internal transcribed spacer polymerase chain reaction (PCR) products amplified from leaf lesions^a

Leaf sample	No. of lesions	Nested PCR ^b	Clones				Accession no. ^c
			<i>M. cryptica</i>	<i>M. nubilosa</i>	<i>M. grandis</i>	Other	
1	10	CGNV	18	5	...	9	DQ465671
2	1	C	4	4	...
3	1	1	13	...
4	1	G	7	21	...
5	1	CG	31	1	...
6	12	GNV	...	8	...	8	DQ465671
7	1	CGTV	24	...	2	3	DQ465672, DQ465674
8	1	GNTV	...	1	...	15	DQ465672, DQ465675
9	2	CGNV	2	9	DQ465673
10	6	C	1	13	...

^a A variable number of clones from each leaf sample was tested by nested PCR to determine the number of clones from each *Mycosphaerella* sp. At least one representative of each species from each leaf sample was sequenced to confirm species designation, including one or more clones that were negative for all five *Mycosphaerella* spp. tested.

^b Results for nested PCR: positive for C = *Mycosphaerella cryptica*, G = *M. grandis/parva*, N = *M. nubilosa*, T = *M. tasmaniensis*, and V = *M. vespa*.

^c No clones containing *M. vespa* or *M. tasmaniensis* sequences were recovered; therefore, taxon-specific amplicons for these species were sequenced directly to confirm that the product came from the target species. Because the *M. vespa* sequences obtained from samples 1 and 6, and samples 7 and 8, were identical, only one GenBank submission was made for each of these sequences.

TABLE 6. Frequency of detection for *Mycosphaerella* spp. on leaves of *Eucalyptus globulus*, *E. nitens*, and *E. regnans* and stems of *E. globulus*

Tree species	Sample type, number	Frequency (%)				
		<i>Mycosphaerella cryptica</i>	<i>M. grandis</i>	<i>M. nubilosa</i>	<i>M. tasmaniensis</i>	<i>M. vespa</i>
<i>E. globulus</i>	Leaf, 60	92	100	83	35	62
<i>E. nitens</i>	Leaf, 13	85	100	8	77	23
<i>E. regnans</i> ^a	Leaf, 4	Y	Y	Y	N	Y
<i>E. globulus</i>	Stem, 7	14	100	0	71	0

^a Due to the small sample size from the native regrowth site, only presence or absence (Y or N, respectively) of *Mycosphaerella* spp. has been presented.

ubiquity of *M. grandis/parva* in plantation samples reinforces the need for taxonomic clarification. Additional unnamed isolates and another species from a non-Myrtaceae host (*M. bellula*) also have high ITS sequence similarity to *M. grandis* and *M. parva* (Fig. 1). Whether this is a single, variable species with a broad host range or many species with highly conserved ITS sequences is unclear. The anamorphs of *M. grandis* and *M. parva* are unknown and there is uncertainty regarding their taxonomic differentiation (15). There is need for a simplified, workable taxonomy (20); however, in the interim, analysis of *Mycosphaerella* spp. on eucalypts and other woody hosts must accommodate an evolving taxonomic framework.

There is potential to increase the range of tests by developing further primers for more taxa, though additional groups, such as *M. aurantia* and *M. africana* (Fig. 1), also may be difficult to distinguish using ITS sequences. The failure of most *Mycosphaerella* spp. from *Eucalyptus* spp. to produce teleomorphs in culture complicates taxonomic investigations (62). Comparative analysis involving cross inoculations under a range of conditions onto various hosts may indicate variation in teleomorph characteristics of individual isolates. These investigations also could confirm anamorph relationships to teleomorphs, especially for isolates that do not form anamorphs in culture. If further taxonomic studies confirm the validity of currently recognized species that are not differentiated by ITS sequences, another genomic region may be able to provide greater differentiation among those species.

Increasingly, β -tubulin, elongation factor, actin, calmodulin, histone, and DNA-dependent RNA polymerase II genes are being used in phylogenetic studies of fungi. Phylogenetic analysis based on multiple genes was able to differentiate several species previously all identified as *Botryosphaeria dothidea* (69). Analysis of elongation factor 1- α and β -tubulin genes supported the synonymy of *M. juvenis* with *M. nubilosa* (18). Any region that provides phylogenetic discrimination is a suitable candidate for species-specific detection. For example, the calmodulin gene was included in a multiple gene phylogeny that provided greater phylogenetic discrimination than ITS sequences for *Fusarium* spp. in the *Gibberella fujikuroi* species complex (57). Subsequently, the calmodulin gene was exploited successfully for species-specific detection of two toxigenic *Fusarium* spp. in asparagus plants (54). As yet, none of these regions have been examined in a large number of *Mycosphaerella* spp.

The occurrence of highly similar ITS sequences in isolates of distinct species from different host genera raises questions about past evolutionary relationships or recent ones involving new host relationships. This is highlighted by the high similarity among *M. aurantia*, *M. africana* (*Eucalyptus* spp. hosts), *M. confusa* (*Rubus* spp.), and *M. pini* (*Pinus* spp.). However, the group is distinct from other species infecting *Eucalyptus* spp. and *M. dearnessii*, another species infecting *Pinus*, is very different from *M. pini*. In addition, there is significant support for a group containing *M. intermedia*, *M. marksii* (both from eucalypts), and *M. musae* (from banana) (Fig. 1). In contrast, three poplar-infecting species, *M. populi*, *M. populicola*, and *M. populorum*, form a distinct group. A multiple gene molecular phylogenetic study on carefully isolated and identified isolates of a broad cross-section

of *Mycosphaerella* spp., including those from members of Myrtaceae and other hosts, could be valuable in elucidating evolutionary relationships, possible host jumps, and some of the current taxonomic quandaries.

