Eur J Plant Pathol (2009) 125:425–433
DOI 10.1007/s10658-009-9491-6

Mycosphaerella species occurring on *Eucalyptus globulus* in Portugal

Márcia Silva · Helena Machado · Alan J. L. Phillips

Received: 5 January 2009 / Accepted: 14 May 2009 / Published online: 14 June 2009

Abstract Mycosphaerella leaf disease (MLD) is caused by species of *Mycosphaerella* and several anamorphic form genera that have been connected to *Mycosphaerella*. Until recently, MLD of eucalypts was largely ignored in Portugal. However, serious damage to *Eucalyptus globulus* has been reported since 1999 when frequent and severe defoliation of young trees was observed. The severity of this disease prompted a preliminary study of the *Mycosphaerella* species associated with major symptoms of a leaf blotch disease in commercial plantations of *E. globulus* in Portugal, which is presented here. The species were identified by molecular methods based on the ITS1-5.8S-ITS2 cluster, together with morphological characters. In addition to confirming the species previously recorded, *Mycosphaerella vespa* is reported for the first time from Portugal, while the status of *Mycosphaerella grandis* remains to be resolved.

M. Silva (✉) · H. Machado
Instituto Nacional dos Recursos Biológicos,
Edifício da ex-Estação Florestal Nacional,
Quinta do Marquês,
2780-159 Oeiras, Portugal
e-mail: marcia.silva@efn.com.pt

A. J. L. Phillips
Centro de Recursos Microbiológicos,
Faculdade de Ciências e Tecnologia,
Universidade Nova de Lisboa,
2829-516 Caparica, Portugal

Keywords *Ascomycetes* · ITS · Mycosphaerella leaf disease · Pathology · Ribosomal RNA operon

Introduction

Commercial plantations of eucalypts in Portugal are almost exclusively of *Eucalyptus globulus*, covering 646,700 ha, which represents about 21% of the total forested area of the country (AFN 2007). Plantations are concentrated in the wetter coastal regions where productivity is higher and attack by pathogens is generally low. However, serious damage to *E. globulus* has been reported since 1999 when repeated and serious defoliation of young *E. globulus* trees was observed in Aveiro. The principal cause of this disease was suspected to be a *Mycosphaerella* species (Carlos Valente, *personal communication*).

Approximately 62 species of *Mycosphaerella* and about 34 anamorphs have been reported associated with Mycosphaerella leaf disease (MLD) of *Eucalyptus* spp. worldwide (Carnegie and Keane 1998; Crous 1998; Dick and Dobbie 2001; Maxwell et al. 2003; Crous et al. 2004, 2006; Hunter et al. 2004; Burgess et al. 2007; Carnegie et al. 2007). Morphological identification of *Mycosphaerella* species and their anamorphs is complicated by problems of preparing single-spore cultures and the slow growth of the fungus in culture. Furthermore, morphological characteristics and ascospore germination patterns are

similar for several species (Crous et al. 2004) and some anamorphs do not sporulate easily in culture, or do not sporulate at all. For these reasons, it is often impossible to identify *Mycosphaerella* species on eucalypts without DNA sequences analyses (Crous et al. 2006). An additional complication is that several species can occur on the same lesion (Crous et al. 2004).

The first *Mycosphaerella* species was described by Persoon in 1794 on dead leaves of *Corylus* as *Sphaeria corylea* (Aptroot 2006). Until the 20th century there were only four species recorded, *M. molleriana*, *M. cryptica*, *M. suttoniae* described as *Cercospora epicoccoides* and *M. nubilosa* (Crous et al. 1995; Crous and Wingfield 1997a, b; Crous 1998; Aptroot 2006). Later, in the 20th century > 27 species were recorded (Park and Keane 1982a, 1984; Crous et al. 1993a, b, 1998; Carnegie and Keane 1994, 1997, 1998; Crous and Alfenas 1995; Crous and Swart 1995; Crous and Wingfield 1996, 1997a, b; Crous 1998) and in the last seven years approximately 31 species were recorded as new in *Eucalyptus* (Dick and Dobbie 2001; Maxwell et al. 2003; Crous et al. 2004, 2006; Hunter et al. 2004; Burgess et al. 2007; Carnegie et al. 2007). This increase in the number of described species in recent years is directly related to the application of molecular techniques that can differentiate morphologically similar species.

The first report of *Mycosphaerella* on eucalypts outside Australia was when Von Thümen described *M. molleriana* from Portugal (Crous and Wingfield 1997a). During the 1990s two further species were reported from Portugal, namely, *M. africana* and *M. walkeri* (Crous 1998). In 2004, *M. madeirae* was described as a new species from Madeira (Crous et al. 2004). Two years later, *M. communis*, *M. heimii*, *M. lateralis*, *M. marksii*, *M. nubilosa* and *M. parva* were included in the work of Crous et al. (2006) from Portugal. The above reports of *Mycosphaerella* species on Eucalypts in Portugal were based on mainly random collections and there has been no systematic survey of these pathogens in this country. There is therefore, the need for a first study of *Mycosphaerella* that considers this new relevant disease in Portugal. This work is the first study of *Mycosphaerella* species in commercial plantations of Portuguese eucalypts.

