Multiple gene genealogies reveal important relationships between species of *Phaeophleospora* infecting *Eucalyptus* leaves

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Abstract

The majority of *Eucalyptus* species are native to Australia, but worldwide there are over 3 million ha of exotic plantations, especially in the tropics and subtropics. Of the numerous known leaf diseases, three species of *Phaeophleospora* can cause severe defoliation of young *Eucalyptus*; *Phaeophleospora destructans*, *Phaeophleospora eucalypti* and *Phaeophleospora epicoccoide*s. *Phaeophleospora destructans* has a major impact on seedling survival in Asia and has not, as yet, been found in Australia where it is considered a serious threat to the biosecurity of native eucalypts. It can be difficult to distinguish *Phaeophleospora* species based on symptoms and micromorphology and an unequivocal diagnostic tool for quarantine purposes would be useful. In this study, a multiple gene genealogy of these *Phaeophleospora* species and designed specific primers has been constructed to detect their presence from leaf samples. The phylogenetic position of these *Phaeophleospora* species within *Mycosphaerella* was established. They are closely related to each other and to other important *Eucalyptus* pathogens, *Mycosphaerella nubilosa*, *Mycosphaerella cryptica* and *Colletogloeopsis zuluensis*. The specific primers developed can now be used for diagnostic and screening purposes within Australia.

Introduction

Eucalyptus species are highly favoured for the establishment of plantations. This is due to their rapid growth, ease of cultivation and their adaptation to a wide variety of different growing conditions (Turnbull, 2000). The timber of these trees is an important source of fibre for the international paper and pulp industry (Turnbull, 2000). In Australia, plantation forestry is rapidly increasing in size (National Forestry Inventory, 2004) and a number of fungal foliar pathogens have been reported to impact negatively on yields of these plantations. Among the most important of these pathogens are *Mycosphaerella* spp. (Carnegie *et al*., 1997; Park *et al*., 2000; Barber *et al*., 2003; Maxwell *et al*., 2003) and their incidence and severity is increasing as the areas under cultivation expand (Park *et al*., 2000; Maxwell *et al*., 2003).

Phaeophleospora Rangel is an anamorph genus assigned to some species of *Mycosphaerella* (Crous, 1998; Crous *et al*., 2001, 2004; Maxwell *et al*., 2003). Six species are known to cause disease on leaves of *Eucalyptus* species These are *Phaeophleospora epicoccoides* (Cooke & Massee) Crous, Ferreira & Sutton, *Phaeophleospora destructans* (MJ Wingf & Crous) Crous, Ferreira & Sutton, *Phaeophleospora eucalypti* (Cooke & Massee) Crous, Ferreira & Sutton, *Phaeophleospora lilianie* (Walker, Sutton & Pascoe) Crous, Ferreira & Sutton, *Phaeophleospora delegatensis* Park & Keane (Crous, 1998) and the recently described *Phaeophleospora toledana* Crous & G. Bills (Crous *et al*., 2004). Of these species, *P. epicoccoides, P. destructans* and *P. eucalypti* are considered important pathogens (Park *et al*., 2000). *Phaeophleospora lilianie* has been found only on yellow bloodwood (*Eucalyptus eximia*) in New South Wales and little is known regarding its importance (Chippendale, 1988). *Phaeophleospora delegatensis* is the anamorph of *Mycosphaerella delegantesis* (Park & Keane, 1984) isolated from the leaves of *Eucalyptus delegantensis* and *Eucalyptus obliqua* in Australia. It occasionally causes premature defoliation if the infection levels are severe. Both *P. liliane* and *P. delegatensis* have poor survival in culture and they have thus have never been successfully stored. *Phaeophleospora toledana* is the anamorph of *Mycosphaerella toledana* (Crous *et al*., 2004) named for its location of origin and it is not considered as a serious leaf pathogen.

Phaeophleospora destructans is an aggressive and often devastating pathogen that causes distortion of infected

leaves and blight of young leaves, buds and shoots (Wingfield *et al.*, 1996). This pathogen was first discovered in Indonesia in 1996 and has subsequently spread to Thailand, China, Vietnam and Timor (Old *et al.*, 2003a, b; Barber 2004; Burgess *et al.*, 2006). While most *Phaeophleospora* species infecting *Eucalyptus* leaves are known from Australia, *P. destructans*, the most pathogenic of these fungi has not been found in this country. Thus, the potential impact of *P. destructans* on native eucalypt forests is unknown, but of concern.

Phaeophleospora epicoccoides is the anamorph of *Mycosphaerella suttoniae* (Crous *et al.*, 1997) and it occurs worldwide infecting almost all eucalypt species (Sankaran *et al.*, 1995). This species is well known on native *Eucalyptus* species in Australia and it has most likely been spread to other countries with germ-plasm used to establish plantations. *Phaeophleospora epicoccoides* is a relatively weak pathogen typically infecting older leaves and stressed trees (Knipscheer *et al.*, 1990). *Phaeophleospora eucalypti*, a native pathogen in Australia, has in the past resulted in complete defoliation of juvenile leaves of *Eucalyptus nitens* in New Zealand, the only country where it is known to have been introduced (Dick, 1982; Hood *et al.*, 2002a, b). The worst affected *E. nitens* stands in New Zealand are currently being converted back to farmland (Hood *et al.*, 2002b).

The appearance and severity of lesions on *Eucalytpus* leaves are generally used to recognize the species of *Phaeophleospora* responsible for disease. However, depending on host and climate, the symptoms associated with infection by *P. epicoccoides*, *P. eucalypti* and *P. destructans* can be almost identical (Fig. 1) and incorrect diagnosis is a common problem. In addition, identification of *P. eucalypti* and *P. destructans* based on conidial morphology can be difficult because spore size varies depending on host species. A simple and accurate molecular diagnostic technique to distinguish between these important species would compliment traditional morphological diagnosis.

The aim of this study was to construct multiple gene genealogies for *P. epicoccoides*, *P. destructans* and *P. eucalypti*, the most common and destructive species occurring on *Eucalyptus*. Thus, partial sequences for six protein coding genes were generated to elucidate the phylogenetic relationships between these *Phaeophleospora* species. Following the construction of the phylogenies, species specific primers were then designed for diagnostic purposes.

