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A specific primer PCR and RFLP assay for the rapid detection and differentiation *in planta* of some *Mycosphaerella* species associated with foliar diseases of *Eucalyptus globulus*

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It is difficult to accurately identify *Mycosphaerella* species associated with leaf diseases of *Eucalyptus* based on morphological characters, as there is considerable overlap between very similar species and subspecies, and isolation from the host is not easy. Thus, a PCR and RFLP assay based on the ITS region of nr DNA was developed for the rapid detection and differentiation of *M. nubilosa*, *M. cryptica* and two non-sporing unidentified *Mycosphaerella* species isolated from the foliage of trees in resistant and susceptible families of *E. globulus* in a seed orchard at Kinglake West, Victoria, Australia. The *M. nubilosa* primer pair MNF/MNR was highly specific. A PCR-RFLP system based on the primer pair MCF/MCR, coupled with two restriction enzymes (*Dde*I and *Tru*1I), differentiated *M. cryptica*, *M. nubilosa*, *M. tasmaniensis* and *M.* aff. *vespa*. One of the unidentified field-isolated *Mycosphaerella* species was identified as *M. grandis* on the basis of ITS sequence data while the other species remains unidentified. A PCR-RFLP system based on the primer pair U1F/U1R, coupled with the restriction enzyme *Sty*I, differentiated between the two unidentified species. Unexpectedly, unlike isolation and culture studies, these assays detected *M. nubilosa*, *M. cryptica* and *M. grandis* in all single lesions examined on both juvenile and adult leaves, and on both highly resistant and highly susceptible *E. globulus* trees at this site.

INTRODUCTION

Leaf disease caused by *Mycosphaerella* species (MLD) has been a major problem in nurseries and young plantations of eucalypts and is the most important disease of *Eucalyptus globulus* (blue gum) plantations in southern Australia (Park & Keane 1982a, Carnegie *et al.* 1998, Milgate *et al.* 2001, Maxwell *et al.* 2003) as well as in other countries (Dick 1982, Crous & Wingfield 1991). It is capable of causing severe leaf blight and defoliation of seedlings and saplings and is highly detrimental to the growth and form of the plants (Dick & Gadgil 1983, Lundquist 1985, Lundquist & Purnell 1987, Carnegie *et al.* 1994, Carnegie & Ades 2003). *E. globulus* is one of the most susceptible species (Carnegie *et al.* 1994, 1998).

So far 31 species of *Mycosphaerella* have been described on *Eucalyptus* species in Australia and elsewhere (Crous 1998, Carnegie & Keane 1998, Dick & Dobbie 2001, Maxwell *et al.* 2003). Of the 16 species recorded in Australia, *M. cryptica* and *M. nubilosa* are the most damaging in blue gum plantations, are the species most commonly associated with MLD (Carnegie *et al.* 1994, Dungey *et al.* 1997, Milgate *et al.* 2001, Carnegie & Ades 2002) and may occur together on *E. globulus*. A range of other *Mycosphaerella* species, including an unidentified species (Paul A. Barber, La Trobe University, unpubl.), *M. grandis* (Carnegie & Keane 1994), *M. aurantia* (Maxwell *et al.* 2003), *M. ambiphylla* (Maxwell *et al.* 2003) and the saprotrophic *M. parva* (Park & Keane 1982b) also occur in association with these species. *M. cryptica* commonly infects both juvenile and adult foliage of several *Eucalyptus* species. *M. nubilosa* has been commonly found only on juvenile foliage of *E. globulus* and related species (Park & Keane 1982a, Carnegie & Ades 2002), although it has recently been found on rare occasions infecting adult foliage of *E. globulus* in Western Australia (Maxwell, Hardy & Dell 2001) and Victoria (Barber, unpubl.).

Differentiation of *Mycosphaerella* species encountered on *Eucalyptus* relies on characters such as ascospore morphology and dimensions of perithecia, asci and ascospores, which vary within and overlap considerably between species (Park & Keane 1982b, Carnegie & Keane 1994, Crous & Wingfield 1996, Crous 1998). In addition, the size and shape of lesions (Carnegie & Ades 2002), distribution of perithecia on the leaf (Carnegie & Ades 2002), mode of ascospore germination (Park & Keane 1982b, Crous 1998), cultural

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characteristics (Park & Keane 1984, Crous & Wingfield 1996) and anamorph characters (Crous 1998) are important. Identification based on these criteria has been difficult and uncertain and has led to confusion in the differentiation of species. Identification is further complicated by the occurrence of more than one species from a leaf, sometimes even in a single lesion, and by the difficulty of isolating the species into pure culture (Crous 1998). Isolation is most successfully achieved by establishing single ascospore cultures. However, this procedure is difficult as the growth of cultures is extremely slow, the fungi do not sporulate readily in culture and the cultures are prone to contamination and poor revival after storage (Crous *et al*. 2001). A technique for rapid detection and accurate identification of these pathogens in infected plant material would be of great value in routine diagnosis in disease monitoring programmes and for epidemiological research.

Difficulties encountered in the use of classical taxonomy have led to the investigation of molecular techniques for the differentiation of *Mycosphaerella* species on *Eucalyptus*. Crous *et al*. (2001) showed that nucleotide sequences of ITS and large subunit (LSU) regions of rDNA can differentiate many *Mycosphaerella* species occurring on *Myrtaceae*, including *M. cryptica* and *M. nubilosa*. Previous work by Carnegie, Ades & Ford (2001) has shown that randomly amplified polymorphic DNA (RAPD) differentiated between *M. cryptica*, *M. nubilosa*, *M. gregaria* and *M. marksii*. These methods have the limitation that they can only be used with pure cultures of fungi because plant DNA also produces bands. In order to detect the fungi in infected plant tissue, specific primers with no homology to plant DNA are required.

The ITS regions of nuclear rDNA have been good targets for the identification, differentiation and phylogenetic analysis of fungi using molecular techniques (White *et al*. 1990, Lee & Taylor 1992, Sreenivasaprasad *et al*. 1996, Crous *et al*. 2001, Dunne *et al*. 2002). The ITS regions are less well conserved sequences nested between the highly conserved 18S, 5.8S and 28S rDNA genes. Differences between species in the nucleotide sequence of the rDNA, in particular the ITS1 and ITS2 regions, have been used to develop species-specific primers for fungi, and this has become a common approach in molecular identification strategies. PCR with such primers has detected pathogens by selectively amplifying fungal DNA from infected plant tissues. This approach has been used for the detection and differentiation of many closely related fungal pathogens such as *Mycosphaerella* species on banana leaves (Johanson & Jeger 1993). Crous *et al*. (2001) showed differences between ITS sequences of most species of *Mycosphaerella* from *Eucalyptus* and suggested that these could be used to simplify the identification of the fungi. Furthermore, Milgate, Vaillancourt & Mohammed (in Milgate *et al*. 2001) used ITS sequence data to confirm the identifications of pure cultures of *M. nubilosa*, *M. cryptica*, *M. grandis* and *M. vespa* isolated from *Eucalyptus* species in Tasmania. Such a method has potential to identify these fungi not only in pure culture but also in infected tissue, and to resolve anomalies such as the apparent absence of certain *Mycosphaerella* species on some *Eucalyptus* species (Milgate *et al*. 2001).

The objective of this study was to design species-specific primers to differentiate *Mycosphaerella* species associated with MLD on *E. globulus* both in culture and infected leaf tissue. An *E. globulus* seed orchard at Kinglake West, Victoria, Australia (37° 30′ S, 145° 14′ E) with a significant level of disease associated with *M. nubilosa*, *M. cryptica*, and two unidentified *Mycosphaerella* species was used as the source of material. The seed orchard had previously been assessed for the incidence and severity of disease, and the 44 'families' ranked according to the level of resistance, from highly resistant to highly susceptible (Barber & Keane 2001).

MATERIALS AND METHODS

Fungal isolates and extraction of genomic DNA

The isolates of *Mycosphaerella* and other fungi used in this study are listed in Table 1. All *Mycosphaerella* isolates were obtained from leaf lesions by establishing single ascospore cultures (Crous 1998,) and are stored in the culture collection at La Trobe University. Species were identified using classical morphological (e.g. ascospore morphology) and culture characters. Isolates that did not sporulate and could not be identified were labelled as 'unidentified species'; isolates 5, 6, 9 and 22 were morphologically like *M. grandis*, but isolate 8 differed in cultural morphology and germination pattern. Cultures of *Mycosphaerella* isolates were grown in half-strength potato dextrose-malt extract liquid medium in deep Petri dishes on a rotary shaker at 21 °C for up to a month depending on the growth rate of each fungus. Cultures of *Alternaria alternata Epicoccum nigrum*, *Penicillium expansum* and *P. glabrum* isolated from seed of *Eucalyptus delegatensis* were grown in potato dextrose liquid medium and incubated similarly. Mycelia were harvested, washed with sterile distilled water, blotted dry, frozen in liquid nitrogen in 100 mg aliquots and stored at −20 ° until used for DNA extraction.

For DNA extraction, mycelia (approx. 100 mg) were ground in liquid nitrogen in a mortar and pestle to produce a fine powder and total genomic DNA was extracted using the QIAGEN™ Dneasy Plant MiniKit (Qiagen, Clifton Hill, VIC) according to the manufacturer's instructions. DNA yields were estimated by electrophoresis using 1.4% agarose gels containing 0.2 μg ml^{-1} ethidium bromide with Tris-borate EDTA (89 mM Tris, 89 mM Boric acid, 4 ml 0.5 M EDTA at pH 8) as the running buffer by comparison with a 100 bp ladder DNA standard (MBI Fermentas™, Progen Industries, Richlands B.C., QD). Final DNA

Table 1. Isolates of *Mycosphaerella* from *Eucalyptus* from various locations in Victoria, Australia, and other fungi used in this study.