Materials and methods

Isolates

Two young *E. globulus* plantations were selected in two separate regions: Torres Vedras and Aveiro, where MLD symptoms were observed during spring 2004. During autumn 2004 and spring 2005, as soon as the first lesions appeared, 50 symptomatic leaves were collected randomly from each plantation. Lesions were examined with a stereomicroscope and characterised on the basis of colour, dimensions and shape. Where possible, 30 ascospores were measured at a magnification of × 600. Germination patterns of ascospores were determined after 24 h on 2% malt extract agar (MEA) at 24°C in the dark and single-ascospore cultures obtained. Linear growth of single-spore cultures was assessed after 1 month on 2% MEA at 24°C in the dark. Colony colours of the top surface and reverse were recorded (Carnegie and Keane 1998; Crous 1998 and Crous et al. 2004). A collection of 43 isolates (Table 1) was maintained on 2% MEA slopes at 24°C in the dark and deposited in the culture collection of ex-Estação Florestal Nacional, Instituto Nacional dos Recursos Biológicos, Oeiras, Portugal (EFN).

DNA extraction and amplification

Molecular characterisation of single-spore isolates was based on sequence analysis of part of the nuclear rRNA operon spanning the 3' end of the 18S rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second internal transcribed spacer (ITS2) and the 5' end of 28S rRNA gene. Genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen GmbH, Germany) following the manufacturer's instructions. The ITS1-5.8S-ITS2 cluster was amplified with primers ITS1: 5' TCC GTA GGT GAA CCT GCG G and ITS4: 5' TCC TCC GCT TAT TGA TAT GC (White et al. 1990). The cycling conditions were 96°C for 5 min for the initial denaturation and cycles of 96°C, 30 s, 55°C, 30 s, 72°C, 90 s repeated 30 times with a final elongation step of 7 min at 72°C (Crous et al. 2004). PCR products were separated by electrophoresis on 1% agarose gels and visualised with UV light after staining with ethidium bromide. The amplification products were purified and sequenced in

both directions. PCR reactions were prepared in a total volume of 50 µl including 1.5 µl of genomic 1/10 dilution DNA, 1.25 U of *Taq* DNA polymerase (ABgene), 1x reaction buffer (100 mM KCl, 20 mM Trix-HCl, pH 8.0 (at 25°C), 0.1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM DTT (dithiothreitol), 0.5% Tween 20, 0.5% Nonidet P40, 50% (*v*/*v*) glycerol) (ABgene) 20 pmol of each primer, 0.2 mM of each dNTP and 2.5 mM $MgCl_2$. PCR amplification products were purified using JETquick spin column (Genomed GmbH) and sequencing reactions prepared with primers ITS1 and ITS4 with Kit BDT v1.1 (Applied Biosystems). Separation of PCR products was by capillary electrophoresis at ABI PRISM 3700 (Applied Biosystems Inc., Foster City, California) and the results were analysed with Software Sequencing Analysis 3.7.

Phylogenetic analyses

The phylogenetic analyses included sequences of 43 isolates from this study and 22 sequences retrieved from GenBank including the outgroup (Table 1). All sequences were checked with BioEdit version 7.0.5.3 (Hall 1999). The internal transcribed spacer (ITS) sequences generated in this study were added to sequences of other *Mycosphaerella* species obtained from GenBank and aligned with Clustal X (Thompson et al. 1997). All sequences generated in this study were deposited in GenBank (Table 1) and alignments were deposited in TreeBASE (submission ID number: SN4338).

Phylogenetic analyses of sequence data were done using PAUP version 4.0b10 (Swofford 2002). All characters were unordered and of equal weight and alignment gaps were treated as a fifth character state. Maximum parsimony (MP) analyses were performed using the heuristic search option with 1000 random taxon addition and TBR (tree bisection and reconnection) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious, trees were saved. Robustness of the branches was evaluated by 1000 bootstrap replications. Other measures included tree length (TL), consistency index (CI), retention index (RI) and homoplasy index (HI). The Kimura-2-parameter nucleotide substitution model (Kimura 1980) was used for distance analysis. Bootstrap values were obtained from 1000 NJ bootstrap replicates.

Results

The dataset consisted of 65 isolates including the outgroup isolate (Table 1). Incomplete parts at the start and end of the sequences were excluded from the analyses. After alignment the dataset consisted of 536 characters including alignment gaps, of which 292 characters were constant while 58 were variable and parsimony-uninformative. MP analysis of the remaining 186 parsimony-informative characters resulted in a single tree (TL=409, CI=0.846, RI=0.978, HI=0.154). NJ analysis yielded a tree with similar topology and similar bootstrap support for the branches.