Materials and methods

Fungal isolates

Phaeophleospora species were isolated under a dissecting microscope by collecting conidia exuding from single pycnidia, on the tip of a sterile needle. The spores were placed on malt extract ($20\,g\,L^{-1}$) agar (MEA), in a single spot and allowed to hydrate for 5 min. Conidia were then drawn across the agar surface with a sterile needle and single spores were picked off the agar and transferred to new MEA plates. Spores were left to germinate, which usually occurred within 24 h. Cultures were maintained at 20 °C on MEA. Isolates made for this study were compared with those of other closely related species (Table 1). All isolates are maintained in the collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa or the Murdoch University culture collection (MUCC), Perth, Western Australia.

DNA extraction

Isolates were grown on 2% MEA at 20 °C for 4 weeks and the mycelium was harvested, frozen in liquid nitrogen, ground to a fine powder and genomic DNA extracted using a hexadecyl trimethyl ammonium bromide (CTAB) protocol from Graham *et al.* (1994) modified by the addition of $100\,\mu g\,mL^{-1}$ Proteinase K and $100\,\mu g\,mL^{-1}$ RNAse A to the extraction buffer.

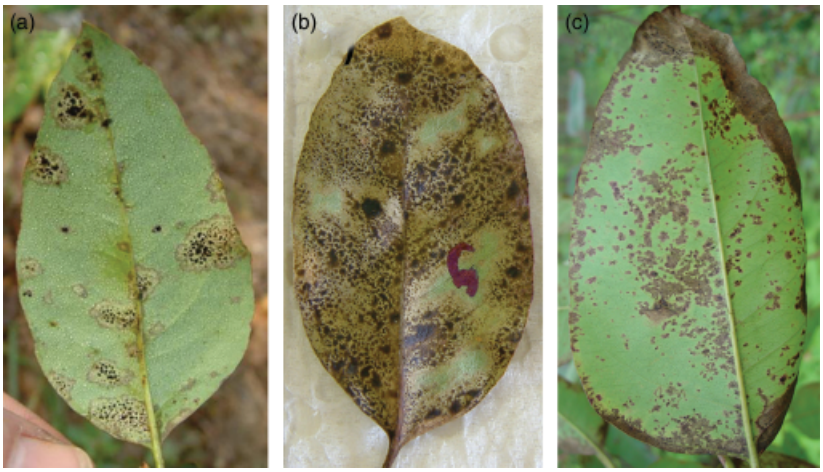


Fig. 1. Comparison of symptoms produced on juvenile *Eucalyptus grandis* leaves infected with (a) *Phaeophleospora destructans*, (b) *Phaeophleospora eucalypti* and (c) *Phaeophleospora epicoccoides* showing the similarity of symptoms associated with these fungi.

Table 1. Species and isolates considered in the phylogenetic study

							GenBank accession nos†		
Culture no.*	Teleomorph	Anamorph	Host	Location	Collector	ITS	β-tubulin	EF-1α	CHS
STE-U 1454 CMW 5351		*Phaeophleospora eugeniae*	*Eugenia uniflora*	Brazil	MJ Wingfield	AF309613 DQ632710			
STE-U1366 CMW 5219		*P. destructans*	*Eucalyptus grandis*	Sumatra, Indonesia	MJ Wingfield	AF309614 DQ632699			
CMW 7127		*P. destructans*	*Eucalyptus sp*	Sumatra, Indonesia	MJ Wingfield	**DQ632698**			
CMW 19906		*P. destructans*	*E. grandis*	Sumatra, Indonesia	PA Barber	**DQ632700**			
CMW 22553		*P. destructans*	*E. grandis*	Sumatra, Indonesia	PA Barber	**DQ632667**	**DQ632625**	**DQ632732**	**DQ632646**
CMW 17918		*P. destructans*	*E. grandis*	Sumatra, Indonesia	PA Barber	**DQ632666**	**DQ632624**	**DQ632731**	**DQ632645**
CMW 19832		*P. destructans*	*E. grandis*	Sumatra, Indonesia	PA Barber	**DQ632665**	**DQ632623**	**DQ632730**	**DQ632644**
CMW 17919		*P. destructans*	*E. urophylla*	Guangzhou, China	TI Burgess	**DQ632701**	**DQ632622**	**DQ632729**	**DQ632643**
MUCC 433		*P. eucalypti*	*E. nitens*	Victoria, Australia	PA Barber	**DQ632661**	**DQ632631**	**DQ632726**	**DQ632650**
CMW 17915		*P. eucalypti*	*E. nitens*	Victoria, Australia	PA Barber	**DQ632664**	**DQ632626**	**DQ632727**	**DQ632653**
MUCC 432		*P. eucalypti*	*E. grandis x E. tereticornis*	New South Wales	AJ Carnegie	**DQ632660**	**DQ632627**	**DQ632724**	**DQ632648**
MUCC 434		*P. eucalypti*	*E. grandis x E. tereticornis*	New South Wales	AJ Carnegie	**DQ632662**	**DQ632632**	**DQ632728**	**DQ632651**
CMW 17917		*P. eucalypti*	*E. grandis x E. tereticornis*	New South Wales	AJ Carnegie	**DQ632711**	**DQ632630**	**DQ632725**	**DQ632649**
MUCC 435		*P. eucalypti*	*E. grandis x E. camaldulensis*	Queensland	AJ Carnegie	**DQ632663**	**DQ632629**	**DQ632723**	**DQ632652**
CMW 17916		*P. eucalypti*	*E. grandis x E. camaldulensis*	Queensland	AJ Carnegie	**DQ632659**	**DQ632628**	**DQ632722**	**DQ632647**
CMW 11687		*P. eucalypti*	*E. nitens*	New Zealand	M Dick	DQ240001	**DS890168**	DQ235115	**DQ890167**
NZFS85C/23		*P. eucalypti*	*E. nitens*	New Zealand	M Dick	AY626988			
NZFS85C/1		*P. eucalypti*	*E. nitens*	New Zealand	M Dick	AY626987			
MUCC 422	*M. suttoniae*	*P. epicoccoides*	*E. grandis x E. camaldulensis*	Queensland	G Hardy	**DQ632656**			
MUCC 424	*M. suttoniae*	*P. epicoccoides*	*E. grandis x E. camaldulensis*	Queensland	G Hardy	**DQ632703**	**DQ632617**	**DQ632712**	**DQ632633**
MUCC 428	*M. suttoniae*	*P. epicoccoides*	*E. grandis x E. camaldulensis*	Queensland	TI Burgess	**DQ632707**	**DQ632618**	**DQ632717**	**DQ632638**
MUCC 430	*M. suttoniae*	*P. epicoccoides*	*E. grandis*	Queensland	G Whyte	**DQ632708**			
MURU 327	*M. suttoniae*	*P. epicoccoides*	*E. globulus*	Western Australia	S Jackson	**DQ632702**	**DQ632619**	**DQ632716**	**DQ632639**
MUCC 426	*M. suttoniae*	*P. epicoccoides*	*E. globulus*	Western Australia	S Jackson	**DQ632704**	**DQ632620**	**DQ632715**	**DQ632637**
CMW 22482	*M. suttoniae*	*P. epicoccoides*	*E. grandis*	Sumatra, Indonesia	PA Barber	**DQ632658**	**DQ632621**	**DQ632719**	**DQ632636**
MUCC 425	*M. suttoniae*	*P. epicoccoides*	*E. grandis*	New South Wales	TI Burgess	**DQ632655**	**DQ632613**	**DQ632713**	**DQ632634**
MUCC 429	*M. suttoniae*	*P. epicoccoides*	*E. grandis*	New South Wales	TI Burgess	DQ530226			
MUCC 431	*M. suttoniae*	*P. epicoccoides*	*E. grandis*	New South Wales	TI Burgess	DQ530227			
CMW 22484	*M. suttoniae*	*P. epicoccoides*	*E. urophylla*	China	TI Burgess	**DQ632705**	**DQ632616**	**DQ632714**	**DQ632635**
CMW 22486	*M. suttoniae*	*P. epicoccoides*	*E. urophylla*	China	TI Burgess	**DQ632706**	**DQ632615**	**DQ632720**	**DQ632642**
CMW 17920	*M. suttoniae*	*P. epicoccoides*	*E. urophylla*	China	TI Burgess	**DQ632654**	**DQ632612**	**DQ632721**	**DQ632641**
CMW 22483	*M. suttoniae*	*P. epicoccoides*	*E. grandis*	Indonesia	PA Barber	**DQ632709**			
CMW 5348 STE-U 1346	*M. suttoniae*	*P. epicoccoides*	*Eucalyptus* sp.	Indonesia	MJ Wingfield	AF309621	DQ240117	DQ240170	DQ890166
SA12	*M. suttoniae*	*P. epicoccoides*	*E. fragrata*	South Africa	MN Cortinas	**DQ632657**	**DQ632614**	**DQ632718**	**DQ632640**
STE-U 10840 CPC 10840	*M. toledana*	*P. toledana*	*E. globulus*	Spain	PW Crous	AY725580			