Isolate no.	Species	Isolate name	Host	Location[e]
1	*M. cryptica*[a]	KLJ 01.02a	*E. globulus*	Kinglake West
2	*M. cryptica*[a]	KLJ 04.3	*E. globulus*	Kinglake West
3	*M. nubilosa*[a]	KLJ 04.1	*E. globulus*	Kinglake West
4	*M. nubilosa*[a]	KLJ 02.03	*E. globulus*	Kinglake West
5	*Mycosphaerella* sp. 1[a,b]	KLJ 01.03	*E. globulus*	Kinglake West
6	*Mycosphaerella* sp. 2[a,b]	KLJ 02.03-1a	*E. globulus*	Kinglake West
7	*M. cryptica*	KLJ 01.02b	*E. globulus*	Kinglake West
8	*Mycosphaerella* sp.	KLJ 04.2	*E. globulus*	Kinglake West
9	*Mycosphaerella* sp.	KLJ 02.03-1b	*E. globulus*	Kinglake West
10	*M. cryptica*	KLJ 04.4	*E. globulus*	Kinglake West
11	*M. cryptica*	MB 0.01	*E. delegatensis*	Mt. Buffalo
12	*M.* aff. *vespa*	MB 0.04a	*E. delegatensis*	Mt. Buffalo
13	*M. cryptica*	TC 0.01	*E. obliqua*	Heywood
14	*M. cryptica*	TC 0.04	*E. ovata*	Heywood
15	*M. cryptica*	TC 0.09	*E. ovata*	Heywood
16	*M. cryptica*	TC 0.17	*E. globulus*	Deans Marsh
17	*M. cryptica*	GR 0.07	*E. camaldulensis*	Grampians
18	*M. nubilosa*	PAB 0.01	*E. globulus*	Dartmoor
19	*Mycosphaerella* sp.[c]	TC 0.37	*E. globulus*	Digby
20	*Mycosphaerella* sp.[c]	TC 0.36	*E. globulus*	Digby
21	*M. tasmaniensis*	TC 0.39	*E. globulus*	Digby
22	*Mycosphaerella* sp.	TC 0.19b	*E. globulus*	Digby
23	*M.* aff. *vespa*	MB 0.04b	*E. globulus*	Mt. Buffalo
24	*Mycosphaerella* sp.	ssp 2	*E. obliqua*	Cabbage Tree Ck.
25	*M. cryptica*	ssp 3	*E. obliqua*	Cabbage Tree Ck.
26	*Epicoccum nigrum*[d]			
27	*Alternaria alternata*[d]			
28	*Penicillium glabrum*[d]			
29	*P. expansum*[d]			

[a] Isolates used for designing species-specific primers.

[b] Isolates morphologically resembled each other on ½ PDA and ½ MEA, but ITS sequence data and BLASTN search results provided evidence that they are two different species.

[c] Isolates that morphologically resembled *M. nubilosa* in culture and were isolated from lesions on adult leaves of *E. globulus*.

[d] Isolated from *Eucalyptus* seed and likely to be common epiphytes on *Eucalyptus* foliage. Preserved cultures in sterile distilled water were used to inoculate potato dextrose liquid medium for the extraction of DNA.

[e] Location (latitude, longitude): Kinglake West (37° 28′ S, 145° 14′ W), Mt Buffalo (36° 46′ S, 146° 46′ W), Heywood (38° 08′ S, 141° 37′ W), Deans Marsh (38° 23′ S, 143° 53′ W), Grampians (37° 15′ S, 142° 26′ W), Dartmoor (37° 55′ S, 141° 16′ W), Digby (37° 48′ S, 141° 31′ W), Cabbage Tree Creek (37° 42′ S, 148° 42′ W).

concentration of each isolate was adjusted to 1–2 ng μl^{-1}.

Extraction of genomic DNA from diseased leaves

Diseased samples of juvenile and adult leaves were harvested from *Eucalyptus globulus* trees in the seed orchard. Leaf samples were frozen at −20 ° after harvest. Single leaf lesions (1–8 mm diam) were ground in liquid nitrogen with 20 µl of PVPP (polyvinylpolypyrrolidone 10 % w/v) (Matheson Coleman & Bell, Norwood, OH) to precipitate polyphenols and total DNA was extracted using a QIAGEN™ Dneasy Plant MiniKit.

Crude DNA extracted from lesions on adult leaves was further purified by passing through Bio-Spin polypropylene columns (Bio-Rad Laboratories, North Ryde, NSW) filled with sepharose CL-6B (Fluka, Castle Hill, NSW) using a method modified from Cullen & Hirsch (1998) to remove phenolic compounds and pigments, the presence of which inhibited DNA amplification. Approx. 1 ml sepharose CL-6B (pre-swollen in 20 % ethanol) equilibrated in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.2) was loaded onto the Bio-spin polypropylene columns, which were fitted with 1.5 ml microcentrifuge tubes and centrifuged at 1600 ***g*** for 6 min in a Beckman® J2-21M/E centrifuge (Beckman Instruments, Palo Alto, CA) to remove the excess ethanol and TE buffer. Columns were conditioned by the addition of 100 µl of sterile Milli-Q water and centrifuged similarly. Crude DNA extracts (100 µl) were loaded onto the semi-dried sepharose columns, the beads were allowed to expand for 5 min and centrifuged at 1600 ***g*** for 6 min at 10 ° on two successive occasions to collect purified DNA. DNA yields were estimated as previously described and final DNA concentrations adjusted to 0.5–1.0 ng μl^{-1}.

rDNA sequence analysis and primer design

The rDNA-ITS regions of two isolates each of *Mycosphaerella cryptica* (isolate nos. 1 and 2), *M. nubilosa* (isolate nos. 3 and 4) and the unidentified *Mycosphaerella* species (isolate nos. 5 and 6) obtained from

Table 2. Nucleotide sequences of species-specific primers designed for *Mycosphaerella* species based on rDNA–ITS sequences and the specific PCR reaction conditions.

Species	Primer name	Nucleotide sequence (5′–3′)	PCR conditions		Expected fragment size (bp)
			Primer concentration (μM)	Annealing temperature (°C)	
Mycosphaerella cryptica	MCF	TTTTCCAACCATGTTGCC	0.8	45	267
	MCR	TGTAATGACGCTCGAACAG	0.8		
Mycosphaerella nubilosa	MNF	CGTCGGAGTAATACAACC	0.2	50	199
	MNR	AGGCTGGAGTGGTGAAATG	0.2		
Mycosphaerella sp. 1	U1F	GCAGCGAAATGCGATAAG	0.8	45	222
	U1R	TCACAAGCGGATGATTAAAC	0.8		
Mycosphaerella sp. 2	U2F	TTCCGACCTCTTGTTGCCTC	0.5	50	165
	U2R	CATTTCGCTGCGTTCTTCATC	0.5		

diseased *E. globulus* trees in the seed orchard (Table 1) were amplified by PCR using ITS1 and ITS4 primers (White *et al*. 1990), sequenced and compared (see later) to design species-specific primers for each species. Reaction mixtures consisted of 2.5 μl 10× PCR buffer (Biotech International, Subiaco, WA), 1.5 μl 25 mM $MgCl_2$ (Biotech International), 0.5 μl 10 mM dNTP mix (Promega Corporation, Madison, WI), 1.0 unit of *Taq* DNA polymerase (Biotech International), 0.25 μl of each of 50 mM ITS1 and ITS4 primers (Oligonucleotide Synthesis Facility, Monash University, Melbourne), 1 μl of template DNA (1–2 ng μl^{-1}) and sterile Milli-Q water to make the volume up to 25 μl. DNA amplifications were performed in a Perkin Elmer® 2400 thermal cycler (Perkin Elmer, Teddington, UK) programmed for 10 min initial denaturation at 94 ° followed by 35 cycles of denaturation for 30 s at 94 °, annealing for 30 s at 55 °, extension for 1 min at 72 ° and final extension for 10 min at 72 °. PCR amplified products (5 μl) were electrophoresed on a 1.4% agarose gel containing 0.2 μg ml^{-1} ethidium bromide at 100 V for approx. 1 h with Tris-borate EDTA as the running buffer, visualised with a Chromato-Vue® UV transilluminator (Model TM 36) (Ultra-violet Products, Gabnel, CA) and photographed with Polaroid (No. 667) film.

PCR amplified rDNA-ITS regions were analysed for nucleotide sequence differences by digestion with restriction endonucleases, *Eco*RI, *Hin*dIII, *Hha*I, *Hae*III and *Pst*I (Promega Corporation) according to the manufacturer's instructions. The digests were electrophoresed on 2% agarose gels and visualised and photographed as described above. The PCR product of each isolate was purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions and sequenced in both directions according to the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit Protocol (Perkin Elmer Corporation). DNA was precipitated according to the ethanol/sodium acetate precipitation protocol (Perkin Elmer Corporation) and sequenced (DNA Sequencing Facility, Monash University).

The rDNA-ITS sequences for each isolate were searched for homology in GenBank using the search program BLASTN (Altschul *et al*. 1997) to confirm their identity. Each sequence was edited so that only ITS1, 5.8S and ITS2 regions were included and aligned with ITS sequences of closely matching species obtained from GenBank using ECLUSTALW (Thompson, Higgins & Gibson 1994). A similarity matrix was also created from this alignment using the HOMOLOGIES program (Jack A.M. Leunissen, www.cmbi.kun.nl/bioinf/EGCGdoc/Evolutionary_Analysis/homologies.html). Specific primer pairs were selected from those generated by the PRIME program on WEBANGIS™ by aligning them with the ITS sequences of all isolates used. Primer pairs were designed for *M. cryptica*, *M. nubilosa* and for each unidentified *Mycosphaerella* isolate. Oligonucleotides were synthesised in the Oligonucleotide Synthesis Facility, Monash University.

Specific PCR amplification

Specificity of each primer pair was tested against *Mycosphaerella* isolates, including those used for primer design, obtained from various *Eucalyptus* hosts, and against saprotrophic fungi that are commonly encountered on eucalypt seed (Table 1). PCR reactions followed a procedure similar to those with ITS1 and ITS4 primers, with minor modifications to the primer concentration and annealing temperature to suit each primer pair (Table 2). All PCR assays contained a sample without the template DNA as the negative control and a sample of pure fungal DNA targeted by each specific primer pair as the positive control. In addition, each DNA sample was assayed with ITS1 and ITS4 primers under similar PCR reaction conditions for each primer pair to ensure that the quality and concentration of DNA was adequate for amplification. All PCR assays were repeated at least twice.

RFLP analysis of the specific primer products of **Mycosphaerella cryptica** *and* **Mycosphaerella** *sp. 1*

PCR of fungal DNA with the primer pair designed for *Mycosphaerella cryptica* produced a non-specific cross-reaction with *M. nubilosa*. Similarly, the primer pairs

designed for each of the unidentified *Mycosphaerella* spp. 1 and 2 resulted in non-specific cross-reactions with the reciprocal unidentified *Mycosphaerella* species. Possible restriction sites of the PCR products were explored with the MAPSORT program in WebANGIS™ and two restriction enzymes, *Dde*I and *Tru*1I (Promega Corporation), were selected to produce polymorphic profiles to differentiate the two species. The restriction enzyme *Sty*I (MBI Fermentas™, Progen Industries, Richlands B.C., QD) was selected to differentiate between the two unidentified *Mycosphaerella* species (isolate nos. 5 and 6) (Table 1) using the PCR products from the *Mycosphaerella* sp. 1 (isolate no. 5) primer pair. Restriction digests with each enzyme were performed according to the manufacturer's instructions. Digests were electrophoresed on 2% agarose gels, visualised and analysed as previously described. All RFLP assays were repeated at least twice.