The MP tree (Fig. 1) could be resolved into three clades. Clade 1 was well-supported with bootstrap values of 100% in both MP and NJ analyses and included isolates identified as *M. molleriana* and *M. vespa* in one sub-clade and *M. nubilosa* and *M. juvenis* in a second sub-clade. Two isolates (EFN NX7A and EFN Y16F) closely clustered with *M. vespa* from Australia and Tasmania (DQ267590 and DQ303059, respectively) than with *M. molleriana* isolated from Portugal (AF309620) while four isolates (EFN M17B; EFN M7A; EFN Y8B; EFN M32) clustered with *M. nubilosa* and *M. juvenis*. Morphologically (Fig. 2) these four isolates correlated well with the descriptions of both *M. nubilosa* (Crous 1998) and *M. vespa* (Carnegie and Keane 1998).

Clade 2 was also well resolved with a bootstrap value of 100% in both analyses and contained isolates previously identified as *M. grandis* and *M. parva*. Most of the isolates from this study (29 isolates) clustered in this clade. Isolate EFN X40D formed a branch separate from the main clade. Only 2 bp differences separated this branch from other isolates in clade 2. Sub-clades within clade 2 were separated by short branches with low bootstrap support. The morphology of all these isolates agrees with the descriptions of *M. grandis* by Carnegie and Keane (1994) and *M. parva* by Park and Keane (1982a, b).

The third major clade (Clade 3), supported by high bootstrap value of 100% in both analyses, contained representatives of *M. lateralis* and *M. communis*. These two species resided in two separate, well-supported (> 90% MP and 100% NJ) clades. Two of the EFN isolates clustered with *M. lateralis* and six with *M. communis*. Morphologically these isolates

Table 1 Isolates included in the sequence analysis of *Mycosphaerella* species

Species	Isolate No.	Origin	Host plant	Date	Collector	GenBank Accession No.	Source
M. communis	EFN X8	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515737	This study
	EFN NX8A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515736	This study
	EFN NX30A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515734	This study
	EFN NX30B	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515739	This study
	EFN NX30C	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515735	This study
	EFN X38A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515738	This study
	CPC 11700	Spain	*E. globulus*	–	P. Mansilla	DQ302948	Crous et al. 2006
M. communis	CBS 114238	Spain	*E. globulus*	–	J.P.M. Vazquez	AY725541	Crous et al. 2004
M. grandis	EFN X4A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515719	This study
	EFN X21C	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515720	This study
	EFN X21D	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515722	This study
	EFN X29A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515718	This study
	EFN X53A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515721	This study
	CMW 8557	Chile	*E. globulus*	–	A. Rotella	DQ267583	Hunter et al. 2006
	CMW 8554	Chile	*E. globulus*	–	M.J. Wingfield	DQ267584	Hunter et al. 2006
M. juvenis	CBS 115669	South Africa	*E. nitens*	–	M.J. Wingfield	AY725548	Crous et al. 2004
M. lateralis	EFN NX6B	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515732	This study
	EFN X13A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515733	This study
	CPC 11789	Portugal	*Eucalyptus sp.*	–	J.P. Sampaio	DQ302975	Crous et al. 2006
	CPC 11706	Spain	*E. globulus*	–	P. Mansilla	DQ302972	Crous et al. 2006
M. lateralis	CMW 4935	Zambia	*Eucalyptus sp*	–	–	AF309625	Hunter et al. 2004
M. madeirae	CBS 112895	Portugal (Madeira)	*E. globulus*	–	S. Denman	AY725553	Crous et al. 2004
	CPC 3746	Portugal (Madeira)	*E. grandis*	–	S. Denman	DQ302976	Crous et al. 2006
M. molleriana	CBS111164	Portugal	*E. globulus*	–	S. McCrae	AF309620	Hunter et al. 2006
M. nubilosa	EFN Y8B	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515730	This study
	EFN M7A	Portugal	*E. globulus*	Autumn 2004	L. Neves	FJ515729	This study
	EFN M17B	Portugal	*E. globulus*	Autumn 2004	L. Neves	FJ515728	This study
	EFN M32	Portugal	*E. globulus*	Autumn 2004	L. Neves	FJ515731	This study
M. nubilosa	CBS116005	Australia	*E. globulus*	–	A.J. Carnegie	AF309618	Crous 1998
M. nubilosa	CPC 11246	Spain	*E. globulus*	–	M.J. Wingfield	DQ302992	Crous et al. 2006
	CPC 11723	Portugal	*E. globulus*	–	A.C. Alfenas	DQ302996	Crous et al. 2006
	CPC 11767	Portugal	*E. globulus*	–	A.J.L. Phillips	DQ302998	Crous et al. 2006
	CPC 11882	Portugal	*E. globulus*	–	A.J.L. Phillips	DQ302999	Crous et al. 2006
	CPC 11885	Portugal	*Eucalyptus sp.*	–	A.J.L. Phillips	DQ303000	Crous et al. 2006
M. parva	EFN X6	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515702	This study
	EFN X9C	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515697	This study
	EFN NX13A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515707	This study
	EFN X15A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515717	This study
	EFN X21A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515709	This study
	EFN X22A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515710	This study
	EFN X27A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515699	This study
	EFN X37A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515708	This study
M. parva	EFN NX46A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515706	This study
	EFN X49C	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515701	This study
	EFN X50B	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515703	This study

