Márcia Filipa Lopes Rosendo de Castro Silva

MSc

Studies on the Eucalyptus Leaf Disease Complex in Portugal

Dissertação para obtenção do Grau de Doutor em
Biologia (especialidade em Microbiologia)

Orientador: Doutor Alan John Lander Phillips, FCT/UNL
Co-orientador: Doutora Maria Helena Neves Machado, INIAV

FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Para os meus meninos
To my little boys

"In God's garden of grace, even a broken tree can bear fruit."
Rick Warren

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To God, for Him, and with His help, I did and can do all things (Philippians 4:13)!

ABSTRACT

Native from south eastern Australia, *Eucalyptus globulus* is the main species in eucalypts plantations in Portugal. The most serious foliar disease in eucalypt plantations is linked to *Mycosphaerella senso lato*, which affects young trees in the juvenile phase foliage causing leaf necrosis. This disease results in reduced growth rate of the host and lower wood volume, thus causing significant productivity losses. The most common name for this disease was Mycosphaerella Leaf Disease that became inappropriate when most of the pathogens on eucalypts were re-distributed into several genera. The term "Eucalyptus Leaf Disease Complex" is now more appropriate. The overall aim of this thesis was to investigate the Eucalyptus Leaf Disease Complex in Portugal, focusing on species diversity, taxonomy and the role played by each species in the disease complex on *Eucalyptus globulus*. Literature on the Eucalyptus Leaf Disease Complex was reviewed and the species were distributed into several genera. A survey based on symptomatic leaves collected from several *Eucalyptus globulus* plantations and characterized by morphological and molecular tools provided an overview of species incidence and of the most frequent species in the disease complex. The present work reveals additional species of *Mycosphaerella senso lato* associated with eucalypt plantations in Portugal. Thus, five new records of *Teratosphaeria* and phylogenetically related species were added to the Iberian Peninsula, namely, *Neodevriesia hilliana*, for the first time on *Myrtaceae*; *Quasiteratosphaeria mexicana*, *Teratosphaericola pseudoafricana*, *Teratosphaeria pluritubularis* and *Teratosphaeria lusitanica*, a new species. Furthermore, new anamorphic structures were found and two new combinations were made. Regarding other genera, some species were observed for the first time, such as *Cladosporium cladosporioides*, *Fusicladium eucalypti*, *Mycosphaerella madeirae*, in the mainland. In addition to leave diseases, *Teratosphaeria gauchensis* was found causing a severe stem and trunk canker on *Eucalyptus globulus*. The aggressiveness of several species was compared to evaluate each species individually in the complex, permitting to distinguish different behaviours, from primary to secondary pathogens. *Cladosporium cladosporioides*, *M. communis* and *M. lateralis*, appeared to be more aggressive than *Teratosphaeria nubilosa*. In fact, contrary to the prevailing views on this disease complex, *Teratosphaeria nubilosa* is not the only species responsible for the disease, which clearly involves a complex of species acting together.

Keywords: Leaf Disease; Canker; *Mycosphaerella*; *Teratosphaeria*; Eucalypts and *Eucalyptus globulus*.

RESUMO

Originário do sudoeste da Austrália, *Eucalyptus globulus* é a principal espécie de eucalipto em plantações em Portugal. A doença mais grave nas folhas de eucalipto está relacionada com espécies de *Mycosphaerella senso lato* as quais afetam árvores jovens com folhagem na sua fase juvenil, resultando na diminuição da sua taxa de crescimento e do volume de madeira produzido, o que causa perdas significativas de produtividade. O nome mais comum desta doença é Doença das Manchas das Folhas do Eucalipto (Mycosphaerella Leaf Disease) que se tornou inapropriado uma vez que as diversas espécies envolvidas na doença foram reorganizadas em diversos géneros, sendo agora o uso do termo "Complexo da Doença das Folhas do Eucalipto" (Eucalyptus Leaf Disease Complex) o mais adequado. O objetivo geral desta tese é investigar o Complexo da Doença das Folhas do Eucalipto em Portugal, especialmente a diversidade de espécies, a taxonomia e o papel de cada espécie no complexo da doença. O Complexo da Doença das Folhas do Eucalipto foi recentemente revisto e as espécies envolvidas distribuídas em diversos géneros. Efetuou-se a prospeção de folhas sintomáticas em diversas plantações de *Eucalyptus globulus* e as espécies detetadas foram caracterizadas morfologicamente e com ferramentas moleculares, permitindo uma análise geral das espécies que se encontram no complexo da doença. O presente trabalho indica um aumento do número de espécies de *Mycosphaerella senso lato* associadas às plantações de eucalipto em Portugal. Em relação ao género *Teratosphaeria* e espécies filogeneticamente próximas, foram adicionadas cinco novas observações na Península Ibérica, *Neodevriesia hilliana*, pela primeira vez em *Myrtaceae*; *Quasiteratosphaeria mexicana*, *Teratosphaericola pseudoafricana*, *Teratosphaeria pluritubularis* e *Teratosphaeria lusitanica*, uma nova espécie; novas estruturas do estado assexuado e duas novas reclassificações de géneros foram também acrescentadas. Relativamente a outros géneros, foram observadas algumas espécies pela primeira vez, tal como *Cladosporium cladosporioides*, *Fusicladium eucalypti*, *Mycosphaerella madeirae*, no continente. Foi também descrita a primeira observação de *Teratosphaeria gauchensis* que causa um cancro severo em *Eucalyptus globulus*. Foi comparada a agressividade de diversas espécies, de modo a avaliar o contributo de cada espécie no complexo da doença, permitindo distinguir diferentes comportamentos, desde agente patogénico primário a secundário. As espécies de *Cladosporium cladosporioides*, *Mycosphaerella communis* e *Mycosphaerella lateralis*, destacam-se por um comportamento mais agressivo relativamente à *Teratosphaeria nubilosa*. De facto, a espécie *Teratosphaeria nubilosa* não é a única responsável pela doença mas envolve claramente um complexo de espécies que atuam em conjunto.

Palavras-chave: Doença foliar; Cancro; *Mycosphaerella*; *Teratosphaeria*; Eucaliptos e *Eucalyptus globulus*.

Publications arising from the current thesis

Peer reviewed journals

Silva M.R.C., E. Diogo, H. Bragança, H. Machado and A.J.L. Phillips, 2015. *Teratosphaeria gauchensis* associated with trunk, stem and foliar lesions of *Eucalyptus globulus* in Portugal. *Forest Pathology* 45, 224–234. Doi: 10.1111/efp.12160.

Silva M.R.C., H. Machado, L. Neves, C. Araujo and A.J.L. Phillips, 2012. *Mycosphaerella* and *Teratosphaeria* species associated with *Mycosphaerella* Leaf Disease on *Eucalyptus globulus* in Portugal. *Forest Systems* 21, (2) 300-305. ISSN: 2171-5068, http://dx.doi.org/10.5424/fs/2012212-02211

International Conference posters

Silva M.R.C., H. Machado, L. Neves, C. Valente and A.J.L. Phillips, 2012. Distribution of Mycosphaerella leaf disease on *Eucalyptus* in Portugal. IUFRO WP 7.02.02 2011 Global Change and Forest Diseases: New Threats, New Strategies, Montesclaros Monastery in Cantabria (Spain) from 23rd-28th of May 2011. *Journal of Agricultural Extension and Rural Development*, Conference Proceedings 4, (9) 298.

Silva M.R.C., H. Machado and A.J.L. Phillips, 2010. *In vitro* pathogenicity tests with *Mycosphaerella* species isolated from Portuguese *Eucalyptus* plantations. 9th International Mycological Congress – The Biology of Fungi, 1-6 August 2010, Edinburgh-UK. Delegate CD-ROM [P3.264].

National Conference posters

Silva M.R.C., H. Machado and A.J.L. Phillips, 2014. Eucalyptus Leaf Disease Complex in Portugal and its recent taxonomy. 1º Simpósio SCAP "Novos desafios na Proteção das Plantas e 7º Congresso da SPF. P03.13. p.53, 20 e 21 Novembro, Oeiras.

Silva M.R.C., E. Diogo, H. Bragança, H. Machado and A.J.L. Phillips, 2014. New disease linked with trunk and stem canker of *Eucalyptus* in Portugal. 1º Simpósio SCAP "Novos desafios na Proteção das Plantas e 7ºCongresso da SPF. P03.12. p.52, 20 e 21 Novembro, Oeiras.

Machado, H.*, H. Bragança, M.R.C. Silva and E. Diogo, 2013. Doenças do eucalipto em Portugal – novas ameaças e desafios. 7º Congresso Florestal Nacional "Florestas – Conhecimento e Inovação", Vila Real/Bragança, 5-8 junho. Livro de Resumos p. 197.

Silva M.R.C., H. Machado, B.N. Gonzalez, C. Valente, L. Neves and A.J.L. Phillips, 2010. Survey of *Mycosphaerella* species complex on *Eucalyptus globulus* in Portugal. 9th Conference of the European Foundation for Plant Pathology/6th Congress of the Sociedade Portuguesa de Fitopatologia – Integrated Plant Disease Management, 15-18 November, Évora-Portugal. Book of Abstracts P8.3, p 139.

Submitted papers

Silva M.R.C., H. Machado and A.J.L. Phillips. Review Paper - Eucalyptus Leaf Disease Complex. *Phytopathologia Mediterranea* (submitted).

Silva M.R.C., H. Machado and A.J.L. Phillips. New records, species and combinations in *Teratosphaeria* on *Eucalyptus* in Portugal. *Forest Pathology* (submitted).

Silva M.R.C., H. Machado and A.J.L. Phillips. Pathogenicity of species in the Eucalyptus Leaf Disease complex. *European Journal of Plant Pathology* (submitted).

Silva M.R.C., H. Machado, L. Neves and A.J.L. Phillips. Species in the *Eucalyptus* Leaf Disease Complex in Portugal. *Forest Pathology* (submitted).

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Chapter 6 Pathogenicity of species in the Eucalyptus Leaf Disease complex

Appendix B Plantations, Symptomatic leaves and Pattern of Ascospore Germination and Cultures

List of **Abbreviations and Acronyms**

ACT	Actin
ANOVA	Analysis of variance
ATPase 6 gene	mitochondrial adenosine triphosphate 6 gene
BLAST	Basic Local Alignment Search Tool
bs	bootstrap support
CBS	Culture collection of Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, The Netherlands
cf.	*confer* – "compare"
CI	Consistency index
CMW	Culture collection of M.J. Wingfield, housed at Forestry and Agricultural Biotechnology Institute
comb. nov.	*combinatio nova* – "new combination"
CPC	Culture collection of P.W. Crous, housed at CBS
diam.	in diameter
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetra acetic acid
EF-1α	Translation elongation factor 1-alpha
EFN	culture collection of ex-Estação Florestal Nacional, Instituto Nacional dos Recursos Biológicos, Oeiras, Portugal, now housed at LISFA
e.g.	*exempli gratia* – "for example"
ELDC	Eucalyptus Leaf Disease Complex
et al.	*et alii* – "and co-workers"
gen. nov.	*genus novum* – "new genus"
HI	Homoplasy index
INIA	National Institute for Agrarian and Veterinary Research
ITS	Internal transcribed spacer
ITS1-5.8S-ITS2 cluster	Cluster of Internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2
LISFA	Herbarium Code of Unit for Research and Services on Agricultural and Forestry Systems and Plant Health, INIA I.P., Oeiras, Portugal
LSD	Least Significant Diference

List of Abbreviations and Acronyms (Cont.)

LSU	Large subunit of 28S rDNA
MEA	Malt extract agar
ML	Maximum-likelihood
MLD	Mycosphaerella leaf disease
MP	Maximum parsimony
MUCC	Murdoch University culture collection, Australia
NCBI	National Center for Biotechnology Information
NJ	Neighbor joining
PCR	Polymerase chain reaction
RAPD	randomly amplified polymorphic DNA
RAxML	Randomized Axelerated Maximum Likelihood
rDNA	Ribosomal DNA
RH	relative humidity
RI	Retention index
SE	Standard error of the mean
s. l.	*sensu lato* – "in the wide or broad sense"
sp. nov.	*species nova* – "new species"
sp., spp. (plural)	species
SSU	small subunit of 18S rDNA
STE-U	Culture collection of the Department of Plant Pathology, Stellenbosch University, South Africa.
TL	Tree length
TLD	Teratosphaeria Leaf Disease
UV	Ultraviolet
vs	against (versus)
w/v	weight per volume

List of *Mycosphaerellaceae, Neodevriesiaceae and Teratosphaeriaceae* species and their synonyms used in this thesis

Current name	Synonyms	Family
Amycosphaerella africana	≡ *Mycosphaerella africana* (*Basionym*) ≡ *Teratosphaeria africana* ≡ *Mycosphaerella ellipsoidea* ≡ *Mycosphaerella aurantia*	*Mycosphaerellaceae*
Amycosphaerella quasicercospora	≡ *Mycosphaerella quasicercospora* (*Basionym*) ≡ *Teratosphaeria quasicercospora*	*Mycosphaerellaceae*
Austroafricana parva	≡ *Mycosphaerella parva* (*Basionym*) ≡ *Teratosphaeria parva* ≡ *Mycosphaerella grandis*	*Teratosphaeriaceae*
Neodevriesia hilliana	≡ *Devriesia hilliana* (*Basionym*)	*Neodevriesiaceae*
Pallidocercospora heimii	≡ *Mycosphaerella heimii* ≡ *Pseudocercospora heimii*	*Mycosphaerellaceae*
Paramycosphaerella marksii	≡ *Mycosphaerella marksii* (*Basionym*)	*Mycosphaerellaceae*
Quasiteratosphaeria mexicana	≡ *Mycosphaerella mexicana* (*Basionym*) ≡ *Teratosphaeria mexicana*	*Teratosphaeriaceae*
Teratosphaeria lusitanica	*Sp. nov.*	*Teratosphaeriaceae*
Teratosphaeria molleriana	≡ *Sphaerella molleriana* (*Basionym*) ≡ *Mycosphaerella molleriana* ≡ *Colletogloeopsis molleriana* ≡ *Readeriella molleriana* ≡ *Mycosphaerella vespa* ≡ *Mycosphaerella ambiphylla* ≡ *Teratosphaeria xenocryptica*	*Teratosphaeriaceae*
Teratosphaeria nubilosa	≡ *Sphaerella nubilosa* (*Basionym*) ≡ *Mycosphaerella nubilosa* ≡ *Mycosphaerella juvenis*	*Teratosphaeriaceae*
Teratosphaeria pluritubularis	≡ *Mycosphaerella pluritubularis* (*Basionym*)	*Teratosphaeriaceae*
Teratosphaericola pseudoafricana	≡ *Mycosphaerella pseudoafricana* (*Basionym*) ≡ *Teratosphaeria pseudoafricana*	*Teratosphaeriaceae*
Teratosphaeriopsis pseudoafricana	*Sp. nov.*	*Teratosphaeriaceae*

List of genera abbreviations used in this thesis

Am.	*Amycosphaerella*
A.	*Austroafricana*
M.	*Mycosphaerella*
P.	*Paramycosphaerella*
Pc.	*Pallidocercospora*
Ph.	*Phaeophleospora*
Pth.	*Phaeothecoidea*
Pp.	*Parapenidiella*
Ps.	*Pseudocercospora*
Pseu.	*Pseudoteratosphaeria*
R.	*Readeriella*
S.	*Septoria*
So.	*Sonderhenia*
Su.	*Suberoteratosphaeria*
T.	*Teratosphaeria*
U.	*Uwebraunia*
X.	*Xenomycosphaerella*
Z.	*Zasmidium*

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I

General Introduction

General Introduction

This study is mainly focused on morphological and molecular identification of species associated with the Eucalyptus Leaf Disease Complex (ELDC) and others species involved. Some attention was also centred on understanding the role of each species on the complex.

An overview of the background knowledge on *Mycosphaerella* s.l. is presented and developed in the Literature Review (Chapter 1). The general objectives of the study are displayed forward, aiming to unravel some of the prevailing unknown species.

Background knowledge

Approximately 700 species of fungi have been linked to *Eucalyptus* leaves and stems. The disease associated with *Mycosphaerella* s.l. teleomorphs and several anamorph genera started to be studied in detail at the end of the 1990s (Crous, 1998). Previous names related to this disease complex were *Mycosphaerella* Leaf Spot, *Mycosphaerella* Leaf Blotch, Crinkle Leaf Blight and *Mycosphaerella* Leaf Disease because many different anamorphic fungi were collected from affected leaves and many of the fungi were associated with the teleomorph genus *Mycosphaerella* (Park *et al.*, 2000). Between 2006 and 2009 there was a taxonomic reorganization of *Mycosphaerella* s.l. that resulted in a redistribution of the genera and species into different families. Thus the name *Mycosphaerella* Leaf Disease became particularly unsuitable since most of the important pathogens on eucalypts were transferred from *Mycosphaerella* to *Teratosphaeria* (e.g., *T. nubilosa* and *T. cryptica*) and for a short time the disease was called *Teratosphaeria* Leaf Disease (TLD) (Schoch *et al.*, 2006; Crous *et al*., 2007; Crous *et al.*, 2009a, 2009b, 2009c). Thereafter it became clear that many different genera were associated with the disease and the application of a fungal genus name to the disease was not suitable. Furthermore, it became clear that this disease was not caused by a single pathogen but a complex of species related to similar symptoms. For all those reasons it was decided that in this thesis the term "Eucalyptus Leaf Disease Complex" (ELDC) will be used, and recommend that this term be used in all future studies.

Earlier studies used 14 different ascospore germination patterns as a character in morphological identification of *Mycosphaerella* s.l. (Fig. B.2, B.3). However, it is now known that different species share similar patterns. The morphology of a lesion has also been used as another character for species identification. Since several different species can co-exist in the same lesion and some lesions coalesce, this character is clearly unsuitable for identifications. In

addition, many of these species are difficult to grow in culture, while others grow very slowly and do not form asexual spores in culture. Some of the species are host-specific and produce small fruiting structures with very conserved morphology (Crous, 1998; Hunter *et al.*, 2006). As a result, morphological identification is extremely difficult and it is essential to confirm the identity by molecular means. Sequence data of the ITS region provides enough resolution to differentiate most taxa (Hunter *et al.*, 2006).

In 1829 eucalypts were introduced into Portugal as ornamental trees and began to be exploited commercially for its wood quality for pulp production on the last half part of XXth Century (Potts, 2004). Today these species represent the first continental forestry area with 812 000 ha, about 26% (ICNF 2013) particularly planted in the coastal regions (Valente *et al.*, 2008).

Eucalypts benefited from the "absence" of pests and diseases before 1970 (Crous and Wingfield, 1997; Valente *et al.*, 2008). The earliest report of *Mycosphaerella s. l.* outside of Australia was reported in 1881 (*T. molleriana*) in Portugal on leaves of *Eucalyptus globulus* (Crous and Wingfield, 1997). However this disease was unnoticed in Portugal before 1999, due to the absence of severe defoliation on young trees. Early studies reported about 10 species on eucalypts in Portugal, but this was based on informal collections (Crous and Wingfield, 1997; Crous, 1998; Crous *et al.*, 2006). This disease affects mainly young trees in the juvenile foliage phase, resulting in reduced growth rate of the trees and lower wood volume, which causes significant productivity losses.

Some studies of epidemiology and pathogenicity have been made on *Mycosphaerella* s. l. on *Eucalyptus* in particular on *Austroafricana parva, Teratosphaeria cryptica* and *T. nubilosa* (e.g. Park & Kean, 1982a, 1982b) the last two were considered the most virulent on eucalypts (Fig. B.4, B.5). However, there have been no such studies on others species belonging to this complex.

All these subjects are described in detail in Chapter 1 – Literature Review.

This work

In Chapter 1 the complex of species of *Mycosphaerella s. l.* was reviewed, in particular the latest developments about re-evaluation of genera and how the species have been re-distributed into several genera, including *Teratosphaeria*. This provides an update on the disease and its damages, focusing on some countries. In particular, for Portugal, the

geographical distribution of the disease, the taxonomy of the pathogens and new potential pathogens that can also cause diseases on eucalypts.

A survey of species associated with ELDC in Portugal is reported in Chapter 2. Symptomatic leaves were collected from *E. globulus* plantations, fungi were isolated and characterized in terms of their morphology (Table B.1; Fig. B.1). DNA sequences of the ITS region were used to give an indication of the species that are associated with the disease and to indicate the most frequent species in the disease complex.

Teratosphaeria species and some phylogenetically closely species are the subject of Chapter 3. In this chapter five new records for the Iberian Peninsula were added: *Neodevriesia hilliana* was reported for the first time on *Myrtaceae*; *Quasiteratosphaeria mexicana*, *Teratosphaericola pseudoafricana* and *Teratosphaeria pluritubularis*, the last one only in Portugal and *Teratosphaeria lusitanica* was introduced as a new species in the Iberian Peninsula. New anamorphic structures were included and described for *A. parva*, *Q. mexicana*, *T. pluritubularis* and *T. pseudoafricana*. Furthermore, two new combinations were made, namely *Amycosphaerella quasicercospora* and *Quasicercospora mexicana*. Thus an update of *Teratosphaeria* species and allies on *E. globulus* in Portuguese plantations is presented.

With the aim of determining the role played by the species present in the complex of the lesions studied, and based on the ascospores germination patterns with similar culture morphology, several isolates were confirmed by molecular characterization and their phylogenetic analysis was accomplished. Furthermore, species present in a single lesion were quantified and the results compared to severity levels in order to evaluate the composition of the complex. Some species were reported for the first time related to the disease symptoms including *Cladosporium cladosporioides*, *Fusicladium eucalypti*, *Mycosphaerella madeirae* (first report from mainland Portugal) and *Venturiaceae* sp. All the other species previously reported were also found (Chapter 4).

During the surveys, attention was made to check for new symptoms on eucalypts leaves and to determine if any cankers are involved (Chapter 5). Regarding the cankers observed, severe damage on *E. globulus* has been observed for the first time in Portugal. The cause of this canker was identified from sequence data of the ITS1-5.8S-ITS2 and EF-1α clusters and morphological characteristics and determined to be *Teratosphaeria gauchensis*.

Leaf spots on *Eucalyptus* are normally attributed to species like *T. nubilosa* and *T. cryptica*, which are considered the most virulent ones, but many other species can inhabit the same lesions with a saprobic or endophytic behaviour. Therefore, it was considered important to

evaluate the individual behaviour of the several species found in the leaf disease complex and compare their capability to colonize leaf tissues and cause leaf necrosis. Such a study is reported in Chapter 6.

Objectives

The purpose of this work is to explore some of the knowledge gaps related to the "Eucalyptus leaf disease complex" in Portugal aiming to identify the species found during a survey.

The specific objectives of this thesis are:

- To compile a literature review on *Mycosphaerella s. l.* (Chapter 1).
- To conduct a survey of fungi associated with symptomatic leaves collected from *E. globulus* plantations, identify them in terms of morphology and DNA phylogeny and determine the most frequent species in the disease complex (Chapter 2 and 4).
- To evaluate morphologically and phylogenetically the existence of new species, make new combinations where necessary (Chapter 3).
- To evaluate the relative frequency of species in *E. globulus* plantations during several seasons, its combinations in symptomatic lesions and the relation of the complex composition with disease severity (Chapter 4).
- To observe collections for new leaf symptoms and for canker development (Chapter 5).
- To characterize aggressiveness of the species in the complex (Chapter 6).

Each chapter included in this thesis is presented as an independent unit (including: Summary, Introduction, Materials and Methods, Results, Discussion and References), corresponding to the complete versions of the manuscripts published (Chapters 2 and 5) or currently being prepared for publication (Chapter 1, 3, 4 and 6) in international, peer-reviewed, scientific journals.

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II

Literature Review

CHAPTER 1

Eucalyptus Leaf Disease Complex

Chapter 1

REVIEW PAPER

Eucalyptus Leaf Disease Complex

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Summary

Eucalypts are the second most important forest plantation species grown worldwide. A complex of species of *Mycosphaerella sensu lato* causes leaf disease on *Eucalyptus* that results in significant economic losses wherever eucalypts are grown. Although the causal agents were included mainly in *Mycosphaerella* this genus was recently re-evaluated and the species distributed into several genera, including *Teratosphaeria* and others. This review provides an update on the disease, the damage caused, impact, influence of weather conditions, disease control, and pathogenicity, focussing on selected countries especially Portugal. Also reviewed are the geographical distribution of the disease, taxonomy of the pathogens and includes new and other possible leaf diseases that can also cause cankers on eucalypts.

Key words. *Mycosphaerella*, *Teratosphaeria*, *Capnodiales*, *Dothideomycetes*, eucalypts.

Introduction

Host

Eucalypts, commonly known as gum trees are a important forest plantation species grown worldwide. The genus *Eucalyptus* was introduced by L'Héritier de Brutelle (1789) for a single species, *E. obliqua*. In the following 200 or more years, many species names have been published in the genus (Brooker, 2000). Eucalypts are found principally in the southern hemisphere and are native to Australia, but since the late 18th century they have been spread around the world (Potts, 2004).

Eucalypts are well-known for their straight form, fast growth, also on hard habitat, facility in vegetative propagation, adaptation to soils and to a wide range of climates including high rainfall, semi-arid, sea level and alpine tree line zones (Old *et al.*, 2003; Potts, 2004). Commercially they are important for their special wood properties and for pulp production, these attractive product qualities have induce the extensive installation of eucalypt plantations in many countries (Old *et al.*, 2003).

A genetically heterogeneous forest community offers an important shield against disease epidemics which their native environments host an extensive variety of fungal pathogens. In opposition, industrial plantations are mostly single species or hybrid plantings, planted in large areas with identical clones in order to get high product quality and an uniform and rapid growth.

This may cause widespread epidemic fungi and pathogens can be transmitted on infected seeds or planting stocks (Old *et al.*, 2003). Some studies on the Leaf Disease Complex, (predominantly species of *Mycosphaerella* and *Teratosphaeria*) and involving different species of eucalypts point to *E. globulus* as the most susceptible species (Carnegie *et al.*, 1994; Dungey *et al.*, 1997; Tejedor, 2004).

Species concepts

Before discussing the taxonomy of *Mycosphaerella* is necessary to outline the concepts that are used to circumscribe the species. Several criteria have been used to contribute to taxonomical studies of fungi. First of all it is important to distinguish between species concept and species criteria. Species concept is a description of the kind of entity that constitutes a species, while criteria that delimit a particular species, i.e. the practical standards for the recognizing whether individuals should be considered members of the same species, are called species criteria (Taylor *et al.*, 2000; Cai *et al.*, 2011).

The diverse criteria that allow the delimitation of species can be characterized as morphological, physiological, intersterility, host specificitation, and phylogenetic. These species recognition criteria try to recognize evolutionary independent lineages (Taylor *et al.*, 2000; Cai *et al.*, 2011). These comprise the Morphological Species Concept (MSC); the Biological Species Concept (BSC); the Ecological Species Concept (ESC); the Phylogenetic Species Concept (PSC) and the Genealogical Concordance Phylogenetic Species Recognition (GCPSR); Polyphasic Method (PM) and finally the Consolidated Species Concept (CSC). However searching for a single species criterion valid for all cases is basically impossible (Giraud *et al.*, 2008).

The MSC was considered by Linnaeus (1758) with the typological Species Concept that the species were a 'type' of organism. Here the species are considered as groups of individuals sharing similar morphology traits. This concept can be acceptable for animals and plants, however for fungi, particularly the microscopic, the morphological traits are few, so there are several limitations. Thus, differences in morphology might not inevitably correspond to different species, but certainly correspond to an extensive phenotypic variation inside the same species. In addition, some individuals may appear to be morphologically similar but they belong to different species. However many of the fungal species described by Saccardo and others are still accepted as valid.

The BSC was proposed by Ernst Mayr (1942) and considers species as "groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups". The capacity to mate resulting in a fertile progeny is the major aim of this species concept. This concept can potentally be applied to fungi that have a sexual stage in their life cycle. However, mating and production of the sexual state of most fungi in culture has not been possible, and many species are known to be purely asexual. Therefore, the BSC is not a feesible concept for fungi.

The ecological species concept (ESC) is used to describe populations that are adapted to certain ecological niches and due to their adaptations will form discrete morphological clusters. According to the ESC, populations form the discrete phenetic clusters that we identify as species because the ecological and evolutionary processes controlling how resources are divided up tend to produce those clusters (Ridley, 2003). Giraude *et al.* (2010) emphasizes the adaptation to a particular ecological niche is also linked to the emergence of novel fungal diseases of plants. Thus, host shift speciation is one of the primordial paths for emergence of new fungal diseases and is a particular case of ecological speciation.

ESC has the advantage of recognizing the role played by the environment in controlling morphological development. On the other hand, it has disadvantages such as potentially ignoring cryptic species (two or more distinct species classified as a single species) and not easily defining ecological niches as a whole. In addition, ESC encompasses the idea of host association and naming species according to the host on which they occur. This can result in an enormous proliferation of names, many of which are synonyms.

Over the last years, the phylogenetics approach became a rapid DNA tool that could resolve fungal taxa that BSC could not resolve (Taylor *et al.,* 2000; Hunter *et al.,* 2006b). The PSC became well suited for fungi because it relates both sexual and asexual organisms and emphasizes a nucleotide divergence between monophyletic lineages (Taylor *et al.,* 2000). The problem with this criterion is that it is based on the assumption that the whole genome is represented by a gene that should have the same evolutionary history, which in some cases did not occur.