Phylogenetic analysis

Nucleotide sequences of rDNA-ITS region of the *Mycosphaerella* isolates obtained from the seed orchard and the isolates of *M. tasmaniensis* and *M.* aff. *vespa* (isolate no. 23) used in this study (Table 1) were analysed together with previously published sequences of *Mycosphaerella* species from *Eucalyptus* to compare their relative phylogenetic status. Nucleotide sequences of the entire ITS1–5.8S–ITS2 region of these isolates were obtained and aligned with sequences of the same region of *Mycosphaerella* isolates downloaded from GenBank using the alignment program ECLUSTALW (Thompson *et al.* 1994) and adjusted manually where necessary to maximise the alignment. The ITS sequence of *Neurospora crassa* (GenBank accession no. NCRGITR) downloaded from GenBank was used as the outgroup. An ITS sequence of *M. fijiensis* (GenBank accession no. AF297225) was also included in this analysis for comparison. The aligned sequences were reformatted using the software Seaview (Galtier *et al.* 1996), subjected to phylogenetic analysis using the neighbour-joining method (Saitou & Nei 1987) in MEGA Version 1.02 (Kumar, Tamura & Nei 1993) and the resulting tree was viewed using Tree Explorer Version 2.12 (Tamura 1999). Clade stability was evaluated using 500 neighbour-joining bootstrap replications (Felsenstein 1985). The sequences generated in this study have been deposited in GenBank (AY500246, AY53422–AY53434).

PCR detection of Mycosphaerella *species in infected plant tissue*

DNA from five lesions on juvenile leaves was screened with the specific primer PCR and RFLP system in a preliminary experiment to investigate if this assay was effective in detecting *Mycosphaerella* spp. in infected plant tissue. All specific primer PCRs and RFLPs followed similar procedures to those previously described for fungal DNA but using approx. 0.5–1.0 ng DNA from each leaf lesion in PCRs. However, a nested PCR was performed to assess the infected leaf material with the specific primer pair for *M. cryptica*, as the reactions of DNA from leaf lesions with this primer pair were too faint for RFLP analysis. The fungal specific primer ITS1F (Gardes & Bruns 1993) in combination with ITS4 (White *et al.* 1990) was used to amplify fungal DNA from leaf lesions in the first-round PCR. Approx. 0.5–1.0 ng DNA was used with the same procedure and the PCR cycle was as described previously with ITS1 and ITS4 except for an annealing temperature of 50 °. A 100-fold dilution of the products obtained in the first round PCR was used as the template (approx. 0.40–0.60 ng μl^{-1} DNA) for the second round PCR with *M. cryptica* specific primers. This PCR was performed as described previously with appropriate primer concentration and annealing temperature (Table 2) and the products were analysed with restriction enzymes *Dde*I and *Tru*1I as described for fungal DNA.

Subsequently, lesions from juvenile and adult leaves on resistant and susceptible *E. globulus* trees in the seed orchard (Barber & Keane 2001) were screened using this specific-primer PCR and RFLP system to compare the occurrence of fungal species on the particular leaves. Only lesions occurring singly on a leaf were used. Ten such lesions on separate leaves (five from juvenile and five from adult leaves) were used from each of five trees from a highly resistant 'family' (Family No. 7) and five trees from a highly susceptible family (Family No. 3) (Barber & Keane 2001). PCRs with primers MNF/MNR and UIF/U1R and RFLP assay were performed as previously described for fungal DNA but using approx. 0.5–1.0 ng DNA from each leaf lesion in PCRs. Assays with MCF/MCR were performed following the nested PCR procedure described above and the products were analysed with *Dde*I and *Tru*1I as described for fungal DNA.

RESULTS

rDNA sequence analysis and primer design

The six isolates of *Mycosphaerella* used for primer design resulted in single fragments of approx. 550 bp in the PCR with ITS1 and ITS4 primers (data not shown). The rDNA-ITS sequences of the two isolates of *M. cryptica* showed 100% similarity with published *M. cryptica* ITS sequences (GenBank accession nos. AY045494, AY045495, AY045498) whereas the two isolates of *M. nubilosa* resulted in 98–99% similarity with the published *M. nubilosa* ITS sequences (GenBank accession nos. AY045507, AY045508, AY045509). *Mycosphaerella* sp. 2 (isolate no. 6) (Table 1) showed 98% similarity with the published ITS sequences of *M. grandis* (GenBank accession nos. AY045513, AY045514, AY045516), whereas *Mycosphaerella* sp. 1 (isolate no. 5) (Table 1) showed 95%

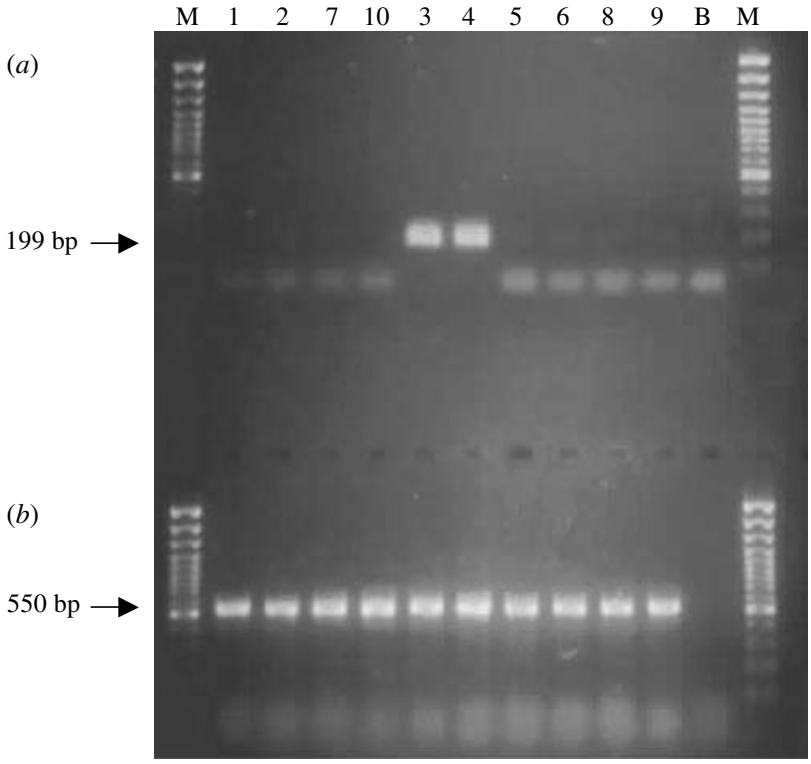

Fig. 1. PCR amplified DNA of *Mycosphaerella* isolates from the seed orchard with MNF/MNR (*a*) and ITS1/ITS4 (*b*) primers. Lane numbers refer to isolate numbers as indicated in Table 1. Lanes M, 100 bp DNA ladder; 1–2, 7, 10, *M. cryptica*; 3–4, *M. nubilosa*; 5, *Mycosphaerella* sp. 1; 6, *Mycosphaerella* sp. 2; 8–9, *Mycosphaerella* spp.; and B, negative control.

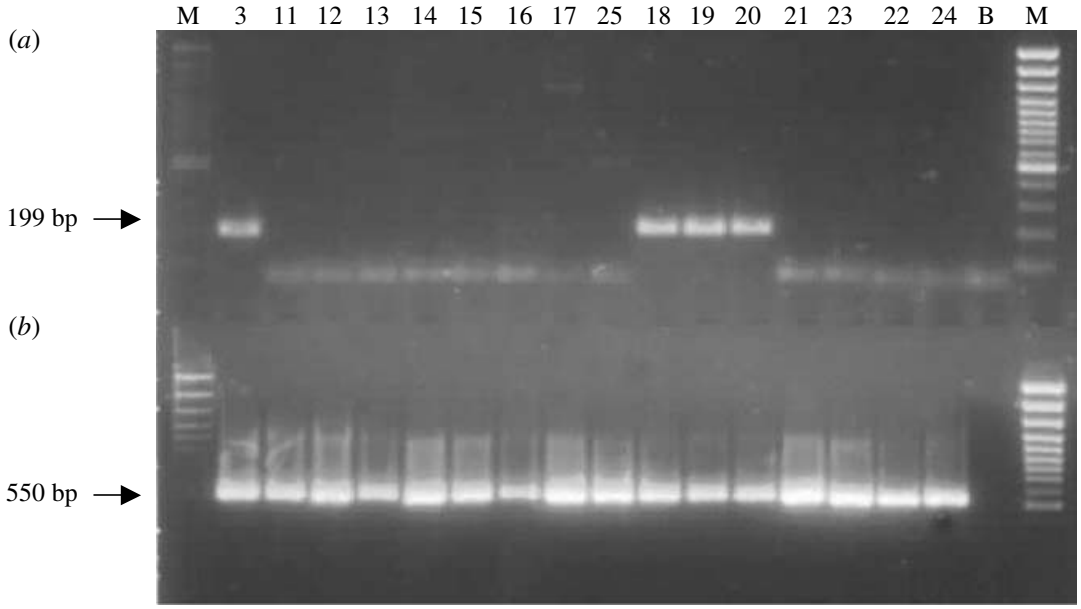

Fig. 2. PCR amplified DNA of *Mycosphaerella* isolates from *Eucalyptus* species at various locations in Victoria, Australia with MNF/MNR (*a*) and ITS1/ITS4 (*b*) primers. Lane numbers refer to isolate numbers as indicated in Table 1. Lanes: M, 100 bp DNA ladder; 3, *M. nubilosa* (positive control); 11, 13–17, 25, *M. cryptica*; 18, *M. nubilosa*; 19–20, unidentified *Mycosphaerella* spp. (from adult *E. globulus* leaves); 21, *M. tasmaniensis*; 12 & 23, *M. vespa*; 22 & 24, unidentified *Mycosphaerella* spp.; and B, negative control.

similarity with *M. grandis*. The similarity between the two unidentified *Mycosphaerella* isolates was 94%. Based on the BLASTN search results and their ITS homology of <99%, the two unidentified *Mycosphaerella* isolates (isolate nos. 5 and 6) were treated as two different species in the rest of this study.