Table 1 (continued)

Species	Isolate No.	Origin	Host plant	Date	Collector	GenBank Accession No.	Source
	EFN Y2B	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515698	This study
	EFN Y27D	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515705	This study
	EFN Y32B	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515704	This study
	EFNG21126BC	Portugal	*E. globulus*	Autumn 2004	L. Neves	FJ515700	This study
	CPC 11273	Spain	*E. globulus*	–	M.J. Wingfield	DQ303001	Crous et al. 2006
	CPC 11888	Portugal	*Eucalyptus sp.*	–	A.J.L. Phillips	DQ303005	Crous et al. 2006
	EFN NX7B	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515711	This study
	EFN X29B	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515714	This study
	EFN Y10A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515715	This study
	EFN Y10B	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515724	This study
	EFN Y12B	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515716	This study
	EFN Y23A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515723	This study
	EFN Y27A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515713	This study
	EFN Y27C	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515712	This study
	EFN X40D	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515725	This study
M. vespa	EFN NX7A	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515726	This study
	EFN Y16F	Portugal	*E. globulus*	Spring 2005	H. Machado	FJ515727	This study
	CBS117924	Tasmania	*E. globulus*	–	Unknown	DQ267590	Hunter et al. 2006
M. vespa	CMW 11558	Australia	*Eucalyptus sp.*	–	Unknown	DQ303059	Crous et al. 2006
Botryosphaeria parva	STE-U 4438	–	–	–	–	AY343467	Crous et al. 2006

Ex-type strains are indicated in **bold** print

CBS Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; *CPC* culture collection of Pedro Crous, housed at CBS; *CMW* culture collection of Mike Wingfield, housed at Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; *EFN* culture collection of ex-Estação Florestal Nacional, Instituto Nacional dos Recursos Biológicos, Oeiras, Portugal; *STE-U* culture collection of Stellenbosch University, South Africa. Isolate numbers from Crous (1998)

were similar to *M. communis* (Crous et al. 2004) and *M. lateralis* (Crous and Wingfield 1996).

Discussion

In this study species of *Mycosphaerella* found during a preliminary survey of MLD in commercial *Eucalyptus* plantations in Portugal were characterised. The identifications were complemented with information on the collection sites, dates and host, which contributes to a better understanding of the distribution and relative importance of the species. Previous reports of *Mycosphaerella* species in Portugal did not include such data (e.g. Crous et al. 2006).

Since 1995 only *M. africana*, *M. molleriana* and *M. walkeri* were recorded from Portugal. In 2004 *M. madeirae* was reported from Madeira and then in 2006 isolates of *M. communis, M. heimii*, *M. lateralis*, *M. marksii*, *M. nubilosa* and *M. parva* from Portugal were included in the work of Crous et al. (2006). In addition to this last report, with the exception of *M. heimii* and *M. marksii*, *M. vespa* was reported as a new record for Portugal. Thus, until now, a total of 11 species of *Mycosphaerella* have been recorded on Portuguese eucalypts.

In the phylogenetic analysis, five species were clearly distinguished with high bootstrap support in both MP and NJ analyses. However, isolates previously identified under different names clustered within each species clade. The status of some of these species is not entirely clear. For example, Carnegie and Keane (1994) described *M. grandis* in Australia as a new species because of its ascospore