Table 1. Continued

							GenBank accession nos†		
Culture no.*	Teleomorph	Anamorph	Host	Location	Collector	ITS	β-tubulin	EF-1α	CHS
CBS 113313 CMW 14457	*M. toledana*	*P. toledana*	*E. globulus*	Spain	PW Crous	AY725581	**DQ658235**	DQ235120	**DQ658226**
AMR 051	*M. nubilosa*		*E. globulus*	Western Australia	A Maxwell	AY509777			
AMR 057	*M. nubilosa*		*E. globulus*	Western Australia	A Maxwell	AY509778			
CMW 11560	*M. nubilosa*		*E. globulus*	Tasmania	A Milgate	**DQ658232**	**DQ658236**	DQ240176	**DQ658230**
CMW 6211	*M. nubilosa*		*E. nitens*	South Africa	G Hunter	AF449094			
CMW 9003	*M. nubilosa*		*E. nitens*	South Africa	G Hunter	AF449099			
AMR 118	*M. cryptica*	*Colletogloeopsis nubilosum*	*E. globulus*	Western Australia	A Maxwell	AY509753			
AMR 115	*M. cryptica*	*C. nubilosum*	*E. globulus*	Western Australia	A Maxwell	AY509754			
CMW 3279	*M. cryptica*	*C. nubilosum*	*E. globulus*	Australia	AJ Carnegie	AY309623	**DQ658234**	DQ235119	**DQ658225**
CMW 4915		*C. zuluensis*	*E. grandis*	South Africa	MJ Wingfield	AY244421			
CBS 117262 CMW 7449		*C. zuluensis*	*E. grandis*	South Africa	L Van Zyl	DQ240021	DQ240102	DQ240155	**DQ658224**
CBS 113399 CMW 13328		*C. zuluensis*	*E. grandis*	South Africa	L Van Zyl	DQ240018	**DQ658233**	DQ240172	**DQ658223**
CBS 110499 CMW 13704	*M. ambiphylla*	*Phaeophleospora* sp.	*E. globulus*	Western Australia	A Maxwell	AY150675	DQ240116	DQ240169	**DQ658229**
STE-U 784	*M. molleriana*	*C. molleriana*	*Eucalyptus* sp.	USA		AF309619			
CMW 4940 CPC1214	*M. molleriana*	*C. molleriana*	*Eucalyptus* sp.	Portugal	MJ Wingfield	DQ239969	DQ240115	DQ240168	**DQ658228**
A/1/8	*M. vespa*	*Coniothyrium ovatum*	*Eucalyptus* sp.	Tasmania	A Milgate	AY045499			
CMW 11588	*M. vespa*	*Co. ovatum*	*E. globulus*	Tasmania	A Milgate	DQ239968	DQ240114	DQ240167	**DQ658227**
CMW 6210	*M. vespa*	*Co. ovatum*	*E. globulus*	New South Wales	MJ Wingfield	AF449095			
CBS 110906		*Coniothyrium* sp.	*E. cladocalyx*	South Africa	PW Crous	AY725513			
CBS 111149		*Coniothyrium* sp.	*E. cladocalyx*	South Africa	PW Crous	AY725514			
CBS 113621		*Coniothyrium* sp.	*E. cladocalyx*	South Africa	PW Crous	AY725515			
CBS 116427		*Coniothyrium* sp.	*Eucalyptus* sp.	South Africa	PW Crous	AY725516			
CPC 18		*Coniothyrium* sp.	*E. cladocalyx*	South Africa	PW Crous	AY725517			
CBS 116428		*Coniothyrium sp.*	*Eucalyptus* sp.	South Africa	PW Crous	AY725518			
CBS 113265 CMW 13333	*M. punctiformis*	*Ramularia endophylla*	*Quercus robor*	Netherlands		AY490763			
CMW 9091	*M. marksii*	*Pseudocercospora epispermogonia*	*Eucalyptus* sp.	South Africa	G Hunter	AF468871			
STE-U 796 CBS 680.95	*M. africana*		*Eucalyptus* sp.	South Africa	PW Crous	AF173314			
STE-U 1084	*M. keniensis*		*Eucalyptus* sp.	Kenya	MJ Wingfield	AF173300			
CBS 110500 AMR 221	*M. aurantia*		*E. globulus*	Western Australia	A Maxwell	AF509743			
CBS 110969 STE-U1106	*M. colombiensis*	*Ps. colombiensis*	*Eucalyptus* sp.	Colombia	MJ Wingfield	AF309612			
CBS 110503 AMR 251	*M. parva*		*E. globulus*	Western Australia	A Maxwell	AF509782			
NZs	*M. suberosa*				A Milgate	AY045503			
CBS 110949	*M. ohnowa*		*E. grandis*	South Africa	MJ Wingfield	AY725575			
STE-U 1225	*M. ellipsoidea*	*Uwebraunia ellipsoidea*	*Eucalyptus* sp.	South Africa	MJ Wingfield	AF173303			
CMW 9098	*M. ellipsoidea*	*U. ellipsoidea*	*Eucalyptus* sp.	South Africa	MJ Wingfield	AF468874			
CMW 7774	*Botryosphaeria obtusa*		*Ribes* sp.	New York, USA	B Slippers	AY236953			
CMW 7773	*B. ribis*		*Ribes* sp.	New York, USA	B Slippers	AY236936	AY808170	AY236878	**DQ658231**