Specific PCR amplifications and RFLP analysis with fungal cultures

The primer pair MNF/MNR designed for *Mycosphaerella nubilosa* (Table 2) amplified a single 199 bp fragment from all the *M. nubilosa* isolates (Figs 1a and 2a). No product was amplified from any other

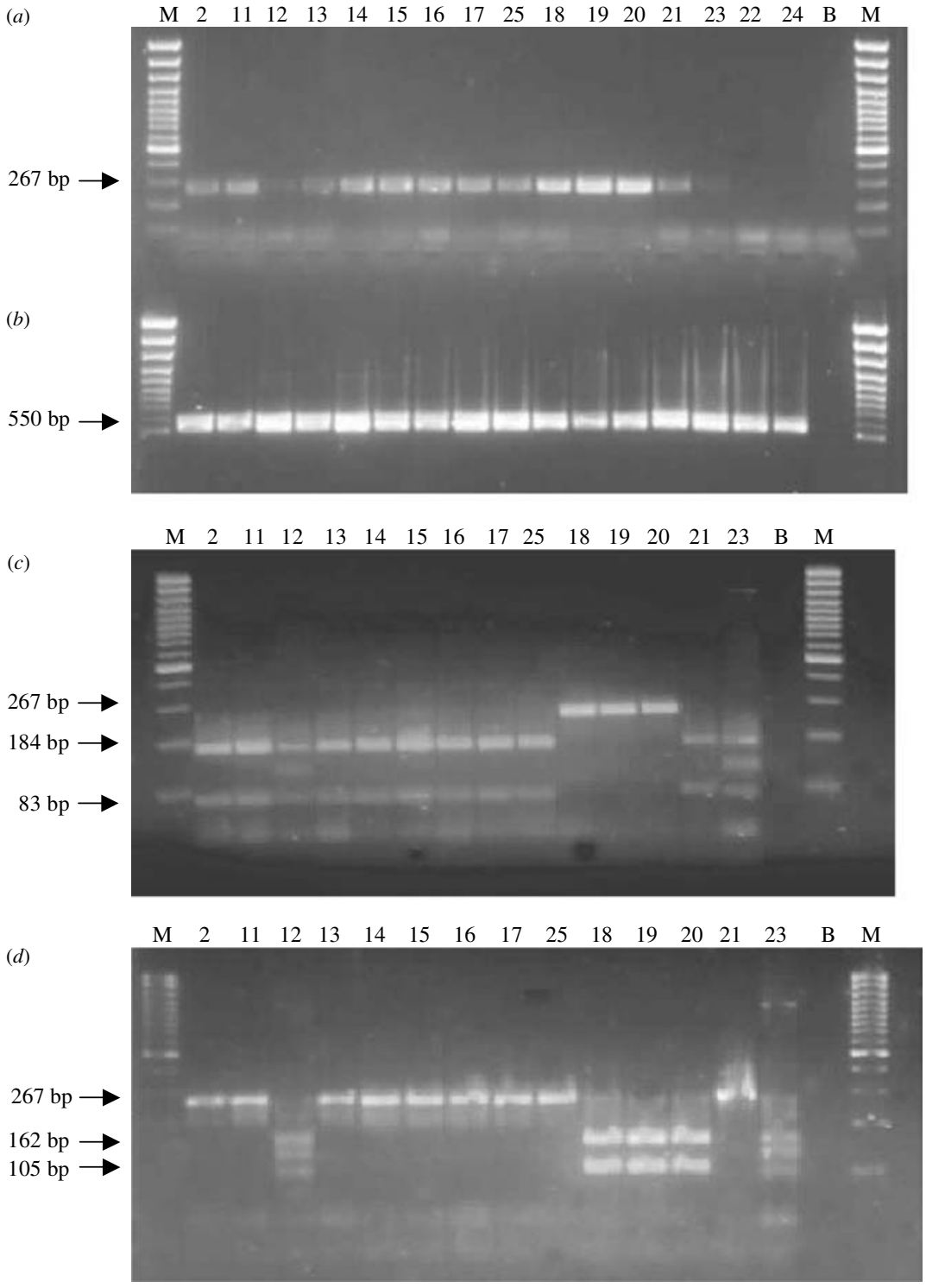

Fig. 3. PCR amplified DNA of *Mycosphaerella* isolates from *Eucalyptus* species at various locations in Victoria, Australia with MCF/MCR (*a*) and ITS1/ITS4 (*b*) primers and the subsequent restriction digestions of the MCF/MCR products with *Dde*I (*c*) and *Tru*1I (*d*). Lane numbers refer to isolate numbers as indicated in Table 1. Lanes: M, 100 bp DNA ladder; 2, *M. cryptica* (positive control); 11, 13–17, 25, *M. cryptica*; 18, *M. nubilosa*; 19–20, unidentified *Mycosphaerella* spp. (from adult *E. globulus* leaves) (*M. nubilosa* – see Fig. 6); 21, *M. tasmaniensis*; 12 & 23, *M.* aff. *vespa*; 22 & 24, unidentified *Mycosphaerella* spp.; and B, negative control. The MCF/MCR primer pair did not react with DNA of isolate nos. 22 and 24 and therefore restriction digestions for these two isolates are not shown in Figs (*c*) and (*d*).

DNA. The ITS1/ITS4 primers resulted in approx. 550 bp fragments with all DNA samples (Figs 1b and 2b). Further, the MNF/MNR primer pair amplified DNA from the two non-sporulating unidentified *Mycosphaerella* isolates (isolate no. 19 and 20) (Table 1) that were collected from adult *E. globulus* leaves in plantations in western Victoria and which morphologically resembled *M. nubilosa* (Fig. 2a). The ITS sequences of

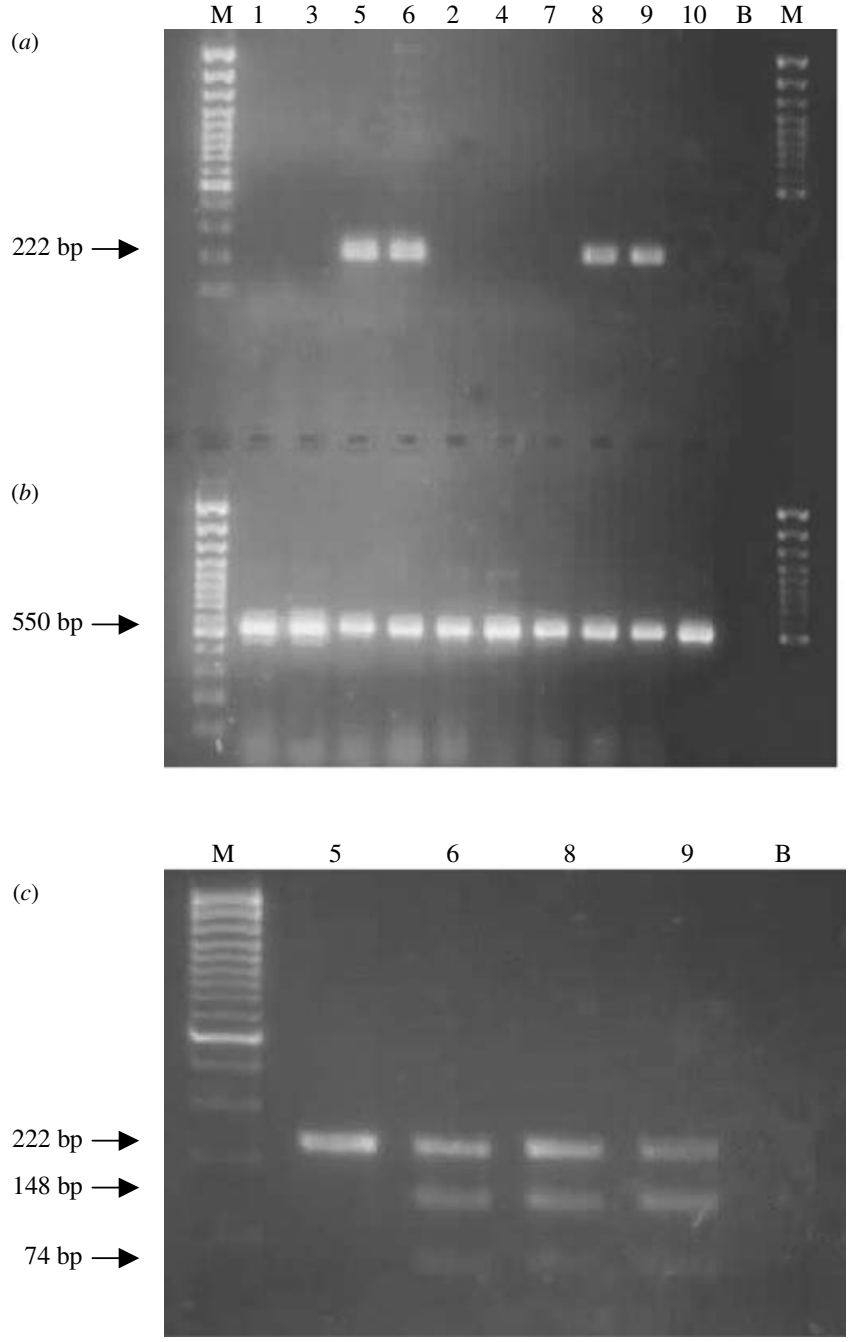

Fig. 4. PCR amplified DNA of *Mycosphaerella* isolates from the seed orchard with U1F/U1R (*a*) and ITS1/ITS4 (*b*) primers and the subsequent restriction digestion of the U1F/U1R products with *Sty*I (*c*). Lane numbers refer to isolate numbers as indicated in Table 1. Lanes: M, 100 bp DNA ladder; 1, 2, 7, 10, *M. cryptica*; 3–4, *M. nubilosa*; 5, *Mycosphaerella* sp. 1; 6, *Mycosphaerella* sp. 2; 8–9, unidentified *Mycosphaerella* spp.; and B, negative control.

both of these isolates showed 100% homology with the same published ITS sequences of *M. nubilosa* as mentioned previously (Fig. 6).