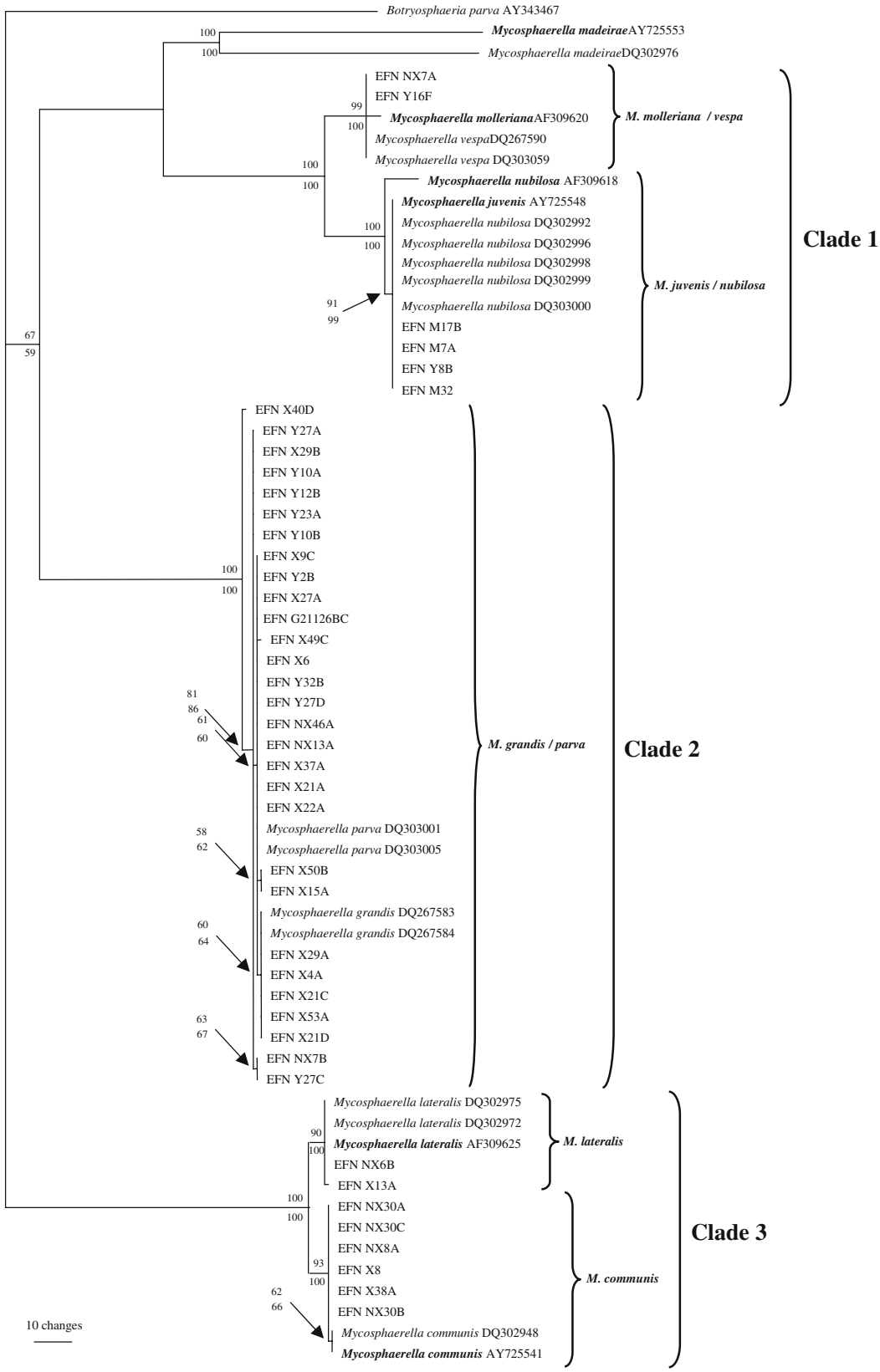
Botryosphaeria parva AY343467
Mycosphaerella madeirae AY725553
Mycosphaerella madeirae DQ302976
EFN NX7A
EFN Y16F
Mycosphaerella molleriana AF309620
Mycosphaerella vespa DQ267590
Mycosphaerella vespa DQ303059
M. molleriana / vespa
Mycosphaerella nubilosa AF309618
Mycosphaerella juvenis AY725548
Mycosphaerella nubilosa DQ302992
Mycosphaerella nubilosa DQ302996
Mycosphaerella nubilosa DQ302998
Mycosphaerella nubilosa DQ302999
Mycosphaerella nubilosa DQ303000
EFN M17B
EFN M7A
EFN Y8B
EFN M32
M. juvenis / nubilosa
Clade 1
EFN X40D
EFN Y27A
EFN X29B
EFN Y10A
EFN Y12B
EFN Y23A
EFN Y10B
EFN X9C
EFN Y2B
EFN X27A
EFN G21126BC
EFN X49C
EFN X6
EFN Y32B
EFN Y27D
EFN NX46A
EFN NX13A
EFN X37A
EFN X21A
EFN X22A
Mycosphaerella parva DQ303001
Mycosphaerella parva DQ303005
EFN X50B
EFN X15A
Mycosphaerella grandis DQ267583
Mycosphaerella grandis DQ267584
EFN X29A
EFN X4A
EFN X21C
EFN X53A
EFN X21D
EFN NX7B
EFN Y27C
M. grandis / parva
Clade 2
Mycosphaerella lateralis DQ302975
Mycosphaerella lateralis DQ302972
Mycosphaerella lateralis AF309625
EFN NX6B
EFN X13A
M. lateralis
EFN NX30A
EFN NX30C
EFN NX8A
EFN X8
EFN X38A
EFN NX30B
Mycosphaerella communis DQ302948
Mycosphaerella communis AY725541
M. communis
Clade 3
10 changes