*Designation of isolates and culture collections: CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW, Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; STE-U, Stellenboch University, South Africa; MUCC, Murdoch University, Perth, Western Australia.

†Sequences in bold were obtained during this study.

PCR amplification

This study included partial amplification of the18S gene, the complete internal transcribed spacer (ITS) region 1, the 5.8S rRNA gene and the complete ITS region 2 and the 5′ end of the 26S (large subunit) rRNA gene, part of the β-tubulin gene region, part of elongation factor 1α gene (EF-1α), part of Chitin synthase 1 gene (CHS), part of the RNA polymerase II subunit (RPB2) and part of ATPase gene (ATP-6). Primers used for amplification of these regions are listed in (Table 2). The PCR reaction mixture (25 μL), PCR conditions and visualization of products were as described previously (Cortinas *et al.*, 2006) except that 1 U of Taq polymerase (Biotech International, Needville, TX) was used in each reaction. For failed amplifications, the Mg concentration was increased to 4 mM, and primer concentration to 0.9 pmol and the following PCR conditions were used; 7 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 45 °C, 2 min at 72 °C and final elongation step of 10 min at 72 °C. RPB2 degenerate primers were tested at a range of temperatures, but failed to amplify the DNA of some representative isolates. Therefore, two successful amplicons were sequenced and primers redesigned and named RPB2-myco-6F and RPB2-myco-7R (Table 2). The PCR products were purified with Ultrabind®DNA purification kit (MO BIO Laboratories, Solana Beach, CA) following the manufacturer instructions. Amplicons were sequenced as described previously (Burgess *et al.*, 2005)

Phylogenetic analyses

In order to compare *Phaeophleospora* isolates used in this study with other closely related species, additional sequences were obtained from GenBank (Table 1). Sequence data were assembled using SEQUENCE NAVIGATOR version 1.01 (Perkin Elmer) and aligned in CLUSTALX (Thompson *et al.*, 1997) Manual adjustments were made visually by inserting gaps where necessary. All sequences obtained in this study have been deposited in GenBank and accession numbers are shown in Table 1.

The initial analysis was performed on an ITS dataset alone and subsequent analyses were performed on a combined dataset of ITS, β-tubulin, CHS and EF-1α sequence, after a partition homogeneity test (PHT) had been performed in phylogenetic analysis using parsimony (PAUP) version 4.0b10 (Swofford, 2003) to determine whether sequence data from

Table 2. Primer sets and annealing temperature used to amplify *Phaeophleospora* spp

Region	Oligos	Oligo Sequence (5′–3′)	Amplicon size (bp)	AT (°C)	Reference
ITS	ITS-1F	CTTGGTCATTTAGAGGAAGTAA	600	50	Gardes & Bruns (1993)
	ITS-4	TCCTCCGCTTATTGATATGC			
ITS	ITS-3	GTATCGATGAAGAACGCAGC	250	55	White *et al.* (1990)
	ITS-4	TCCTCCGCTTATTGATATGC			
β-tubulin	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	680	45–55	Glass & Donaldson (1995)
	Bt2a	ACCCTCAGTGTAGTGACCCTTGGC			
EF-1α	EF1-728F	CATCGAGAAGTTCGAGAAGG	350	45–55	Carbone & Kohn (1999)
	EF1-986R	TACTTGAAGGAACCCTTACC			
CHS	CHS-79F	TGTGGGCAAGGATGCTTGGAAGAAG	300	55	Carbone & Kohn (1999)
	CHS-354R	TGGAAGAACCATCTGTGAGAGTTG			
RPB2	RPB2-6F	CAAGGTCTTCACAGATGC	1400	45–55	Liu *et al.* (1999)
	RPB2-7R	CCCATRGCTTGYTTRCCCAT			
RPB2myco	RPB2myco-6F	CAAGGTCTTCACAGATGC	650	50–55	This study
	RPB2myco-7R	CAGGATGAATCTCGCAATG			
ATP6	ATP6-1	ATTAATTSWCCWTTAGAWCAATT	600	45	Kretzer & Bruns (1999)
	ATP6-2	TAATTCTANWGCATCTTTAATRTA			
β-tubulin (*P. destructans*)	PdBt-F	GTAACCAAATCGGTGCTGCT	198	62	This study
	PdBt-R	CAAAGTGGCTGCTCCGGGCG			
EF-1α (*P. destructans*)	Pd-EF-F	CGAGAAGTTCGAGAAGGTCAG	204	62	This study
	Pd-EF-R	GCGAGGGCTCTGTCGAAG			
β-tubulin (*P. eucalypti*)	Pey-Bt-F	GTAACCAAATCGGTGCTGCT	203	62	This study
	Pey-Bt-R	GAGTACAAGTGGCTGCTTAG			
EF-1α (*P. eucalypti*)	Pey-EF-F	CGAGAAGTTCGAGAAGGTCAG	229	62	This study
	Pey-EF-R	CTCTATCTGAAAGTCTTGGC			
β-tubulin (*P. epicoccoides*)	Pep-Bt-F	CGACGGCTCAGGCGTGTATG	218	62	This study
	Pep-Bt-R	GCGTTAGTGGTGTTGCTTGA			
EF-1α (*P. epicoccoides*)	Pep-EF-F	CCTACACACCCGCTGGTTAC	173	62	This study
	Pep-EF-R	CGGCGATCCTCCATAATCT			