Taylor *et al.,* (2000) described GCPSR as an extension of the PSC and offered a better discrimination for delimiting species. Thus, GCPSR tries to define the limits of sexual species. To do this it uses the phylogenetic concordance of multiple unlinked genes to designate a lack of genetic exchange and the evolutionary independence of lineages. One practical result is that it is more useful to distinguish closely related sibling species (Cai *et al.,* 2011).

During the last decade, the polyphasic approach of combining Biological, Morphological and Phylogenetic Species Concepts has revolutionised the taxonomy of fungi (Lombard *et al.*, 2010). More recently Quaedvlieg *et al.,* (2014) proposed a formal change in the name of the polyphasic approach, which they called the Consolidated Species Concept (CSC).

Taxonomy of *Mycosphaerella* and related genera

Persoon (1794) described *Sphaeria corylea* (Pers.) on dead leaves of *Corylus*. Over the next 3 years he discovered more related species and then (Persoon, 1797) transferred them all to *Sphaeria maculiformis* (Pers.) relegating the species epithet to the status of variety. Subsequently, Saccardo transferred all species of *Sphaeria* with 1-septate, hyaline ascospores to *Sphaerella* (Saccardo, 1882). However, the genus name *Sphaerella* was already occupied by green algae and all *Sphaerella* species were placed in *Mycosphaerella* (Aptroot, 2006). Thus, the first generic description for *Mycosphaerella* Johanson (1884) was that of *Sphaerella* (1882).

Early mycologists frequently described new species based on the host with which they were associated. This lead to a proliferation of species names, many of which were later reduced to synonymy (Von Arx, 1949; Barr, 1972; Tomilin, 1979; Corlett, 1991). Aptroot (2006) re-examined more than 10 000 taxa in *Mycosphaerella* and recognized about 3000 species.

The first phylogenetic studies, based on ITS sequence data, suggested that *Mycosphaerella* is monophyletic (Crous *et al*., 2000; Goodwind *et al*., 2001). Later, however, Maxwell (2004) showed that based on ITS sequence data many of the anamorph genera within *Mycosphaerella* are polyphyletic.

Müller and Oehrens (1982) characterized the genus *Teratosphaeria* as "globose, perithecium-like ascomata growing inside the living leaf-tissue, with bitunicate asci and brownish, bicellular ascospores". Taylor *et al.,* (2003) resolved the relationships among members of the *Mycosphaerellaceae* by phylogenetic analysis of ITS sequence data and concluded that *Teratosphaeria* was a synonym of *Mycosphaerella*. Furthermore, phylogenetically it was monophyletic based on ITS (Crous *et al*., 2001).

Crous *et al.,* (2007) used combined ITS and LSU sequence data and demonstrated polyphyly (within *Teratosphaeria*) and paraphyly (within the *Capnodiales*) in *Mycosphaerella*. Thus several species were retained in *Mycosphaerella* while others were transferred to *Teratosphaeria* and even to others families.

Crous *et al.* (2007a) recognized a subset of isolates, representing various species as morphologically different from *Mycosphaerella s. str.* (Crous, 2009). That study positioned *Teratosphaeria* within the *Teratosphaeriaceae* because of the distinct asexual morphs and DNA phylogenetic data, also the presence of pseudoparenchymatal remnants in ascomata, ascospores that turn brown and verruculose while still in the asci, and ascospores with a mucoid sheath. Thus, polyphyly was demonstrated in a phylogeny based on combined ITS and partial large subunit (LSU) of the nuclear rRNA operon sequence data. Generic concepts were stabilized when *Teratosphaeria* was reinstated and several species of *Mycosphaerella* were transferred to *Teratosphaeria*. All genera, except *Schizothyrium*, were characterized by pseudothecial ascomata (Table 1.1). Furthermore, *Colletogloeopsis*, *Kirramyces* and *Readeriella* anamorphs were transferred to *Teratosphaeria* (Crous *et al*, 2009a, 2009d).

Basically, between 2006 and 2009, phylogenetic studies based on partial LSU gene sequences supported by anamorph and teleomorph morphologies revealed that the *Mycosphaerella* complex resides in several different families (*Davidiellaceae*, *Dissoconiaceae*, *Mycosphaerellaceae*, *Teratosphaeriaceae* and *Schizothyriaceae*) and are represented by several genera (Table 1.2) (Schoch *et al.*, 2006; Crous *et al.*, 2009b, 2009c, 2009d).

In 2009, Crous *et al*. (2009c) considered generic boundaries in the *Teratosphaeriaceae and Mycosphaerellaceae*. Thus, *Mycosphaerellaceae* has *Cercospora*, *Cercosporella*, *Dothistroma*, *Lecanosticta*, *Phaeophleospora*, *Polythrincium*, *Pseudocercospora*, *Ramularia* (*Mycosphaerella* s. s.), *Ramulispora*, *Septoria*, *Sonderhenia* and *Zasmidium* anamorphs. The genera *Ramichloridium* and *Dissoconium* were excluded from the *Mycosphaerellaceae* and shown to represent an undefined family.

In 2013, Hyde *et al.* (2013) added 10 more families to the *Dothideomycetes*, 7 new orders, 38 asexual genera within *Mycosphaerellaceae*, and 22 asexual plant pathogenic and extremophilic genera in *Teratosphaeriaceae*. Furthermore, Crous *et al.* (2013a) studied *Pseudocercospora* (an anamorphic state with mycosphaerella-like teleomorphs) and recognised 14 clades, six of which cluster in *Mycosphaerellaceae*. *Pseudocercospora s. str.* corresponds to a distinct clade, sister to *Passalora eucalypti*, and a clade representing the genera *Scolecostigmina*, *Trochophora* and *Pallidocercospora*, taxa formerly accommodated in the *Mycosphaerella heimii* complex. Also, Quaedvlieg *et al.* (2013) studied *Septoria*, which was shown to be a different genus in the *Mycosphaerellaceae*, which has mycosphaerella-like sexual morphs. A total of 47 genera were resolved, with the introduction of 14 new genera, 36 new species, and 19 new combinations.

In 2009, Crous (2009) reviewed the taxonomy and phylogeny of *Mycosphaerella* genus and their anamorphs and sum up several old studies like Klebahn (1918), Laibach (1922), Müller and von Arx (1962), Von Arx (1983) and Crous (1998).

The genus *Mycosphaerella* is placed in *Capnodiales* (Schoch *et al.*, 2006) as a large genus of ascomycetous, mostly leaf infecting fungi. *Capnodiales* is inserted in *Dothideomycetes* that have approximately 115 families and include a highly varied range of fungi characterized mainly by asci with two wall layers (bitunicate asci) and frequently with fissitunicate dehiscence (Hyde *et al.*, 2013).

Table 1.1 Morphological characteristics of several genera of *Mycosphaerella sensu lato* (Crous *et al.*, 2007a).

Genera	Morphological Characters
Mycosphaerella-like Genera	
Davidiella	- Ascospores with irregular, angular lumens
Dissoconium	- Actively discharged conidia, conidiophores solitary, pale brown, giving rise to primary and secondary
Mycosphaerella s. str.	- Conidiomata variable from solitary conidiophores to sporodochia, fascicles to pycnidia, but conidia not actively discharged
Teratosphaeria	- Ascospores turning brown in asci often observed, hamathecial tissue, ascospore sheath, multi-layered endotunica, prominent periphysoids, ascomata usually linked by superficial stroma;
Schizothyrium	- Ascomata thyrothecial
***Teratosphaeria* (*Teratosphaeriaceae*)**	
Cibiessia Pseudotaeniolina	- With hyphae submerged to superficial, disarticulating into arthroconidia.
Cibiessia	- Hyphae superficial, brown to green-brown, smooth, disarticulating to form pale brown, cylindrical, 0–3-septate conidia with subtruncate ends, frequently with a *Readeriella* synanamorph
Pseudotaeniolina	- Mature, brown hyphae disarticulating into thick-walled, spherical, smooth to verruculose 0(–2) transversely septate, brown conidia
Batcheloromyces Capnobotryella Catenulostroma Devriesia Hortaea Nothostrasseria Penidiella Phaeothecoidea Readeriella Staninwardia	- Hyphae not disarticulating into arthroconidia and with endoconidia absent (except *Phaeothecoidea*)
Capnobotryella Devriesia Hortaea	- Conidiogenous cells integrated in hyphae; well-developed conidiomata or long, solitary, macronematous, terminally penicillate conidiophores absent

Genera	Morphological Characters
Capnobotryella Hortaea	- Conidia solitary on indistict to well defined phialides on hyphae.
Batcheloromyces, Catenulostroma, Penidiella, Readeriella, Staninwardia	- Conidia brown, but basal appendages lacking, amero- to scolecospores
Readeriella, Staninwardia.	- Conidiomata pycnidial to acervular
Batcheloromyces Catenulostroma Penidiella	- With conidiomata not enclosed by host tissue, fasciculate to sporodochial or solitary, hyphomycetous.
Batcheloromyces Catenulostroma	- Conidiophores not penicillate, without a branched conidiogenous apparatus, *in vivo* fasciculate to sporodochial.
Batcheloromyces	- Biotrophic; fruiting composed of sporodochia and radiating layers of hyphae arising from the stromata, conidiophores arising from superficial sporodochia and radiating hyphae, conidiogenous cells unilocal, with conspicuous annellations, conidia solitary or in fragile disarticulating chains, aseptate or transversely 1–3-septate, usually with distinct frills, secession rhexolytic.
Capnobotryella	- Conidiogenous cells integrated in the distal ends of hyphae; conidia thick-walled, brown, smooth, 1 –septate
Catenulostroma	- Biotrophic, leaf-inhabiting, with distinct, subepidermal to erumpent, well-developed sporodochia, or saxicolous, saprobic, sometimes causing opportunistic human infections; radiating layers of hyphae arising from sporodochia; conidiogenous cells without annellations; conidia in true simple or branched basipetal chains, transversely 1- to pluriseptate or with longitudinal and oblique septa (dictyosporous), occasionally distoseptate.
Devriesia	- With conidia in chains, holoblastic, pseudocladosporium-like in morphology, but scars and hila not excessively thickened, nor refractive, producing chlamydospores in culture; species are mostly heat resistant.
Hortaea	- Conidiophores short and frequently reduced to conidiogenous cells that proliferate percurrently via wide necks, giving rise to hyaline, 0(–2)- septate, broadly ellipsoidal conidia.
Nothostrasseria	- Conidia brown, with hyaline basal appendages; conidiomata pycnidial, conidiogenous cells phialidic, but also percurrent, subhyaline.
Penidiella	- Conidiophores usually solitary, rarely densely fasciculate to synnematous (*in vivo*), penicillate, with a branched, apical conidiogenous apparatus giving rise to ramoconidia and branched chains of secondary conidia; scars not to slightly thickened and darkened-refractive.
Phaeothecoidea	- Hyphal ends forming endoconidia; hyphae pale to medium brown, verruculose, end cells dividing into several brown, verruculose, thick-walled, ellipsoid to obovoid endoconidia.
Readeriella	- Conidia solitary, dry, without mucilaginous sheath
Staninwardia	- Conidia catenulate, with persistant mucilaginous sheath.

Table 1.2 Phylogenetic studies that have analysed the *Mycosphaerella* complex between 2006 and 2009.

Families	Genera	References
Davidiellaceae	*Davidiella* (*Cladosporium*)	Schoch *et al.*, 2006
Dissoconiaceae	*Dissoconium* (=*Uwebraunia*) *Ramichloridium*	Crous *et al.*, 2004a Crous *et al.*, 2009b
Mycosphaerellaceae	*Cercospora* *Cercosporella* *Dothistroma* *Lecanosticta* *Phaeophleospora* *Polythrincium* *Pseudocercospora*	Crous *et al.*, 2009c
	Mycosphaerella s.s. (*Ramularia*) *Ramulispora* *Septoria* *Sonderhenia* *Zasmidium*	Crous *et al.*, 2009d
Teratosphaeriaceae	*Baudoinea* *Capnobotryella* *Devriesia* *Penidiella* *Phaeothecoidea* *Readeriella* *Staninwardia*	Crous *et al.*, 2009c, 2009d
	Teratosphaeria (*Collectogloeopsis, Kirramyces, Readeriella*)	Crous *et al.*, 2009a
Schizothyriaceae	*Schizothyrium (Zygophiala)*	Crous *et al.*, 2009d

Disease

In earlier studies the disease was called *Mycosphaerella* Leaf Spot / *Mycosphaerella* Leaf Blotch / Crinkle Leaf Blight and *Mycosphaerella* Leaf Disease because many of the anamorphic fungi isolated from symptomatic leaves were related to the teleomorph genus *Mycosphaerella* (Park *et al.*, 2000). After the taxonomic rearrangement of *Mycosphaerella* (Schoch *et al.*, 2006; Crous *et al.*, 2009b, 2009c, 2009d) and consequent redistribution of the genera and species into different families (*Davidiellaceae*, *Dissoconiaceae*, *Mycosphaerellaceae*, *Teratosphaeriaceae* and *Schizothyriaceae*) the name MLD was clearly inappropriate. Most of the significant foliar pathogens of eucalypts had been moved from *Mycosphaerella* to *Teratosphaeria* (Crous *et al.*, 2007a). As a result, the name *Teratosphaeria* Leaf Disease (TLD) began to be used (*e. g.* Andjic *et al.*, 2010a; Taole *et al.*, 2012, Balmelli *et al.*, 2013). Recently, however, Quaedvlieg *et al.*,

2014 introduced 23 novel genera to accommodate many of the species that were included in *Teratosphaeria*. Thus, the name Teratosphaeria leaf disease is also inappropriate. For this reason we prefer to refer to the disease as the "Eucalyptus leaf disease complex", and this term will be used in this review.

More than 150 species have been reported associated with Eucalyptus leaf diseases (Crous, 1998; Dick and Dobbie, 2001; Taylor *et al.*, 2001; Maxwell, *et al.*, 2003; Hunter *et al.*, 2004b; Burgess, *et al.*, 2007b; Carnegie, *et al.*, 2007; Cheewangkoon *et al.*, 2008; Crous *et al.*, 2004, 2006, 2007a, 2009a, 2009d; Pérez *et al.*, 2014; Quaedvlieg *et al.*, 2014) (Table 1.3).

Several species can occur on a individual lesion (Crous *et al.*, 2004; Hunter *et al.*, 2006b; Silva *et al.*, 2009). Park and Keane (1982b) only found *T. nubilosa* (pathogenic) alone on young blighted lesions while *Austroafricana parva* was observed only on older lesions where it was considered to be a saprobe. It was also assumed that *T. nubilosa* is a primary pathogen that infected the leaves and only later did other species arrive and develop on the spots caused by *T. nubilosa*. On the other hand, *A. parva* was regularly observed alone or linked with older lesions caused by *T. cryptica*, *Phaeophleospora gregaria*, *Mycosphaerella lateralis*, *Paramycosphaerella marksii*, *Quasicercospora mexicana*, *T. nubilosa* and *Parapenidiella tasmaniensis* (Milgate *et al.*, 2001; Maxwell, 2004; Silva *et al.*, 2009).

With regard to hyperparasitism, *M. lateralis* is the only species that has been studied and there was no evidence that it could parasitise *T. cryptica* or *T. nubilosa* (Jackson *et al.*, 2004). Nevertheless its ecological position needs to be determined (Crous *et al.*, 2006). Field observations suggest that the division of *Mycosphaerella* s.l. into different life trophic groups, i.e. parasitic vs saprobic, would be artificial and could result in a strong correlation between the trophic life and diverse climatic zones (Crous *et al.*, 2000).

Table 1.3 Genera and species of fungi occurring on *Eucalyptus* leaves and stems.

Species	Geographic distribution	Hosts	Reference
Amycosphaerella africana	Australia, Colombia, Portugal, South Africa, Spain, Uruguay, Zambia	*E. cladocalyx*, *E. deanei*, *E. dunnii*, *E. globulus*, *E. grandis*, *E. nitens*, *E. radiata*, *E. viminalis*, *Eucalyptus* sp.	Crous and Wingfield, 1996 Crous, 1998 Maxwell *et al.*, 2003 Hunter *et al.*, 2004b Otero *et al.*, 2007a Jackson *et al.*, 2008 Perez *et al.*, 2009 Andjic *et al.*, 2010b De Blas *et al.*, 2011 Quaedvlieg *et al.*, 2014
Am. quasicercospora	Tanzania	*E. maidenii*	Crous *et al.*, 2006, 2007a Silva *et al.*, b
Austroafricana parva	Australia, Ethiopia, Portugal, South Africa, Spain	*E. agglomerata*, *E. botryoides*, *E. cypellocarpa*, *E. delegatensis*, *E. dunnii*, *E. grandis* × *E. camaldulensis*, *E. globulus*, *E. globulus* × *E. urophylla*, *E. grandis*, *E. grandis* × *E. camaldulensis*, *E. moluccana*, *E. nitens*, *E. obliqua*, *E. pellita*, *E. pilularis*, *E. regnans*, *E. saligna*	Park and Keane, 1982a Carnegie, 2000 Maxwell *et al.*, 2003 Jackson *et al.*, 2005 Crous *et al.*, 2004 Gezahgne *et al.*, 2006 Carnegie, 2007a Otero *et al.*, 2007a Crous *et al.*, 2008 Silva *et al.*, 2009 Carnegie *et al.*, 2011 Quaedvlieg *et al.*, 2014 Silva *et al.*, b
Catenulostroma eucalyptorum	Australia	*E. laevopinea*	Crous *et al.*, 2011a
Euteratosphaeria verrucosiafricana	Australia, Indonesia	*Eucalyptus* sp., *E. tereticornis*	Crous *et al.*, 2006 Carnegie *et al.*, 2011
Kirramyces delegatensis	Australia	*E. delegatensis*, *E. obliqua*	Park and Keane, 1984 Crous, 1998
Mycosphaerella aggregata	Australia	*E. grandis*	Carnegie and Keane, 1994
M. ambiphylla	Australia	*E. globulus*	Maxwell *et al.*, 2003
M. communis	Portugal Spain	*E. globulus*, *Eucalyptus* sp.	Crous *et al.*, 2004a Crous *et al.*, 2006
M. davisoniellae	Australia	*E. marginata*	Crous *et al.*, 2006
M. endophytica	South Africa, Spain	*Eucalyptus* sp., *E. grandis*, *E. nitens*	Crous, 1998 De Blas *et al.*, 2009
M. eucalypti	Australia	*Eucalyptus* sp.	Park and Keane, 1984 Crous, 1998 Carnegie *et al.*, 2011
M. irregulari	Thailand	*Eucalyptus* sp., *E. globulus*	Cheewangkoon *et al.*, 2008

Table 1.3 Genera and species of fungi ocurring on *Eucalyptus* leaves and stems (cont.).

Species	Geographic distribution	Hosts	Reference
M. juvenis	Kenya, South Africa, Tanzania, Zambia	*E. globulus* *E. grandis* *E. nitens*	Crous and Wingfield, 1996 Crous, 1998 Crous *et al.*, 2004a
M. keniensis	Kenya	*E. grandis*	Crous, 1998
M. konae	Thailand	*E. camaldulensis*	Crous *et al.*, 2007b
M. lateralis	Australia, Portugal, South Africa, Spain, Zambia	*E. globulus*, *E. grandis x saligna*, *E. saligna*, *E. nitens*, *E. grandis*, *E. maidenii*, *Eucalyptus* sp.	Crous and Wingfield, 1996 Crous, 1998 Maxwell *et al.*, 2000 Crous *et al.*, 2006 Silva *et al.*, 2008, 2009, 2012
M. longibasalis	Colombia	*E. grandis*	Crous, 1998
M. madeirae	Madeira, Portugal, Spain	*E. globulus*	Crous *et al.*, 2004a Aptroot, 2006 Otero *et al.*, 2007a Silva *et al.*, 2012
M. medusae	Australia	*E. alba*	Carnegie *et al.*, 2011
M. pseudoendophytica	South Africa	*E. nitens*	Crous *et al.*, 2006
M. pseudomarksii	Thailand	*Eucalyptus* sp.	Cheewangkoon *et al.*, 2008
M. pseudovespa	Australia	*E. biturbinata*	Carnegie *et al.*, 2007
M. quasiparkii	Thailand	*Eucalyptus* sp.	Cheewangkoon *et al.*, 2008
M. sumatrensis	Indonesia	*Eucalyptus* sp.	Crous *et al.*, 2006
M. swartii	Australia, New Zealand	*E. coccifera*, *E. delegatensis*, *E. dives*, *E. elata*, *E. fastigata*, *E. globoidea*, *E. leucoxylon*, *E. nitens*, *E. obliqua*	Park and Keane, 1984 Crous, 1998
M. tumulosa	Australia	*C. variegata*, *Eucalyptus* sp., *E. acmeniodes*, *E. amplifolia*, *E. melanophloia*, *E. moluccana*, *E. seeana*, *E. tereticornis*	Carnegie, 2007a Carnegie *et al.*, 2007
M. vietnamensis	Vietnam	*E. camaldulensis*, *E. grandis*, *E. grandis* hybrid	Burgess *et al.*, 2007a
Myrtapenidiella eucalypti	Thailand	*E. camaldulensis*	Cheewangkoon *et al.*, 2008
M. tenuiramis	Australia	*E. tenuiramis*	Crous *et al.*, 2009c
Neodevriesia hilliana	Portugal	*E. globulus*	Silva *et al.*, b
Neotrimmatostroma excentricum	Australia	*E. agglomerata*, *C. torelliana x C. variegata* *C. variegata*	Crous *et al.*, 2007b Carnegie, 2007a Carnegie *et al.*, 2011 Quaedvlieg *et al.*, 2014
Pallidocercospora acaciigena	Australia, Venezuela	*Eucalyptus* sp., *E. camaldulensis* × *E. urophylla*	Crous *et al.*, 2007b Quaedvlieg *et al.*, 2014

Table 1.3 Genera and species of fungi ocurring on *Eucalyptus* leaves and stems (cont.).

Species	Geographic distribution	Hosts	Reference
Pc. colombiensis	Colombia	*E. urophylla*	Crous, 1998 Quaedvlieg *et al.*, 2014
Pc. crystallina	Brazil, South Africa	*Eucalyptus* sp., *E. bicostata*, *E. grandis* × *E. camaldulensis*	Crous and Wingfield, 1996 Crous, 1998 Quaedvlieg *et al.*, 2014
Pc. heimii	Australia, Brazil, Colombia Madagascar, Portugal, Thailand, Uruguay, Venezuela	*E. camaldulensis*, *E. dunnii*, *E. obliqua*, *E. platyphylla*, *E. urophylla*	Crous, 1998 Whyte *et al.*, 2005 Crous *et al.*, 2007b Perez *et al*., 2009 Quaedvlieg *et al.*, 2014
Pc. heimioides	Indonesia	*Eucalyptus* sp.	Crous and Wingfield, 1997b Crous, 1998
Pc. irregulariramosa	South Africa	*E. grandis*, *E. saligna*	Crous and Wingfield, 1996, 1997b Crous, 1998 Hunter *et al.*, 2004b
Pc. thailandica	Thailand	*E. camaldulensis*	Crous *et al.*, 2007b
Paramycosphaerella intermedia	New Zealand	*E. saligna*	Dick and Dobbie, 2001 Quaedvlieg *et al.*, 2014
P. marksii	Australia, China, Ethiopia, Indonesia, Madagascar, Portugal, South Africa, Spain, Uruguay	*Eucalyptus* sp., *E. bicostata* *E. botryoides*, *E. camaldulensis*, *E. cloeziana*, *E. diversicolor*, *E. dunnii*, *E. fraxinoides*, *E. grandis*, *E. grandis* × *E. camaldulensis*, *E. grandis* × *E. resinifera*, *E. grandis* × *E. saligna*, *E. globulus*, *E. globulus* × *E. camaldulensis*, *E. longirostrata*, *E. maidenii*, *E. nitens*, *E. pellita*, *E. pilularis*, *E. propinqua*, *E. quadrangulata*, *E. resinifera*, *E. rudis*, *E. saligna*, *E. scias*, *E. smithii*, *E. tereticornis*, *C. maculata*, *C. torelliana* × *C. variegat*, *Eucalyptus* sp.	Carnegie and Keane, 1994 Crous and Wingfield, 1996 Crous, 1998 Crous *et al.*, 2004a Hunter *et al.*, 2004b Jackson *et al.*, 2005 Gezahgne *et al.*, 2006 Carnegie, 2007a Perez *et al*., 2009 Carnegie *et al*., 2011 Zhou and Wingfield, 2011 Quaedvlieg *et al.*, 2014
Parapenidiella pseudotasmaniensis	Australia	*E. globulus*	Crous *et al.*, 2009c Quaedvlieg *et al.*, 2014

Table 1.3 Genera and species of fungi ocurring on *Eucalyptus* leaves and stems (cont.).

Species	Geographic distribution	Hosts	Reference
Pp. tasmaniensis	Australia	*E. globulus*, *E. nitens*	Crous *et al.*, 1998 Jackson *et al.*, 2008 Quaedvlieg *et al.*, 2014
Passalora eucalypti	Brazil	*E. saligna*	Crous, 1998
P. intermedia	Madagascar	*E. camaldulensis*	Crous *et al.*, 2009e
P. leptophlebiae	Brazil	*E. leptophlebia*	Crous *et al.*, 2011c
P. zambiae	Zambia	*E. globulus*	Crous *et al.*, 2004a
Phaeophleospora gregaria	Australia, South Africa	*Eucalyptus* sp., *E. botryoides*, *E. cladocalyx*, *E. globulus*, *E. grandis*, *E. saligna*, *C. maculata*	Carnegie and Keane, 1997 Maxwell *et al*., 2003 Crous, 1998 Quaedvlieg *et al.*, 2014
Ph. scytalidii	Brazil, Colombia, Uruguay	*E. dunnii*, *E. globulus* *E. grandis*, *E. urophylla*	Crous *et al.*, 2006 Perez *et al*., 2009 Quaedvlieg *et al.*, 2014
Ph. stonei	Australia	*Eucalyptus* sp.	Crous *et al.*, 2007b
Ph. stramenti	Brazil	*Eucalyptus* sp.	Crous *et al.*, 2006 Quaedvlieg *et al.*, 2014
Phaeothecoidea intermedia	Australia	*E. botryoides*	Crous *et al.*, 2009c
Pth. eucalypti	Australia	*E. botryoides*	Crous *et al.*, 2007b
Pth.. minutispora	Australia	*E. botryoides*	Crous *et al.*, 2009c
Pseudocercospora acerosa	New Zealand	*E. baxteri*, *E. nitens*	Braun and Dick, 2002
Ps. basiramifera	Thailand	*E. camaldulensis*, *E. pellita*	Crous, 1998
Ps. basitruncata	Colombia	*Eucalyptus* sp., *E. grandis*	Crous, 1998
Ps. chiangmaiensis	Thailand	*E. camaldulensis*	Cheewangkoon *et al*., 2008
Ps. crousii	Australia, New Zealand	*E. delegatensis*, *E. dendromorpha*, *E. fastigata*, *E. muelleriana*, *E. obliqua*, *E. oreades*, *E. pilularis*, *E. regnans*, *E. regnans* × *E. obliqua*, *E. stenostoma*	Braun and Dick, 2002 Carnegie *et al*., 2007
Ps. cubae	Cuba	*Eucalyptus* sp.	Crous, 1998
Ps. deglupta	Malaysia, Papua New Guinea	*E. deglupta*, *E. delegatensis*	Crous, 1998 Braun, 2001 Braun and Dick, 2002
Ps. denticulata	Dominican Republic, Japan	*Eucalyptus* sp., *E. globulus*	Crous, 1998 Braun and Dick, 2002
Ps. epispermogonia	South Africa	*E. grandis* × *E. saligna*	Crous and Wingfield, 1996 Braun and Dick, 2002

Table 1.3 Genera and species of fungi ocurring on *Eucalyptus* leaves and stems (cont.).

Species	Geographic distribution	Hosts	Reference
Ps. eucalyptorum	Australia, China, Germany, Italy, Kenya, Madagascar, New Zealand, Portugal, South Africa, Spain, United Kingdom	*E. aggregata*, *E. alba*, *E. albens*, *E. amygdalina*, *E. bicolour*, *E. blakelyi*, *E. bosistoana*, *E. botryoides*, *E. bridgesiana*, *E. camaldulensis*, *E. camphora*, *E. cinerea*, *E. crebra*, *E. dalrympleana*, *E. glaucescens*, *E. globulus*, *E. gomphocephala*, *E. goniocalyx*, *E. grandis*, *E. gunnii*, *E. melliodora*, *E. nitens*, *E. occidentalis*, *E. ovata*, *E. paniculata*, *E. ployanthemos*, *E. populnea*, *E. punctata*, *E. resinifera*, *E. robusta*, *E. rubida*, *E. rudis*, *E. saligna*, *E. scoparia*, *E sideroxylon*, *E. stellulata*, *E. tereticornis*, *E. trabutii*, *E. viminalis*, *Eucalyptus* sp.	Crous, 1998 Crous *et al.*, 1989b Braun and Dick, 2002 Crous *et al.*, 2004a Carnegie *et al.*, 2011 Crous *et al.*, 2013a
Ps. flavomarginata	Thailand	*E. camaldulensis*	Hunter *et al.*, 2006b
Ps. fori	Australia, South Africa, Spain, Uruguay	*E. globulus*, *E. grandi*, *Eucalyptus* sp.	Hunter *et al.*, 2004b Jackson *et al.*, 2008 Márquez et al. 2011
Ps. gracilis	China, Indonesia, Spain	*E. globulus*, *E. urophylla*, *Eucalyptus* sp.	Crous and Alfenas 1995 De Blas *et al.*, 2009
Ps. irregularis	Peru	*Eucalyptus* sp.	Crous, 1998
Ps. madagascariensis	Madagascar	*E. camaldulensis*	Crous *et al.*, 2009e
Ps. natalensis	South Africa	*E. nitens*	Crous, 1998
Ps. norchiensis	Italy, Uruguay	*Eucalyptus* sp., *E. grandis*, *E. globulus*	Crous *et al.*, 2007b Perez *et al.*, 2009
Ps. paraguayensis	Brazil, Israel, Paraguay, Taiwan	*Eucalyptus* sp., *E. globulus*, *E. nitens*	Crous, 1998
Ps. pseudobasitruncata	New Zealand	*E. nitens*	Braun and Dick, 2002

Table 1.3 Genera and species of fungi ocurring on *Eucalyptus* leaves and stems (cont.).