The *Eucalyptus* plantation industry is relatively new to Australia and the study of *Mycosphaerella* spp. in Australia has only recently received attention. Native Australian eucalypt forests mainly consist of mixtures of species of uneven age, in which MLD is usually a minor disease. The establishment of increasing areas of even-aged monocultures provides ideal conditions for epidemics. Crous et al. (19) have suggested that certain *Mycosphaerella* spp. found on eucalypts in plantations in South Africa, South America, and Europe probably originated in Australasia although, as yet, not all have been found in Australia. The possibility that many species currently known only from overseas are yet to emerge on eucalypt plantations in Australia implies that more serious disease may yet occur.

The capacity to detect *Mycosphaerella* spp. or taxon groups in samples from plantations and natural forests and to provide detailed and spatially explicit information on the species present within and between trees in a plantation clearly was demonstrated. In our case, this information provided baseline information prior to establishing a rotational length trial for the long-term monitoring of disease impact and the efficacy of different control options. Using standard techniques based on lesion attributes, ascospore germination, and cultural characteristics, rapid determination of the *Mycosphaerella* spp. associated with the large number of lesions sampled in this study would be impractical. Considering that all the stems and many of the leaves analyzed in the present study had lesions without reproductive structures, the use of classical techniques to rapidly identify species also would be impossible. Spore release from mature fruiting bodies is highly variable, with clusters of spores released by only one or two sporulating species per lesion. Therefore, in the past, *Mycosphaerella* spp. damage usually has been attributed to these one or two sporulating species (11,14,25,38,47,50,51,61). Although the species detected at the trial site and elsewhere in Tasmania support the findings of a seasonally and geographically more comprehensive survey (51), the detection frequency for all species was much higher in this study, presumably due to the sensitivity and specificity of the molecular detection methods, the ability of the test to detect species in tissue without reproductive structures and to test large numbers of samples. It is evident from this survey that, in Tasmania, where *E. globulus* is indigenous in native forests and *E. nitens*, which is indigenous to mainland Australia, has been grown in plantations for over 30 years, species compositions are far more complex than original surveys in eucalypt plantations using classical techniques have suggested.

There is substantial evidence from this study to indicate that the co-occurrence of several pathogenic and saprophytic species in a *Mycosphaerella* leaf lesion is typical for *Eucalyptus* spp. in Tasmania. Several *Mycosphaerella* spp. previously have been identified on the same lesions on Myrtaceae and Proteaceae hosts (16). The co-occurrence of several *Mycosphaerella* spp. in a small leaf volume raises the issue of species-species interaction and their roles as primary or secondary pathogens or saprophytes. In other systems, the co-occurrence of pathogenic species is

TABLE 7. Coexistence of multiple *Mycosphaerella* spp. in single leaves of *Eucalyptus nitens*, *E. regnans*, and *E. globulus*

Tree species	Sample type, number	Percentage of samples with the corresponding number of <i>Mycosphaerella</i> spp.				
		1	2	3	4	5
<i>E. nitens</i>	Leaf, 13	0	22	67	11	0
<i>E. globulus</i>	Leaf, 60	0	10	37	33	20
<i>E. regnans</i> ^a	Leaf, 4	N	Y	Y	N	N
<i>E. globulus</i>	Stem, 7	29	57	14	0	0

^a Due to the small sample size from the native regrowth site, only presence or absence (Y or N, respectively) of *Mycosphaerella* spp. has been presented.

common. These highly characterized disease systems include *Fusarium* head blight in cereals (55,56), *Melampsora* rust on willow and poplar (66), sooty blotch in apple trees (41), and the Dutch elm disease complex of *Ophiostoma ulmi*, *O. novo-ulmi*, and *O. himal-ulmi* (36). There is limited knowledge of the pathogenicity of most *Mycosphaerella* spp., on eucalypts, including *M. tasmaniensis* and *M. vespa*, which have been isolated frequently in past surveys in Tasmania (51,77). The ubiquity of *M. grandis/parva* in the leaf samples tested raises questions about its possible role in pathogenicity. *M. parva* is considered to be endophytic and there are doubts about the taxonomic distinction between *M. parva* and *M. grandis* (15). *M. grandis* was described as a leaf pathogen by Carnegie and Keane (12). The detection of only *M. grandis/parva* in two stem lesions indicates their potential role as a pathogen in stems.

This study has illustrated that nested PCR is a suitable technique for differentiating *Mycosphaerella* spp. in pre-necrotic and prereproductive lesions and has provided a rapid and accurate method for *Mycosphaerella* spp. identification to validate disease surveys and research trials. Unlike classical methods, the detection technique enables rapid processing of large sample numbers and provides more comprehensive and reliable data. The nested PCR is a reliable and cost-effective method, useful for studies on the developmental ecology and epidemiology of MLD. It also may provide valuable information for the advance of management strategies for MLD on eucalypt plantations. The species-specific primer pairs described here also meet the requirements of an effective quarantine tool, providing rapid, direct detection of pathogens of concern from asymptomatic tissue, including germ plasm (74). This capability also may assist in nursery quality assurance that *Mycosphaerella* pathogens are eliminated and not merely suppressed prior to certification of disease-free seedlings and planting stocks. Such a move may help minimize the risk to industry of unwittingly introducing disease, particularly cryptic infections, into plantations at their most vulnerable stage.

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