In contrast, the primer pair designed for *M. cryptica* (MCF/MCR) amplified the expected product of 267 bp with DNA from all isolates of *M. cryptica*, *M. nubilosa*, *M. tasmaniensis* and *M.* aff. *vespa* (Fig. 3a) even at an annealing temperature of 55 °. The subsequent restriction digestion of the PCR products with *Dde*I and *Tru*1I produced polymorphic profiles for each *Mycosphaerella* species (Figs 3c and 3d). *Dde*I produced a unique restriction pattern for each *Mycosphaerella* species (Fig. 3c). The two fragments produced by *Dde*I for *M. cryptica* and *M. tasmaniensis* were very similar but comparison of nucleotide sequences revealed that the smaller fragment was 13 bp longer in *M. tasmaniensis* than that of *M. cryptica*. By contrast, for *M.* aff. *vespa* this enzyme produced a restriction profile consisting of three fragments (Fig. 3c). *Tru*1I resulted in a unique restriction pattern for each of *M. nubilosa* and *M.* aff. *vespa*; however, it did not cut the PCR product of *M. cryptica* or *M. tasmaniensis* (Fig. 3d).

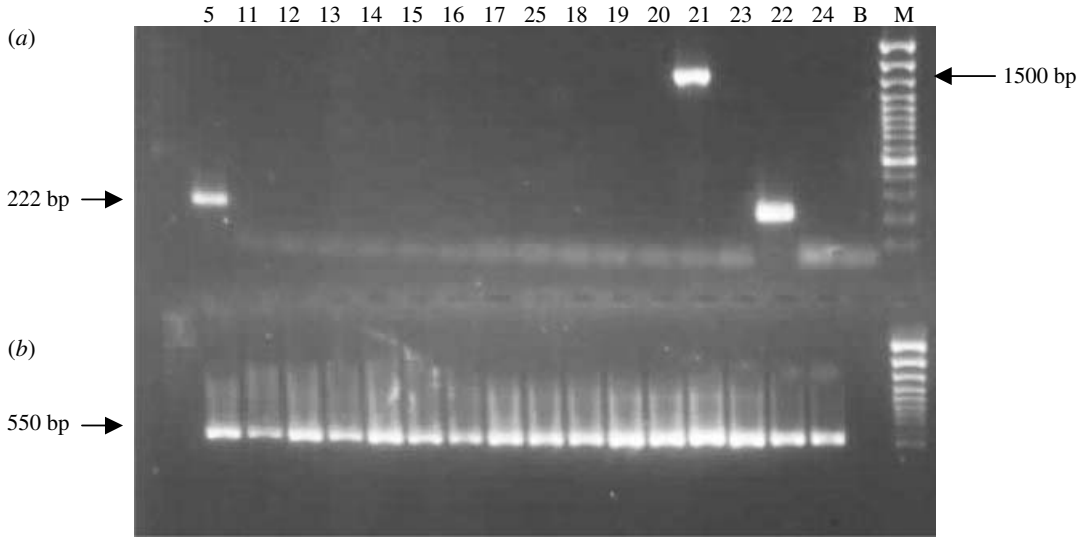

Fig. 5. PCR amplified DNA of *Mycosphaerella* isolates from *Eucalyptus* species at various locations in Victoria, Australia with U1F/U1R (*a*) and ITS1/ITS4 (*b*) primers. Lane numbers refer to isolate numbers as indicated in Table 1. Lanes: M, 100 bp DNA ladder; 5, *Mycosphaerella* sp. 1 (positive control); 11, 13–17, 25, *M. cryptica*; 18, *M. nubilosa*; 19–20, unidentified *Mycosphaerella* spp. (from adult *E. globulus* leaves); 21, *M. tasmaniensis*; 12 & 23, *M. vespa*; 22 & 24, unidentified *Mycosphaerella* spp.; and B, negative control.

The primer pair designed for *Mycosphaerella* sp. 1 (U1F/U1R) produced a single 222 bp fragment, only with all the unidentified *Mycosphaerella* isolates (nos. 5, 6, 8 and 9) obtained from the seed orchard (Fig. 4a). *Sty*I resulted in incomplete digestion of the U1F/U1R primer PCR products of *Mycosphaerella* sp. 2 (isolate no. 6) and two unidentified *Mycosphaerella* isolates (isolate nos. 8 and 9), resulting in two fragments of 148 bp and 74 bp (Fig. 4c). The U1F/U1R primers also resulted in a 222 bp product for the unidentified *Mycosphaerella* sp. (isolate no. 22) (Table 1, Fig. 5a), but *Sty*I did not cut this product (data not shown). The U1F/U1R primer pair amplified a single unique fragment of approx. 1500 bp with *M. tasmaniensis* (Fig. 5a).

The primer pair (U2F/U2R) designed for *Mycosphaerella* sp. 2 (isolate no. 6) produced a single 165 bp fragment, only with the unidentified *Mycosphaerella* isolates (nos. 5, 6, 8 and 9) from the seed orchard, isolate 22 and *M. tasmaniensis* (data not shown).

None of the specific primers produced any PCR product with the four common epiphytes (*Alternaria alternata*, *Epicoccum nigrum* and two *Penicillium* species) associated with *E. globulus* (Park & Keane 1982a).

Phylogenetic analysis

All *Mycosphaerella nubilosa* isolates used in the phylogenetic analysis formed a separate clade supported by a bootstrap value of 95% (Fig. 6). The *M. cryptica* isolates grouped together with isolates of *M. cryptica* from Australia and New Zealand with a bootstrap value of 96%, except for GenBank sequence AF309623. The unidentified *Mycosphaerella* isolates (isolate nos. 5, 6, 8 and 9) grouped together with *M. grandis* isolates within one clade but with a bootstrap value of 71%. The isolate of *M.* aff. *vespa* (isolate no. 23) grouped closest to a *M. vespa–M. molleriana* clade formed by these two species with a bootstrap support of 82%. The *M. tasmaniensis* isolate (isolate no. 21) grouped together with the isolates of the same species from Australia in a clade with 100% bootstrap support.

PCR detection of Mycosphaerella *species in infected plant tissue*

The PCR-RFLP system detected each *Mycosphaerella* species in leaf lesions, as they produced fragments with the expected sizes directly from DNA extracted from plant material, except for the U2F/U2R primers. None of the primer pairs amplified DNA from healthy (without visible lesions) *E. globulus* leaves.

The *M. nubilosa* specific primers (MNF/MNR) amplified a product of 199 bp for most of the DNA samples obtained directly from leaf lesions, whether from juvenile or adult leaves, on either resistant or susceptible plants (Figs 7a and 8a). Similarly the *Mycosphaerella* sp. 1 primers (U1F/U1R) resulted in a product of 222 bp for all these DNA samples (Figs 9a and 10a). The ITS1/ITS4 primers with each DNA sample produced two fragments of approx. 550 and 800 bp, representing fungal and plant DNA respectively, under similar PCR conditions (Figs 7b, 8b, 9b and 10b). Restriction digests of the U1F/U1R PCR products with *Sty*I produced the expected RFLP profile for *Mycosphaerella* sp. 2 (222, 148 and 74 bp) for all samples together with undigested products as for the pure cultures (Figs 9c and 10c).

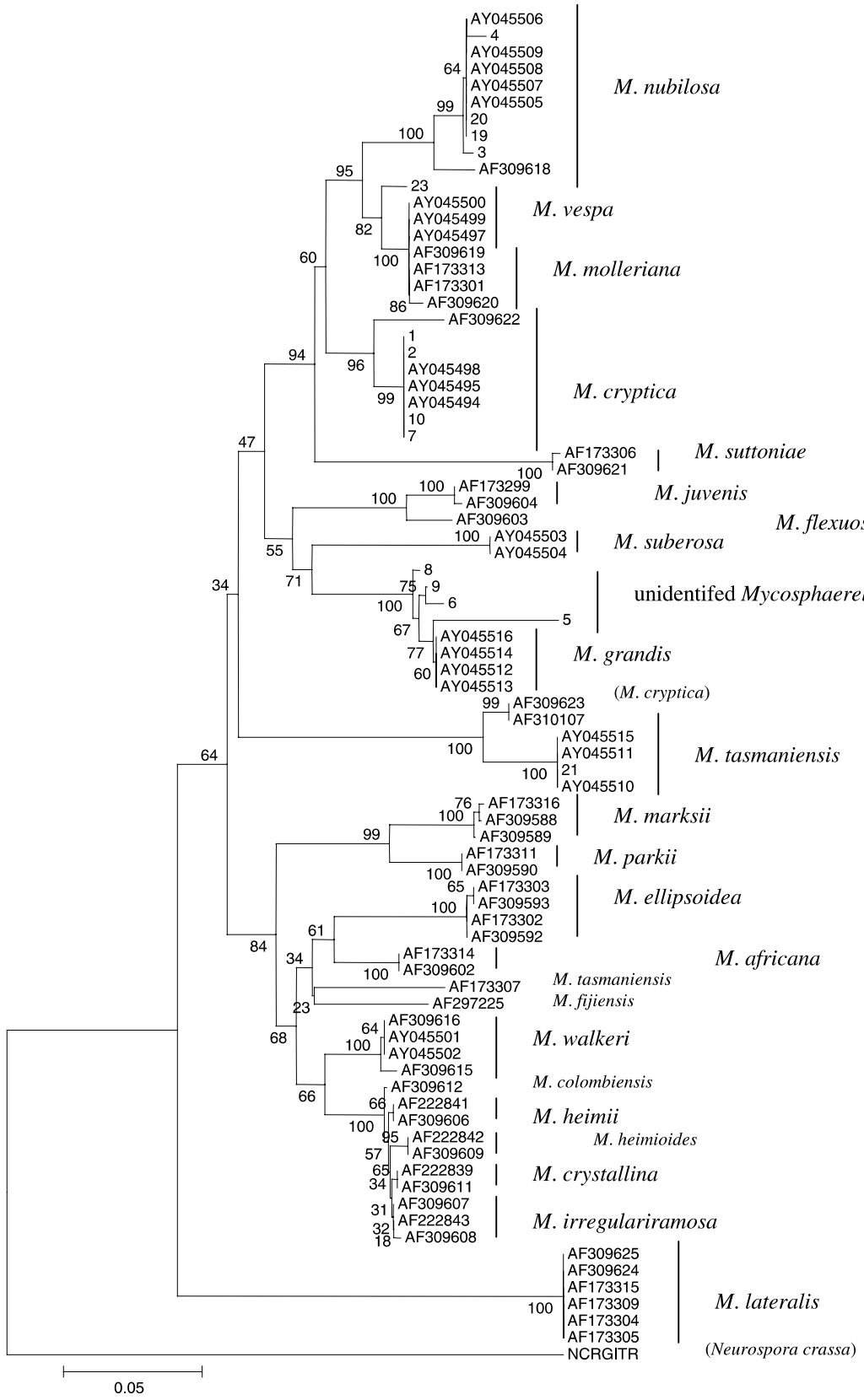

Fig. 6. Phylogenetic tree for ITS1–5.8S–ITS2 rDNA sequences of *Mycosphaerella* species observed on *Eucalyptus* constructed using the neighbour-joining method. Bootstrap values (500 replicates) are indicated in the nodes. Isolates with numbers from 1–10, 19–21 and 23 refer to isolate numbers in Table 1.