Species	Geographic distribution	Hosts	Reference
Ps. robusta	Malaysia	*E. robusta*, *E. robur*	Crous, 1998
Ps. schizolobii	Thailand	*E. camaldulensis*	Crous *et al.*, 2009d
Ps. sphaerulinae	Chile	*E. globulus*, *E. nitens*	Crous *et al.*, 2003
Ps. subulata	Australia, New Zealand	*E. botryoides*	Crous *et al.*, 2006 Carnegie *et al.*, 2007
Ps. tereticornis	Australia	*E. nitens*, *E. tereticornis*	Crous *et al.*, 2009c
Pseudoteratosphaeria flexuosa	Colombia, Portugal, South Africa, Spain Zambia	*E. globulu*, *Eucalyptus* sp.	Crous, 1998 De Blas *et al.*, 2009
Pseu. gamsii	India	*Eucalyptus* sp.	Crous *et al.*, 2006 Quaedvlieg *et al.*, 2014
Pseu. ohnowa	Australia, South Africa, Uruguay	*E. dunnii*, *E. grandis*, *E. smithii*, *E. viminalis*	Crous *et al.*, 2004a Crous *et al.*, 2007b Perez *et al.*, 2009 Quaedvlieg *et al.*, 2014
Pseu. parkiiaffinis	Venezuela	*E. urophylla*	Crous *et al.*, 2007b Quaedvlieg *et al.*, 2014
Pseu. perpendicularis	Colombia	*E. eurograndis*	Crous *et al.*, 2006 Crous *et al.*, 2007a
Pseu. secundaria	Brazil, Colombia	*Eucalyptus* sp.	Crous *et al.*, 2006
Pseu. stramenticola	Brazil	*Eucalyptus* sp.	Crous *et al.*, 2006
Quasiteratosphaeria mexicana	Australia, Mexico, Portugal, USA	*Eucalyptus* sp., *E. globulus*	Crous, 1998 Maxwell *et al.*, 2003 Crous *et al.*, 2007b Silva *et al.*, b
Ramularia eucalypti	Australia, Italy	*Eucalyptus* sp., *E. grandiflora*	Crous *et al.*, 2007b
Readeriella angustia	Australia	*E. delegatensis*, *E. regnans*	Crous *et al.*, 2009c
R. brunneotingens	Australia	*E. tereticornis*	Crous *et al.*, 2007a
R. callista	Australia	*Eucalyptus* sp., *E. cannonii*, *E. deanei*, *E. haemastroma*, *E. multicaulis*, *E. sclerophylla*	Crous *et al.*, 2009d
R. considenianae	Australia	*E. consideniana*	Taylor *et al.*, 2011
R. deanei	Australia	*E. deanei*	Quaedvlieg *et al.*, 2014
R. dendritica	Australia	*E. deanei*, *E. globulus*, *E. nitens*	Crous *et al.*, 2007b, Quaedvlieg *et al.*, 2014
R. dimorphospora	Australia	*E. nitens*	Crous *et al.*, 2007b

Table 1.3 Genera and species of fungi ocurring on *Eucalyptus* leaves and stems (cont.).

Species	Geographic distribution	Hosts	Reference
R. eucalypti	Australia, Spain	*E. dunnii*, *E. globulus*, *E. grandis*, *E. grandis* × *E. camaldulensis*, *E. gunnii*, *E. haemastoma*, *E. microcorys*, *E. miniata*, *E. pilularis*, *E. parvula*, *E. saligna*	Barber *et al.*, 2003 Carnegie, 2007a Quaedvlieg *et al.*, 2014
R. eucalyptigena	Australia	*E. dives*	Crous *et al.*, 2009c
R. limoniforma	Australia	*Eucalyptus* sp.	Quaedvlieg *et al.*, 2014
R. menaiensis	Australia	*E. oblonga*	Crous *et al.*, 2009c
R. mirabiliaffinis	Australia	*E. delegatensis*	Quaedvlieg *et al.*, 2014
R. mirabilis	Australia	*E. capitellata*, *E. cinerea*, *E. globulus*, *E. nicholii*, *E. pilularis*	Barber *et al.*, 2003 Carnegie, 2007a Crous *et al.*, 2009c
R. nontingens	Australia	*E. molucanna*, *E. oblonga*, *E. tereticornis*	Crous *et al.*, 2007b
R. novezelandiae	New Zealand	*E. botryoides*	Crous *et al.*, 2004a
R. patrickii	Australia	*E. amygdalina*	Crous *et al.*, 2009d
R. pseudocallista	Australia	*E. prominula*	Crous *et al.*, 2009c
R. readeriellophora	Spain	*E. globulus*	Crous *et al.*, 2004a
R. tasmanica	Australia	*E. delegatensis*	Crous *et al.*, 2009c
Septoria eucalyptorum	India	*Eucalyptus* sp.	Crous *et al.*, 2006
S. provencialis	France	*Eucalyptus* sp.	Crous *et al.*, 2006
Sonderhenia eucalypticola	Australia, Chile,Colombia, Ecuador, New Zealand, Portugal, Spain	*E. globulus*, *E. globoidea*, *Eucalyptus* sp., *E. cladocalyx*, *E. gomphocephala*, *E. nitens*, *E. polyanthemos*	Park and Keane, 1984 Wingfield *et al.*, 1995 Crous, 1998 Carnegie, 2000 Crous *et al.*, 2006

Table 1.3 Genera and species of fungi ocurring on *Eucalyptus* leaves and stems (cont.).

Species	Geographic distribution	Hosts	Reference
So. eucalyptorum	Australia, New Zealand	*E. cameronii*, *E. coccifera*, *E. delegatensis*, *E. dives*, *E. elata*, *E. fastigata*, *E. globoidea*, *E. leucoxylon*, *E. nitens*, *E. obliqua*, *E. agglomerata*, *E. amygdalina*, *E. baxteri*, *E. consideniana*, *E. dalrympleana*, *E. fastigata*, *E. fraxinoides*, *E. grandis*, *E. johnstonii*, *E. melliodora*, *E. muellerana*, *E. pauciflora*, *E. phaeotricha*, *E. radiata*, *E. regnans*, *E. sieberi*, *E. smithii*, *E. tereticornis*	Dick, 1982 Park and Keane, 1984 Crous, 1998 Carnegie, 2000
Staninwardia suttonii	Australia	*E. robusta*	Summerell *et al.*, 2006
Suberoteratosphaeria pseudosuberosa	Uruguay	*Eucalyptus* sp.	Crous *et al.*, 2006
Su. suberosa	Australia, Brazil, Colombia, Indonesia, New Zealand, Spain	*E. agglomerata*, *E. cloeziana*, *E. dunnii*, *E. globulus*, *E. grandis*, *E. grandis* × *E. camaldulensis*, *E. laevopinea*, *E. moluccana*, *E. nitens*, *E. nitens* × *E. nobilis*, *E. muelleriana*, *E. punctata*, *E. saligna*, *E. tereticornis*, *E. viminalis*	Crous *et al.*, 1993a Carnegie *et al.*, 1997 Crous *et al.*, 2006 Carnegie, 2007a Dick and Dobbie, 2001
Su. xenosuberosa	Australia	*E. molucana*	Quaedvlieg *et al.*, 2014
Teratosphaeria alboconidia	Australia	*E. miniata*	Crous *et al.*, 2009c
T. associata	Australia	*E. dunnii*, *Corymbia henryii*, *C. variegata*, *E.tereticornis*	Carnegie, 2007a Crous *et al.*, 2007b Carnegie *et al.*, 2011
T. aurantia	Australia	*E. grandis*	Andjic *et al.*, 2010b
T. australiensis	Australia	*E. ficifolia*	Crous *et al.*, 2009d
T. biformis	Australia	*E. globulus*	Andjic *et al.*, 2010b
T. blakelyi	Australia	*E. blakelyi*	Crous *et al.*, 2009d

Table 1.3 Genera and species of fungi ocurring on *Eucalyptus* leaves and stems (cont.).

Species	Geographic distribution	Hosts	Reference
T. complicata	Australia	*E. miniata*	Crous *et al.*, 2009c
T. crispata	Australia	*E. bridgesiana*	Carnegie *et al*., 2011
T. cryptica	Australia, Chile, New Zealand, Spain	*E. acmenoides*, *E. alba*, *E. bridgesiana*, *E. camaldulensis*, *E. cinerea*, *E. cloeziana*, *E. consideniana*, *E. cordata*, *E. crenulata*, *E. delegatensis*, *E. diversicolor*, *E. dunnii*, *E. fastigata*, *E. fraxinoides*, *E. globulus*, *E. globulus* × *E. nitens*, *E. grandis*, *E. grandis* × *E. camaldulensis*, *E. grandis* × *E.* *urophylla*, *E. gunnii*, *E. laevopinea*, *E. longirostrata*, *E. marginata* *E. micrantha*, *E. microcorys*, *E. moluccana*, *E. nitens*, *E. nova-anglica*, *E. obliqua*, *E. ovata*, *E. patens*, *E. parvula*, *E. pellita*, *E. pilularis*, *E. propinqua*, *E. pulverulenta*, *E. punctata*, *E. regnans*, *E. saligna*, *E. saligna* × *E. tereticornis*, *E. scorparia*, *E. tereticornis*, *E. urophylla*	Ganapathi and Corbin, 1979 Dick, 1982 Cheah and Hartill, 1987 Crous *et al.*, 1995 Carnegie *et al*., 1997, 2011 Crous, 1998 Carnegie and Ades, 2002c Barber *et al.*, 2003 Jackson *et al.*, 2005 Carnegie, 2007a De Blas *et al.*, 2009
T. destructans	Australia, China, Indonesia	*Eucalyptus* spp., *E. camaldulensis*, *E. grandis* × *E. urophylla*, *E. urophylla*	Wingfield *et al.*, 1996a Burgess *et al.*, 2007a Crous *et al.*, 2009d Zhou and Wingfield, 2011
T. dimorpha	Australia	*Eucalyptus* sp., *E. caesia*, *E. nitens*	Crous *et al.*, 2009a

Table 1.3 Genera and species of fungi ocurring on *Eucalyptus* leaves and stems (cont.).

Species	Geographic distribution	Hosts	Reference
T. eucalypti	Argentina, Australia,Brazil,India, Italy, Peru,Paraguay,New Zealand,Taiwan, Zaire	*E. aggregata*, *E. alba*, *E. albens*, *E. amygdalina*, *E. blakelyi*, *E. bosistoana*, *E. botryoides*, *E. bridgesiana*, *E. camaldulensis*, *E. camphora*, *E. cephalocarpa*, *E. cinerea*, *E. crebra*, *E. cypellocarpa*, *E. dalrympleana*, *E. fasitgata*, *C. ficifolia*, *E. gardneri*, *E. globulus*, *E. gomphocephala*, *E. goniantha*, *E. goniocalyx*, *E. grandis*, *E. gunnii*, *E. largiflorens*, *E. leucoxylon*, *E. longiflora*, *E. melliodora*, *E. moluccana*, *E. nitens*, *E. nutans*, *E. obliqua*, *E. occidentalis*, *E. oreades*, *E. ovata*, *E. paniculata*, *E. pauciflora*, *E. paulistana*, *E. perriniana*, *E. platypus*, *E. ployanthemos*, *E. populnea*, *E. pulchella*, *E. punctata*, *E. regnans*, *E. resinifera*, *E. robusta*, *E. rostrata*, *E. rubida*, *E. rudis*, *E. saligna*, *E. sideroxylon*, *E. stellulata*, *E. stenostoma*, *E. tereticornis*, *E. trabutii*, *E. viminalis*	Gadgil and Dick, 1983 Crous, 1998 Crous *et al.*, 2007a
T. fimbriata	Australia	*Corymbia* sp.	Crous *et al.*, 2007
T. foliensis	Australia	*E. globulus*	Andjic *et al.*, 2010b

Table 1.3 Genera and species of fungi ocurring on *Eucalyptus* leaves and stems (cont.).

Species	Geographic distribution	Hosts	Reference
T. gauchensis	Argentina, Ethiopia, Hawai, Portugal Uganda Uruguay	*E. camaldulensis*, *E. grandis*, *E. globulus*, *E. maidenii*, *E. tereticornis*	Gezahgne *et al.*, 2003, 2005 Cortinas *et al.*, 2004, 2006a Perez *et al*., 2009 Silva *et al.*, 2015
T. hortaea	Madagascar	*E. camaldulensis*	Crous *et al.*, 2009e
T. juvenalis	South Africa	*E. cladocalyx*	Crous *et al.*, 2009a
T. keanei	Australia	*E. globulus* × *E. camaldulensis*	Carnegie *et al.*, 2011
T. lilianiae	Australia	*E. eximia*	Walker *et al.*, 1992
T. lusitanica	Portugal	*E. globulus*	Silva *et al.*, b
T. majorizuluensis	Australia	*E. botryoides*	Crous *et al.*, 2009c
T. mareebensis	Australia	*E. alba*	Crous *et al.*, 2011a
T. micromaculata	Australia	*E. globulus*	Andjic *et al.*, 2010b
T. miniata	Australia	*E. miniata*	Crous *et al.*, 2009c
T. multiseptata	Australia	*E. subvelutina*	Carnegie *et al.*, 2007 Crous *et al.*, 2009d
T. nubilosa	Australia, Brazil, Ethiopia, Kenya, New Zealand, Portugal, South Africa, Spain, Tanzania, Uruguay, Zambia	*Eucalyptus* sp., *E. bicostata*, *E. botryoides*, *E. bridgesiana*, *E. camaldulensis*, *E. cypellocarpa*, *E. dalrympleana*, *E. dunnii*, *E. globulus*, *E. grandis*, *E. grandis* × *E. nitens*, *E. grandis* × *E. resinifera*, *E. macarthurii*, *E. maidenii*, *E. nitens*, *E. nova-anglica*, *E. quadrangulata*, *E. saligna*, *E. smithii*, *E. stuarrtiana*, *E. tereticornis*, *E. urophylla* × *E. globulus*, *E. viminalis*	Dick, 1982 Crous *et al.*, 1989a Crous, 1998 Maxwell *et al.*, 2001 Crous *et al.*, 2004a Hunter *et al.*, 2004b Jackson *et al.*, 2005 Carnegie, 2007a Gezahgne *et al.*, 2006 Perez *et al*., 2009, 2009b Silva *et al.*, 2008, 2009, 2012
T. obscuris	Indonesia, Vietnam	*Eucalyptus* sp., *E. pellita*	Burgess *et al.*, 2007b
T. ovata	Australia, South Africa, New Zealand	*E. cladocalyx*, *E. dives*, *E. lehmannii*, *E. leucoxylon*, *E. macrohyncha*, *E. melliodora*, *E. obliqua*, *E. phoenicea*, *E. regnans*	Crous *et al.*, 1989a Crous, 1998 Crous *et al.*, 2009a

Table 1.3 Genera and species of fungi ocurring on *Eucalyptus* leaves and stems (cont.).

Species	Geographic distribution	Hosts	Reference
T. pluritubularis	Australia, Portugal, Spain, Uruguay	*E. globulus*	*Crous et al.*, 2006 Perez *et al*., 2009 Carnegie *et al*., 2011 Silva *et al.*, b
T. praelongispora	Australia	*Eucalyptus* sp., *E. dives*, *E. dunnii*	Carnegie *et al*., 2011
T. profusa	Australia	*E. nitens*	Crous *et al.*, 2009c
T. pseudocryptica	New Zealand	*Eucalyptus* sp.	Crous *et al.*, 2006
T. pseudoeucalypti	Australia	*E. grandis* × *E. camaldulensis*	Andjic *et al.*, 2010a
T. pseudonubilosa	Australia	*E. globulus*	Pérez *et al.*, 2014
T. stellenboschiana	France, South Africa	*Eucalyptus* sp., *E. punctata*	Crous *et al.*, 2006 Crous *et al.*, 2009d
T. suttonii	Argentina, Australia, Bhutan, Brazil, China, Ethiopia, Hong Kong, India, Indonesia, Italy, Madagascar, Malawi, Myanmar, New Zealand, Philippines, South Africa, Taiwan, Tanzania, USA, Vietnam, Zambia	*E. amplifolia*, *E. camaldulensis*, *C. citriodora*, *E. cladocalyx*, *E. crebra*, *E. dealbata*, *E. delegatensis*, *E. drepanophylla*, *E. dunnii*, *E. exserta*, *E. globulus*, *E. grandis*, *E. longifolia*, *E. macarthurii*, *C. maculata*, *E. major*, *E. microcorys*, *E. nitens*, *E. nova-anglica*, *E. pellita*, *E. platypus*, *E. punctata*, *E. quadrangulata*, *E. radiata*, *E. resinifera*, *E. robusta*, *E. rostrata*, *E. saligna*, *E sideroxylon*, *E. tereticornis*, *E. urophylla*, *E. viminalis*	Crous and Wingfield, 1997b Crous *et al.*, 1998 Carnegie, 2007a Crous, 2007a Jackson *et al.*, 2008 Zhou and Wingfield, 2011
T. tinara	Australia	*Corymbia* sp.	Andjic *et al.*, 2010b
T. toledana	Spain	*Eucalyptus* sp.	Crous *et al.*, 2004a
T. veloci	Australia	*E. miniata*	Crous *et al.*, 2009a
T. verrucosa	South Africa	*Eucalyptus* sp., *E. cladocalyx*	Crous *et al.*, 2009a
T. viscidus	Australia	*Eucalyptus* sp., *E. grandis*, *E. grandis* × *E. camaldulensis*	Andjic *et al.*, 2007 Crous *et al.*, 2009d
T. xenocryptica	Chile	*Eucalyptus* sp.	Crous *et al.*, 2009c

Table 1.3 Genera and species of fungi ocurring on *Eucalyptus* leaves and stems (cont.).

Species	Geographic distribution	Hosts	Reference
T. zuluensis	China, Malawi, Mexico, South Africa, Thailand, Uganda, Vietnam, Zambia	*E. camaldulensis*, *E. cloeziana*, *E. grandis*, *E. urophylla*	Wingfield *et al.*, 1996b Van Zyl *et al.*, 2002 Roux *et al.*, 2002 Gezahgne *et al.*, 2003, Cortinas *et al.*, 2006a, 2006b Chungu *et al.*, 2010 Jimu *et al.*, 2014
T. molleriana	Australia Portugal, Spain, Uruguay, USA	*E. globulus*, *E. viminalis*, *Eucalyptus* sp.	Crous and Wingfield, 1997a Carnegie and Ades, 1998 Milgate *et al.*, 2001 Crous *et al.*, 2004a, 2007b Jackson *et al.*, 2008 Perez *et al.*, 2009 Silva *et al.*, 2009, 2012
Teratosphaericola pseudoafricana	Portugal, Zambia	*E. globulus*	Crous *et al.*, 2006 Quaedvlieg *et al.*, 2014 Silva *et al.*, b
Teratosphaeriopsis pseudoafricana	South Africa	*Eucalyptus* sp.	Quaedvlieg *et al.*, 2014
Uwebraunia australiensis	Australia	*E. platyphylla*	Li *et al.*, 2012
U. commune	South Africa	*E. nitens*	Li *et al.*, 2012
U. dekkeri	Australia	*E. molucana*	Li *et al.*, 2012
Xenomycosphaerella elongata	Venezuela	*E. camaldulensis* × *E. urophylla*	Crous *et al.*, 2007b Quaedvlieg *et al.*, 2014
X. yunnanensis	China	*E. urophylla*	Burgess *et al.*, 2007b Quaedvlieg *et al.*, 2014
Zasmidium aerohyalinosporum	Australia	*E. tectifica*	Crous *et al.*, 2009c
Z. eucalypti	Australia	*E. tereticornis*	Crous *et al.*, 2007b
Z. eucalyptorum	Indonesia	*Eucalyptus* sp.	Crous *et al.*, 2006 Quaedvlieg *et al.*, 2014
Z. nabiacense	Australia	*Eucalyptus* sp.	Crous *et al.*, 2009c
Z. parkii	Brazil, Colombia, Indonesia, Spain	*E. globulus*, *E. grandis*, *E. saligna*	Crous *et al.*, 1993b Crous and Alfenas, 1995 Crous, 1998 De Blas *et al.*, 2009
Z. pseudoparkii	Colombia	*Eucalyptus* sp.	Crous *et al.*, 2006
Z. xenoparkii	Indonesia	*E. grandis*	Crous *et al.*, 2006

Leaf disease complex according to country

Eucalypts were introduced into France in 1804, Chile in 1823, Brazil in 1825, South Africa in 1828, Portugal in 1829, India in 1843, Italy and New Zealand in 1849, Uruguay in 1853, Argentina in 1857, Spain in 1860 and China in 1890 probably brought from Australia or other countries (Doughty *et al.*, 2000; Potts *et al.*, 2004b; Borralho *et al.*, 2007). For several decades after their introduction, eucalypts remained free of diseases or pests. Indeed, the initial success of eucalypts as exotics was due to the nonexistence of pests and pathogens affecting

them in their native regions of origin. As the areas of plantations enlarged, disease problems also increased (Wingfield, 1999). Presently, pests and diseases are known to seriously threaten *Eucalyptus* plantations throughout the world (Wingfield *et al.*, 2008). The Leaf Disease Complex has caused widespread damage in Australasia, South America, Western Europe, Southern Africa and South-East Asia (Park *et al.*, 2000) and it is expected to become gradually more important worldwide in the future (Crous *et al.*, 2004a).

Australia

In 2013, *Pinus radiata* (75.4 %) and southern pines (14.7 %) dominated the softwood plantation estate. Both species are primarily managed for sawlog production. The hardwood plantation estate is dominated by *E. globulus* (blue gum) (55.1 %) and *E. nitens* (shining gum) (24.2 %). Both species are primarily managed for wood pulp production. Most southern *E. globulus* plantations are in Western Australia and in the Green Triangle (located in the South East of South Australia and South Western Victoria, flanked by Melbourne and Adelaide and covers an area of 6 million ha) while most *E. nitens* plantations are in Tasmania. *Eucalyptus dunnii* (Dunn's white gum) is primarily managed for wood pulp production in South East Queensland and managed for sawlog and wood pulp production in the North Coast region. *Eucalyptus pilularis* (Blackbutt) and *E. grandis* (flooded gum) (2.7 %) and *Corymbia maculata* (spotted gum) are primarily managed for sawlog production. Most *E. pilularis*, *E. grandis* and *C. maculata* are in the north coast region of New South Wales (Gavran, 2013).

In Australia the most severe foliar disease of eucalypt plantations is the Leaf Disease Complex, previously known as MLD (Park and Keane, 1984; Park, 1988a; Park *et al.*, 2000; Maxwell *et al.*, 2003; Carnegie, 2007a). In 2000, specifically, the most common foliar fungi in eucalypt plantations were *Aulographina eucalypti*, *T. cryptica* and *T. suttonii* (Park *et al.*, 2000). Previously, *T. nubilosa* was only reported on juvenile foliage (Park and Keane, 1982a; Park *et al.*, 2000). Then in 2004, *T. nubilosa* was isolated on some occasions from adult foliage but was mainly responsible for causing disease on juvenile foliage (Maxwell, 2004). Also, in south-western Australia *T. cryptica* was reported on juvenile and adult phase foliage of *E. globulus*, and was the major contributor to disease on adult leaves of *E. globulus* followed by *P. marksii*, *T. molleriana, Q. mexicana*, *A. parva* and *Suberoteratosphaeria suberosa*. All these species were also found on juvenile leaves (Maxwell, 2004).

In New South Wales, during a forest health survey between 1996–2005, 21 species of foliar and shoot fungi of young eucalypt plantations were recorded (Carnegie, 2007b). These

included: *A. parva*, *Aulographina eucalypti*, *Cryptosporiopsis eucalypti*, *Lembosina corymbiae*, *L. eucalypticola*, *M. tumulosa*, *Neofusicoccum eucalyptorum*, *Phaeothyriolum microthyrioides*, *Pilidiella eucalypticola*, *P. marksii*, *Quambalaria eucalypti*, *Q. pitereka*, *So. eucalypticola*, *Su. suberosa*, *T. alcornii*, *T. cryptica*, *T. corymbiae*, *T. eucalypti*, *T. nubilosa*, *T. suttonii*, and *Readeriella eucalypti*.

In recent years, 28 species were reported from New South Wales and Queensland. *Teratosphaeria cryptica* was the most frequently recorded species, followed by *P. marksii*. New records were reported including *Euteratosphaeria verrucosiafricana*, *Neotrimmatostroma excentricum*, *Ps. eucalyptorum*, *T. multiseptata*, *T. nubilosa*, *T. pluritubularis*, *Su. suberosa* and in Queensland (Carnegie *et al.*, 2011).

Brazil

Over the last decades the area planted with eucalypts has increased in Brazil and currently stands at 5 102 030 ha (ABRAF, 2013) in 2012. In 2004, several species in the leaf spot complex were reported namely *P. marksii*, *Su. suberosa*, *T. suttonii* and *Zasmidium parkii* but there were reports of severe losses to the host (Alfenas *et al.*, 2004). In 2006, Crous *et al.*, (2006) added *Pallidocercospora heimii*, *Phaeophleospora scytalidii*, *Pseudoteratosphaeria secundaria*, *Pseu. stramenticola* and *T. suttonii* (already reported) to the list of species. Subsequently, Perez *et al.*, (2009b) reported *T. nubilosa* from leaf spots and blotches on both juvenile and adult foliage in the province of Rio Grande do Sul. In 2010, two plantations in Brazil (Santa Catarina and Rio Grande do Sul) were surveyed and the species observed were *Pseu. perpendicularis*, *Ph. scytalidii*, *M. lateralis*, *T. ohnowa*, *T. pseudoafricana*, *T. flexuosa* and *T. nubilosa* (Teodoro, 2010; Teodoro *et al.*, 2012).

Portugal

In Portugal, mostly dominated by *Eucalyptus globulus*, eucalypts represents the main continental forestry area (812 000 ha; 26%) followed by *Quercus suber* (737 000 ha; 23%) and *Pinus pinaster* (714 000 ha; 23%). The total area of eucalypts increased 13% between 1995 and 2010. Contributing to this growth was the loss of 70 000 ha of *Pinus pinaster* in 1995, 13 500 ha of shrublands and grasslands and 12 000 ha of agricultural areas. However, about 8000 ha of eucalypt forest in 1995 are now for urban usage (ICNF 2013). *Eucalyptus* plantations are

located mainly in the coastal regions where productivity is higher and where the attack by pathogens is generally low.

The first report of *Mycosphaerella* s.l. on eucalypts outside of Australia was in 1881 when *T. molleriana* was described in Portugal (Crous and Wingfield, 1997a). Until 1999 leaf disease complex on eucalypts was virtually unknown in Portugal when serious damage on *Eucalyptus globulus* characterized by a frequent and severe defoliation of young trees close to the coast was reported (Silva *et al.*, 2009). The increased severity of this Leaf Disease Complex encouraged the cooperation between the Instituto Superior de Agronomia (ISA), the Instituto Nacional de Investigação Agrária e Veterinária (ex-Estação Florestal Nacional), the Institute RAIZ and Altri Florestal (ex-Silvicaima), and the Forestry and the Agricultural Biotechnology Institute, FABI, South Africa on project AGRO 550 (2004-2007) (Silva, 2007). During this project, about 170 industrial plantations throughout Portugal were evaluated by RAIZ and the outcome was that the Leaf Disease Complex on eucalypts is limited to the central-north coastal regions (Carlos Valente, personal communication). Meanwhile, species isolated from some industrial plantations were characterized morphologically and on a molecular basis to identify the role of species in the complex (Silva, 2007; Silva *et al.*, 2008, 2009). From 1997 until 2006, 10 species were reported on *Eucalyptus* in Portugal (Crous and Wingfield, 1997a; Crous, 1998; Crous *et al.*, 2004, 2006). However, these reports were based on casual collections and did not give a clear indication of the frequency or distribution of the pathogens in wild populations of the host or commercial plantations (Silva *et al.*, 2009). In 2008, Silva *et al.* (2008) used morphological characters and reported the presence of *Amycosphaerella africana*, *A. parva*, *M. communis*, *M. lateralis*, *P. marksii*, *So*. eucalypticola, *T. molleriana*, *T. nubilosa*. Silva *et al.* (2009) analysed more isolates by morphological characteristics and by molecular methods based on the ITS cluster to confirm the species previously recorded. In recent years, following a survey throughout the country, more species were reported including *M. madeirae* (in the mainland) *Cladosporium cladosporioides*, *Fusicladium eucalypti*, *Neodevriesia hilliana*, *Q. mexicana*, *T. pluritubularis*, *Teratosphaericola pseudoafricana* and a new species, *T. lusitanica* (Silva *et al.*, b, d submitted).