Base codes: R (AG), Y (CT), K (GT), W (AT).

the four separate gene regions were statistically congruent (Farris *et al.*, 1995; Huelsenbeck *et al.*, 1996). The most parsimonious trees were obtained using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option, off. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (Hillis & Huelsenbeck, 1992). Characters were unweighted and unordered, branch and branch node supports were determined using 1000 bootstrap replicates (Felsenstein, 1985), characters were sampled with equal probability. Trees were rooted to *Botryosphaeria ribis* and *Botryosphaeria obtusa*, which were treated as the outgroup taxa.

Baysian analysis was conducted on the same aligned combined dataset. First MRMODELTEST v2.2 (Nylander, 2004) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with MRBAYES v3.1 (Ronquist & Heuelsenbeck, 2003) applying a general time reversible (GTR) substitution model with gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites. The Markov Chain Monte Carlo (MCMC) analysis of four chains started from random tree topology and lasted 10 000 000 generations. Trees were saved each 10 000 generations, resulting in 10 000 saved trees. Burn-in was set at 500 000 generations after which the likelihood values were stationary, leaving 9950 trees from which the consensus trees and posterior probabilities were calculated. PAUP 4.0b10 was used to reconstruct the consensus tree and maximum posterior probability assigned to branches after a 50% majority rule consensus tree was constructed from the 9950 sampled trees.

Specific primer design and validation

To design species-specific primers, the gene regions with the greatest sequence difference between *P. epicoccoides, P. eucalypti* and *P. destructans* were targeted. Only two gene regions, β-tubulin and EF-1α, were sufficiently variable between *P. eucalypti* and *P. destructans* to allow for primer design.

Repeatability of the specific primers was tested using at least 10 isolates of each *Phaeophleospora* species (*P. destructans*, CMW17918, 17919, 19832, 19844, 19886, 19906, 19909, 19910, 19936, 22553; *P. eucalypti*, CMW17912, 17915, 19916, MUCC432, 433, 434, 435, 436, 437, 438; *P. epicoccoides*, CMW5348, 22482, 22984, 22485, 22486, MUCC327, 424, 425, 426, 427). The isolates were amplified using specific β-tubulin and EF-1α primers (Table 2) and the same PCR conditions as (Cortinas *et al.*, 2006). Thereafter, primers were tested for their specificity, primarily to closely related species, but also to four less related *Mycosphaerella* spp. (Table 3).

The ability of the primers to amplify DNA directly from fruiting bodies from infected leaves was determined. The samples were frozen in liquid nitrogen, ground and DNA extracted with CTAB as described previously (Wittzell, 1999). DNA was then subjected to nested PCR, first using general β-tubulin and EF-1α primers and then the initial PCR product was diluted 1 : 5 and nested PCR conducted using the specific primers.

Table 3. Specific primers test results

		P. destructans		*P. eucalypti*		*P. epicoccoides*	
Test species	Code	β-tubulin 198 bp	EF1-α 204 bp	β-tubulin 203 bp	EF1-α 229 bp	β-tubulin 218 bp	EF1-α 173 bp
P. destructans	CMW17919	+	+	+	–	–	–
P. eucalypti	CMW17916	–	–	+	+	–	–
P. epicoccoides	CMW5348	–	–	–	–	+	+
M. cryptica	CMW3279	–	–	–	–	–	–
M. vespa	CMW11588	–	–	–	–	–	–
M. toledana	CMW14457	–	–	+	–	–	–
C. zuluensis	CMW7449	–	+(500 bp)	–	–	–	–
M. nubilosa	CMW11560	–	–	–	–	–	–
M. molleriana	CMW4940	–	–	+	–	–	–
M. ambiphylla	CMW13704	–	–	+	–	–	–
P. eugeniae	CMW5351	–	+(400 bp)	+		–	–
M. aurantia	MUCC258	–	–	–	–	–	–
M. marksii	MUCC214	–	Multiple bands	–	–	–	–
M. grandis	MUCC216	–	–	+	–	–	–
M. lateralis	MUCC436	–	–	+	–	–	–

Shaded cells indicate where the primers amplified nonspecific DNA.