The nested PCR with the MCF/MCR primer pair and the RFLP system detected the banding patterns typical of both *M. cryptica* and *M. nubilosa* in about two-thirds of the DNA samples obtained from leaf lesions from juvenile and adult leaves on resistant and susceptible plants (Figs 11 and 12), although there

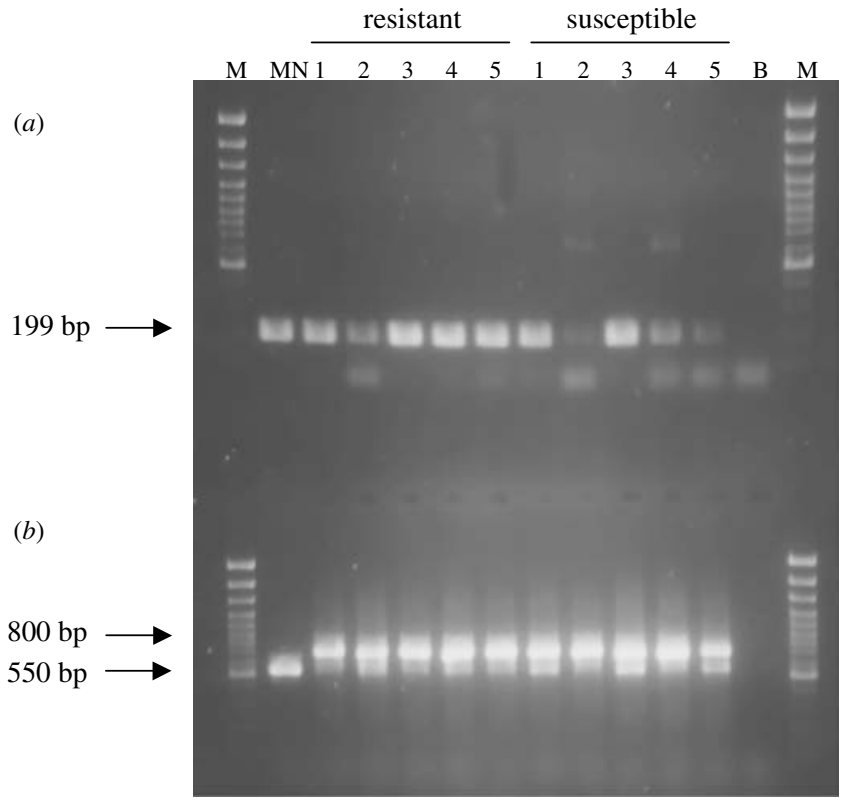

Fig. 7. PCR products of DNA from lesions on juvenile leaves of *Eucalyptus globulus* trees resistant or susceptible to Mycosphaerella leaf disease in seed orchard with MNF/MNR (*a*) and ITS1/ITS4 (*b*) primers. Lanes: M, 100 bp DNA ladder; MN, genomic DNA of *M. nubilosa* (positive control); 1–5, DNA of single leaf lesions; and B, negative control.

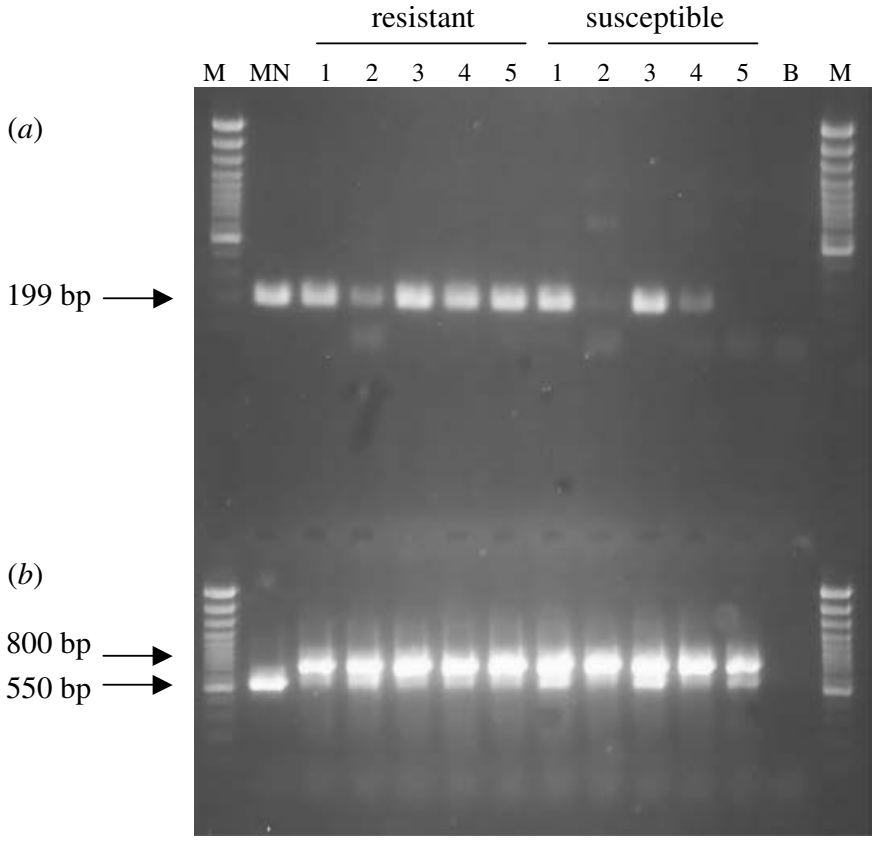

Fig. 8. PCR products of DNA from lesions on adult leaves of *Eucalyptus globulus* trees resistant or susceptible to Mycosphaerella leaf disease in the seed orchard with MNF/MNR (*a*) and ITS1/ITS4 (*b*) primers. Lanes: M, 100 bp DNA ladder; MN, genomic DNA of *M. nubilosa* (positive control); 1–5, DNA of single leaf lesions; and B, negative control.

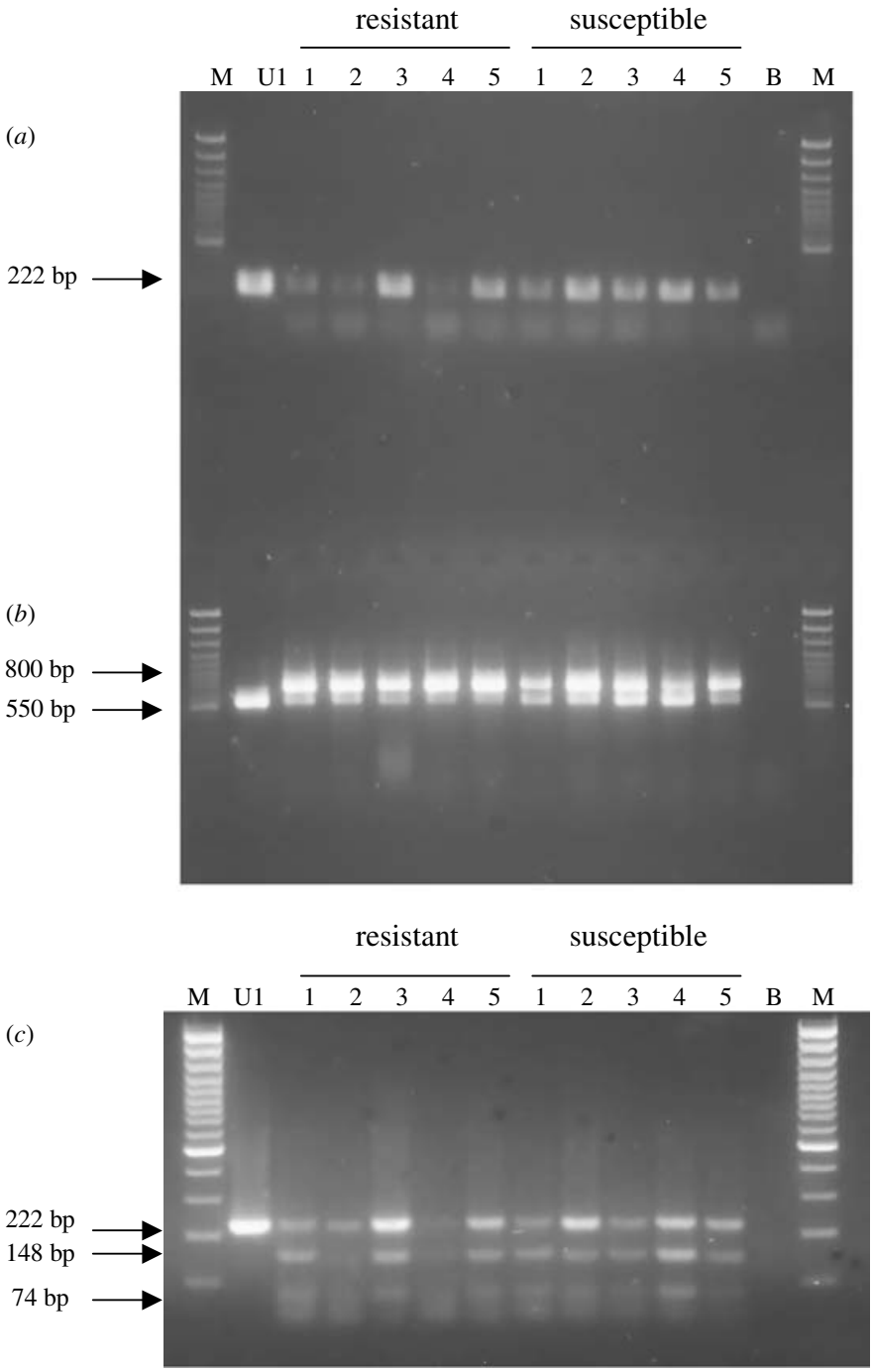

Fig. 9. PCR products of DNA from lesions on juvenile leaves of *Eucalyptus globulus* trees resistant or susceptible to Mycosphaerella leaf disease in the seed orchard with U1F/U1R (*a*) and ITS1/ITS4 (*b*) primers and the subsequent restriction digestions of the U1F/U1R PCR products with *Sty*I (*c*). Lanes: M, 100 bp DNA ladder; U1, genomic DNA of *Mycosphaerella* sp. 1 (positive control); 1–5, DNA of single leaf lesions; and B, negative control.

was a consistent problem with partial digestions. *Dde*I showed the RFLP pattern expected for *M. cryptica* with most leaf lesions (Figs 11c and 12c) and *Tru*1I showed the expected RFLP pattern for *M. nubilosa*, again with most leaf lesions (Figs 11d and 12d).