South Africa

In the 1930's the impact of the Leaf Disease Complex in South Africa was so severe that the establishment of *E. globulus* plantations was suspended (Crous, 1998). In 1989, several foliage pathogens were reported including *Fairmaniella leprosa* (cause of shoot and leaf

necrosis with distinct corky circular lesions), *Harknessia globosa* (leaf spot disease), *Harknessia eucalypti* (leaf and stem necrosis) and *Neofusicoccum ribis* (on lesions) (Crous *et al.*, 1989b). In nurseries, several pathogens affecting *Eucalyptus* spp. foliage, such as *Botrytis cinerea*, *Cylindrocladium scoparium*, *Colletotrichum gloeosporioides*, *Coniella castaneicola*, *Hainesia lythri*, *Harknesia hawaiiensis* and *Phaeoseptoria eucalypti* were found later (Viljoen *et al.*, 1992).

The predominance of *T. nubilosa* on *E. nitens* suggested it to be the only species that causes disease typical of the Leaf Disease Complex in South Africa (Purnell and Lundquist, 1986; Crous *et al.*, 1989a). But in the 1990's, two new species were reported, namely *Am. africana* and *Pallidocercospora crystallina*, (Crous and Wingfield, 1996; Crous 1998). In 2004, five more species were identified namely *M. lateralis*, *Pallidocercospora irregulariramosa*, *P. marksii*, *Pseudocercospora fori*, *T. nubilosa*. The last one was the species most commonly isolated from commercial plantations, particularly on *E. nitens*, and appears to be the dominant species contributing to the Leaf Disease Complex (Hunter *et al.*, 2004a, 2004b).

Spain

In Spain the first report of *Mycosphaerella* was in 1980 (Ruperez and Munoz, 1980). In 2000 in Galicia this disease began to appear, albeit sporadically and without causing major damage. Nowadays, however, in Cantabria this disease causes massive defoliation on *Eucalyptus* at the juvenile leaf stage, significant growth reduction and widespread death of trees in areas exposed to frost or other limiting factors such as inadequate soil conditions. This disease has become the biggest obstacle to the regeneration of *Eucalyptus* in this region. Species identified were *P. marksii*, *So. eucalypticola* and *T. nubilosa.* This last one was present in about 90% of the identifications (Tejedor, 2007).

Between 2004 and 2013, 24 species were reported (Crous *et al.*, 2004, 2006; Otero *et al.*, 2007; De Blas *et al.*, 2009; Sánchez Márquez *et al.*, 2011; Aguín *et al.*, 2013). Most of the species reported in Portugal have also been reported in Spain, except for *Pc. heimii*. In Spain species diversity is greater, with 14 more species detected. Thus, twice as many species have been observed in Spain than in Portugal and one important point to emphasize is the absence of *T. cryptica* from Portugal (*Silva et al.*, 2012).

In more than 60% of the plantations in Spain only *T. nubilosa* was observed. In a recent survey in Galicia, *Am. africana*, *M. madeirae* and *P. marksii* were found only on juvenile stage

leaves. On juvenile and adult stage leaves, were reported (Aguin *et al.*, 2013; Manzilla *et al.*, 2013).

Uruguay

In this country there are 1 402 144 ha of forests, of which 53% corresponds to natural forest, 40% are plantation forest, the remainder are parks, coastal forests, and shelter. In plantation forests *E. globulus* is the principal species with 244 760 ha (45%), *Pinus* with 174 275 ha (31%), *E. grandis* 135 389 ha (24%) and 77 285 ha of other eucalypts. The main forestry areas are in the Rivera and Tacuerembó departments (Petraglia and Dell'Acqua, 2006). The pulp and paper industry is the primary interest of these plantations (Perez *et al.*, 2009a).

In 2004, several species were reported, without severe losses, like *P. marksii*, *So. eucalypticola, T. molleriana* and *Su. suberosa*, (Balmelli *et al.*, 2004), *T. gauchensis* (Cortinas *et al.*, 2006b) *T. pseudosuberosa* and *T. suttonii* (Crous *et al.*, 2006, 2007a). In recent years, Perez *et al.*, (2009) identified several species associated with leaf spots and stem cankers on *Eucalyptus*. Of these, *A. africana*, *M. lateralis, Ph. scytalidii, Pc. heimii, Pseudocercospora norchiensis*, *Pseudoteratosphaeria ohnowa* and *T. pluritubularis* were newly recorded and new hosts were identified for *M. aurantia*, *M. lateralis*, *P. marksii, Ph. scytalidii*, *P. norchiensis*, *Pseu. ohnowa*, *T. gauchensis*, *T. molleriana* and *T. pluritubularis*. *Teratosphaeria gauchensis*, which has been known only as a stem pathogen, was isolated from leaf spots on *E. maidenii* and *E. tereticornis*.

Symptoms

Photosynthetic area is affected by leaf lesions that consequently cause early defoliation, reduced growth rates and shoot dieback of the affected trees. Many variations are associated with symptom development, resulting in different combinations of lesion size, colour and morphology (Old *et al.*, 2003). Fruiting bodies can be formed on one or both surfaces of the leaves (Crous, 1998). Infected leaves can have an increased amount of spots and blotches and their severity depends on the pathogen species and the susceptibility of the host species. Crinkling leaves with large lesions are developed on the most susceptible hosts (Old *et al.*, 2003) (Fig. 1.1).

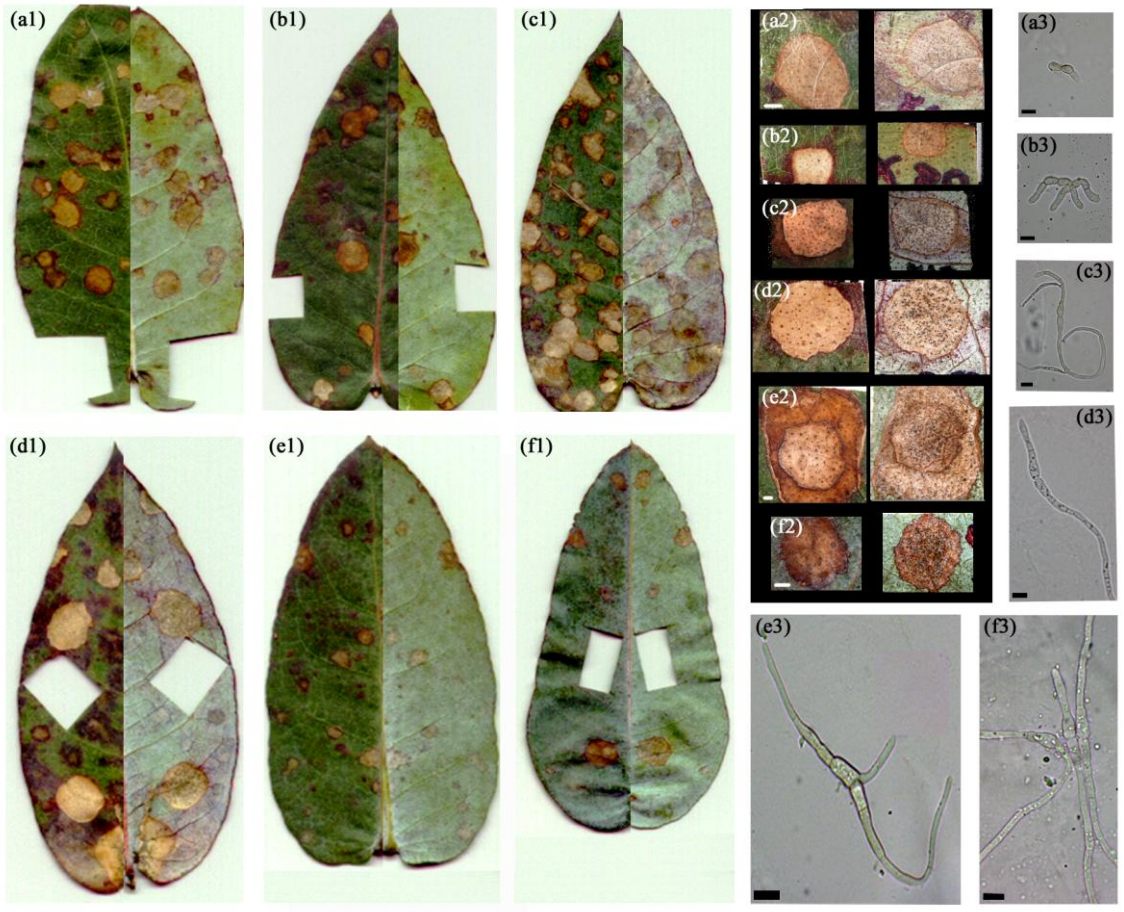

Fig. 1.1 Leaves, lesions and ascospores of *Mycosphaerellacea* and *Teratosphaeriaceae* on *Eucalyptus globulus*: **(a)** *Austroafricana parva* **(b)** *Mycosphaerella communis* **(c)** *Mycosphaerella lateralis* **(d)** *Paramycosphaerella marksii* **(e)** *Teratosphaeria molleriana* and **(f)** *Teratosphaeria nubilosa***.** For each species letter, respectively: **(a1, b1, c1, d1, e1 and f1)** Adaxial leaf surface (on left) Abaxial leaf surface (on right); **(a2, b2, c2, d2, e2 and f2)** Lesion from adaxial leaf surface (on left) and abaxial leaf surface (on right); **(a3, b3, c3, d3, e3 and f3)** Germinating ascospores on MEA. Scale bars: **(a2-d2)** = 10 μm (same bar to all), **(e2)** and **(f2)** = 10 μm, **(a3-f3)** = 10 μm, respectively.

Eucalyptus leaf disease has become one of the main diseases affecting eucalypt plantations worldwide (Crous, 1998; Wingfield *et al.*, 2008). The Leaf Disease Complex causes spots, blotches and blights on the leaves. Nevertheless, other *Teratosphaeria* species, such as *T. gauchensis* and *T. zuluensis*, can cause other symptoms including branch cankers, premature branch death, shoot dieback and in some cases tree death, (Cortinas *et al.*, 2010, Silva *et al.*, a submitted). The characteristic species group that causes disease on leaves and on stems is conserved, nevertheless it is possible to find typical stem-inhabiting species on leaves, acting as

endophytes and it is difficult to know if they could act as saprobes or become pathogenic when their hosts senesce.

This disease results in reduced growth rate and lower wood volume, which can result in significant losses (Lundquist and Purnell, 1987; Carnegie, 2007b; Hunter *et al.*, 2009; Cortinas *et al.*, 2010). Eucalyptus leaf disease affects mainly young trees in the juvenile phase foliage rather than adult foliage (Lundquist and Purnell, 1987; Dungey *et al.*, 1997; Carnegie and Ades, 2005; Pinkard and Mohamed, 2006; Carnegie, 2007a; Hunter *et al.*, 2009).

This complex can reduce eucalyptus growth when the defoliation levels of the juvenile crown are over 25%, and even levels of diseased leaf area less than 10% can cause 17% reduction in height of *E. globulus* (Lundquist and Purnell, 1987; Dungey *et al.*, 1997; Carnegie *et al.*, 1998). However, some of these studies were not similar enough in the parameters evaluated and did not calculate growth for a sufficiently long period to allow confident predictions of impact (Mohamed *et al.*, 2003).

Infection of adult foliage is unusual, but some growth effects have been documented at levels less than 15% (Carnegie and Ades, 2002a). In recent years there have been reports of increased infections of adult foliage in Australia, Uruguay and Brazil (Barber *et al.*, 2008; Pérez *et al.*, 2009a, 2009b). For example, *P. marksii*, have been recorded in Uruguay always on adult leaves (Pérez *et al.*, 2009). This suggests an increasing aggressiveness of these species to certain clones. Also, the attack of adult foliage will have greater impact on tree growth.

In the earlier years of research on this complex *T. cryptica* and *T. nubilosa* were considered the most severe foliar pathogen of several *Eucalyptus* spp. (Park and Kean, 1982a, 1982b, 1984; Hunter *et al.*, 2009). However, in the last few years some other species have been recognised as causing severe disease including *T. destructans*, *T. eucalypti*, *T. gauchensis* and *T. zuluensis* (Andjic *et al.*, 2010).

Weather Conditions

Infection and development of severe disease are favoured by warm wet weather (Park, 1988b; Carnegie & Ades, 2005). Leaf disease reaches significant levels only in areas that have a climate that provides sufficient periods of leaf wetness and temperatures within the optimum range for leaf infection, lesion development, and spore discharge that coincide with a susceptible growth stage of the host (Mohamed *et al.*, 2003). Outbreaks in plantations increase

with high humidity, particularly with long periods of rain, in the meanwhile spores are discharged to affect sensitive foliage, in particular juvenile foliage. In addition, some species produce conidia that providing more inoculum sources for plantations (Old *et al.*, 2003).

Epidemiology

Etiology of disease differs according to the host-pathogen combinations and there is no single pattern of disease development (Old *et al.*, 2003). In terms of disease progress, Park (1988a) reported that with *T. nubilosa* there was a delay of 4–5 months between infection of newly-formed leaves and the disease reaching epidemic disease levels. On the other hand, *T. cryptica* had several generations of conidia and secondary ascospores during each epidemic of the disease (Park, 1988a). Thus, in *T. nubilosa* the primary inoculum is seed-borne or infected leaf litter and the secondary infection rarely occurs during the growing season of the tree, *i. e.*, the pathogen does not spread from tree to tree within one growing season. However, in Portugal a new cycle of disease development ocassionally occurs. Thus *T. nubilosa* normally has only one generation per year and acts as a simple interest disease. In *T. cryptica*, however, disease development follows that of a compound interest disease in most countries where the disease is known, but not in Portugal (Silva et al., 2009, 2012). The pathogen produces enormous amounts of inoculum during the growing season and this is disseminated easily by wind and spread from tree to tree during the same growing season. The percentage of the infected leaf area of *Eucalyptus* was measured with ascospores to *T. cryptica* and *T. nubilosa* and the best temperatures varied between 15°C and 20°C and between five and seven days (Park, 1988a, 1988b).

Ascospores were released at 90% RH or higher for *A. parva* and *T. nubilosa*. The optimum temperature for spore release is 20°C for *T. cryptica*, 25°C for *T. nubilosa* and between 7–25°C for *A. parva* (Park and Keane, 1982b). Ascospores germinate a few hours after deposition on the leaf surface. In *T. nubilosa*, they germinate and penetrate stomata of juvenile leaves often within 24h of inoculation. On the other hand, *A. parva* does not penetrate stomata like *T. nubilosa* or the cuticle like *T. cryptica* but after ascospore germination there is an extensive growth of mycelium on the leaf surface. Mature fructifications develop between 12–16 weeks after inoculation. *Teratosphaeria nubilosa* causes lesions only on leaves that have just expanded, developing between 4–8 weeks. On the other hand, *T. cryptica* forms lesions with ascospores between 3–4 weeks after inoculation (Park and Keane, 1982b). Park & Keane (1987) demonstrated that ascocarps could discharge ascospores for at least for 3 months even after leaf

fall and ascospores remain attached to the leaves for more than 17 months, even if they are non-viable. Thus, the presence of ascospores on the leaves decreases over time, and ascocarps are productive for 6–8 months until the leaves decay. There is no release of ascospores below 10°C or above 30°C.

Disease Control

Species associated with the Leaf Disease Complex of eucalypts seem to be specific and are considered to be carried by seeds (Burgess *et al.*, 2007b). Exclusion or eradication is not a viable control option because these pathogens are already dispersed extensively worldwide (Mohamed *et al.*, 2003). However, the use of fungicides to control the *Eucalyptus* Leaf Disease Complex in nurseries can be useful (Carnegie, 2000). There are few studies on the use of fungicides to control Eucalyptus Leaf Disease Complex in experimental plantations (Carnegie and Ades, 2002b; Mohamed *et al.*, 2003; Silva *et al.*, 2008). For example, Silva *et al.* (2008) evaluated the susceptibility of two experimental plantations where the resistance to this disease was evaluated. Two treatments were applied, with and without fungicide treatment (bitertanol or tolyfluanid). In both plantations fungicide applications did not completely control disease progress but they did reduce disease severity. Although, all the studies mentioned reach the same conclusion, the use of fungicides on a large-scale in plantations is not economically or environmentally viable.

Some studies on disease resistance have been done on eucalypts (Mohamed *et al.*, 2003; Potts *et al.*, 2004a; Milgate *et al.*, 2005; Balmelli *et al.*, 2014). There are some examples of studies in Silviculture like Stone and Birk (2001); Carnegie and Ades (2002b); Gonçalves *et al.*, (2013). In silvicultural treatments, Mohamed *et al.*, (2003) considered that disease resistance offers good prospects for control of the disease, it is operationally feasible and potentially economical viable. However, applicability needs to be validated in field conditions. In the fungicide trials of Silva *et al.* (2008) analysis of results from the untreated plot distinguished some *E. globulus* clones and hybrid clones with resistance to the disease. In the absence of fungicide applications susceptibility of *E. globulus* clones ranged from highly susceptible to highly resistant. This experiment showed the potential benefits of selecting resistant *E. globulus* material for plantations in areas where this disease is a problem. Indeed, Old *et al.* (2003) suggested that selection of resistant planting material would be an effective control measure if epidemics of the disease appear in the future.

Host Effects on Infection

It is well known that there is a clear distinction between young and mature foliage of *E. globulus*, and the distinction includes variation in texture, size, insertion and position (Johnson, 1926). Leaf characteristics of *E. globulus* depend on the environmental conditions, plant genotype and ontogenetic position. Adult foliage of *E. globulus* has lower stomatal density and thicker cuticles than juvenile leaves (James & Bell, 2001). There is no difference in total phenolics concentration, but there is a higher tannin activity in juvenile leaves (Gras *et al.*, 2005). After juvenile leaves are infected by pathogens of the Leaf Disease Complex it is suggested that characteristics such as cuticle thickness, palisade mesophyll density and wax coverage of stomata are linked to disease resistance (Smith *et al.*, 2006, 2007). This can explain why incidence and severity is higher in juvenile-phase foliage and why they are more prone to infection by pathogens and endophytes (Dungey *et al.*, 1997; Pinkard & Mohammed, 2006; Hunter *et al.*, 2009; Márquez *et al.*, 2011). Most species in the Leaf Disease Complex are more frequently found in young leaves. For this reason the most severe epidemics are normally observed in plantations with trees at the juvenile foliage stage of growth. For example, juvenile-phase or adult foliage can be colonized by *Am. africana*, *M. madeirae*, *P. marksii* and *So. eucalypticola* while adult foliage can be colonized by *M. lateralis*, *P. marksii*, *Pc. heimii*, *T. cryptica*, *T. molleriana* and *T. nubilosa* (Carnegie & Keane, 1997; Maxwell *et al.*, 2000; Milgate *et al.*, 2001; Pérez, 2008; Márquez *et al.*, 2011).

Pathogenicity

Normally a group of leaf pathogens is found together in an area although only a small number, principally *T. nubilosa* and *T. cryptica* seem to be responsible for significant damage (Park *et al.*, 2000). Recently, a leaf spot on a *Eucalyptus* was studied and from it a total of 41 single conidial propagules were obtained for morphological studies and DNA sequencing. This study underlines the degree of fungal biodiversity that can occur within a small and very specific niche (Crous *et al.*, 2009e). At least five species of fungi associated with Leaf Disease Complex can occur on a small leaf lesion (Crous *et al.*, 2004). On a eucalypt leaf lesion the mechanisms that permit several species to occupy the same niche are not known. No doubt some species just take on an opportunity that places them on a substrate that is not necessarily its ideal host (Crous *et al.*, 2009e).

Until now, only a few studies of epidemiology and pathology have been made on the Leaf Disease Complex on Eucalyptus, and most are derived from research done in Australia with *A.*

parva, *T. nubilosa* and *T. cryptica* (Cheah, 1977; Beresford, 1978; Ganapathi, 1979; Park & Kean, 1982b, 1987; Cheah & Hartill, 1987; Park, 1988a, 1988b; Carnegie, 2000). These two last species were found to be the most virulent leading to some studies on pathogenicity and epidemiology (Park & Kean, 1982a, 1982b, 1984).

The combination of pathogenicity tests of these fungi associated with this complex with the analysis of the effects and the single species aggressiveness on *E. globulus* is important to understand the complexity of this disease (Maxwell *et al.*, 2003). Recently, Silva *et al.* (submitted) analysed several species found associated with the Eucalyptus Leaf Disease Complex and evaluated their individual pathogenicity on the same host material. *Mycosphaerella communis* confirmed its higher virulence, causing rapid necrosis and showing preference for non-wounded leaves, indicating a primary pathogenic behaviour of this species. A similar behaviour is observed with *M. lateralis*, *T. nubilosa*, *C. cladosporioides*, *T. lusitanica*, *T. molleriana* and *N. hilliana*. All these species grow better on non-wounded than on wounded leaves. *T. nubilosa* and *A. parva* need more time to start necrosis and both attain similar development on non-wounded leaves. In non-wounded leaves *A. parva* shows reduced on necrosis development supporting field observations where this species is commonly associated with old lesions. In secondary or opportunistic pathogens group we found, for example, *M. madeirae* and *Q. mexicana* (Silva *et al.*, c submitted, *unpublished data*).

Identification

Park and Keane (1982a) established ascospore germination patterns as an instrument to facilitate species classification. This was followed by Crous (1998) who showed that ascospore germination patterns, represented by 14 patterns, provide the most useful morphological characteristics for species identification.

Morphological identification of *Mycosphaerella* and related species is complex due to problems of preparing single-spore cultures and the slow growth of the fungus in culture (Silva *et al.*, 2009). In addition, morphological description and ascospore germination patterns are similar for several species, 4 to 5 different species frequently inhabit the same lesion and the lesions often partly coalesce (Crous *et al.*, 2004a). Furthermore, they are host-specific pathogens, with weak growth in culture, generate small fruiting structures with very conserved morphology (Hunter *et al.*, 2006b). *Mycosphaerella* s.l. produces specialized sexual fruiting bodies, named pseudothecia, that contain asci with ascospores. Morphologically, the asexual states are extremely diverse and the conidiomata are variable forming solitary conidiophores

(*Dissoconium*) or sporodochia and fascicles or pycnidia (Crous *et al.*, 2007a). Thus, morphological identification is difficult and molecular tools are essential to confirm an identifcation.

Diverse approaches to molecular characterization have been used. Most of the initial studies were based on the ITS region (Crous *et al.*, 2001a, 2001b, 2004a, 2006; Hunter *et al.*, 2004b). Carnegie *et al.* (2001) used RAPD to analyse differences in *Mycosphaerella* species and Kularatne *et al.* (2004) designed species-specific primers for RFLP analysis to differentiate *Mycosphaerella* species associated with the disease complex on *E. globulus*. Furthermore, Maxwell *et al.* (2005) developed species-specific primers based on the ITS cluster for identification and detection, especially for *A. parva, M. lateralis, P. marksii, T. cryptic* and *T. nubilosa.* Hunter *et al.* (2006b) showed that sequences of the ITS region give sufficient resolution to differentiate most taxa except for critical taxa and used sequences of ITS, EF-1α, actin (ACT) and LSU and combined data from all regions. Subsequently, Hunter *et al.*, (2006a) with the intention to help advance the knowledge of the genetics and distribution of *T. nubilosa* populations, used polymorphic microsatellite markers. Recently, the ITS region and LSU regions were used by Crous *et al.* (2009a), Cheewangkoon *et al.* (2009) and by Quaedvlieg *et al*. (2014).

Other Leaf Diseases and Cankers on Eucalypts

Eucalyptus species are affected by a canker caused by *Teratosphaeria gauchensis* and *T. zuluensis* that cause serious damage and affects the growth of trees (Cortinas *et al.*, 2010, 2011).

This canker was reported for the first time in South Africa on *E. grandis* and the cause attributed to *Coniothyrium zuluensis* (Wingfield *et al.*, 1996b). It was subsequently found to affect a wide range of *Eucalyptus* species and hybrids (Cortinas *et al.*, 2006b). This canker disease has never been observed in the native range of *Eucalyptus* in Australia, but it does affect eucalypts in several countries (Table 1.4). In South Africa the disease increased rapidly, with losses to the forest industry and caused damage in the most susceptible hosts in the first years after its detection (Cortinas *et al.*, 2006b). In South America, because of the rapid increase in the number of eucalypt plantations *T. gauchensis* has became a growing threat to the industry (Cortinas *et al.*, 2011).

Cankers caused by these two species are morphologically nearly identical (Cortinas *et al.*, 2011). The first symptoms are discrete necrotic spots on young green stems at the top of the

trees. The lesions on twigs increase into cankers in the interior which then enlarge and coalesce to form large, dark, oval-shaped cankers on the stems and trunks. Transverse sections through the trunk show the presence of concentric kino pockets. Water transport to terminal shoots is disrupted resulting in reduced growth and malformation of soft tissues (Cortinas *et al.*, 2006b, 2011).

Both species associated with canker disease are strictly linked and have adapted in a different way supported by some ecological differences. Morphologically, *T. gauchensis* has slighter longer conidia then *T. zuluensis*, and sympodial polyphialidic conidiogenous cells, whereas conidiogenous cells of *T. zuluensis* are monophialidic. The easiest way to differentiate them is by comparing DNA sequences. Phylogenetically they differ at 26 fixed nucleotide positions. For instance, 5 fixed positions in ITS; 11 fixed positions on exons 3 to 6 and the respective introns of the β-tubulin gene (BT2); 9 positions on the intron sequence of the EF1-α gene and one fixed position on intron 2 and exon 3 of the ATP6 gene (ATP6) (Cortinas *et al.*, 2006b).

Initially it was assumed that this canker disease had its origin in South Africa (Wingfield *et al.*, 1996b). However, according to Hunter *et al.*, (2009) and Cortinas *et al.*, (2010) the South African population is most probably not the centre of the origin because it has a lower degree of genetic diversity than populations in other countries. Cortinas *et al.*, (2010) analysed genetic diversity in three populations of *T. zuluensis*, one from South Africa, one from China and another from Malawi and showed that a moderate to high diversity and clonal reproduction is predominant. They also confirmed that the genetic diversity of the populations in South Africa has increased over time. Their data indicates that the populations in South Africa, Malawi and China appear to have begun separately and from an unidentified origin. Furthermore, an unexpected difference in two South Africa populations was observed. Finally it was concluded that *T. zuluensis* might have its origin in South-East Asia (Cortinas *et al.*, 2010).

A study of genetic diversity in *T. gauchensis* was conducted on two South America populations, one in Uruguay and the other in Argentina (Cortinas *et al.*, 2011). The diversity was higher than previously supposed and inconsistent. It has been demonstrated that the two populations did not present a genetic structure as expected in recently introduced pathogen and these populations are considered to have arisen the same genetic pool (Cortinas *et al.*, 2011). Furthermore, Cortinas *et al.*, (2011) formulated two hypotheses to support that *T. gauchensis* is not a newly found pathogen: (1) this fungus could have originated from native host stock (Australia) and has not been found there or (2) host jumps where *Eucalyptus* is an exotic species.

Table 1.4 Teratosphaeria canker records by year, country and host.

Species	Year	Country	Host	Reference
Teratosphaeria zuluensis	1996	South Africa	*E. grandis*	Wingfield *et al.*, 1996b
	1996	Thailand	*E. camaldulensis*	Van Zyl *et al.*, 2002
	2000	Mexico	*E. grandis*	Roux *et al*., 2002
	2000	Vietnam	*E. urophylla*	Gezahgne *et al.*, 2003
	2004	Malawi	*E. grandis*	Cortinas *et al.*, 2006b
	2004	China	*E. urophylla*	Cortinas *et al.*, 2006a
	2010	Zambia	*E. cloeziana* and *E. grandis*	Chungu *et al.*, 2010
	2012	Uganda	*E. grandis*	Jimu *et al.*, 2014
T. gauchensis	2001	Uganda	*E. grandis*	Cortinas *et al.*, 2006b
	2001	Ethiopia	*E. camaldulensis*	Gezahgne *et al*., 2005
	2001	Argentina	*E. grandis*	Gezahgne *et al*., 2003
	2001	Uruguay	*E. grandis*,	Cortinas *et al.*, 2006b;
	2002	Hawaii	*E. grandis*	Cortinas *et al.*, 2004
	2006	Portugal	*E. globulus*	Silva *et al.*, 2015a

What is new about *Mycosphaerellaceae* and *Teratosphaeriaceae*

Until 2006, the only multi-locus approach was that of Hunter *et al.* (2006b) who used ITS, EF-1α, ACT and LSU. Quaedvlieg *et al.* (2014) constructed a phylogeny based on partial DNA sequence data of the 28S rRNA and RNA polymerase II second largest subunit (RPB2) gene that supported the division of *Mycosphaerellaceae* and *Teratosphaeriaceae*. It also indicated two further families - *Extremaceae* and *Neodevriesiaceae*. They also constructed a phylogeny based on ITS, LSU, β-tubulin gene (Btub), ACT, RPB2, EF-1α and calmodulin (Cal), which revealed 48 new combinations, 23 novel genera and five new species in the Eucalyptus Leaf Disease Complex (Table 1.5). Quaedvlieg *et al.* (2014) further concluded that the ITS gene provides a primary barcode approach with a wide dataset available, while Btub, EF-1α or RPB2 offer a secondary barcoding locus for these families (except for *Pseudocercospora* species).