Fig. 1 MP tree using bootstrap method with heuristic search (no. replicates = 1000): random taxon sampling; branch-swapping algorithm (tree-bisection-reconnection). TL=409, CI=0.846, RI=0.978, HI=0.154 from DNA sequence alignment on Internal Transcribed Spacer (ITS) rDNA data of *Mycosphaerella* spp. occurred on *Eucalyptus* spp. leaves. The tree was rooted to *Botryosphaeria parva*. MP bootstrap values are given above the branch with NJ bootstrap values below the branches. The scale bar shows 10 changes. Ex-type strains are indicated in bold print

morphology and lesion characteristics. On the other hand Crous (1998), Crous et al. (2004), Maxwell et al. (2005) and Hunter et al. (2006) regarded it as a synonym of *M. parva*. In the present study these two species clustered with *M. grandis* in a subclade with low bootstrap support (60% in MP). Isolates of *M. parva* from Spain and Portugal, showed type N germination patterns; ascospores and germ tubes became uniformly brown, distorted and verruculose as described by Crous (1998). *Mycosphaerella grandis* clustered with specimens from Chile, and germination patterns were similar to those described by Carnegie and Keane (1994). Observations of germination patterns suggested that these could represent two distinct species. However, this will need to be confirmed by a more detailed study.

The distinction between *M. molleriana* and *M. vespa* is unclear. In a phylogenetic study, Hunter et al. (2006) regarded these two species as synonyms. However, the authentic single isolate of *M. molleriana* included in the phylogeny presented here formed a branch distinct from two authentic isolates of *M. vespa* and the two strains isolated in this study (EFN NX7A and EFN Y16F). Furthermore, the germination patterns observed for specimens EFN NX7A and EFN Y16F were similar to those described for *M. vespa* (Carnegie and Keane 1998) and ascospore morphology was unlike that of *M. molleriana* (Crous 1998) (Fig. 2).

Mycosphaerella lateralis and *M. communis* were reported from Portugal for the first time by Crous et al. (2006). In the present study these two species were clearly distinguished from one another both phylogenetically and morphologically.

Mycosphaerella nubilosa was first described as *Sphaerella nubilosa* by Cooke (1891). Subsequently Park and Keane (1984) examined various collections of *M. molleriana* and concluded that it resembled *M. nubilosa* in general morphology, and Crous et al. (1991) showed that it is impossible to distinguish the two species on the basis of their morphology. Crous and Wingfield (1996) described *M. juvenis* as a new species that is commonly associated with leaf spots on juvenile leaves of *E. nitens.* Crous et al. (2001) supported *M. juvenis* as a species distinct from *M. nubilosa* and *M. molleriana* based on ITS phylogeny, possibly because the ex-type strain of *M. juvenis* was not included in that phylogenetic analysis (Crous et al. 2004). Subsequently, Crous et al. (2004) used ex-type cultures of *M. juvenis* and showed that they were identical to *M. nubilosa* and some strains of *M. juvenis* produced an *Uwebraunia* anamorph, despite the fact that *M. nubilosa* did not. Finally, the confusion surrounding *M. juvenis* and *M. nubilosa* was resolved when Crous et al. (2004) showed that ascospore germination patterns of *M. juvenis* on MEA at 24°C were type C (germinating from both ends parallel to germ tubes) as seen in *M. nubilosa*. When

Fig. 2 *Mycosphaerella vespa* (EFN Y16F). Germinating ascospores determined after 24 h on MEA 2% at 24°C in the dark. Scale bar = 10 μm

germination patterns were re-evaluated after 24 h spores became distorted as in type F, similar to those observed for *M. juvenis* in Crous (1998).

Of particular concern is the confirmation of the presence of *M. nubilosa* in Portugal. This species is well known as an aggressive pathogen of eucalypts (Carnegie et al. 1997) and was reported from Portugal in 2006 (Crous et al. 2006). It is possible that *M. nubilosa* has not been detected previously. However, it is most likely that it is a recent introduction on propagation material during the 1990s because as an aggressive pathogen it could not stay undetected for so many years like, e. g., *M. molleriana*, which has not been reported since 1881. Considering that *M. nubilosa* is now confirmed to be present in Portugal it is important that rapid screening tools are developed to aid in the detection of this pathogen. It is also important to select tolerant planting material in order to reduce its impact.

More than one species was frequently found on a lesion and in the same leaf. *Mycosphaerella communis* and *M. nubilosa* always occurred alone on the lesion, while *M. grandis* and *M. vespa* were observed either alone or with *M. parva* on the same lesion. Park and Keane (1982b) showed that *M. parva* was isolated only from older lesions and *M. nubilosa* was isolated from young blighted lesions. It was also observed that *M. nubilosa* was only found alone, maybe before other species arrived or developed on spots caused by *M. nubilosa*. *Mycospherella parva* also occurred alone, while *M. lateralis* always occurred in association with *M. parva*. Jackson et al. (2004) showed that there was no evidence that *M. lateralis* could parasitise *M. cryptica* or *M. nubilosa*. However, *M. lateralis* is frequently found associated with other *Mycosphaerella* species leaf spots. Nevertheless its ecological position needs to be determined (Crous et al. 2006). The species *M. grandis* was regularly observed linked with older lesions caused by *M. tasmaniensis*, *M. nubilosa* and *M. cryptica* (Milgate et al. 2001) and occurred alone or with one or a combination of *M. cryptica*, *M. gregaria*, *M. marksii, M. nubilosa* or *M. mexicana* on the same lesion (Maxwell 2004). In the present study *M. grandis* was observed in combination only with *M. parva*. Carnegie and Keane (1994) suggested that *M. grandis* is a pathogen and *M. parva* a saprophyte on old *M. nubilosa* lesions. It is important in future pathogenicity tests to analyse these relationships and relate them to individual virulence and aggressiveness of the coexisting species.