The specific-primer PCR-RFLP system detected patterns corresponding to *M. nubilosa*, *M. cryptica* and unidentified *Mycosphaerella* sp. 2 (isolate no. 6) in 67–95% of DNA samples from leaf lesions (Table 3). Although problems were experienced with partial digestions, especially with the U1F/U1R primer PCR product, this PCR-RFLP system showed no difference in the presence of the four *Mycosphaerella* species between resistant and susceptible plants, on either juvenile or adult leaves (Table 3).

DISCUSSION

The rDNA ITS based specific primer PCR-RFLP assay developed in this study successfully differentiated

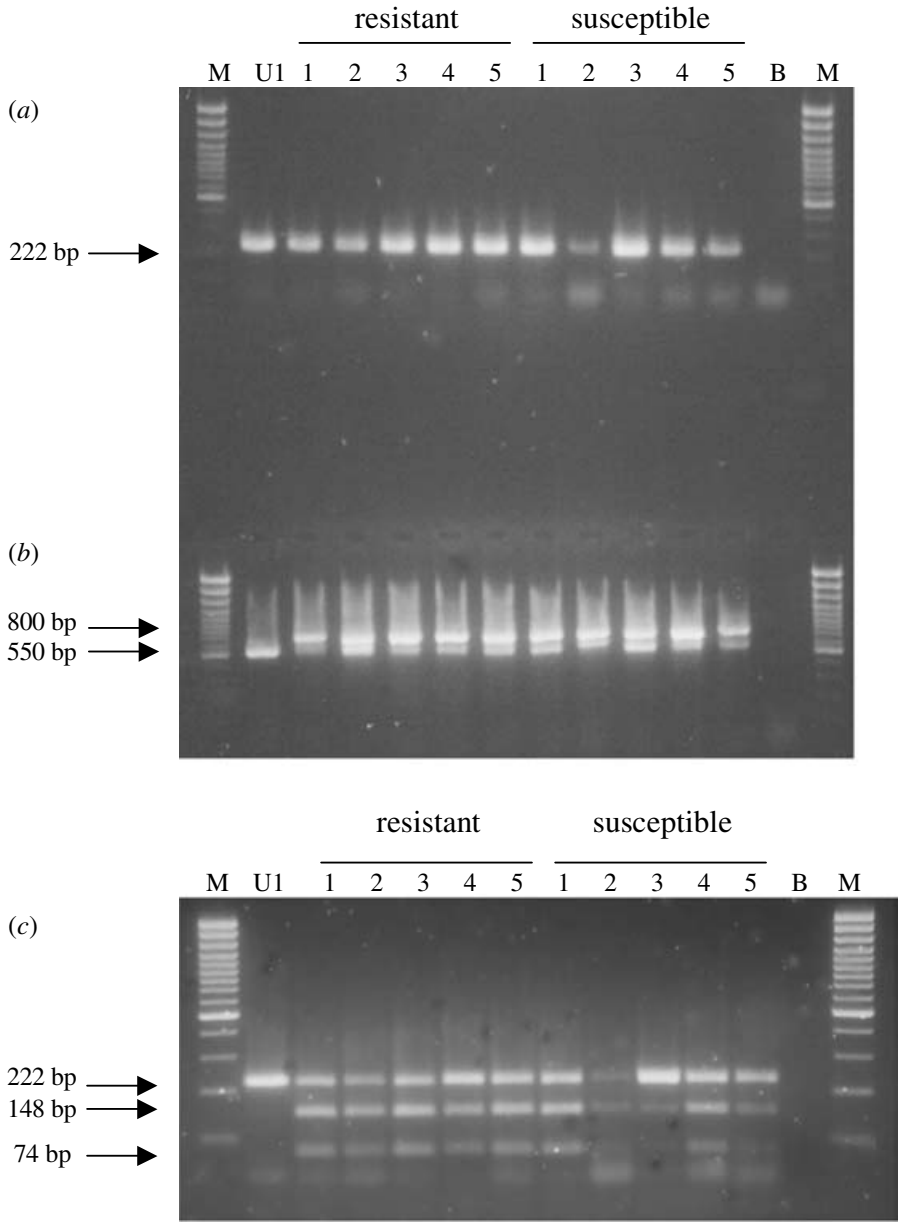

Fig. 10. PCR products of DNA from lesions on adult leaves of *Eucalyptus globulus* trees resistant or susceptible to Mycosphaerella leaf disease in the seed orchard with U1F/U1R (*a*) and ITS1/ITS4 (*b*) primers and the subsequent restriction digestions of the U1F/U1R PCR products with *Sty*I (*c*). Lanes: M, 100 bp DNA ladder; U1, genomic DNA of *Mycosphaerella* sp. 1 (positive control); 1–5, DNA of single leaf lesions; and B, negative control.

Mycosphaerella cryptica, *M. nubilosa* and the unidentified *Mycosphaerella* species encountered in the *Eucalyptus globulus* seed orchard. Further, this assay detected and differentiated between each of these fungi in infected leaf material, even in barely visible lesions (1 mm diam). Therefore, it can be used as a routine, sensitive, rapid and reliable alternative to the identification of these fungi by classical methods in leaf material, which has always been difficult.

The primer pair (MNF/MNR, Table 2) designed for *M. nubilosa* in this study was highly specific, as it did not amplify DNA from other *Mycosphaerella* species or any other fungi tested. It was highly sensitive in detecting the fungus in infected leaf material and can therefore be used as a fast and accurate method for detecting this common foliar pathogen on blue gums. The primer pair (MCF/MCR, Table 2) designed for *M. cryptica*, when used in combination with the restriction enzymes *Dde*I and *Tru*1I and the primer pair (U1F/U1R, Table 2) designed for *Mycosphaerella* sp. 1, when used in combination with *Sty*I differentiated *M. cryptica*, *M. nubilosa* and the unidentified *Mycosphaerella* species, in both culture and infected leaf tissue. The MCF/MCR primer pair in combination with the restriction enzyme *Dde*I differentiated between *M. tasmaniensis* and *M.* aff. *vespa*. Similar PCR-RFLP approaches have been widely used to differentiate between closely related fungal species (Chen, Hoy & Schneider 1992, Phan *et al.* 2002). As this assay was sensitive enough to identify

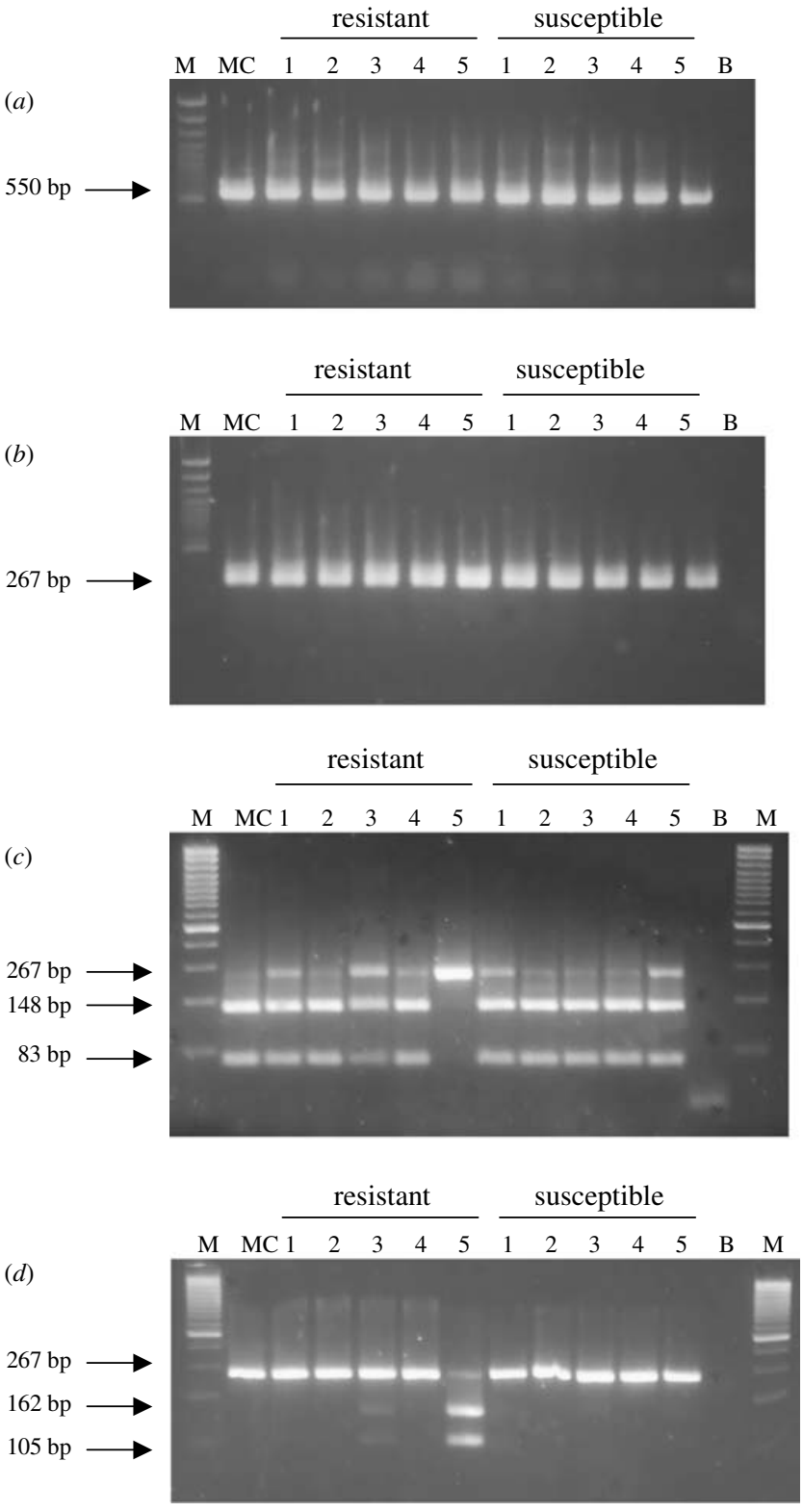

Fig. 11. PCR products of DNA from lesions on juvenile leaves of *Eucalyptus globulus* trees resistant or susceptible to Mycosphaerella leaf disease in the seed orchard with ITS1F/ITS4 (*a*) and MCF/MCR (*b*) primers and the subsequent restriction digestions of the MCF/MCR primer PCR products with *Dde*I (*c*) and *Tru*1I (*d*). Lanes: M, 100 bp DNA ladder; MC, genomic DNA of *M. cryptica* (positive control); 1–5, DNA of single leaf lesions; and B, negative control.

the presence of *Mycosphaerella* species in infected leaf tissue and to differentiate between species, it can be used as an accurate method of detection of these fungi *in planta*.