Table 1.5 Recent new families that left *Mycosphaerellaceae* and *Teratosphaeriaceae* and new genera, combinations on *Mycosphaerellaceae* and *Teratosphaeriaceae* and the respective original name.

New family *fam. nov.*	**New genus** *(gen. nov.)*	**New combination** *(comb. nov.)*	**Original Name** *(Basionym)*	**References**
Extremaceae	***Extremus*** Quaedvlieg & Crous	***Extremus adstrictus*** (Egidi & Onofri) Quaedvlieg & Crous	*Devriesia adstricta*	Quaedvlieg *et al.*, 2014
		Extremus antarcticus (Selbmann & de Hoog) Quaedvlieg & Crous	*Devriesia antarctica*	
Incertae sedis (Capnodiales)	***Mucomycosphaerella*** Quaedvlieg & Crous	***Mucomycosphaerella eurypotami*** (Kohlm., Volkm.-Kohlm. & O.E. Erikss.) Quaedvlieg & Crous	*Mycosphaerella eurypotami*	
***Mycosphaerellaceae* (1)**	***Neopenidiella*** Quaedvlieg & Crous	***Neopenidiella nectandrae*** (Crous, U. Braun & R.F. Castañeda) Quaedvlieg & Crous,	*Penidiella nectandrae*	
	Amycosphaerella Quaedvlieg & Crous	***Amycosphaerella africana*** (Crous & M.J. Wingf.) Quaedvlieg & Crous	*Mycosphaerella africana* *Teratosphaeria africana* *Mycosphaerella ellipsoidea* *Mycosphaerella aurantia*	
	Paramycosphaerella Crous	***Paramycosphaerella intermedia*** (M.A. Dick & K. Dobbie) Quaedvlieg & Crous	*Mycosphaerella intermedia*	Crous *et al.*, 2013b; Quaedvlieg *et al.*, 2014
		Paramycosphaerella marksii (Carnegie & Keane) Quaedvlieg & Crous	*Mycosphaerella marksii*	Quaedvlieg *et al.*, 2014

Table 1.5 Recent new families that left *Mycosphaerellaceae* and *Teratosphaeriaceae* and new genera, combinations on *Mycosphaerellaceae* and *Teratosphaeriaceae* and the respective original name (Cont.).

New family *fam. nov.*	**New genus** *(gen. nov.)*	**New combination** *(comb. nov.)*	**Original Name** *(Basionym)*	**References**
	Phaeophleospora Rangel (1)	***Phaeophleospora gregaria*** (Carnegie & Keane) Quaedvlieg & Crous	*Mycosphaerella gregaria* *Mycosphaerella aggregata*	
		Phaeophleospora scytalidii (Crous & M.J. Wingf.) Quaedvlieg & Crous	*Mycosphaerella scytalidii*	
		Phaeophleospora stramenti (Crous & Alfenas) Quaedvlieg & Crous	*Mycosphaerella stramenti*	
	Pseudocercospora Spegazzini	***Pseudocercospora eucalyptorum*** Crous, M.J. Wingf., Marasas & B. Sutton (1)	*Pseudocercospora pseudoeucalyptorum*	Crous *et al*., 2013a
	Xenomycosphaerella Quaedvlieg & Crous	***Xenomycosphaerella elongata*** (Crous & M.J. Wingf.) Quaedvlieg & Crous	*Mycosphaerella elongata*	Quaedvlieg *et al*., 2014
		Xenomycosphaerella yunnanensis (Barber & T.I. Burgess) Quaedvlieg & Crous	*Mycosphaerella yunnanensis*	
	***Zasmidium* (1)**	***Zasmidium eucalyptorum*** (Crous & M.J. Wingf.) Quaedvlieg & Crous	*Mycosphaerella eucalyptorum*	
Neodevriesiaceae Quaedvlieg & Crous	***Neodevriesia*** Quaedvlieg & Crous	***Neodevriesia hilliana*** (Crous & U. Braun) Quaedvlieg & Crous	*Devriesia hilliana*	
		Neodevriesia xanthorrhoeae (Crous, Pascoe & Jacq. Edwards) Quaedvlieg & Crous	*Devriesia xanthorrhoeae*	
***Teratosphaeriaceae* (1)**	***Austroafricana*** Quaedvlieg & Crous	***Austroafricana associata*** (Crous & Carnegie) Quaedvlieg & Crous	*Mycosphaerella associata* *Teratosphaeria associata*	
		Austroafricana keanei (Carnegie & G.S. Pegg) Quaedvlieg & Crous	*Teratosphaeria keanei*	
		Austroafricana parva (R.F. Park & Keane) Quaedvlieg & Crous,	*Mycosphaerella parva* *Mycosphaerella grandis* *Teratosphaeria parva*	
	Eupenidiella Quaedvlieg & Crous	***Eupenidiella venezuelensis*** (Crous & U. Braun) Quaedvlieg & Crous	*Penidiella venezuelensis*	
	Euteratosphaeria Quaedvlieg & Crous	***Euteratosphaeria verrucosiafricana*** (Crous & M.J. Wingf.) Quaedvlieg & Crous	*Mycosphaerella verrucosiafricana*	

Table 1.5 Recent new families that left *Mycosphaerellaceae* and *Teratosphaeriaceae* and new genera, combinations on *Mycosphaerellaceae* and *Teratosphaeriaceae* and the respective original name (Cont.).

New family *fam. nov.*	**New genus** *(gen. nov.)*	**New combination** *(comb. nov.)*	**Original Name** *(Basionym)*	**References**
	Myrtapenidiella Quaedvlieg & Crous	***Myrtapenidiella corymbia*** (Cheew. & Crous) Quaedvlieg & Crous	*Penidiella corymbia*	
		Myrtapenidiella eucalypti (Cheew., K.D. Hyde & Crous) Quaedvlieg & Crous	*Penidiella eucalypti*	
		Myrtapenidiella tenuiramis (Crous & Summerell) Quaedvlieg & Crous	*Penidiella tenuiramis*	
	Neocatenulostroma Quaedvlieg & Crous	***Neocatenulostroma abietis*** (Butin & Pehl) Quaedvlieg & Crous	*Trimmatostroma abietis* *Catenulostroma abietis*	
		Neocatenulostroma germanicum (Crous & U. Braun) Quaedvlieg & Crous	*Catenulostroma germanicum*	
		Neocatenulostroma microsporum (Joanne E. Taylor & Crous) Quaedvlieg & Crous	*Trimmatostroma microsporum* *Catenulostroma microsporum* *Teratosphaeria microspora*	
	Neohortaea Quaedvlieg & Crous	***Neohortaea acidophila*** (Hölker, Bend, Pracht, Tetsch, Tob. Müll., M. Höfer & de Hoog) Quaedvlieg & Crous	*Hortaea acidófila*	
	Neophaeothecoidea Quaedvlieg & Crous	***Neophaeothecoidea proteae*** (Crous) Quaedvlieg & Crous	*Phaeothecoidea proteae*	
	Neotrimmatostroma Quaedvlieg & Crous	***Neotrimmatostroma bifarium*** (Gadgil & M.A. Dick) Quaedvlieg & Crous	*Trimmatostroma bifarium*	
		Neotrimmatostroma excentricum (B. Sutton & Ganap.) Quaedvlieg & Crous	*Trimmatostroma excentricum* *Catenulostroma excentricum* *Mycosphaerella excêntrica* *Teratosphaeria excentrica*	
	Apenidiella Quaedvlieg & Crous	***Apenidiella strumelloidea*** (Milko & Dunaev) Quaedvlieg & Crous	*Cladosporium strumelloideum* *Penidiella strumelloidea*	

Table 1.5 Recent new families that left *Mycosphaerellaceae* and *Teratosphaeriaceae* and new genera, combinations on *Mycosphaerellaceae* and *Teratosphaeriaceae* and the respective original name (Cont.).

New family *fam. nov.*	**New genus** *(gen. nov.)*	**New combination** *(comb. nov.)*	**Original Name** *(Basionym)*	**References**
	Parateratosphaeria Quaedvlieg & Crous	***Parateratosphaeria altensteinii*** (Crous) Quaedvlieg & Crous	*Teratosphaeria altensteinii*	
		Parateratosphaeria bellula (Crous & M.J. Wingf.) Quaedvlieg & Crous	*Mycosphaerella bellula* *Teratosphaeria bellula*	
		Parateratosphaeria karinae (Crous) Quaedvlieg & Crous	*Teratosphaeria karinae*	
		Parateratosphaeria marasasii (Crous) Quaedvlieg & Crous	*Teratosphaeria marasasii*	
		Parateratosphaeria persoonii (Crous & L. Mostert) Quaedvlieg & Crous	*Teratosphaeria persoonii*	
	Pseudoteratosphaeria Quaedvlieg & Crous	***Pseudoteratosphaeria flexuosa*** (Crous & M.J. Wingf.) Quaedvlieg & Crous	*Mycosphaerella flexuosa* *Teratosphaeria flexuosa*	
		Pseudoteratosphaeria gamsii (Crous) Quaedvlieg & Crous	*Mycosphaerella gamsii*	
		Pseudoteratosphaeria ohnowa (Crous & M.J. Wingf.) Quaedvlieg & Crous	*Mycosphaerella ohnowa* *Teratosphaeria ohnowa*	
		Pseudoteratosphaeria perpendicularis (Crous & M.J. Wingf.) Quaedvlieg & Crous	*Mycosphaerella perpendiculares* *Teratosphaeria perpendicularis*	
		Pseudoteratosphaeria secundaria (Crous & Alfenas) Quaedvlieg & Crous	*Mycosphaerella secundaria* *Teratosphaeria secundaria*	
		Pseudoteratosphaeria stramenticola (Crous & Alfenas) Quaedvlieg & Crous	*Mycosphaerella stramenticola* *Teratosphaeria stramenticola* *Mycosphaerella parkiiaffinis* *Teratosphaeria parkiiaffinis*	
	Queenslandipenidiella Quaedvlieg & Crous	***Queenslandipenidiella kurandae*** (Crous & J.K. Stone) Quaedvlieg & Crous	*Penidiella kurandae*	

Table 1.5 Recent new families that left *Mycosphaerellaceae* and *Teratosphaeriaceae* and new genera, combinations on *Mycosphaerellaceae* and *Teratosphaeriaceae* and the respective original name (Cont.).

New family *fam. nov.*	**New genus** *(gen. nov.)*	**New combination** *(comb. nov.)*	**Original Name** *(Basionym)*	**References**
	Readeriella Syd. & P. Syd (1)	***Readeriella deanei*** Quaedvlieg, Summerell & Crous (2)	-	
		Readeriella limoniforma Quaedvlieg, Summerell & Crous (2)	-	
		Readeriella mirabiliaffinis Quaedvlieg, Summerell & Crous (2)	-	
	Suberoteratosphaeria Quaedvlieg & Crous	***Suberoteratosphaeria pseudosuberosa*** (Crous & M.J. Wingf.) Quaedvlieg & Crous	*Mycosphaerella pseudosuberosa* *Teratosphaeria pseudosuberosa*	
		Suberoteratosphaeria suberosa (Crous, F.A. Ferreira, Alfenas & M.J. Wingf.) Quaedvlieg & Crous	*Mycosphaerella suberosa* *Teratosphaeria suberosa*	
		Suberoteratosphaeria xenosuberosa Quaedvlieg, Carnegie & Crous		
	***Teratosphaeria* (1)**	***Teratosphaeria molleriana*** (Thüm.) Crous & U. Braun (1)	*Sphaerella molleriana* *Mycosphaerella molleriana* *Colletogloeopsis molleriana* *Readeriella molleriana* *Mycosphaerella vespa* *Mycosphaerella ambiphylla* *Teratosphaeria xenocryptica*	
	Teratosphaericola Quaedvlieg & Crous	***Teratosphaericola pseudoafricana*** (Crous & T.A. Cout.) Quaedvlieg & Crous	*Mycosphaerella pseudoafricana* *Teratosphaeria pseudoafricana*	
	Teratosphaeriopsis Quaedvlieg & Crous	***Teratosphaeriopsis pseudoafricana*** Quaedvlieg & Crous (2)	-	
	Xenopenidiella Quaedvlieg & Crous	***Xenopenidiella rigidophora*** (Crous, R.F. Castañeda & U. Braun) Quaedvlieg & Crous	*Penidiella rigidophora*	
	Xenoteratosphaeria Quaedvlieg & Crous	***Xenoteratosphaeria jonkershoekensis*** (P.S. van Wyk, Marasas & Knox-Dav.) Quaedvlieg & Crous	*Mycosphaerella jonkershoekensis* *Teratosphaeria jonkershoekensis*	

(1) Not a gen. nov. or a comb. nov. on the reference
(2) Not a *comb. nov.* but a *sp. nov.*

Conclusions

Close to 700 species of fungi have been reported on *Eucalyptus* leaves and stems (Hyde *et al*., 2007). Since 2006 phylogenetic studies based on DNA sequence data in the *Mycosphaerella* complex have revealed constant changes in their taxonomy, in families (Schoch *et al.*, 2006; Crous *et al*., 2007a, 2009b, 2009c, 2009d) in genera and in new species (Crous *et al.*, 2013a; Quaedvlieg *et al.*, 2013, 2014).

Over the last years, disease problems have increased worldwide because of this complex and it is predicted that they will become progressively more significant in the future due to climate change (Crous *et al.*, 2004a). Several studies have been developed because of the constant reports of these new species (Alfenas *et al*., 2004; Balmelli *et al.*, 2004; Hunter *et al.*, 2004a, 2004b; Maxwell, 2004; Cortinas *et al.*, 2006b; Carnegie, 2007b; Perez *et al.*, 2009; Teodoro, 2010; Carnegie *et al.*, 2011; Aguin *et al.*, 2013; Manzilla *et al.;* 2013, Silva *et al.*, 2007, 2008, 2009, b, d).

A less studied subject of these fungi is pathogenicity. To understand the role of each species in the complex it is essential to study interactions within the complex and there is large scope for more research in the future.

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III

Status of Eucalyptus Leaf Disease Complex in Portugal

CHAPTER 2

Mycosphaerella and *Teratosphaeria* species associated with Mycosphaerella Leaf Disease on *Eucalyptus globulus* in Portugal

Chapter 2

RESEARCH ARTICLE

Mycosphaerella and *Teratosphaeria* species associated with *Mycosphaerella* Leaf Disease on *Eucalyptus globulus* in Portugal

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Summary

Plantations of *Eucalyptus globulus* represent the main source of wood for the pulp and paper industry. This disease affect mainly young trees with juvenile-phase foliage, causing premature defoliation in Portugal and are affected by the complex of *Mycosphaerella* and *Teratosphaeria* species *(Mycosphaerella* leaf disease), which is an important foliage disease worldwide, decreased growth and wood production. Species of *Mycosphaerella sensu lato* reported on eucalypts in Portugal are *M. communis, M. heimii, M. lateralis, M. madeirae, M. marksii M. walkeri, T. africana, T. molleriana, T. nubilosa* and *T. parva.* In order to complete the survey, symptomatic leaves were collected from *Eucalyptus globulus* plantations. Morphological and molecular characterization was used to give an indication of the species occurrence and most frequent species (*T. nubilosa*) and the composition of the MLD complex that did not change after the latest review.

Key words: *Mycosphaerella*; *Teratosphaeria*; Leaf Disease; MLD.

Resumen

Especies de *Mycosphaerella* y *Teratosphaeria* asociadas con la enfermedad *Mycosphaerella* de las hojas en *Eucalyptus globulus* en Portugal

Las plantaciones de *Eucalyptus globulus* representan la principal fuente de madera para la industria de pasta y papel en Portugal y se ven afectadas por el complejo de especies de *Mycosphaerella* y *Teratosphaeria* (enfermedad *Mycosphaerella* de las hojas), que es una enfermedad importante en todo el mundo. Esta enfermedad afecta principalmente a los árboles con fase juvenil de follaje, causando defoliación prematura, disminución del crecimiento y de producción de madera. Las especies de *Mycosphaerella sensu lato* descritas sobre el eucalipto en Portugal son *M. communis, M. heimii,M. lateral, M. madeirae, M. marksii, M walkeri, T. africana, T. molleriana, T. nubilosa* y *T. parva*. Con el fin de completar el inventario, se obtuvieron hojas sintomáticas de plantaciones de *Eucalyptus globulus*. Se ha utilizado una caracterización morfológica y molecular para identificar la presencia de especies, la especie más frecuente (*T. nubilosa*) y la composición del complejo de MLD que no cambió después de esta última revisión.

Palabras clave: *Mycosphaerella*; *Teratosphaeria;* Enfermedad *foliar;* MLD.

Introduction

Mycosphaerella sensu lato is represented by more than 10,000 taxa (Crous, 2009) and some of them cause significant economic losses worldwide on plant hosts. M*ycosphaerella* leaf disease (MLD) on eucalypts is a significant disease worldwide that can reduce the growth of *Eucalyptus* when defoliation of the juvenile crown reaches more than 25% (Lundquist and Purnell, 1987; Dungey *et al.,* 1997) thus affecting the pulp and paper industry. Commercial plantations of eucalypts in Portugal are almost exclusively of *Eucalyptus globulus*, covering 646,700 ha representing 20.6% of the total forested area (AFN, 2007). MLD is considered to be the most damaging leaf disease of *Eucalyptus* in Australia (Park and Keane, 1984; Park, 1988; Park *et al.*, 2000; Carnegie, 2007) and is expected to become gradually more important worldwide in the future (Crous *et al.*, 2004). In recent years adult foliage were infected more often, like e.g. *T. nubilosa* in Australia (Barber *et al.*, 2008), in Uruguay (Perez *et al.*, 2009a), in Brazil (Perez *et al.*, 2009b) and *M. marksii* recorded in Uruguay always on adult leaves (Perez *et al.*, 2009).

In the 19th century, eucalypts were introduced in Portugal and Spain and *Teratosphaeria molleriana* (= *M. molleriana*) reported by von Thumen (1881) in Portugal was the first species of *Mycosphaerella* recorded outside Australia (Crous and Wingfield, 1997). Since 1999, serious damage on *E. globulus* has been reported characterized by a frequent and severe defoliation of young trees in the coastal regions assigned to MLD complex. In subsequent years more species were reported, including *T. africana* and *M. walkeri* (Crous, 1998), *M. madeirae* in Madeira (Crous *et al.*, 2004), *M. communis, M. heimii, M. lateralis, M. marksii, T. nubilosa, T. parva* (Crous *et al.*, 2006). In 2009, based on morphological and molecular analysis was suggested the occurrence of two more species, *M. grandis* and *M. vespa*, considered synonyms of *T. parva* and *T. molleriana* respectively by Hunter *et al.* (2006) (Silva *et al.*, 2009).

In Spain the situation is comparable and to the best of our knowledge the first report was in 1980 by Ruperez and Munoz. Only in 2004 more species were added like *M. communis, M. marksii, M. readeriellophora, M. toledana, T. molleriana, T. nubilosa*; in 2006 *M. lateralis, M. suberosa, M. walkeri, T. parva, T. pluritubularis* and in 2007 *M. aurantia* and *M. madeirae* (Crous *et al.*, 2004, 2006; Otero *et al.*, 2007). In 2009, *M. ellipsoidea, M. endophytica, M. flexuosa, M. gracilis, M. parkii, T. cryptica* were reported by De Blas *et al.* (2009). In 2011, *M. fori, M. punctiformis, T. africana* and *T. dimorpha* were reported by Sánchez Márquez *et al.* (2011).

The aim of this study was to observe species occurrence and the main species associated with MLD in ten locations distributed all over the coastal border in Portugal.

Materials and Methods

During autumn 2009 and spring 2010, ten young *Eucalyptus globulus* stands (1-2 years old) were surveyed throughout the country in regions that have suffered significant outbreaks of MLD (Table 2.1). As soon as the first lesions appeared, early spring or autumn, 5 trees were selected randomly and at least 10 symptomatic leaves were collected from each tree. In this study only the juvenile leaves were analyzed.

Lesions were examined with a stereomicroscope and characterized on the basis of colour, dimensions and shape. Wherever possible, 30 ascospores were measured at a magnification of × 600. Germination patterns of ascospores were determined after 24 h on 2% (w/v) malt extract agar (MEA) at 24 °C in the dark and single ascospore cultures obtained and colony colours of the top and reverse surface were recorded (Crous, 1998). The isolates were maintained on 2% (w/v) MEA slopes at 24 °C in the dark and deposited in the culture collection of LISFA (Herbarium Code of Unidade de Silvicultura e Produtos Florestais, Instituto Nacional de Investigação Agrária, I.P., L-INIA, Oeiras, Portugal).

All the unclear isolates morphological characterized were confirmed by sequence analysis based on the ITS1-5.8S-ITS2 cluster and for a preliminary taxonomical placement, each sequence was submitted to Basic Local Alignment Search Tool (BLAST) against the National Center for Biotechnology Information (NCBI) nucleotide databases. Genomic DNA was extracted (described by Silva *et al.*, 2009) and the ITS1-5.8S-ITS2 cluster was amplified with primers ITS1 and ITS4 (White *et al.*, 1990). Sequencing reactions and purification of amplification products were according to the procedure described by Silva *et al.* (2009).

Results

All isolates were processed for morphological methods and in few cases characterized with molecular methods. The diversity of MLD species by location is presented at Table 2.1 (Valongo, Trofa, Penafiel, Aveiro – further North and Ourém, Santarém, Cadaval, Torres Vedras, Bombarral 1, Bombarral 2 – further South). It showed less species in the MLD complex composition in northern Portugal than in further southern locations. *T. nubilosa* is the most frequent, present in 8 out of the 10 analyzed plantations; followed by *T. parva* and *M. lateralis* (6 of 10), *M. grandis* and *M. vespa* (4 of 10). The other species present were *M. communis* and *M. marksii* (3 of 10) and *T. molleriana* (2 of 10).

T. nubilosa was always isolated from young lesions on juvenile leaves. In contrast, the others species observed were found only on older lesions on juvenile leaves. *T. nubilosa* was the species best represented with 80% of occurrences contrasting with few isolates of *T. molleriana* and *M. vespa* with 20% and 40% isolates correspondingly.

Table 2.1 Locations of the ten plantations studied along the coastal border, ordered from northern to southern, total number of fungal species identified at each location and its presence **(1)** and absence **(0)**: **(C)** *M. communis*; **(G)** *M. grandis*; **(L)** *M. lateralis*; **(MK)** *M. marksii*; **(V)** *M. vespa*; **(MLL)** *T. molleriana*; **(N)** *T. nubilosa*; **(P)** *T. parva* and number of other species unidentified.

Location	Number of species identified	Species presence or absence								
		C	G	L	MK	V	MLL	N	P	Number of other species
Valongo	4	0	1	1	0	0	0	1	1	0
Trofa	1	0	0	1	0	0	0	0	0	0
Penafiel	1	0	0	0	0	0	0	1	0	0
Aveiro	4	1	0	1	0	1	0	1	0	0
Ourém	1	0	0	0	0	0	0	0	1	0
Santarém	1	0	0	0	0	0	0	1	0	0
Cadaval	5	0	1	1	0	1	0	1	1	0
Torres Vedras	11	1	1	1	1	1	1	1	1	3
Bombarral 1	5	0	0	0	1	0	0	1	1	2
Bombarral 2	10	1	1	1	1	1	1	1	1	2
Number of occurrences		3	4	6	3	4	2	8	6	

A summarized description for the main characters used on morphological identification of *M. communis, M. grandis, M. lateralis, M. marksii, M. vespa, T. molleriana, T. nubilosa and T. parva* is presented below.

Germination of *M. communis* ascospores was type F (Crous, 1998) with ascospores not darkening and germinating from both ends, with germ tubes parallel to the long axis of the spore and distorting prominently upon germination. Lesions were sub-circular to circular, 4-12 mm diameter, medium brown, surrounded by a thin, raised, concolorous border. Diameter of cultures were 27-38 mm after 1 month at 24 °C in the dark, irregular, erumpent, uneven, folded, aerial mycelium moderate to sparse, hazel in surface view, olivaceous-black on reverse.

Ascospores and germ tubes of *M. grandis* were type N (Crous, 1998), became dark with gross distortion and produced several germ tubes. Leaf lesions were round and angular, 5-7 mm diameter. Cultures were 16-25 mm diameter after 1 month at 24 °C in the dark, olive in surface view, green on reverse.

Ascospore germination of *M. lateralis* was type I (Crous, 1998) with germination from both ends, germ tubes parallel to the long axis of the spore, not darkening, constricted at septum, developing lateral branches. Leaf spots were subcircular, 3-12 mm diameter, greybrown, surrounded by raised borders, medium brown on the adaxial surfaces concolorous on the lower surfaces. Cultures were 33-37 mm after 1 month at 24 °C in the dark, with an even margin, cream aerial mycelium, grey olivaceous on reverse.

Ascospore germination of *M. marksii* was type B (Crous, 1998), with germination from both ends germ tubes parallel to the long axis of the spore, not darkening or distorting. Leaf spots were subcircular to irregular, 4-20 mm diameter, light brown, delimited by raised borders, medium brown with red-purple margins. Cultures grew 22-26 mm after 1 month at 24 °C in the dark, with an even or uneven margin, aerial mycelium sparse, olivaceous grey on surfaces.

Ascospores of *M. vespa* were type C (Crous, 1998), with germination from both ends, parallel to the long axis of the spore, ends obtusely rounded, slightly constricted. Leaf lesions were circular to irregular, sometimes confluent, light-brown to grey and less than 5 mm. Cultures were 23-38 mm diameter after 1 month at 24 °C in the dark, olivaceous grey on both surfaces.

Ascospore germination of *T. molleriana* was type C (Crous, 1998) germination from one or both ends, germ tubes parallel to long axis of the spore, not darkening, constricted at septum. Leaf spots were subcircular to irregular, 2-11 mm diameter, light brown, medium brown border. Diameter of cultures was 10-39 mm after 1 month at 24 °C in the dark, aerial mycelium absent or sparse, iron gray on bottom and olivaceous-gray on top.

Ascospore germination of *T. nubilosa* was type C (Crous, 1998) with germination from both ends, germ tubes parallel to long axis of spore, not darkening or distorting. Lesions were 3-15 mm diameter, irregular or round, occasionally coalescent, forming larger irregular pale brown blotches, often surrounded by a raised thin brown margin. Cultures grew 15-19 mm after 1 month at 24 °C in the dark, with irregular margins, olivaceous grey on both surfaces.

Ascospores of *T. parva* and its germ tubes became uniformly brown, distorted and verruculose. Leaf spots were subcircular 4-15 mm diameter, light brown, surrounded by a raised

border and thin, dark brown margin. Ascospore germination was type N (Crous, 1998). In this study cultures were 14-18 mm after 1 month at 24 ºC in the dark, olive in surface view, and green on reverse.

The comparison obtained between sequences reads of the ITS1-5.8S-ITS2 cluster and the published sequences of the GenBank nucleotide sequences database confirmed the relation of the isolates with the highest similarity (Table 2.2).

Table 2.2 Species identified on juvenile leaves of *Eucalyptus globulus* based in the percentage of similarity of each isolate to its closest match at the GenBank nucleotide sequences database.

Sequence accession no.	Origin	Sequence-based identification	Maximum identity (%)
FJ515739	Portugal	*Mycosphaerella communis*	100
FJ515719	Portugal	*Mycosphaerella grandis*	100
EU851919	Uruguay	*Mycosphaerella lateralis*	99
EU851931	Uruguay	*Mycosphaerella marksii*	100
FJ515727	Portugal	*Mycosphaerella vespa*	100
EU851932	Uruguay	*Teratosphaeria molleriana*	99
FJ515731	Portugal	*Teratosphaeria nubilosa*	100
AY509779	Australia	*Teratosphaeria parva*	99

Discussion

Morphological characteristics of species identified concurred with descriptions for *M. communis* (Crous *et al.*, 2004), *M. grandis* (Carnegie and Keane, 1994), *M. lateralis* (Crous and Wingfield, 1996), *M. marksii* (Carnegie and Keane, 1994), *M. vespa* (Carnegie and Keane, 1998), *T. molleriana* (Crous and Wingfield, 1997), *T. nubilosa* and *T. parva* (Park and Keane, 1982). *M. grandis* and *M. vespa* were considered synonyms of *T. parva* and *T. molleriana* respectively (Hunter *et al.*, 2006). However, in this study some differences in morphology were described as it was shown by Silva *et al*. (2009).

The composition of species of MLD complex did not change after the latest revision in Portuguese plantations (Silva *et al.*, 2009). *T. nubilosa* is the dominant species and occurred mostly on young lesions on juvenile leaves confirming the aggressiveness of this species on eucalyptus. *T. molleriana* was reported in 1881 (130 years ago), after that only in 1995 in

Abrantes (collected by S. McCrae), in 2005 in Torres Vedras (collected by H. Machado), in 2006 near Lisbon (collected by A.J.L. Phillips) and in this work, in Torres Vedras and Bombarral. All these reports refer to close locations in the centre of the country. It seems that *T. molleriana* did not spread out during all these years and not become problematic and aggressive as *T. nubilosa* did in a few years since the first detection. *M. marksii* is also confined to centre locations. The others reported species are spread out all over the country as *T. nubilosa*.