Further work should be done to clarify these species complexes and focus on determining species barriers between different species of *Mycosphaerella*. Sequence analyses will be a useful tool to study population structure of the pathogen from different regions in Portugal. Furthermore it is likely that more new species will be found since Crous et al. (2006) estimate that only 14% of *Mycosphaerella* species from eucalypts have so far been described.

Acknowledgements The biological material would not have been available without direct collaboration with Carlos Valente (Instituto RAIZ) and Lucinda Neves (Silvicaima) and their teams, who provided infected material. Experimental plantations were supported by programme AGRO 8.1, Project 550, 'Pest management on pine and eucalypts stands minimising environmental impacts and conserving biodiversity'.

References

AFN. (2007). Inventário Florestal Nacional 2005/06: http://www.afn.min-agricultura.pt/portal/ifn/ifn-resultados-preliminares-julho-2006.

Aptroot, A. (2006). *Mycosphaerella* and its anamorphs: 2. Conspectus of *Mycosphaerella*. *CBS Biodiversity Series, 5*, 1–231.

Burgess, T. I., Barber, P. A., Sufaati, S., Xu, D., Hardy, G. E. S. J., & Dell, B. (2007). *Mycosphaerella* spp. on *Eucalyptus* in Asia; new species, new hosts and new records. *Fungal Diversity, 24*, 135–157.

Carnegie, A. J., & Keane, P. J. (1994). Further *Mycosphaerella* species associated with leaf diseases of *Eucalyptus*. *Mycological Research, 98*, 413–418. doi:10.1016/S0953-7562(09)81198-2.

Carnegie, A. J., & Keane, P. J. (1997). A revised *Mycosphaerella gregaria* nom. nov. for *M. aggregata* on *Eucalyptus*. *Mycological Research, 101*, 843–844. doi:10.1017/S095375629600305X.

Carnegie, A. J., & Keane, P. J. (1998). *Mycosphaerella vespa* sp. nov. from diseased Eucalyptus leaves in Australia. *Mycological Research, 102*, 1274–1276. doi:10.1017/S0953756298006431.

Carnegie, A. J., Keane, P. J., & Podger, F. D. (1997). The impact of three species of *Mycosphaerella* newly recorded on *Eucalyptus* in Western Australia. *Australasian Plant Pathology, 26*, 71–77. doi:10.1071/AP97012.

Carnegie, A. J., Burgess, T., Beilharz, V., & Wingfield, M. J. (2007). New species of *Mycosphaerella* from Myrtaceae in plantations and native forests in New South Wales, Australia. *Mycologia, 99*, 461–474. doi:10.3852/mycologia.99.3.461.

Cooke, M. C. (1891). Grevillea - A quarterly record of Cryptogamic Botany and its literature 19, 61.

Crous, P. W. (1998). *Mycosphaerella* spp. and their anamorphs associated with leaf spot diseases of Eucalyptus. *Mycologia Memoir, 21*, 1–170. St. Paul, Minn., USA, APS Press.

Crous, P. W., & Alfenas, A. C. (1995). *Mycosphaerella gracilis* and other species of *Mycosphaerella* associated with leaf spots of Eucalyptus in Indonesia. *Mycologia, 87*, 121–126. doi:10.2307/3760954.

Crous, P. W., & Swart, W. J. (1995). Foliicolous fungi of *Eucalyptus* spp. from eastern Madagascar: implications for South Africa. *South African Forestry Journal, 172*, 1–5.

Crous, P. W., & Wingfield, M. J. (1996). Species of *Mycosphaerella* and their anamorphs associated with leaf blotch disease of *Eucalyptus* in South Africa. *Mycologia, 88*, 441–458. doi:10.2307/3760885.

Crous, P. W., & Wingfield, M. J. (1997a). *Colletogloeopsis*, a new coelomycete genus to accommodate anamorphs of two species of *Mycosphaerella* on *Eucalyptus*. *Canadian Journal of Botany, 75*, 667–674.

Crous, P. W., & Wingfield, M. J. (1997b). New species of *Mycosphaerella* occurring on *Eucalyptus* leaves in Indonesia and Africa. *Canadian Journal of Botany, 75*, 781–790.