Results

DNA sequence comparisons

Initially, 57 isolates representing 24 *Mycosphaerella* species and their anamorphs, including five species of *Phaeophleospora* found on *Eucalyptus* species and *Phaeophleospora eugeniae* the type species of the genus, were compared based on ITS sequence data (Table 1). The aligned data set consisted of 709 characters of which 127 bp were due to a large indel in two isolates of *P. epicoccoides* (MUCC327 and MUCC424) and this indel was excluded from the analyses. Of the remaining characters, 261 were parsimony informative. These data contained significant phylogenetic signal ($P < 0.01$; gl = − 0.41) to allow for meaningful analysis. Initial heuristic searches of unweighted characters in PAUP resulted in three most parsimonious trees of 910 steps (CI = 0.56, RI = 0.85). The *Phaeophleospora* species from *Eucalyptus*; *P. destructans, P. eucalypti, P. epicoccoides, P. toledana* and *Mycosphaerella ambiphylla* (which has a *Phaeophleospora* anamorph) grouped together in a strongly supported clade. This clade also included *Mycosphaerella nubilosa, Mycosphaerella cryptica, Mycosphaerella vespa, Mycosphaerella molleriana, Colleteogloeopsis zuluensis* and various undescribed '*Coniothyrium*' spp. (Fig. 2). The ex-type culture of *P. destructans* (STEU1336 = CMW5219) was resequenced in this study and was distant from the isolate of *P. destructans* on GenBank (AF309614) (Crous *et al.*, 2001). It was also distant from *P. eugeniae*, which is the type species of the genus, but close to *P. eucalypti* (Fig. 2, TreeBASE SN2884). The ex-type culture of *P. eugeniae* (STEU1454 = CMW5351) was also resequenced and, while the new sequence was similar to that on GenBank (AF309613), it differed in the first 50 bp of the ITS1 region. Based on results obtained for analysis of ITS sequence data, only species from the 'nubilosa clade' were retained for further study.

The multiple gene genealogies compared 31 isolates, including five *Phaeophleospora* species from *Eucalyptus*. The data set for the ATP6 region could not be completed because of difficulties encountered in amplifying DNA for all isolates. The RPB2 region proved not to be informative and these two regions were excluded from the combined analysis. The aligned data set for the combined ITS, β-tubulin, CHS and EF-1α sequences consisted of 1259 characters of which 352 were parsimony informative and were included in analysis. The PHT showed significant difference ($P = 0.001$) between the data from the different gene regions (sum of lengths of original partition was 902, range for 1000 randomizations was 902–921). When the data sets were compared in pairs, the incongruence in the complete combined data set was actually due to incongruence between CHS and both the ITS and EF-1α datasets. On closer examination of the individual tree topography, the incongruence was due to the relationship of *M. cryptica* and *C. zuluensis* and not to the positions of the *Phaeophleospora* species (data not shown, sequence alignments are available from TreeBASE SN2884). Despite the fact that the PHT showed significant difference between data sets, they were nonetheless combined as suggested previously (Hognabba & Wedin, 2003).

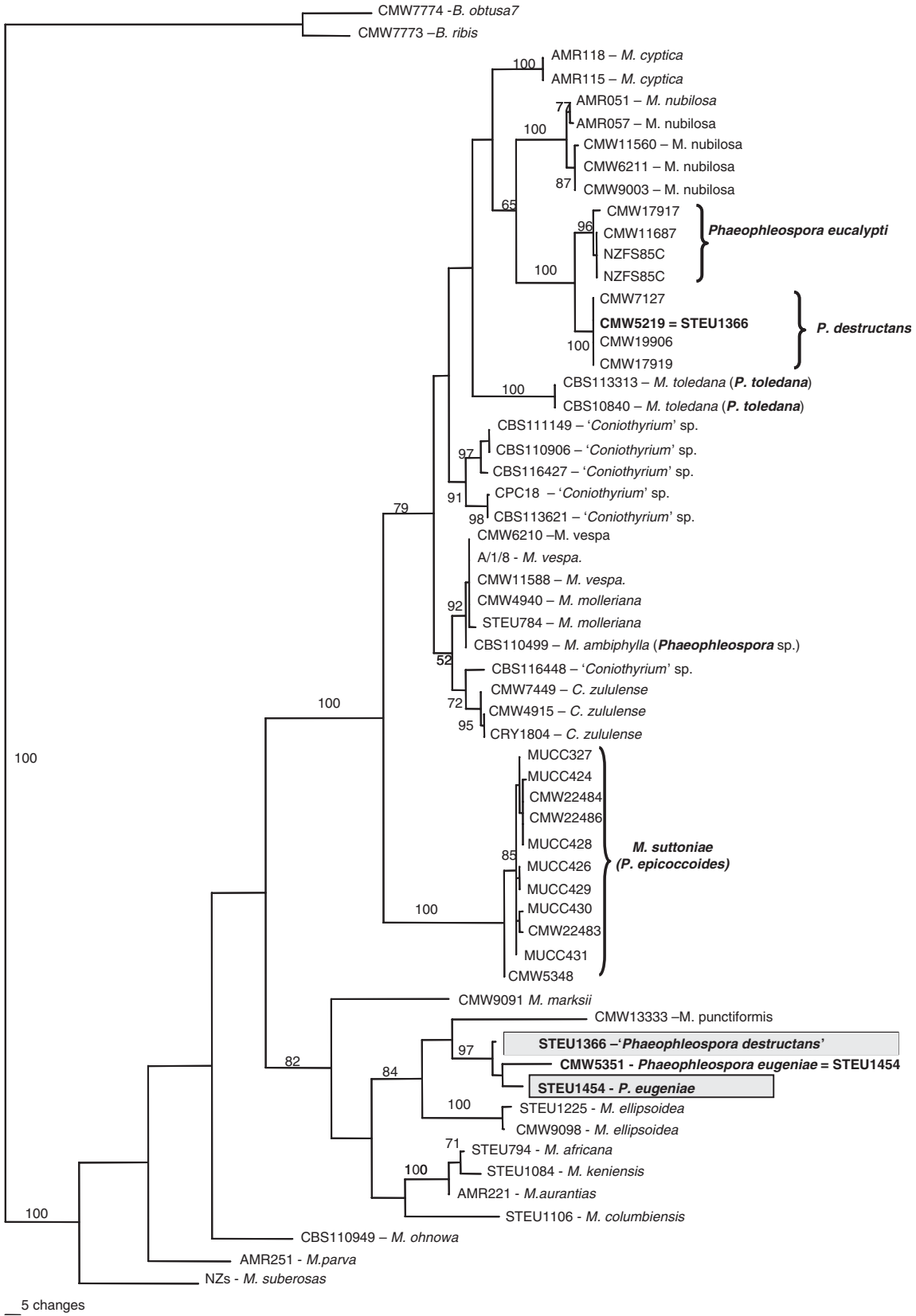
The combined data set contained significant phylogenetic signal ($P < 0.01$, gl = − 0.29). Heuristic search of unweighted characters in PAUP resulted in 18 most parsimonious trees of 937 steps (CI = 0.68, RI = 0.90). In the resultant tree (Fig. 3, TreeBASE SN2884), *M. vespa, M. molleriana* and *M. ambiphylla* grouped together, while *P. destructans* and *P. eucalypti* were separated with 100% bootstrap support. The four isolates of *P. destructans* were identical and no polymorphisms were observed in any of the gene regions. There were eight fixed polymorphic sites in the ITS region, nine in the β-tubulin region and 24 in the EF-1α region separating *P. destructans* and *P. eucalypti*. The variable sites in the β-tubulin and EF-1α regions were used to design specific primers (Table 2). A table of polymorphic sites is available at http://path.murdoch.edu.au/downloads/Andjicetal_Additionals.pdf