All the species reported in Portugal were also observed in Spain, except *M. heimii*. In Spain the diversity of species of MLD complex is higher than in Portugal with more 14 species detected: *M. readeriellophora, M. toledana* (2004)*; M. suberosa, T. pluritubularis* (2006); *M. aurantia* (2007); *M. ellipsoidea, M. endophytica, M. flexuosa, M. gracilis, M. parkii, T. cryptica* (2009) *M. fori, M. punctiformis* and *T. dimorpha* (2011) (Crous *et al.*, 2004, 2006; Otero *et al.*, 2007; de Blas *et al.*, 2009; Sánchez Márquez *et al.*, 2011).

Conclusions

In this study were identified *M. communis, M. grandis, M. lateralis, M. marksii, M. vespa, T. molleriana, T. nubilosa* and *T. parva*, and five more unidentified species on Portuguese plantations, suggesting that the main species composition on MLD complex did not change after the latest revision (Silva *et al.*, 2009).

Until now, there are twice as many species reported from Spain than from Portugal and one particular highlight is the absence of *T. cryptica* in Portugal, since this is one of the most aggressive species of MLD as *T. nubilosa*.

This study suggests that *T. nubilosa* is the main species causing MLD in Portugal. Nevertheless, a continuing effort on survey associated with a complete climatic characterization during different seasons to accurately attribute species aggressiveness will be necessary. Furthermore, the distribution was less diverse in northern than in southern locations thus more research is needed to understand and identify the factors implicated in differences in composition of MLD complex.

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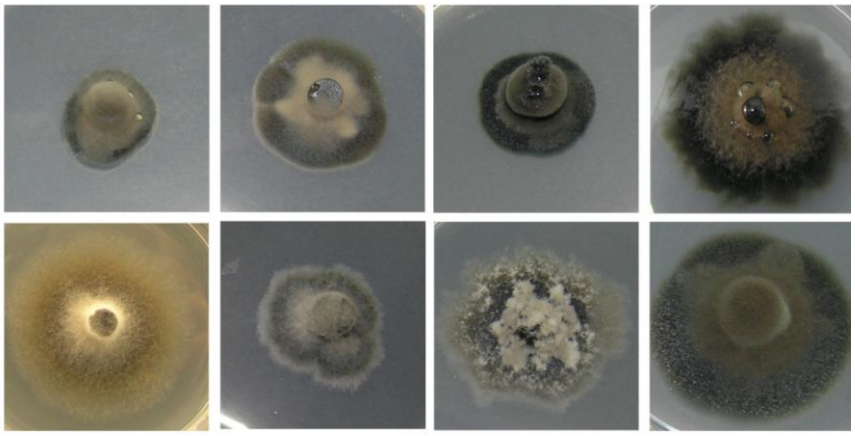
CHAPTER 3

New records, species and combinations in *Teratosphaeria* on *Eucalyptus* in Portugal



Chapter 3

RESEARCH ARTICLE

New records, species and combinations in *Teratosphaeria* on *Eucalyptus* in Portugal

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Summary

Approximately 50 species of *Teratosphaeria* have been reported on eucalypts worldwide and some have recently been transferred to other genera. *Neodevriesia hilliana* (≡*Devriesia hilliana*) is reported for the first time on *Myrtaceae* and *Teratosphaeria lusitanica* is introduced as a new species of *Teratosphaeria. Quasiteratosphaeria mexicana* (≡*T. mexicana*)*; Teratosphaericola pseudoafricana* (≡*Teratosphaeria pseudafricana*) were introduced as new species of *Teratosphaeria* in the Iberian Peninsula and *Teratosphaeria pluritubularis* in Portugal. In addition new structures of *Austroafricana parva* (≡*T. parva*), *Q. mexicana*, *T. pluritubularis* and *T. pseudoafricana* were observed, as well as two new combinations of genera were made *Amycosphaerella quasicercospora* (≡*T. quasicercospora*) and *Q. mexicana*. The aim of this research was to update the *Teratosphaeria* species which have been appearing on *Eucalyptus globulus* in Portuguese plantations.

Keywords

Dothideomycetes, ITS, *Amycosphaerella, Devriesia, Neodevriesia,* eucalypts, *Myrtaceae*.

Introduction

Eucalyptus is the main component of the Portuguese continental forests, covering 812,000 ha and representing 26% of the total forested area, which is 165 300 ha (5.4%) more than in the last inventory in 2005/06. The total area of *Eucalyptus* increased by 13% between 1995 and 2010, representing an area of more 95 000 ha. Approximately 8000 ha that were *Eucalyptus* forest in 1995 became of urban use in 2010 (AFN 2007; ICNF 2013). Commercial plantations of eucalypts in Portugal are almost exclusively of *Eucalyptus globulus* Labill. The superior pulping qualities and fast growth rate are valuable properties of this species. Plantations are concentrated in the coastal regions where production is higher and attack by pathogens is generally reduced. Nevertheless, pests and diseases are known to seriously threaten *Eucalyptus* plantations throughout the world (Wingfield *et al*., 2008).

Mycosphaerella leaf disease (MLD) is one of the most important leaf diseases of eucalypt plantations worldwide, and is caused by a complex of *Teratosphaeria* and *Mycosphaerella* species. Often more than 4 or 5 different species inhabit the same lesion and the lesions

frequently overlap (Crous *et al.*, *2*004). The first case of serious defoliation of young *E. globulus* trees in Portugal was reported in 1999 (Silva *et al.*, 2009).

Prior to 2007, *Mycosphaerella* was considered to be a large genus of approximately 3000 mostly leaf infecting species (Aptroot 2006). Taylor *et al.* (2003) resolved the phylogenetic relationships between members of the *Mycosphaerellaceae* through analysis of DNA sequence data of the ITS1-5.8S-ITS2 cluster (ITS) and concluded that *Teratosphaeria* was a synonym of *Mycosphaerella*. Müller & Oehrens (1982) characterized the genus *Teratosphaeria* by "globose, perithecium-like ascomata growing inside the living leaf-tissue, with bitunicate asci and brownish, bi-cellular ascospores". Crous et al. (2007a) agreed with this circumscription and added that in *Teratosphaeria* the ascospores often turn brown in asci, ascospore with a mucilagenous sheath, multi-layered endotunica, prominent periphysoids, ascomata frequently connected by a superficial stroma (Crous *et al.*, 2007a). Using combined ITS and LSU sequence data Crous *et al.*, (2007a) demonstrated polyphyly (within *Teratosphaeria*) and paraphyly (within Capnodiales) in *Mycosphaerella*. Thus several species were transferred to *Mycosphaerellaceae* and *Teratosphaeriaceae* and other families like *Davidiellaceae*, *Dissoconiaceae* and *Schizothyriaceae* (Schoch *et al*., 2006; Crous *et al*., 2009b, 2009c, 2009d). On the other hand, *Teratosphaeriaceae* contains some genera like *Baudoinea*, *Capnobotryella*, *Devriesia*, *Penidiella*, *Phaeothecoidea*, *Readeriella*, *Staninwardia* and *Teratosphaeria* (Crous *et al.*, 2009c, 2009b). Most of the severe MLD pathogens are included in *Teratosphaeria* eucalypts (Carnegie & Ades, 2002; Carnegie, 2007; Hunter *et al*., 2009).

Until now more than 50 species of *Teratosphaeria* have been reported on eucalypts worldwide (Aptroot, 2006; Crous, 1998; Crous *et al*., 1995, 1993, 2004, 2006, 2007a, 2007b, 2009a, 2009d; 2009e; Crous & Wingfield, 1996, 1997a, 1997b; Park & Keane, 1982a; Carnegie *et al*., 2011). In 2014, Quaedvlieg *et al*. (2014) reported a new species (*Teratosphaeriopsis pseudoafricana*) and more than 20 *Teratosphaeria* were transferred to new genera such as *Amycosphaerella*, *Austroafricana*, *Euteratosphaeria*, *Parateratosphaeria*, *Pseudoteratosphaeria*, *Suberoteratosphaeria*, *Teratosphaericola* and *Xenoteratosphaeria*.

The first report of *Teratosphaeria* on eucalypts outside Australia, *Teratosphaeria molleriana* (Thüm.) Lindau (Crous & Wingfield, 1997a) was reported from Portugal in 1881. More recently Crous (1998) reported *Amycosphaerella africana*, and later *T. nubilosa* and *Austroafricana parva* (Crous *et al*., 2006; Quaedvlieg *et al*., 2014). In contrast, six species of *Mycosphaerella*, i.e., *M. walkeri*, *M. madeirae* (in Madeira island) *M. communis*, *M. heimii*, *M. lateralis*, *Paramycosphaerella marksii* are known from Portugal (Crous, 1998; Crous *et al*., 2004, 2006; Silva *et al*., 2008, 2009; Quaedvlieg *et al*., 2014). The aim of the present study was

to contribute to an update of the *Teratosphaeria* species occurring on *Eucalyptus globulus* in Portuguese plantations.

Materials and methods

Isolates and isolations

Symptomatic leaves were collected from young (1–2 years old) *E. globulus* plantations throughout Portugal where MLD symptoms were observed. Lesions were examined with a stereomicroscope and characterized on the basis of colour, dimensions and shape. Thereafter, lesions were soaked in autoclaved distilled water for approximately 2 h and then placed in the base of Petri dish lids, with pseudothecia facing upwards, with the other half of the dish containing 2 % malt extract agar (MEA) (w/v) (Difco) on top (Crous, 1998). Plates were incubated at 25 °C in the dark and were examined every 24 h for up to 120 h to observe the presence of germinating ascospores. Single germinating ascospores were transferred to fresh plates of 2% MEA. Germinating ascospores were also transferred to microscope slides and mounted in autoclaved distilled water. Observations on micro morphological features were made with an Olympus BX41TF microscope with bright field illumination. Digital images were recorded with a ProgRes Speed XT Core 5 camera. Measurements were made with the ProgRes Capture Pro 2.8.8 Jenoptik. Wherever possible, 30 ascospores or mycelium of each isolate were measured on images taken with the 60x objective lens. Linear growth of single spore cultures was assessed after 1 month on 2% MEA at 25°C in the dark. Cultures were also incubated under continuous near-ultraviolet light at room temperature to promote sporulation. A collection of 65 isolates (Table 3.1) was maintained on 2% MEA slopes at 25°C in the dark and deposited in the culture collection of UEIS-SAFSV (Herbarium Code of Unidade Estratégica Sistemas Agrários e Florestais e Sanidade Vegetal of Instituto Nacional de Investigação Agrária e Veterinária, I.P., Oeiras, Portugal).

Table 3.1 Isolates included in the phylogenetic analyses.

Species	**Strain no.**[1]	**Host**	**Country**	**Collector**	**GenBank** [2]
Amycosphaerella africana	**CMW 4945**	*Eucalyptus*	South Africa		AF309602
Amycosphaerella quasicercospora	**CBS 111161**	*Eucalyptus* sp.	Tanzania	M.J. Wingfield	DQ303012
Austroafricana associata	**CBS 120730**	*Corymbia henryii*	Australia	A.J. Carnegie	EF394826
Austroafricana parva	**CBS 122892**	*E. globulus*	Australia	I. Smith	**EU707875** [a]
≡*M. grandis*	CPC 12249	*Eucalyptus globulus*	Portugal	A.J.L. Phillips	**GQ852820** [a]
	CPC 11888	*Eucalyptus* sp.	Portugal	A.J.L. Phillips	**DQ303005** [a]
	CMW 8554	*E. globulus*	Chile	M.J. Wingfield	**DQ267584** [b]
	EFN X37A	*E. globulus*	Portugal	H. Machado	FJ515708 [a]
	LISFA BBC168	*E. globulus*	Portugal	L. Neves	**a**
	LISFA BBC364	*E. globulus*	Portugal	L. Neves	**a**
	LISFA OR02	*E. globulus*	Portugal	L. Neves	**a**
	LISFA QP03	*E. globulus*	Portugal	M.R.C. Silva	**a**
	LISFA QP89	*E. globulus*	Portugal	M.R.C. Silva	**a**
	LISFA SA04	*E. globulus*	Portugal	L. Neves	**a**
	LISFA VL05	*E. globulus*	Portugal	M.R.C. Silva	**a**
	EFN X4A	*E. globulus*	Portugal	H. Machado	FJ515719[b]
	LISFA BBC34	*E. globulus*	Portugal	L. Neves	**b**
	LISFA BBC268	*E. globulus*	Portugal	L. Neves	**b**
	LISFA QP18	*E. globulus*	Portugal	M.R.C. Silva	**b**
	LISFA SA16	*E. globulus*	Portugal	L. Neves	**b**
	LISFA VL04	*E. globulus*	Portugal	M.R.C. Silva	**b**

Table 3.1 Isolates included in the phylogenetic analyses (Cont.).

Species	**Strain no.**[1]	**Host**	**Country**	**Collector**	**GenBank** [2]
Austroafricana parva	EFN X49C	*E. globulus*	Portugal	H. Machado	FJ515701[c]
	EFN Y27C	*E. globulus*	Portugal	H. Machado	FJ515712[c]
	LISFA BBC7	*E. globulus*	Portugal	L. Neves	[c]
	LISFA BBC16	*E. globulus*	Portugal	L. Neves	[c]
	LISFA BBC252	*E. globulus*	Portugal	L. Neves	[c]
	LISFA BBC278	*E. globulus*	Portugal	L. Neves	[c]
	LISFA BBC280	*E. globulus*	Portugal	L. Neves	[c]
	LISFA BBC309	*E. globulus*	Portugal	L. Neves	[c]
	LISFA BBC311	*E. globulus*	Portugal	L. Neves	[c]
	LISFA BBC343	*E. globulus*	Portugal	L. Neves	[c]
	LISFA BBC350	*E. globulus*	Portugal	L. Neves	[c]
	LISFA BBC354	*E. globulus*	Portugal	L. Neves	[c]
	LISFA BBC357	*E. globulus*	Portugal	L. Neves	[c]
	LISFA BBC366	*E. globulus*	Portugal	L. Neves	[c]
	LISFA PB71	*E. globulus*	Portugal	L. Neves	[c]
	LISFA PB72	*E. globulus*	Portugal	L. Neves	[c]
	LISFA QP8	*E. globulus*	Portugal	M.R.C. Silva	[c]
	LISFA QP20	*E. globulus*	Portugal	M.R.C. Silva	[c]
	LISFA QP70	*E. globulus*	Portugal	M.R.C. Silva	[c]
Batcheloromyces alistairii	**CBS 120035**	*Protea repens*	South Africa	P.W. Crous & A. Smith	DQ885901
Neodevriesia hilliana	CBS 123187	*Macrozamia communis*	New Zealand		GU214633
	LISFA BBC352	*E. globulus*	Portugal	L. Neves	

Table 3.1 Isolates included in the phylogenetic analyses (Cont.).

Species	Strain no.[1]	Host	Country	Collector	GenBank [2]
Neofusicocum parvum (Outgroup)	STE-U 4438				AY343467*****
Neotrimmatostroma excentrica	**CBS 121102**	*Eucalyptus agglomerata*	Australia	G. Price	EF394834
Parateratosphaeria bellula	CPC 14908	*Protea* sp.	South Africa	P.W. Crous	EU707861
Pseudoteratosphaeria flexuosa	**CMW 5224**	*Eucalyptus*	Colombia		AF309603
Pseudoteratosphaeria gamsii	CBS 118495	*Eucalyptus sp*	India	W. Gams	DQ302959
Pseudoteratosphaeria ohnowa	CBS1109492	*E. grandis*	South Africa	M.J. Wingfield	AY725575
Pseudoteratosphaeria parkiiaffinis	**CBS 120737**	*Eucalyptus urophylla*	Venezuela	M.J. Wingfield	EF394846
Pseudoteratosphaeria perpendicularis	**CBS 118367**	*Eucalyptus* sp.	Colombia	M.J. Wingfield	DQ303006
Pseudoteratosphaeria secundaria	**CBS 118507**	*Eucalyptus* sp.	Brazil	A.C. Alfenas	DQ303020
Pseudoteratosphaeria stramenticola	**CBS 118506**	*Eucalyptus* sp.	Brazil	A.C. Alfenas	DQ303043
Quasiteratosphaeria mexicana	CBS 120744	*Eucalyptus* sp.	Hawaii	W. Gams	EF394843
	LISFA PB24	*E. globulus*	Portugal	L. Neves	a
	LISFA QP13	*E. globulus*	Portugal	M.R.C. Silva	a
Teratosphaeria cryptica	**CMW 3279**	*Eucalyptus*	Australia		AF309623
Teratosphaeria fibrilosa	**CBS 121707**	*Protea* sp.	South Africa	P.W. Crous & L. Mostert	EU707862
Teratosphaeria fimbriata	**CBS 120736**	*Corymbia* sp.	Australia	P.W. Crous	EF394836
Teratosphaeria lusitanica	MUCC293	*E. pellita*	Australia	T Burgess	EU301093
	LISFA BBC239	*E. globulus*	Portugal	L. Neves	a
	LISFA QP93	*E. globulus*	Portugal	M.R.C. Silva	a
Teratosphaeria maxii	**CBS 120137**	*P. repens*	South Africa	M. Crous & P.W. Crous	DQ885899

Table 3.1 Isolates included in the phylogenetic analyses (Cont.).

Species	Strain no.[1]	Host	Country	Collector	GenBank [2]
Teratosphaeria molleriana	CMW 4940	*Eucalyptus*	Portugal	S. McCrae	AF309620
	CBS 120746	*Eucalyptus* sp.	Portugal	P.W. Crous & A.J.L. Phillips	EF394844[a]
	LISFA BBC322	*E. globulus*	Portugal	L. Neves	[a]
	LISFA BBC324	*E. globulus*	Portugal	L. Neves	[a]
	LISFA BBC412	*E. globulus*	Portugal	L. Neves	[a]
≡ *M. ambiphylla*	**CBS 110499**	*E. globulus*	Australia	A. Maxwell	AY725530
≡ *M. vespa*	CMW 11558	*Eucalyptus* sp.	Australia	-	DQ303059[b]
	EFN Y16F	*E. globulus*	Portugal	H. Machado	FJ515727[b]
	LISFA Y35I	*E. globulus*	Portugal	H. Machado	[b]
	LISFA QP46	*E. globulus*	Portugal	M.R.C. Silva	[b]
Teratosphaeria nubilosa	**STE-U 937**	*E. globulus*	Australia	A.J. Carnegie	AF309618
	CMW 9000	*E. nitens*	South Africa		AF449096[a]
	CMW 9001	*E. nitens*	South Africa		AF449097[a]
	CBS 116005	*E. globulus*	Australia	A.J. Carnegie	AY725572[a]
	CPC10497	*E. globulus*	New Zealand	W. Gams	AY725574
	EFN M7A	*E. globulus*	Portugal	L. Neves	FJ515729[a]
	EFN M17B	*E. globulus*	Portugal	L. Neves	FJ515728[a]
	EFN M32	*E. globulus*	Portugal	L. Neves	FJ515731[a]
	EFN Y8B	*E. globulus*	Portugal	H. Machado	FJ515730[a]
	LISFA A25	*E. globulus*	Portugal	M.R.C. Silva	[a]
	LISFA A32	*E. globulus*	Portugal	M.R.C. Silva	[a]

Table 3.1 Isolates included in the phylogenetic analyses (Cont.).

Species	Strain no.[1]	Host	Country	Collector	GenBank [2]
Teratosphaeria nubilosa	LISFA A41	*E. globulus*	Portugal	M.R.C. Silva	[a]
	LISFA A47	*E. globulus*	Portugal	M.R.C. Silva	[a]
	LISFA A52	*E. globulus*	Portugal	M.R.C. Silva	[a]
	LISFA A112a	*E. globulus*	Portugal	M.R.C. Silva	[a]
	LISFA A125	*E. globulus*	Portugal	M.R.C. Silva	[a]
	LISFA BBC270	*E. globulus*	Portugal	L. Neves	[a]
	LISFA BBC332	*E. globulus*	Portugal	L. Neves	[a]
	LISFA BBC377	*E. globulus*	Portugal	L. Neves	[a]
	LISFA BBC416	*E. globulus*	Portugal	L. Neves	[a]
	LISFA LN01	*E. globulus*	Portugal	L. Neves	[a]
	LISFA LN02	*E. globulus*	Portugal	L. Neves	[a]
	LISFA RF02	*E. globulus*	Portugal	M.R.C. Silva	[a]
	LISFA VL2	*E. globulus*	Portugal	M.R.C. Silva	[a]
Teratosphaeria pluritubularis	**CBS 118508**	*Eucalyptus globulus*	Spain	P. Mansilla	DQ303007[a]
	LISFA BBC106	*E. globulus*	Portugal	L. Neves	[a]
	LISFA BBC317	*E. globulus*	Portugal	L. Neves	[a]
Teratosphaeria pseudocryptica	**CBS 118504**	*Eucalyptus* sp.	New Zealand	J.A. Stalpers	DQ303010
Teratosphaeria suttonii	**CMW5348**	*Eucalyptus*	Indonesia		AF309621
Teratosphaeria toledana	**CBS 113313**	*Eucalyptus* sp.	Spain	P.W. Crous	AY725580
Teratosphaericola pseudafricana	**CBS 114782**	*Eucalyptus globulus*	Zambia	T.A. Coutinho	DQ303008
	LISFA BBC293	*E. globulus*	Portugal	L.Neves	
Readeriella dendritica	**CBS 120032**	*Eucalyptus deanei*	Australia	B.A. Summerell	EF394829

Table 3.1 Isolates included in the phylogenetic analyses (Cont.).

Species	Strain no.[1]	Host	Country	Collector	GenBank [2]
Readeriella readeriellophora	**CBS 114240**	*E. globulus*	Spain	J.P.M. Vazquez	AY725577
Suberoteratosphaeria pseudosuberosa	**CBS 118911**	*Eucalyptus* sp.	Uruguay	M.J. Wingfield	DQ303011
Suberoteratosphaeria suberosa	**CPC 515**	*E. dunnii*	Brazil	M.J. Wingfield	AY725579
Xenoteratosphaeria jonkershoekensis	**CBS 122897**	*Protea* sp.	South Africa	P.W. Crous & L. Mostert	EU707864

(1) Ex-type cultures **on bold;**
CBS culture collection of Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, The Netherlands;
CPC Culture collection of P.W. Crous, housed at CBS;
CMW culture collection of M.J. 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, now housed at **LISFA** Herbarium Code of collection of UEIS-SAFSV (Herbarium Code of Unit for Research and Services on Agricultural and Forestry Systems and Plant Health, I.P., Oeiras, Portugal);
MUCC Murdoch University culture collection, Australia;
STE-U culture collection of the Department of Plant Pathology, Stellenbosch University, South Africa.
(a)these sequences are exactly identical in this study (within same species)
(b) these 2nd group of sequences are exactly identical in this study (within same species)
(c) these 3rd group of sequences are exactly identical in this study (within same specie)

DNA isolation and amplification

Genomic DNA was isolated from fungal mycelium collected directly from MEA plates using the isolation protocol of DNeasy Plant Mini Kit (Qiagen GmbH, Germany) following the manufacturer's instructions. The ITS1-5.8S-ITS2 cluster was amplified with primers ITS1 and ITS4 (White *et al.*, 1990). PCR conditions were as described by Crous *et al.*, 2004). A negative control consisting of MilliQ water instead of template DNA was included in each set of reactions. Purification of amplification products of PCR was prepared with a GeneJET PCR Purification kit (Fermentas). The PCR reaction conditions and sequencing reactions were according to the procedure described by Silva *et al.* (2009).

Phylogenetic analyses

ITS sequences of the isolates obtained in this study were checked with BioEdit version 7.0.5.3 (Hall, 1999), compiled with ITS sequences retrieved from GenBank, aligned with Clustal X v. 2.0 (Thompson *et al.*, 1997), and manual adjustments were made where necessary. The phylogenetic analyses included sequences of 65 isolates from this study and 56 sequences retrieved from GenBank including the outgroup (Table 3.1). All sequences generated in this study were deposited in GenBank (Table 3.1).

Maximum likelihood analyses (ML) were made with RAxML-HPC BlackBox v. 7.3.2 (Stamatakis, 2006; Stamatakis *et al.*, 2008) run on the CIPRES Science Gateway V. 3.2 (Miller *et al.*, 2010) selecting the gamma model of rate heterogeneity.

Results

Phylogenetic analyses

Amplicons of approximately 640 bases were obtained for the isolates listed in Table 3.1 and the ITS alignment contained 121 sequences, including the outgroup sequence (*Neofusicocum parvum*). Incomplete parts at the start and end of the sequences were excluded from the analyses.

The ML tree (Fig. 3.1) could be resolved into four major clades. Clade 2 included several well-supported sub-clades corresponding to individual species. Clade 2 comprised 13 species of

Teratosphaeria. Three known species (*T. molleriana, T. nubilosa, T. pluritubularis*) were found in Portugal. Morphologically these isolates correlated well with previous descriptions (Carnegie and Keane, 1998; Crous, 1998; Crous *et al.*, 2006). Three isolates formed a well-supported (100%) subclade that was not associated with any known species and the name *T. lusitanica* is introduced to accommodate them.

The third clade also included several well-supported sub-clades corresponding to *Quasiteratosphaeria mexicana* (100%) *Neodevriesia hilliana* (100%) *Teratosphaericola pseudafricana* (100%). The fourth main clade represented mostly *Austroafricana parva* with 87% bootstrap support. The morphology of all these isolates agree with the descriptions of *A. parva* by Park and Keane (1982a, 1982b). The topology of the tree was similar to that of the one published by Quaedvlieg *et al*. (2014) except for *Parateratosphaeria bellula*.

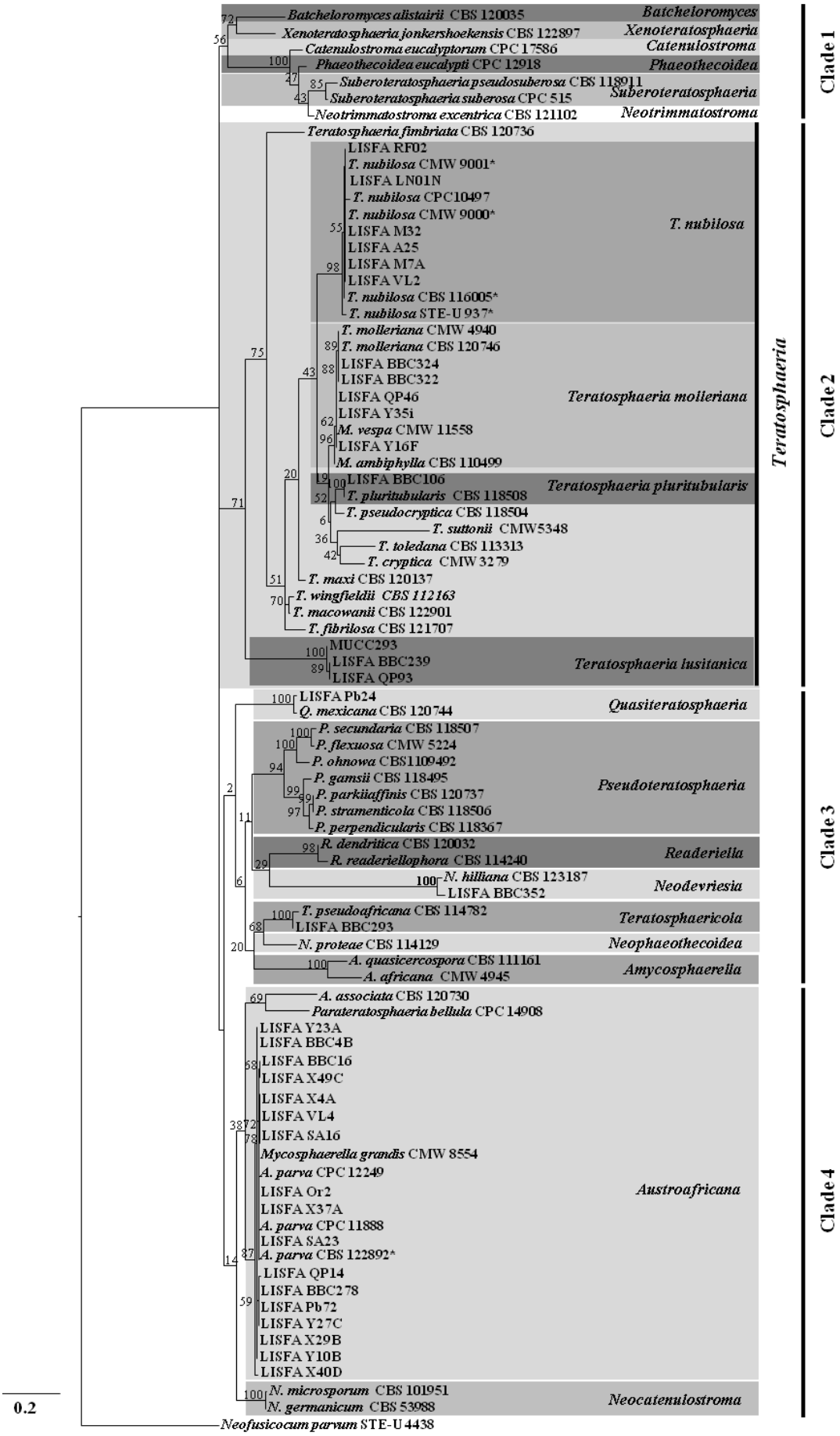

Fig. 3.1 Maximum likelihood (ML) tree constructed from ITS sequence data with RAxML selecting the gamma model of rate heterogeneity for containing all isolates related with Teratosphaeria leaf disease of *Eucalyptus*. Maximum Likelihood bootstrap value is given at the nodes. The scale bar shows 0.2 changes. Ex-type strains are indicated with a (*).