Crous, P. W., Wingfield, M. J., & Park, R. F. (1991). *Mycosphaerella nubilosa*, a synonym of *M. molleriana*. *Mycological Research, 95*, 628–632. doi:10.1016/S0953-7562(09)80079-8.

Crous, P. W., Ferreira, F. A., Alfenas, A., & Wingfield, M. J. (1993a). *Mycosphaerella suberosa* associated with corky leaf spots on *Eucalyptus* in Brazil. *Mycologia, 85*, 705–710. doi:10.2307/3760515.

Crous, P. W., Wingfield, M. J., Ferreira, F. A., & Alfenas, A. (1993b). *Mycosphaerella parkii* and *Phyllosticta eucalyptorum*, two new species from *Eucalyptus* leaves in Brazil. *Mycological Research, 97*, 582–584. doi:10.1016/S0953-7562(09)81181-7.

Crous, P. W., Carnegie, A. J., & Keane, P. J. (1995). IMI descriptions of fungi and bacteria, Set 121. *Mycosphaerella cryptica, M. delegatensis, M. gracilis, M. heimii, M. marksii, M. molleriana, M. parkii, M. suberosa, M. swartii, M. walkeri*. *Mycopathologia, 130*, 43–64. doi:10.1007/BF01104349.

Crous, P. W., Wingfield, M. J., Mohammed, C., & Yuan, Z. Q. (1998). New foliar pathogens of *Eucalyptus* from Australia and Indonesia. *Mycological Research, 102*, 527–532. doi:10.1017/S0953756297005091.

Crous, P. W., Hong, L., Wingfield, B. D., & Wingfield, M. J. (2001). ITS rDNA phylogeny of selected *Mycosphaerella* species and their anamorphs occurring on *Myrtaceae*. *Mycological Research, 105*, 425–431. doi:10.1017/S0953756201003835.

Crous, P. W., Groenewald, J. Z., Mansilla, J. P., Hunter, G. C., & Wingfield, M. J. (2004). Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. *Studies in Mycology, 50*, 195–214.

Crous, P. W., Wingfield, M. J., Mansilla, J. P., Alfenas, A. C., & Groenewald, J. Z. (2006). Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. II. *Studies in Mycology, 55*, 99–131.

Dick, M. A., & Dobbie, K. (2001). *Mycosphaerella suberosa* and *M. intermedia* sp. nov. on *Eucalyptus* in New Zealand. *New Zealand Journal of Botany, 39*, 269–276.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series, 41*, 95–98.

Hunter, G. C., Roux, J., Wingfield, B. D., Crous, P. W., & Wingfield, M. J. (2004). *Mycosphaerella* species causing leaf disease in South African Eucalyptus plantations. *Mycological Research, 108*, 672–681. doi:10.1017/S0953756204009864.

Hunter, G., Wingfield, B. D., Crous, P. W., & Wingfield, M. J. (2006). A multi-gene phylogeny for species of *Mycosphaerella* occurring on *Eucalyptus* leaves. *Studies in Mycology, 55*, 147–161.

Jackson, S. L., Maxwell, A., Neumeister-Kemp, H. G., Dell, B., Hardy, G. E., & St, J. (2004). Infection, hyperparasitism and conidiogenesis of *Mycosphaerella lateralis* on *Eucalyptus globulus* in Western Australia. *Australasian Plant Pathology, 33*, 49–53. doi:10.1071/AP03068.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution, 16*, 111–120. doi:10.1007/BF01731581.

Maxwell, A. (2004). *The taxonomy. Phylogeny and impact of Mycosphaerella species on Eucalypts in South-Western Australia. PhD Dissertation*. Australia: Murdoch University.

Maxwell, A., Dell, B., Neumeister-Kemp, H. G., & Hardy, G. E. S. J. (2003). *Mycosphaerella* species associated with *Eucalyptus* in South-western Australia: new species, new records and a key. *Mycological Research, 107*, 351–359. doi:10.1017/S0953756203007354.

Maxwell, A., Jackson, S. L., Dell, B., Hardy, G. E., & St, J. (2005). PCR-identification of *Mycosphaerella* species associated with leaf diseases of *Eucalyptus*. *Mycological Research, 109*(09), 992–1004. doi:10.1017/S0953756205003539.

Milgate, A. W., Yuan, Z. Q., Vaillancourt, R. E., & Mohammed, C. (2001). *Mycosphaerella* species occurring on *Eucalyptus globulus* and *Eucalyptus nitens* plantations of Tasmania, Australia. *Forest Pathology, 31*, 53–63. doi:10.1046/j.1439-0329.2001.00222.x.

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

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

Park, R. F., & Keane, P. J. (1984). Further *Mycosphaerella* species causing leaf diseases of *Eucalyptus*. *Transactions of the British Mycological Society, 83*, 93–105.

Swofford, D. L. (2002). *PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4.0b10*. Sunderland, MA: Sinauer.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research, 25*, 4876–4882. doi:10.1093/nar/25.24.4876.

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