Phaeophleospora eucalypti isolates were further separated in three subgroups, corresponding to isolates from (a) Queensland, (b) New South Wales and (c) Southern New South Wales, Victoria and New Zealand (Fig. 3). There were 18 polymorphic positions across the four gene regions among isolates of *P. eucalypti* with 2–3 distinct profiles corresponding to geographic regions. *Phaeophleospora epicoccoides* was the basal species of the group and has three strongly supported subgroups (Fig. 3). Although there were 26 polymorphic sites across the four gene regions, there was no geographic association linked to these polymorphisms. A table showing polymorphic sites between isolates of *P. eucalypti* and *P. epicoccoides* is available at http://path.murdoch.edu.au/downloads/Andjicetal_Additionals.pdf

Validation of species-specific primers

Gel photos showing reproducibility of the specific primers for *P. destructans, P. eucalypti* and *P. epicoccoides* are given at http://path.murdoch.edu.au/downloads/Andjicetal_Additionals.pdf

Fig. 2. One of three most parsimonious phylogenetic trees of 977 steps obtained from analysis of ITS sequence data. Branch support (bootstrap values) is given above the branches. The sequences of the ex-type cultures of *Phaeophleospora eugineae* and *Phaeophleospora destructans* from Crous *et al*. (2001) are in a shaded box and those from the present study are in bold type. The tree is rooted to *Botryosphaeria ribis* and *Botryosphaeria obtusa*.



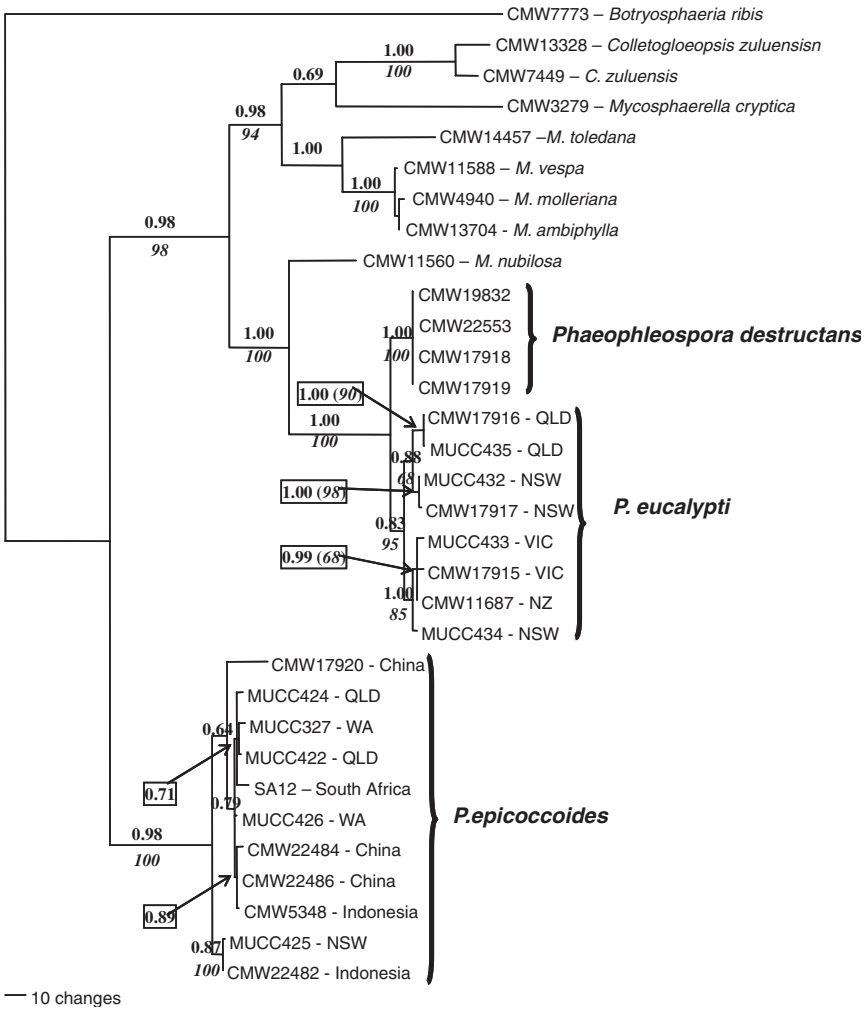


Fig. 3. Consensus phylogram of 9950 trees resulting from Baysian analysis of the combined ITS-2, β-tubulin, EF-1α and CHS sequence data of *Phaeophleospora* isolates. Posterior probabilities of the node are indicated above the branches and bootstrap values from the parsimony analysis are indicated below branches in italics. Not all nodes with high posterior probabilities also have bootstrap support. The tree is rooted to *Botryosphaeria ribis*.

Phaeophleospora destructans

DNA for 10 isolates of *P. destructans* was amplified using the primers specific for β-tubulin and EF1-α. These primers were then tested on 10 closely related *Mycosphaerella* spp. and five less related species and none gave amplification products for the β-tubulin primers specific to *P. destructans*. The EF1-α primer specific to *P. destructans* also amplified DNA of *C. zuluensis*, *P. eugeniae*, *Mycosphaerella marksii*, but the amplicons either contained multiple bands or were larger than the amplicon for *P. destructans* (Table 3). Both specific primer sets detected *P. destructans* directly from spores scraped from the surface of leaves. The β-tubulin primers specific for *P. destructans* also detected the presence of *P. eucalypti*, but the amplicon was larger than that obtained for *P. destructans* and it contained a double band.