Taxonomy

This study presents a new species, *Teratosphaeria lusitanica* and a first report of *Neodevriesia hilliana* on *Myrtaceae*. New reports from the Iberian Peninsula are *Q. mexicana*, *T. pseudafricana and T. pluritubularis,* the last one only for Portugal. Some new combinations are made of *Teratosphaeria mexicana* and *Teratosphaeria quasicercospora* based of tree topology, which are changed the genera to *Quasiteratosphaeria and Amycosphaerella* respectively (Fig. 3.1).

Amycosphaerella quasicercospora (Crous & T.A. Coutinho) Silva, Machado & A.J.L. Phillips *comb. nov.*

≡ *Mycosphaerella quasicercospora* Crous & T.A. Cout., Studies in Mycology 55: 119 (2006)

≡ *Teratosphaeria quasicercospora* (Crous & T.A. Cout.) Crous & U. Braun, Studies in Mycology 58: 11 (2007)

Austroafricana parva (R.F. Park & Keane) Quaedvlieg & Crous, Persoonia 33: 25 (2014) - MycoBank MB 807796

≡ *Mycosphaerella parva* R.F. Park & Keane, Transactions of the British Mycological Society 79 (1): 99 (1982).

= *Mycospherella grandis* Carnegie & Keane, Mycological Research 98: 414 (1994)

≡*Teratosphaeria parva* (R.F. Parke & Keane) Crous & U. Braun, Studies in Mycology 58: 10 (2007)

Colonies sporulation on MEA. *Conidia* in simple or branched basipetal chains, straight to flexuous, sub cylindrical, (10.6–)14.5–21.6(–22.2) x (4.0–)4.2–5.8(–6.0) µm, 2–4 transversely septate, hyaline to light green, thick-walled, verruculose. *Culture characteristics*, colonies on MEA reaching 8–24 mm diam., after 1 month at 24°C in the dark, mycelium olive, colonies erumpent, reverse green.

Specimens examined. Portugal, Lisbon, Torres Vedras, Furadouro, on young leaf of *E. globulus*, H. Machado, Spring 2005, cultures EFN Y10A and EFN Y27D; Portugal, Aveiro, Albergaria, on young leaf of *E. globulus*, Spring 2005, cultures EFN X15A and EFN X21A.

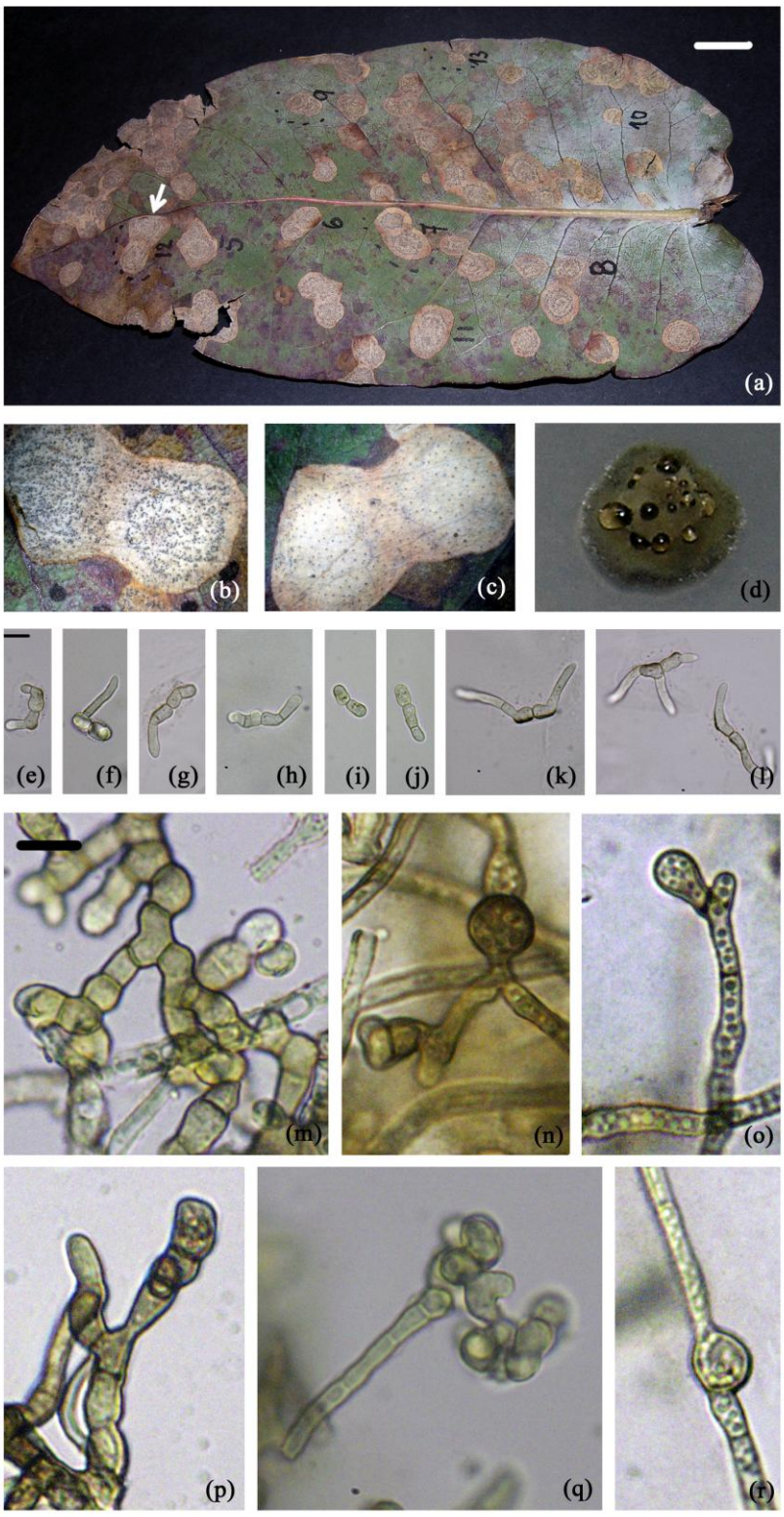

Fig. 3.2 ***Austroafricana parva*****: (a)** Leaf spot **(**abaxial surface) isolation site (white arrow) from *Eucalyptus globulus* **(b)** Lesion from abaxial leaf surface **(c)** Lesion from adaxial leaf surface **(d)** Culture on MEA at 25 °C in the dark after 30 days **(e–l)** Germinating ascospores on MEA **(m–r)** Conidiogenous and conidia, scale bar: **(a)** = 1 cm, **(e–l)** and **(m–r)** = 10µm.

Neodevriesia hilliana (Crous & U. Braun) Quaedvlieg & Crous, Persoonia 33: 24 (2014) - MycoBank MB 807768 Fig. 3.3.

Devriesia hilliana Crous & U. Braun, Studies in Mycology 64: 37 (2009) - MycoBank

Colonies sporulating on MEA. *Conidia* (5.0–)6.3–9.1(–9.9) x (3.7–)3.7–4.8(–5.2) µm, 1–3 transversely septate, hyaline to pale brown, smooth, ellipsoidal, apical conidium. *Culture characteristics*, colonies on MEA reaching 17–22 mm diam. after 1 month at 24°C in the dark, colonies erumpent, regular, surface pale brown to dark brown smooth, sparse aerial mycelium in the central part, reverse dark brown.

Specimen examined. Portugal, Lisbon, Torres Vedras, Bogalheira, on young leaf of *E. globulus*, L. Neves, 14 May 2010, cultures LISFA BBC352.

Notes: This species is reported for the first time on *E. globulus*.

Quasiteratosphaeria mexicana (Crous) Silva, Machado & A.J.L. Phillips *comb. nov.*, Fig. 3.4

≡ *Mycosphaerella mexicana* Crous, Mycologia Memoirs 21: 81 (1998)

≡ *Teratosphaeria mexicana* Crous & U. Braun, Studies in Mycology 58: 10 (2007)

Colonies sporulating on MEA. *Conidia* in simple or branched basipetal chains, straight to flexuous (4.9–)5.2–8.0(–9.8) x (2.72–)3.2–5.0(–5.3) µm, 2–5 transversely septate, hyaline to light brown, in some cases forming a lateral conidium at the tip. *Culture characteristics*, colonies on MEA reaching 16–17 mm diam. after 1 month at 24°C in the dark, dark brown to dark grey, dark brown on reverse.

Specimen examined. Portugal, Leiria, Bombarral, Quinta do Pisão, on young leaf of *E. globulus*, M.R.C. Silva, 28 Jan 2009, cultures LISFA QP13.

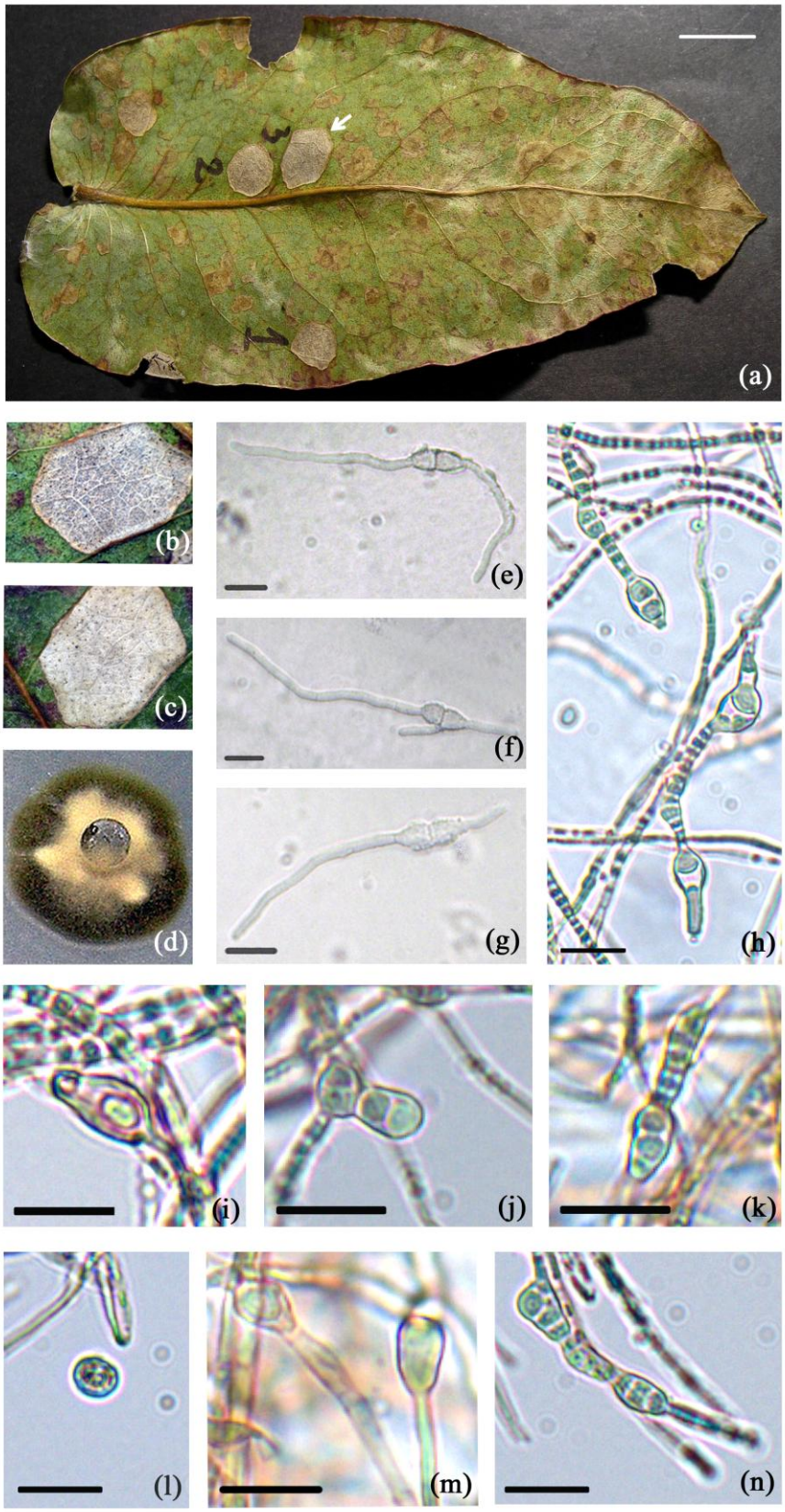

Fig. 3.3 ***Neodevriesia hilliana*****: (a)** Leaf spot **(**abaxial surface) isolation site (white arrow) from *Eucalyptus globulus* **(b)** Lesion from abaxial leaf surface **(c)** Lesion from adaxial leaf surface **(d)** Culture on MEA at 25 °C in the dark after 30 days **(e–g)** Germinating ascospores on MEA **(h–n)** Conidiogenous and conidia, scale bar: **(a)** = 1 cm, **(e–n)** = 10µm.

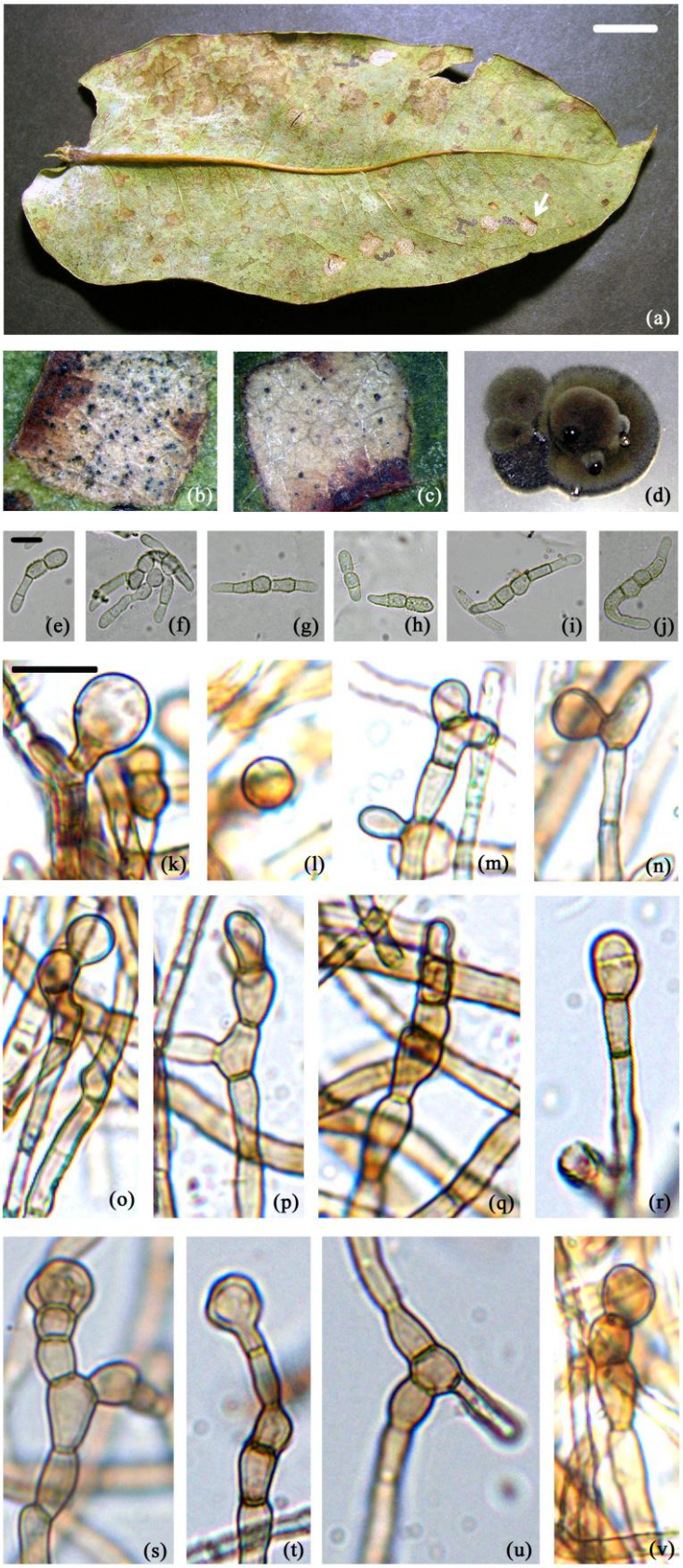

Fig. 3.4 ***Quasiteratosphaeria mexicana*****: (a)** Leaf spot **(**abaxial surface) isolation site (white arrow) from *Eucalyptus globulus* **(b)** Lesion from abaxial leaf surface **(c)** Lesion from adaxial leaf surface **(d)** Culture on MEA at 25 °C in the dark after 30 days **(e–j)** Germinating ascospores on MEA **(h–n)** Conidiogenous and conidia, scale bar: **(a)** = 1 cm, **(e–j)** and **(k–v)** = 10µm.

Teratosphaeria lusitanica Silva, Machado & AJL Phillips *sp. nov.*, Fig. 3.5.

Etymology. Named after the Roman province of Lusitania, which includes most of present day Portugal.

Colonies sporulating on MEA. *Conidia* branched basipetal chains, sub cylindrical, straight to flexuous, medium to dark brown, thick-wall, smooth, transversely septate with some times, 1 oblique septa, (7.3–)8.0–12.4(–15.6) x (4.6–)6.2–8.9(–9.4) µm. *Culture characteristics*, colonies on MEA erumpent, irregular, surface black brown (marron), smooth, sparse aerial mycelium,. margins catenate, dark brown reverse, reaching 29–34 mm diam., after 4 wk on MEA, at 24°C in the dark.

Specimen examined. Portugal, Lisbon, Torres Vedras, Bogalheira, on young leaf of *E. globulus*, L. Neves, 25 Feb 2010, holotype, a dried culture of LISFA BBC239, cultures LISFA BBC239 (ex-type); Portugal, Leiria, Bombarral, Quinta do Pisão, on young leaf of *E. globulus*, M.R.C. Silva, 10 May 2010, cultures LISFA QP93.

Teratosphaeria pluritubularis (Crous & Mansilla) Crous & U. Braun, Studies in Mycology 58: 10 (2007). - MycoBank MB 504490, Fig. 3.6.

≡ *Mycosphaerella pluritubularis* Crous & Mansilla, Studies in Mycology 55: 114 (2006).

Colonies sporulating on MEA. *Conidia***,** hyaline to pale brown, thick-walled, ellipsoidal to oval, becoming transversely 1-septate (5.6–)6.1–7.6(–8.2) x (3.02–)3.2–3.7(–3.95)µm. *Culture characteristics*, colonies on MEA reaching 27–35 mm diam., after 1 month at 24°C in the dark, colonies erumpent, light gray at the centre with sparse aerial mycelium to dark gray at the margin, margin irregular, dark brown to medium brown in the margins on the reverse.

Specimen examined. Portugal, Lisbon, Torres Vedras, Bogalheira, on young leaf of *E. globulus*, 25 Feb 2010, L. Neves, cultures LISFA BBC106; L. Neves, 14 May 2010, cultures LISFA BBC317.

Notes: New report in Portugal.

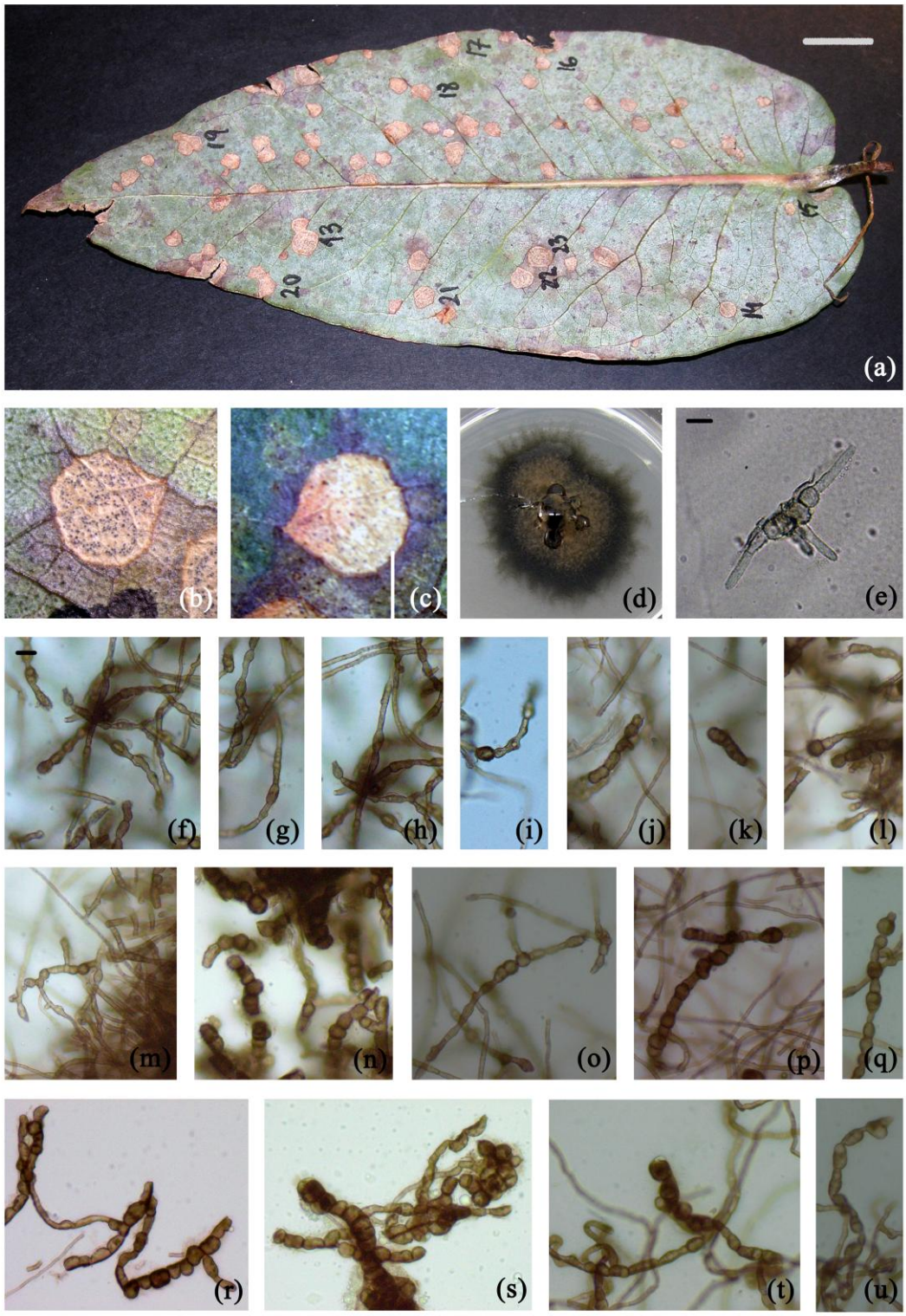

Fig. 3.5 ***Teratosphaeria lusitanica*****: (a)** Leaf spot **(**abaxial surface) from *Eucalyptus globulus* **(b)** Lesion from abaxial leaf surface **(c)** Lesion from adaxial leaf surface **(d)** Culture on MEA at 25 °C in the dark after 30 days **(e)** Germinating ascospore on MEA **(f–u)** Conidiogenous and conidia, scale bar: **(a)** = 1 cm, **(e)** and **(f–u)** = 10µm.

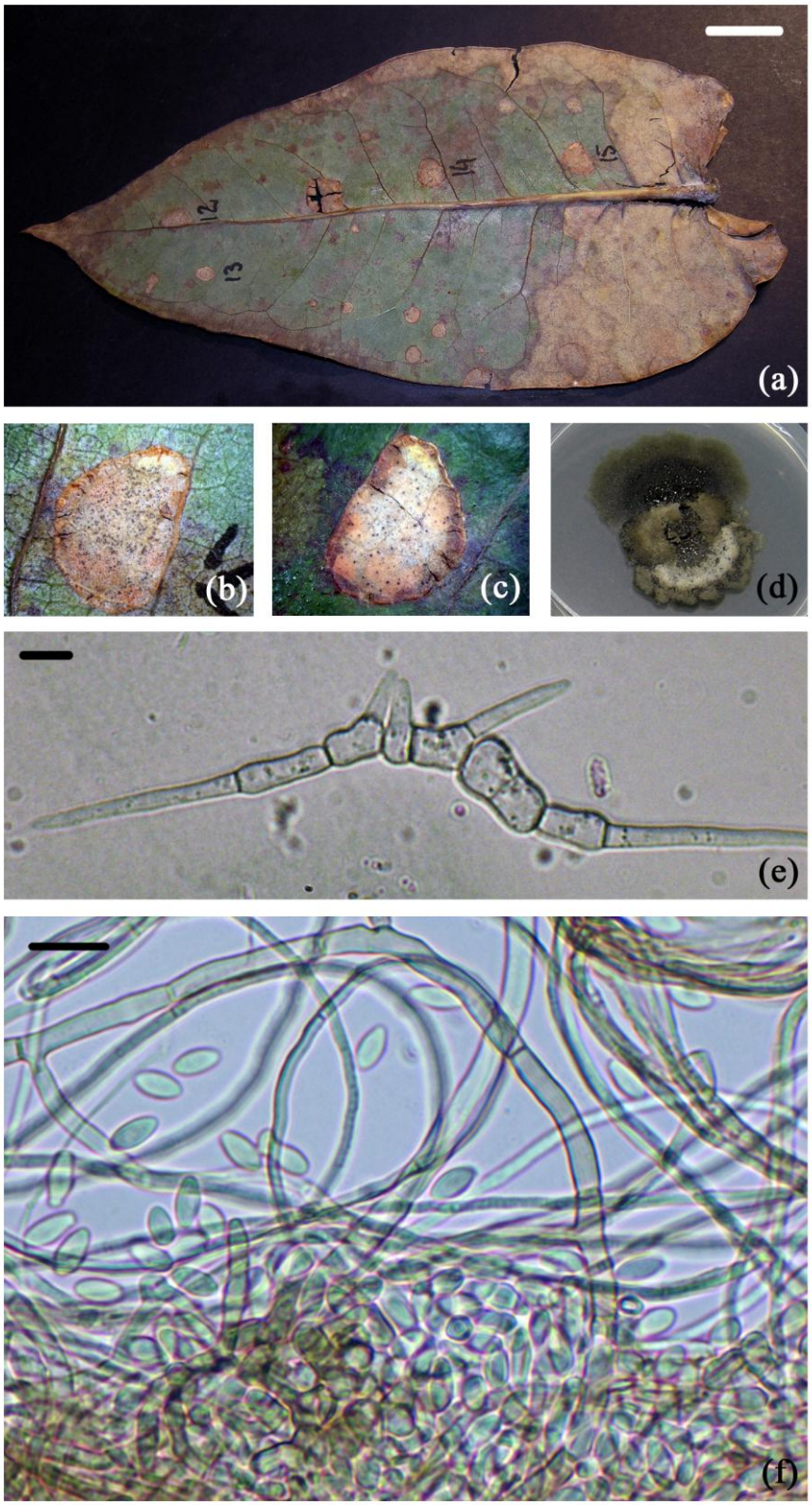

Fig. 3.6 ***Teratosphaeria pluritubularis*****: (a)** Leaf spot **(**abaxial surface) from *Eucalyptus globulus* **(b)** Lesion from abaxial leaf surface **(c)** Lesion from adaxial leaf surface **(d)** Culture on MEA at 25 °C in the dark after 30 days **(e)** Germinating ascospore on MEA **(f)** Conidia, scale bar: **(a)** = 1 cm, **(e)** and **(f)** = 10µm.

Teratosphaeria pseudoafricana (Crous & T.A. Cout.) Crous & U. Braun, Studies in Mycology 58: 11 (2007), Fig. 3.7.

≡ *Mycosphaerella pseudoafricana* Crous & T.A. Cout. Studies in Mycology 55: 115 (2006).

Colonies sporulating on MEA. *Conidia* in simple or branched basipetal chains, subcylindrical, (6.1–)6.4–8.9(–9.9) x (4.3–)4.6–8.4(–10.2) µm, 2–3 transversely septate, hyaline to brown, thick-walled. *Culture characteristics*, colonies on MEA reaching 29–30 mm diam., after 1 month at 24°C in the dark, erumpent, dark brown at the surface with aerial mycelium and dark brown on the reverse.

Specimen examined. Portugal, Lisbon, Torres Vedras, Bogalheira, on young leaf of *E. globulus*, L. Neves, 14 May 2010, cultures **LISFA BBC293**.