The PCR-RFLP assay developed in this study revealed the presence of *M. nubilosa*, *M. cryptica* and at least *Mycosphaerella* sp. 2 (probably *M. grandis*) on adult leaves of *E. globulus* showing symptoms of MLD,

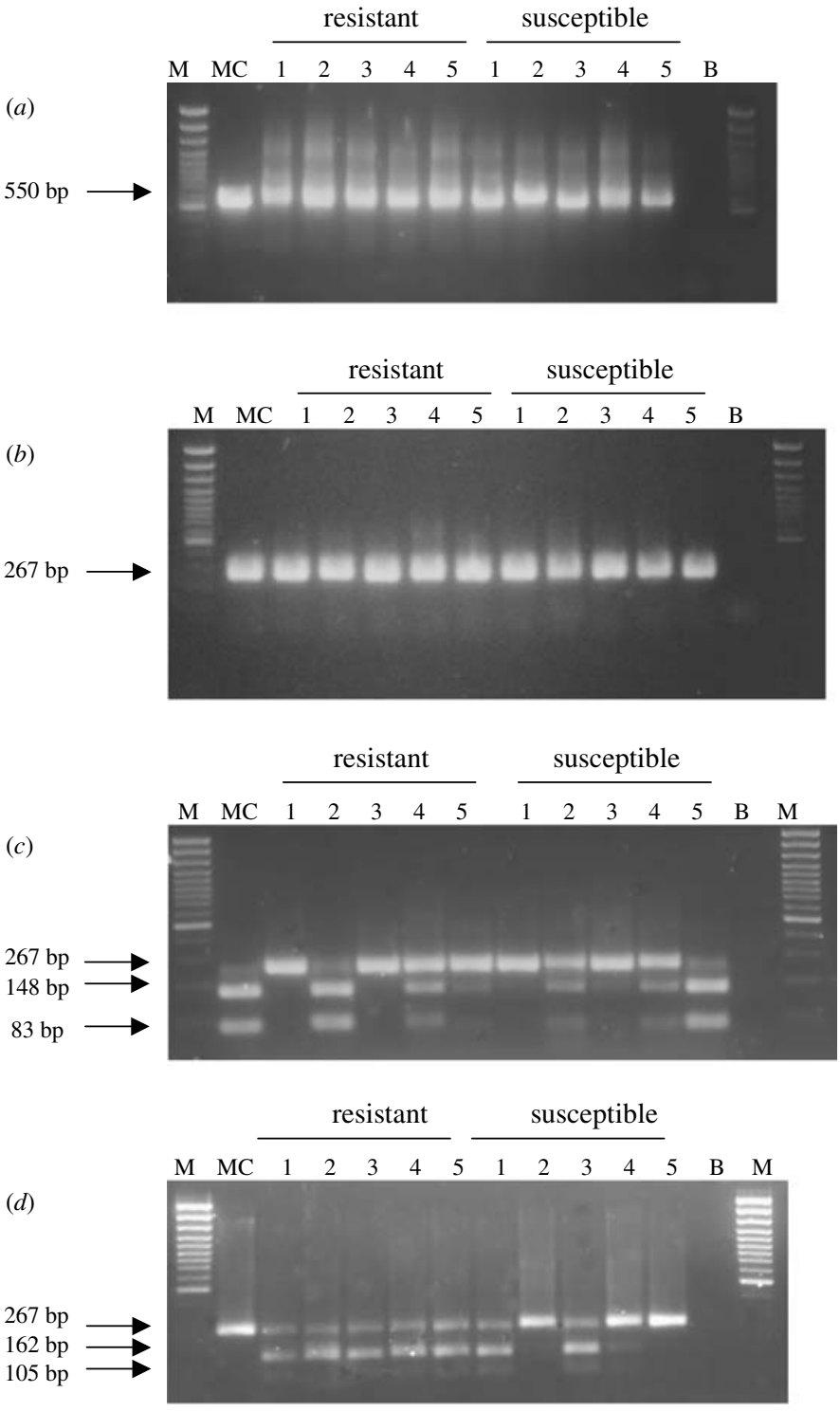

Fig. 12. PCR products of DNA from lesions on adult leaves of *Eucalyptus globulus* trees resistant or susceptible to Mycosphaerella leaf disease in the seed orchard with ITS1F/ITS4 (*a*) and MCF/MCR (*b*) primers and the subsequent restriction digestions of the MCF/MCR primer PCR products with *Dde*I (*c*) and *Tru*1I (*d*). Lanes: M, 100 bp DNA ladder; MC, genomic DNA of *M. cryptica* (positive control); 1–5, DNA of single leaf lesions; and B, negative control.

even though these symptoms were thought to be caused only by *M. cryptica* on the basis of visual observations and isolation and culture of fungi from the leaves. Furthermore, the *M. nubilosa* specific primers and sequencing of the ITS region confirmed that the two unidentified *Mycosphaerella* isolates resembling *M. nubilosa* (isolate nos. 19 and 20) obtained from adult leaves were *M. nubilosa*. Therefore, this study confirmed the regular occurrence of *M. nubilosa* in lesions on adult leaves of *E. globulus*, despite the fact that this species has been considered a pathogen only of juvenile leaves (Park & Keane 1982a).

It was not possible to infer any relationship between the different levels of disease resistance observed

Table 3. Results of the screening with the *Mycosphaerella* species-specific primers and RFLPs from lesions associated with juvenile and adult leaves of *Eucalyptus globulus* families designated as resistant or susceptible to Mycosphaerella leaf disease in the seed orchard.

		Juvenile leaves										Adult leaves									
Primer pair/ restriction		Resistant					Susceptible					Resistant					Susceptible				
enzyme	Target species	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
MNF/MNR	*M. nubilosa*	++	+	+	+	+	+	(+)	+	+	(+)	+	+	+	+	+	+	(+)	+	+	–
ITS1F/ITS4	*Mycosphaerella* spp.	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MCF/MCR	*Mycosphaerella* spp.	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
*Dde*I[a,c]	*M. cryptica*	++	+	+	+	(+)	+	+	+	+	+	–[e]	+	–[e]	+	+	(+)	+	+	+	+
*Tru1*I[b,c]	*M. nubilosa*	(+)	–[e]	+	(+)	+	(+)	–[e]	(+)	–[e]	(+)	+	+	+	+	+	+	(+)	+	+	–[e]
U1F/U1R	*Mycosphaerella* sp. 1 & 2	+	+	+	(+)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
*Sty*I[d]	*Mycosphaerella* sp. 2	+	(+)	+	(+)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

++, strong reaction; +, moderate reaction; (+), faint reaction; and –, no reaction.

[a] Only cut the MCF/MCR primer product of *M. cryptica.*

[b] Only cut the MCF/MCR primer product of *M. nubilosa.*

[c] Incomplete digestion was observed with all samples that gave positive reactions.

[d] Digested the U1F/ U1R primer product of the *Mycosphaerella* sp. 2. Resulted in incomplete digestion with all samples that showed positive reactions suggesting the presence of at least unidentified *Mycosphaerella* sp. 2.

[e] Incomplete digestions were observed with the complementary enzyme. The digestion product may have been very faint and not visible on the agarose gel.

among the two *E. globulus* 'families' and the occurrence of *Mycosphaerella* species on them. The PCR-RFLP assay detected no difference in the occurrence of four *Mycosphaerella* species from resistant or susceptible families. It detected all four species of *Mycosphaerella* on the majority of leaf samples screened.

On the basis of the PCR-RFLP assay, *M. grandis* (*Mycosphaerella* sp. 2) (as identified from GenBank) was found together with *M. cryptica* and *M. nubilosa* in single leaf lesions on juvenile as well as adult leaves. *M. nubilosa* and *M. cryptica*, which each cause distinctive leaf spots and blotches, often occur together on juvenile leaves of *E. globulus*, while *M. parva* was reported to occur in association with *M. nubilosa* and, to a lesser extent, *M. cryptica*, probably as a secondary invader (Park & Keane 1982a). Crous (1998) considered *M. parva* and *M. grandis* to be the same species and reduced *M. grandis* to synonymy under *M. parva*. The consistent occurrence reported here of *M. grandis* together with *M. nubilosa* and *M. cryptica* in single leaf lesions provides strong evidence in support of the synonymy of *M. grandis* under *M. parva*. This will not be resolved until the ITS region of the type specimen of each is sequenced. *Mycosphaerella* sp. 1 (isolate no. 5) from the seed orchard remains unidentified. This could be a new species or a species for which the ITS sequence is not available in GenBank. The PCR-RFLP system at present cannot confirm the presence of *Mycosphaerella* sp. 1 along with *Mycosphaerella* sp. 2 in single leaf lesions due to partial digestions observed with *Sty*I. Further work is necessary to solve this problem.

In conclusion, the PCR-RFLP assay developed in this study has differentiated between four *Mycosphaerella* species. These include *M. cryptica* and *M. nubilosa*, the two most common and destructive pathogens on *E. globulus* in southern Australia, which were distinguished both in culture and as in infected leaf tissue. Detection of the presence of a species in a leaf, however, does not mean that it is causing a high level of infection, as PCR is extremely sensitive, being able to detect even a single molecule of template DNA (Li, Cui & Arnheim 1990). Lee & Taylor (1990) amplified rDNA sequences from a single spore of *Neurospora tetrasperma*. Primers such as MNF/MNR with high sensitivity could therefore potentially detect even a spore of the target DNA in PCR. However, there was no asymptomatic detection of any *Mycosphaerella* species in healthy leaves from the same trees, suggesting that infection is required for detection by this PCR method. Further studies are needed to determine if *M. nubilosa*, *M. grandis* and the unidentified *Mycosphaerella* species are all actively pathogenic on adult *E. globulus* leaves.

This PCR-based direct detection assay provides a very useful tool for pathogen diagnosis, avoiding the difficulties encountered in producing pure cultures and inaccuracies often encountered when attempting to identify *Mycosphaerella* species using classical

taxonomic methods and cultural characteristics. It can be used as a fast and accurate method for detecting these fungi in infected plant material, which will facilitate routine diagnosis of the diseases, disease monitoring and epidemiological research.

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