Phaeophleospora eucalypti

DNA for all 10 isolates of *P. eucalypti* was amplified using specific primers for β-tubulin and EF1-α. None of *Mycosphaerella* spp. tested in this study gave amplification products for the EF1-α primers designed to be specific to *P. eucalypti* (Table 3). The β-tubulin primers designed for *P. eucalypti* were not specific and amplified seven other species, amplifying bands of the same size as those for *P. eucalypti* (Table 3). Only the EF1-α primers detected *P. eucalypti* from spores scraped from leaves.

Phaeophleospora epicoccoides

All ten isolates of *P. epicoccoides* gave amplification products using the β-tubulin and EF1-α primers developed for this species. None of the *Mycosphaerella* spp. tested gave amplification products using these primers (Table 3). In planta, the EF1-α primer set detected the presence of *P. eucalypti* as well as *P. epicoccoides* and the β-tubulin primer set detected presence of *P. epicoccoides* and *P. destructans* on leaf material.

Discussion

The current phylogenetic study has unequivocally shown *P. destructans* to be closely related to *P. eucalypti* and specific

primers have been developed to easily distinguish between these two species. *Phaeophleospora destructans* is unknown in Australia and is considered a major biosecurity threat. However, based on symptoms it is hard to distinguish between *P. eucalypti*, which is well-known in Australia, and *P. destructans*. Thus the specific primers will be very useful for detection and surveillance activities.

In a former study, *Phaeophleospora* species emerged in two separate clades (Crous *et al*., 2001). One of these clades included *P. eucalypti* and *P. epicoccoides* and the other accommodated *P. eugeniae* and *P. destructans* (Crous *et al*., 2001). All the isolates of *P. destructans* that have been examined, including the ex-type culture (STE-U1366 = CMW5219), had identical ITS sequence data, which was different to the single sequence previously lodged in GenBank (isolate STE-U 1366, AF309613). Consequently, all *Phaeophleospora* species from *Eucalyptus* species cluster together and they are closely related to the important *Eucalyptus* pathogens, *C. zuluensis, M. cryptica* and *M. nubilosa*. In contrast, these fungi are distantly related to *P. eugeniae*. A taxonomic re-evaluation of species of *Phaeophleospora* and *Colletogloeopsis* associated with *Eucalyptus* species is currently underway (unpublished data).

The sequence data obtained in this study for four isolates of *P. destructans*, three from Indonesia and one from China, were identical for all six gene regions examined. This finding is unusual as some variability is usually observed in sequence data between isolates of the same species, especially when more than one region of origin is considered. The limited variability among isolates of *P. destructans* supports the hypothesis of selection pressure resulting in the adaptation of a limited number of genotypes to a new host (*Eucalyptus* in Sumatra, Indonesia) followed by dispersal of these genotypes throughout Asia. In the present study, no informative characters in the RPB2 and CHS regions were found that could separate *P. destructans* from *P. eucalypti*. There were, however, a few stable differences between the two species in the sequences for the ITS2 and β-tubulin regions. The most variable gene region was EF1-α where a 22 bp indel separated these species. For ITS2, β-tubulin and CHS gene regions there were more polymorphic sites among isolates of *P. eucalypti* than between *P. destructans* and *P. eucalypti*. This suggests that while *P. destructans* emerged as a major *Eucalyptus* pathogen in Asia, it may have very recently evolved from *P. eucalypti*, to which it is very closely related. Where this adaptation could have occurred, however, remains a mystery as *P. eucalypti* has not been detected in Asia.

The sequence data for different *P. eucalypti* isolates was variable and analysis resulted in the isolates residing in different subgroups based on their origin. As isolates from New Zealand grouped with isolates from Victoria and southern New South Wales, *P. eucalypti* might have been moved to New Zealand from this region. Phylogeographic studies are required to test this hypothesis appropriately (Carbone & Kohn, 2001; Kasuga *et al*., 2003).

Many polymorphic sites were observed amongst the sequence data sets for isolates of *P. epicoccoides*, but the groupings did not reflect any obvious pattern relating to origin or other characteristics of the isolates. Unlike *P. eucalypti*, this species is widely distributed throughout most *Eucalyptus* growing regions of the world. The lack of phylogenetic grouping amongst isolates with variable sequence data, probably reflects anthropogenic movement of germplasm and multiple introductions of the fungus into new areas. *Phaeophleospora epicoccoides* is known to be a morphologically variable species and it may represent a species complex rather than a single taxon (Crous & Wingfield, 1997). Population genetic studies and large numbers of isolates from different locations, especially in Australia are required to resolve this question.

Efforts to develop species specific primers for *P. destructans, P. eucalypti* and *P. epicoccoides* reflected the close relatedness between these species and the variably within the species. Nonetheless a suite of species specific primers have been developed that allow for simple distinction between these species. Primers based on the EF1-α region distinguished between all three species and primers for the β-tubulin regions provided reliable detection of *P. destructans* and *P. epicoccoides*. Specific primers based on EF1-α sequences were able to detect *P. eucalypti* and *P. destructans* directly from plant samples. The β-tubulin primers developed to detect *P. epicoccoides* also showed a faint positive band for *P. destructans*, while EF1-α primers developed to detect *P. epicoccoides* showed a faint band for *P. eucalypti* from leaf material. While this result may be considered confusing, it is believed that this reflects duel infection as *P. epicoccoides* is very often present on the same lesion together with *P. eucalypti* and *P. destructans* (Burgess *et al*., 2006).

Phaeophleospora destructans is a devastating pathogen of *Eucalyptus* as yet undetected in Australia. Since the fungus has been detected in East Timor, which is very close to the Australian border, it is a potential threat to the biosecurity and biodiversity of Australia's vast native *Eucalyptus* forests. Its early detection in Australia is important and the Australian Quarantine and Inspection Service (AQIS) regularly inspects *Eucalyptus* species in Australia and neighbouring countries for pathogens including *P. destructans*. Because the symptoms caused by *P. destructans* can be almost identical to those associated with *P. eucalypti* and *P. eppicocoides*, unequivocal identification procedures are important. The DNA sequence data for many gene regions and the specific markers produced in this study should assist in this process.

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