Notes: New report in Portugal

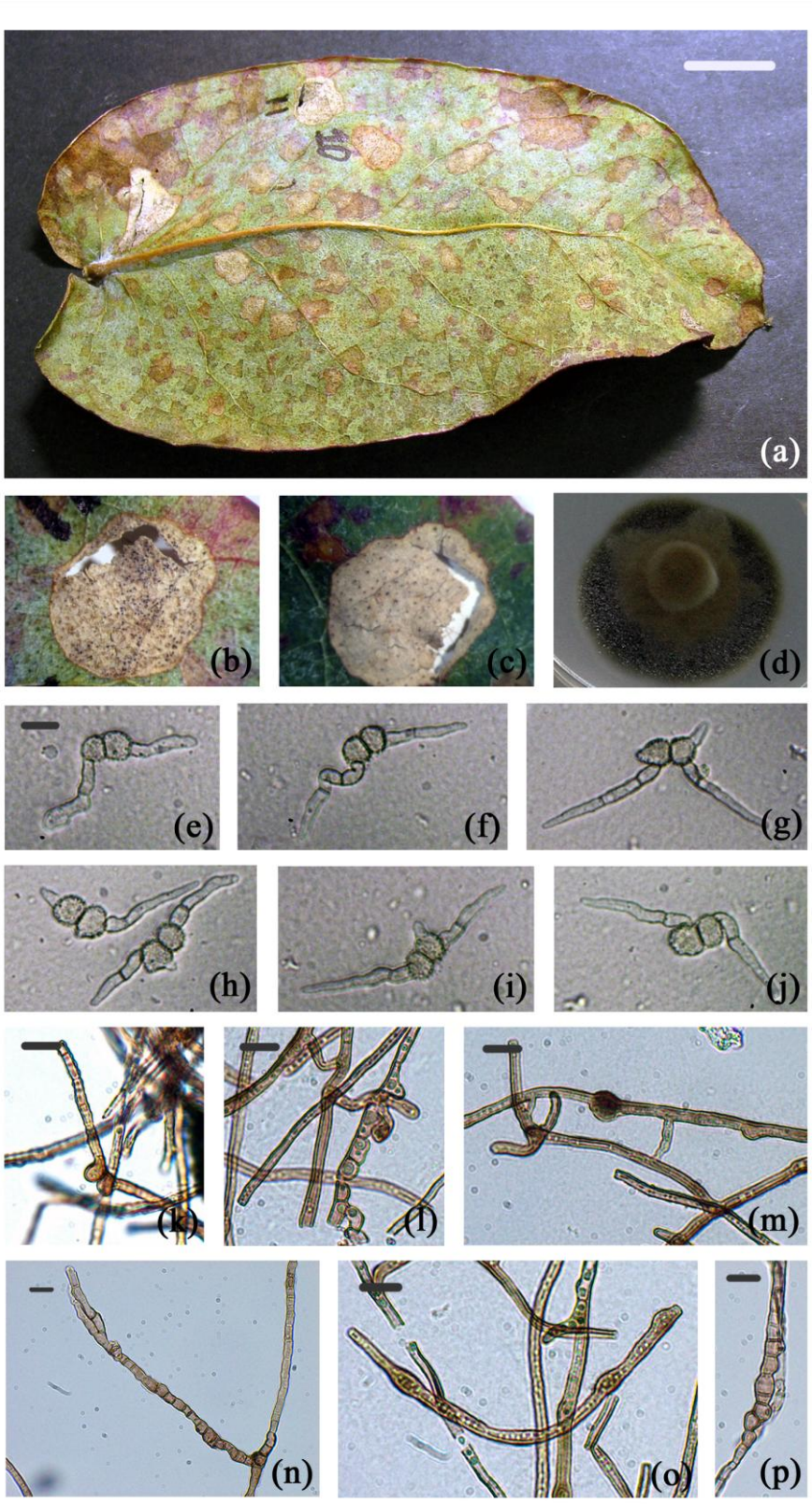

Fig. 3.7 ***Teratosphaericola pseudoafricana*****: (a)** Leaf spot **(**abaxial surface) from *Eucalyptus globulus* **(b)** Lesion from abaxial leaf surface **(c)** Lesion from adaxial leaf surface **(d)** Culture on MEA at 25 °C in the dark after 30 days **(e–j)** Germinating ascospore on MEA **(k–p)** Conidia, scale bar: **(a)** = 1 cm, **(e–j)** and **(k–p)** = 10µm.

Discussion

In the present study five species of *Teratosphaeriaceae* are reported for the first time from Portugal. These are *N. hilliana, Q. mexicana, T. pluritubularis, T. pseudoafricana* and a new species, *Teratosphaeria lusitanica*. With these reports a total of nine species are now known to be associated with leaf spots on *Eucalyptus* in Portugal. In addition, new anamorphic structures are reported for *A. parva, T. pluritubularis, Q. mexicana, T. pseudoafricana* and the descriptions of these species are emended.

In 2004, a phylogenetic analysis based on ITS sequence data revealed that two species originally described as *Cladosporium* (*C. staurophorum, C. chlamydosporis*) and three new species (*Devriesia acadiensis*, *D. thermodurans*, *D. shelburniensis*) formed a monophyletic group that was marginal in the *Mycosphaerellaceae* but phylogenetic distinct from *Cladosporium* sensu strict (Seifert *et al*., 2004). The genus *Devriesia* (*Teratosphaeriaceae*) was introduced for these species and was characterized for not being linked to *Pseudocladosporium*-like (= *Fusicladium*; *Venturiaceae*) but were morphologically close (Crous *et al.,* 2007a). *Devriesia* were regarded as heat-resistant and characterized by simple or branched conidiophores and the formation of abundant chlamydospores in culture. They are predominantly soil-borne and shown to be paraphyletic (Seifert *et al.*, 2004, Crous *et al.*, 2007b). A subsequent phylogenetic analysis of LSU sequences showed that it is paraphyletic and represents several lineages of which only *Devriesia s.str*. is thermo-tolerant (Frank *et al.*, 2010).

Since its initial description 20 species have been described in *Devriesia* and in *Neodevriesia* (Seifert *et al.*, 2004; Crous *et al*., 2007b, 2009b, 2010a, 2010b, 2011b, 2012b; Arzanlou *et al*., 2008; Frank *et al*., 2010; Crous & Groenewald, 2011a, 2012a; Li *et al*., 2013) (Table 3.2). In 2014, Quaedvlieg *et al.* (2014) divided *Devriesia* into two genera, (1) *Neodevriesia*, a folicolous, saprobic or plant pathogenic genus and (2) *Extremus* to accommodate fungal species isolated from rocks. In this study *N. hilliana* was found for the first time on a new host, *Myrtaceae*, namely *E. globulus*.

Quasiteratosphaeria mexicana was first reported from Mexico in 1986 on leaves of *Eucalyptus* sp. (Crous, 1998), in Australia (2000) on older juvenile leaves (*E. globulus*), occurring alone or with a combination of *T. cryptica*, *Paramycosphaerella marksii*, *T. nubilosa* or *A. parva* on the same lesion (Maxwell *et al.,* 2003) in Hawaii (2005) occurring on older lesions with *Aulographina eucalypti*, on *Eucalyptus* leaves (Crous *et al*., 2007c), in Uruguay (Pérez *et al.,* 2009) and now in Portugal as recorded in this work. Due to its position in the phylogenetic tree in this study it was transferred to *Quasiteratosphaeria* genus.

Table 3.2 The *Devriesia* and *Neodevriesia* species and their hosts.

Species	Substrate	References
Devriesia acadiensis	heat-treated soil	Seifert *et al.*, 2004
D. agapanthi	*Agapanthus africanus*	Crous & Groenewald, 2012a
D. americana	isolated from air	Crous *et al.*, 2007b
D. chlamydospora	on decaying *Pinus sylvestris* needle	Seifert *et al.*, 2004
D. ficus	rubber trees (*Ficus elastica*)	Li *et al.*, 2013
D. fraseriae	on a *Myrtaceae*, leaves of *Melaleuca* sp.	Crous *et al.*, 2010b
D. imbrexigena	glazed decorative tiles	Crous *et al.*, 2012b
D. lagerstroemiae	on *Lagerstroemia indica*	Crous *et al.*, 2009b
D. pseudoamericana	Sooty blotch and flyspeck on apples	Frank *et al.*, 2010
D. queenslandica	on leaves of *Scaevola taccada*	Crous *et al.*, 2011b
D. shakazului	from *Aloe*	Crous *et al.*, 2012b
D. shelburniensis	heat-treated soil	Seifert *et al.*, 2004
D. staurophora	on decaying *Pinus sylvestris* needle	Seifert *et al.*, 2004
D. stirlingiae	*Stirlingia*	Crous *et al.*, 2012b
D. strelitziae	on leaves of wild banana	Arzanlou *et al.*, 2008
D. strelitziicola	on leaves of *Strelitzia* sp.	Crous *et al.*, 2009b
D. tardicrescens	on leaf bracts of *Phaenocoma prolifera*	Crous & Groenewald, 2011a
D. thermodurans	heat-treated soil	Seifert *et al.*, 2004
Neodevriesia hilliana ≡ *D. hilliana*	on Australian cycad (*Macrozamia communis*); *Eucalyptus globulus*	Crous *et al.*, 2009b; in this study
N. xanthorrhoeae ≡ *D. xanthorrhoeae*	on leaves of *Xanthorrhoea australis* (*Xanthorrhoeaceae*)	Crous *et al.*, 2010a

Teratosphaeria pluritubularis was first observed in Spain, on leaves of *E. globulus*, in 2004 (Crous *et al.*, 2006), recently in South America (Uruguay) (Pérez *et al.*, 2009) and in New South Wales and Queensland, Australia (Carnegie *et al.*, 2011) and now reported on *E. globulus* in Portugal.

Teratosphaeriopsis pseudoafricana was first isolated in 1995 in Zambia, on leaves of *E. globulus* (Crous *et al.*, 2006) and later in South Brazil (Teodoro *et al.*, 2012). Here we report it from *E. globulus* in Portugal.

In summary, five new records and a new species were added, new anamorphic structures were observed and a new host was found in Portugal. It is expected that incidence and severity of this disease will increase particularly in favorable climate conditions and a possible swap of genetic material when several species colonize the same lesion and also by biological material is exchanged globally.

Acknowledgements

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

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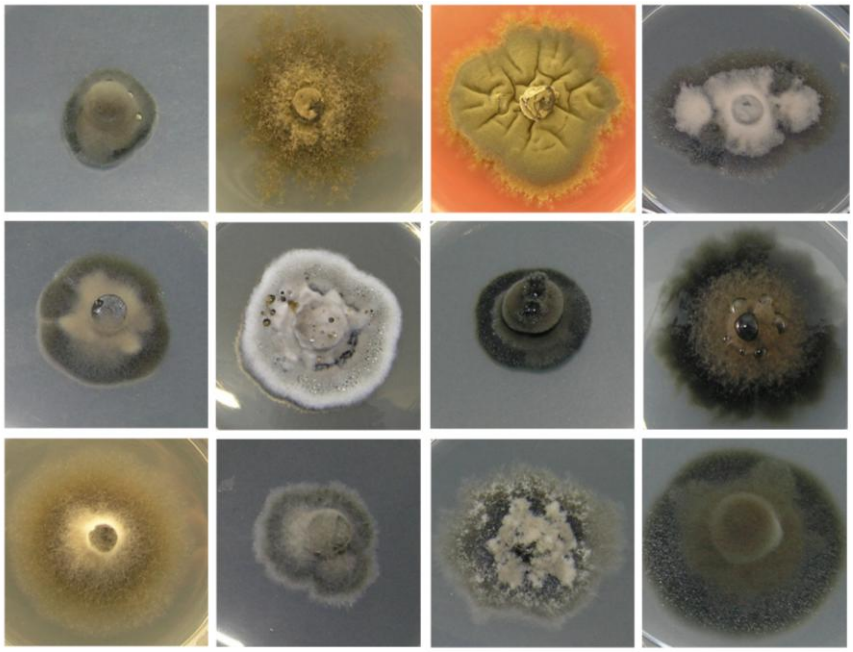
CHAPTER 4

Species in the Eucalyptus Leaf Disease Complex in Portugal



Chapter 4

RESEARCH ARTICLE

Species in the Eucalyptus Leaf Disease Complex in Portugal

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Summary

Several foliar fungi on eucalypts are associated to *Mycosphaerella* s.l., causing leaf spots and significant productivity losses. The relative frequency of species in *E. globulus* plantations was evaluated during several seasons, its combinations in lesions and the relation of the complex composition with disease severity were considered. Morphological and molecular characterization of the species were used to give an indication of the main species in the complex. *Teratosphaeria nubilosa* and *Austroafricana parva* represented 58.3% of all species occurrences followed by a small group of species comprising *Mycosphaerella communis*, *M. lateralis*, *Paramycosphaerella marksii* and *T. molleriana* with 36.6% and also by several species in *Cladosporium*, *Mycosphaerella*, *Fusicladium*, *Neodevriesia*, *Quasiteratosphaeria*, *Teratosphaeria* and *Teratosphaericola* which made up 5.1% of all species occurrences in lesions on symptomatic leaves of *Eucalyptus globulus*. New reports were identified for the first time on the Iberian Peninsula in *Eucalyptus globulus* such as *Cladosporium cladosporioides*, *Fusicladium eucalypti*, *Mycosphaerella madeirae* (first report from mainland Portugal), and an unidentified *Venturiaceae*.

Keywords

Ascomycetes; *Dothideomycetes*; *Mycosphaerella*; *Teratosphaeria*; Leaf spots; eucalypts; species combinations.

Introduction

The most serious foliar disease in eucalypts is associated with species in *Mycosphaerella* s.l. causing leaf necrosis, which results in significant productivity losses. In eucalypts the incidence and severity of the disease is higher in juvenile than in adult stages of the leaf (Dungey *et al.*, 1997) and *Teratosphaeria cryptica* and *T. nubilosa* that in various studies on pathology and epidemiology (Park & Kean, 1984, 1982a, 1982b) are considered the most virulent species. The Eucalyptus Leaf Disease Complex has caused widespread damage in several regions including Australasia, South America, Western Europe, Southern Africa and South-East Asia (Park *et al.*, 2000). Table 4.1 compares reports from several countries of the most commonly foliar fungi in the field.

Some species of *Mycosphaerella* s.l. infect juvenile leaves acting as a primary pathogens parasitizing living tissues, but they can have a part of their life cycle on dead leaves. Thereby several species linked to this genus can be found in leaf litter such as *Austroafricana parva*, *Mycosphaerella fori*, *Paramycosphaerella marksii*, *Teratosphaeria molleriana* and even *T. nubilosa* (Márquez *et al*., 2011).

Symbiotic interactions between fungi and plants can diverge from mutualism to parasitism (Kogel *et al.*, 2006). Several species can occupy the same blighted lesion (Crous *et al.*, 2004, 2009; Hunter *et al.*, 2006; Silva *et al.*, 2009). *A. parva* is considered to be saprobic and is always found in association with older lesions caused by *T. nubilosa* or other species, which is only found alone on young blighted lesions (Park and Keane, 1982b; Milgate *et al.*, 2001; Maxwell, 2004; Hunter *et al.*, 2008; Pérez C. *et al.*, 2009; Silva *et al.*, 2009). Thus, the mechanisms permitting them to inhabit the same niche are not yet understood (Crous *et al.*, 2009). The aim of this work is to evaluate the relative frequency of species in *E. globulus* plantations throughout the year, their combinations in lesions to relate the complex composition with the level of the disease severity.

Materials and methods

During the autumn of 2009 and the autumn of 2010, five young *Eucalyptus globulus* plantations (1–2 years old) were surveyed throughout the country in regions that have suffered significant outbreaks of ELDC. Symptomatic juvenile leaves were collected randomly from ten trees per plantation and approximately ten leaves were selected from each tree. The same trees were used for subsequent leaf collections in autumn, winter, early spring and spring.

Symptomatic juvenile leaf lesions were examined with a stereomicroscope and characterized on the basis of colour, dimensions and shape. Each lesion was characterized by the number and identity of species present. Severity of leaf disease was evaluated as the percentage of necrotic leaf surface area (Milgate *et al*., 2001) on six severity levels: (1) 0–6%; (2) 7–12%; (3) 13–25%; (4) 26–50%; (5) 51–75%; (6) ≥75 %. A matrix of species presence/absence in lesions, per plantation and season was constructed and analysed by Kruskal-Wallis ANOVA and Median test implemented in STATISTICA 6.1.

Table 4.1 Reports of the most commonly found foliar fungi in the field for several countries.

Country	Species reported	References
Australia	***Austroafricana parva***	Park and Keane 1982a
	Aulographina eucalypti	Park *et al.*, 2000
	Paramycosphaerella marksii	Maxwell, 2004
	Quasicercospora mexicana	Carnegie *et al.*, 2011
	Teratosphaeria cryptica	
	Teratosphaeria molleriana	
	Teratosphaeria nubilosa	
	Teratosphaeria suttonii	
	Suberoteratosphaeria suberosa	
Brazil	***Mycosphaerella lateralis***	Alfenas *et al.*, 2004
	Paramycosphaerella marksii	Perez G. *et al.*, 2009
	Phaeophleospora scytalidii	Teodoro, 2010
	Pseudoteratosphaeria perpendicularis	Teodoro *et al.*, 2012
	Teratosphaeria flexuosa	
	Teratosphaeria nubilosa	
	Teratosphaeria ohnowa	
	Teratosphaeria suttonii	
	Teratosphaericola pseudoafricana	
	Suberoteratosphaeria suberosa	
	Zasmidium parkii	
Portugal	***Austroafricana parva***	Silva *et al.*, 2008, 2009, 2012
	Mycosphaerella communis	this work
	Mycosphaerella lateralis	
	Paramycosphaerella marksii	
	Teratosphaeria molleriana	
	Teratosphaeria nubilosa	
South Africa	***Teratosphaeria nubilosa***	Purnell and Lundquist, 1986
		Crous *et al.*, 1989a
Spain	***Austroafricana parva***	Tejedor, 2007
	Mycosphaerella communis	Aguin *et al.*, 2013
	Mycosphaerella lateralis	Manzilla *et al.*, 2013
	Paramycosphaerella marksii	
	Pseudocercospora eucalyptorum	
	Teratosphaeria cryptica	
	Teratosphaeria molleriana	
	Teratosphaeria nubilosa	
	Teratosphaeria readeriellophora	
	Sonderhenia eucalypticola	

The most frequent species in Portugal are in **bold.**

Morphological characterization

Ascospores were discharged from ascomata onto agar plates (2% (w/v) malt extract agar (MEA). Germination patterns of ascospores were analysed at a magnification of × 600 every 24 h for up to 120 h. About 30 ascospores measurements were obtained for each taxonomically informative structure. Linear growth was assessed after 1 month at 24 ºC in the dark on 2% (w/v) MEA and colony colours of the top and reverse surface were recorded (Crous, 1998). Single ascospore cultures were grown on MEA slopes at 24 °C in the dark and deposited in the culture collection of LISFA (Herbarium Code of Unidade Estratégica de Investigação e Serviços, Sistemas Agrários e Florestais e Sanidade Vegetal, Instituto Nacional de Investigação Agrária e Veterinária, I.P., INIAV, Oeiras, Portugal). Observations of micro morphological features were made with an Olympus BX41TF microscope with bright field illumination. Digital images were recorded with a ProgRes Speed XT Core 5 camera. Measurements were made with the ProgRes Capture Pro 2.8.8 Jenoptik. Isolates were grouped into morphotypes, according to micro and macroscopic characteristics of the germination pattern and mycelia. Subsequently, several isolates of each morphotype were identified by DNA sequence analysis.

Molecular characterization

Molecular characterization of single-spore isolates was based on a sequence analysis of ITS1-5.8S-ITS2 cluster and 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). All the unclear isolates morphological characterized were sequenced on to validate the species. Genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen GmbH, Germany) following the manufacturer’s instructions. The PCR amplification products were purified using JETquick spin column (Genomed GmbH) and the PCR reaction conditions and sequencing reactions were according to the procedure described by Silva *et al.* (2009).

All sequences were checked with BioEdit version 7.0.5.3 (Hall 1999) and manual adjustments were made where necessary. The phylogenetic analyses included sequences of 33 isolates from this study and 23 sequences retrieved from GenBank including the outgroup (Table 4.2) and aligned with Clustal X (Thompson *et al.,* 1997). Maximum likelihood analyses (ML) were conducted in CIPRES Science Gateway V. 3.2 (Miller *et al.,* 2010) using RAxML-HPC BlackBox v. 7.3.2 (Stamatakis 2006; Stamatakis *et al.,* 2008) selecting the gamma model of rate heterogeneity.

Table 4.2 Isolates included in the phylogenetic analyses.

Species	Strain no.[1]	Host	Country	Collector	GenBank
Austroafricana parva	**CBS 122892**	*E. globulus*	Australia	I. Smith	**EU707875**
≡*M. grandis*	CPC 12249	*Eucalyptus globulus*	Portugal	A.J.L. Phillips	GQ852820
	CPC 11888	*Eucalyptus* sp.	Portugal	A.J.L. Phillips	DQ303005
	CMW 8554	*E. globulus*	Chile	M.J. Wingfield	DQ267584
	LISFA NX7B	*E. globulus*	Portugal	H. Machado	
	LISFA BBC170	*E. globulus*	Portugal	L. Neves	
	LISFA X27A	*E. globulus*	Portugal	H. Machado	
	EFN X15A	*E. globulus*	Portugal	H. Machado	
	LISFA QP84	*E. globulus*	Portugal	M.R.C. Silva	
	EFN X50B	*E. globulus*	Portugal	H. Machado	
	LISFA Y27A	*E. globulus*	Portugal	H. Machado	
	LISFA Y27D1	*E. globulus*	Portugal	H. Machado	
	LISFA PB77	*E. globulus*	Portugal	L. Neves	
Cladosporium cladosporioides	LISFA Pb15	*E. globulus*	Portugal	L. Neves	
Fusicladium eucalypti	LISFA BBC264	*E. globulus*	Portugal	L. Neves	
Mycosphaerella communis	CBS 114238		Spain	-	AY725541
	LISFA A78		Portugal	M.R.C. Silva	
	LISFA NX30A		Portugal	H. Machado	
	LISFA BBC235		Portugal	L. Neves	
	LISFA QP58C		Portugal	M.R.C. Silva	

Table 4.2 Isolates included in the phylogenetic analyses (Cont.).

Species	Strain no.[1]	Host	Country	Collector	GenBank
Mycosphaerella lateralis	STE-U-825	*Eucalyptus sp*	Zambia	-	AF309625
	STE-U-1233				AF309624
	LISFA A105	*E. globulus*	Portugal	M.R.C. Silva	
	LISFA BBC206	*E. globulus*	Portugal	L. Neves	
	LISFA BBC240	*E. globulus*	Portugal	L. Neves	
	LISFA QP50	*E. globulus*	Portugal	M.R.C. Silva	
	LISFA X13A	*E. globulus*	Portugal	H. Machado	
Mycosphaerella madeirae	LISFA BBC198	*E. globulus*	Portugal	L. Neves	
	LISFA Pb4	*E. globulus*	Portugal	L. Neves	
	CBS 112895				AY725553
Neodevriesia hilliana	CPC 15382	*Macrozamia communis*	New Zealand		GU214633
	LISFA BBC352	*E. globulus*	Portugal	L. Neves	
Neofusicocum parvum	STE-U 4438				AY343467
Paramycosphaerella marksii	STE-U-982		South Africa -	-	AF309589
	LISFA BBC241	*E. globulus*	Portugal	L. Neves	
	LISFA QP38	*E. globulus*	Portugal	M.R.C. Silva	
	LISFA QP40	*E. globulus*	Portugal	M.R.C. Silva	
Quasiteratosphaeria mexicana	CBS 120744	*Eucalyptus* sp.	Hawaii	W. Gams	EF394843
	LISFA PB69	*E. globulus*	Portugal	L. Neves	

Table 4.2 Isolates included in the phylogenetic analyses (Cont.).

Species	Strain no.[1]	Host	Country	Collector	GenBank
Teratosphaeria lusitanica	LISFA QP93	*E. globulus*	Portugal	M.R.C. Silva	
Teratosphaeria molleriana	STE-U-1214	*Eucalyptus*	Portugal	S. McCrae	AF309620
	CBS 120746	*Eucalyptus* sp.	Portugal	P.W. Crous & A.J.L. Phillips	EF394844
≡ *M. ambiphylla*	**CBS 110499**	*E. globulus*	Australia	A. Maxwell	AY725530
≡ *M. vespa*	CMW 11558	*Eucalyptus* sp.	Australia	-	DQ303059
	LISFA QP45	*E. globulus*	Portugal	L. Neves	
	NX7A	*E. globulus*	Portugal	H. Machado	
	LISFA QP46	*E. globulus*	Portugal	M.R.C. Silva	
Teratosphaeria nubilosa	**STE-U -937**	*E. globulus*	Australia	A.J. Carnegie	AF309618
	CMW 9000	*E. nitens*	South Africa		AF449096
	CMW 9001	*E. nitens*	South Africa		AF449097
	CBS 116005	*E. globulus*	Australia	A.J. Carnegie	AY725572
	CPC10497	*E. globulus*	New Zealand	W. Gams	AY725574
	LISFA BBC244	*E. globulus*	Portugal	L. Neves	
	LISFA A77	*E. globulus*	Portugal	M.R.C. Silva	
Teratosphaeria pluritubularis	**CPC 11697**	*Eucalyptus globulus*	Spain	P. Mansilla	DQ303007
	LISFA BBC105	*E. globulus*	Portugal	L. Neves	
	LISFA BBC115	*E. globulus*	Portugal	L. Neves	
Teratosphaericola pseudoafricana	**CBS 114782**	*Eucalyptus globulus*	Zambia	T.A. Coutinho	DQ303008
	LISFA BBC293	*E. globulus*	Portugal	L.Neves	
	LISFA QP47	*E. globulus*	Portugal	M.R.C. Silva	
Unidentified Ventureaceae	LISFA BBC191	*E. globulus*	Portugal	L. Neves	

(1) Ex-type cultures **on bold**; **CBS** Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, The Netherlands; **CPC** Culture collection of P.W. Crous, housed at CBS; **CMW** culture collection of M.J. Wingfield, housed at Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; **LISFA** Herbarium Code of collection of UEIS-SAFSV (Herbarium Code of Sistemas Agrários e Florestais e Sanidade Vegetal of Unidades Estratégicas de Investigação e Serviços of Instituto Nacional de Investigação Agrária e Veterinária, I.P., Oeiras, Portugal); **STE-U** culture collection of the Department of Plant Pathology, Stellenbosch University, South Africa.

Results

A total of 587 isolates were processed and identification obtained from 439 lesions of symptomatic juvenile leaves of *E. globulus*.

Morphological characterization

Morphological characteristics of the species identification agree with the previous descriptions (Park and Keane, 1982b; Carnegie and Keane, 1994, 1997; Crous and Wingfield, 1996, 1997; Crous *et al.,* 2004; Hunter *et al.,* 2006; Silva *et al.,* 2009).

Molecular characterization

Amplicons of approximately 560 bases were obtained for the isolates listed in Table 1, incomplete parts at the start and end of the sequences were excluded from the analysis. The ITS alignment comprised 56 sequences, including *Neofusicocum parvum* (STE-U-4438) as outgroup.

The ML tree (Fig. 4.1) was resolved into six major clades. One clade included a well-supported sub-clade corresponding to individual isolates of *Austroafricana parva* (bootstrap support = 97%). A second clade presented several well-supported sub-clades, 3 species of *Teratosphaeria* and a *Quasiteratosphaeria* species like *T. nubilosa* (sub-clade with bs =99%); *T. pluritubularis* and *T. molleriana* (sub-clade with bs =89%) and *Q. mexicana* (sub-clade with bs =100%). The third clade also included several well-supported sub-clades corresponding to *Mycosphaerella madeirae* and *Paramycosphaerella marksii* (all sub-clades with bs =100%). The fourth clade contained a well-supported sub-clade associated to *Mycosphaerella* species, *M. communis* (bs =100%) and *M. lateralis* (bs =66%). The fifth clade included well-supported sub-clade of *Neodevriesia hilliana* (bs =100%). The sixth clade comprised a well-supported sub-clade of *Teratosphaericola pseudoafricana* (bs =100%).

In this study 15 species were identified on lesions of *E. globulus* as belonging to several genera (Table 4.2).

Several new reports on *E. globulus* from the Iberian Peninsula were found in this study namely *Cladosporium cladosporioides*, *Fusicladium eucalypti*, *Mycosphaerella madeirae* (first report from mainland Portugal), and an unidentified *Venturiaceae*.

Table 4.3 Species identified on juvenile leaves of *Eucalyptus globulus* based on the percentage similarity of each isolate to its closest match in GenBank, but not included in the phylogenetic tree.

Sequence accession no.	Origin	Sequence-based identification	Maximum identity (%)
KM216325	Australia (Tasmania)	*Venturiaceae* sp.	100
KP780438	-	*Cladosporium cladosporioides*	100
HQ599600	Australia	*Fusicladium eucalypti*	99

Combinations of species in the Leaf Disease Complex

Concerning the species prevailing in the total of occurrences (N=587), *T. nubilosa* is the most represented in 29.5% followed by *A. parva* in 28.8% (Table 4.4). About 36.6% was represented by *M. lateralis* (11.9%), *P. marksii* (11.8%), *M. communis* (6.5%) and *T. molleriana* (6.5%). The remaining species are represented in 5.1% of all occurrences. The nuclear group of species in the complex is constituted by 6 species, which represents 94.9% of all the occurrences (*T. nubilosa*, *A. parva*, *M. lateralis*, *P. marksii*, *M. communis* and *T. molleriana*).

Combinations of one to five species per lesion in total of occurrences were observed. Lesions with only one species isolated were dominant representing 56.7% with emphasis on *T. nubilosa* with 23.3% of this case, followed by 16.9% in *A. parva*. The higher diversity of the species grouped was observed in combinations of two species per lesion in 24.9% in total of occurrences. The total of occurrences of three to five species combinations per lesion represents the lower diversity of species with 18.4%.

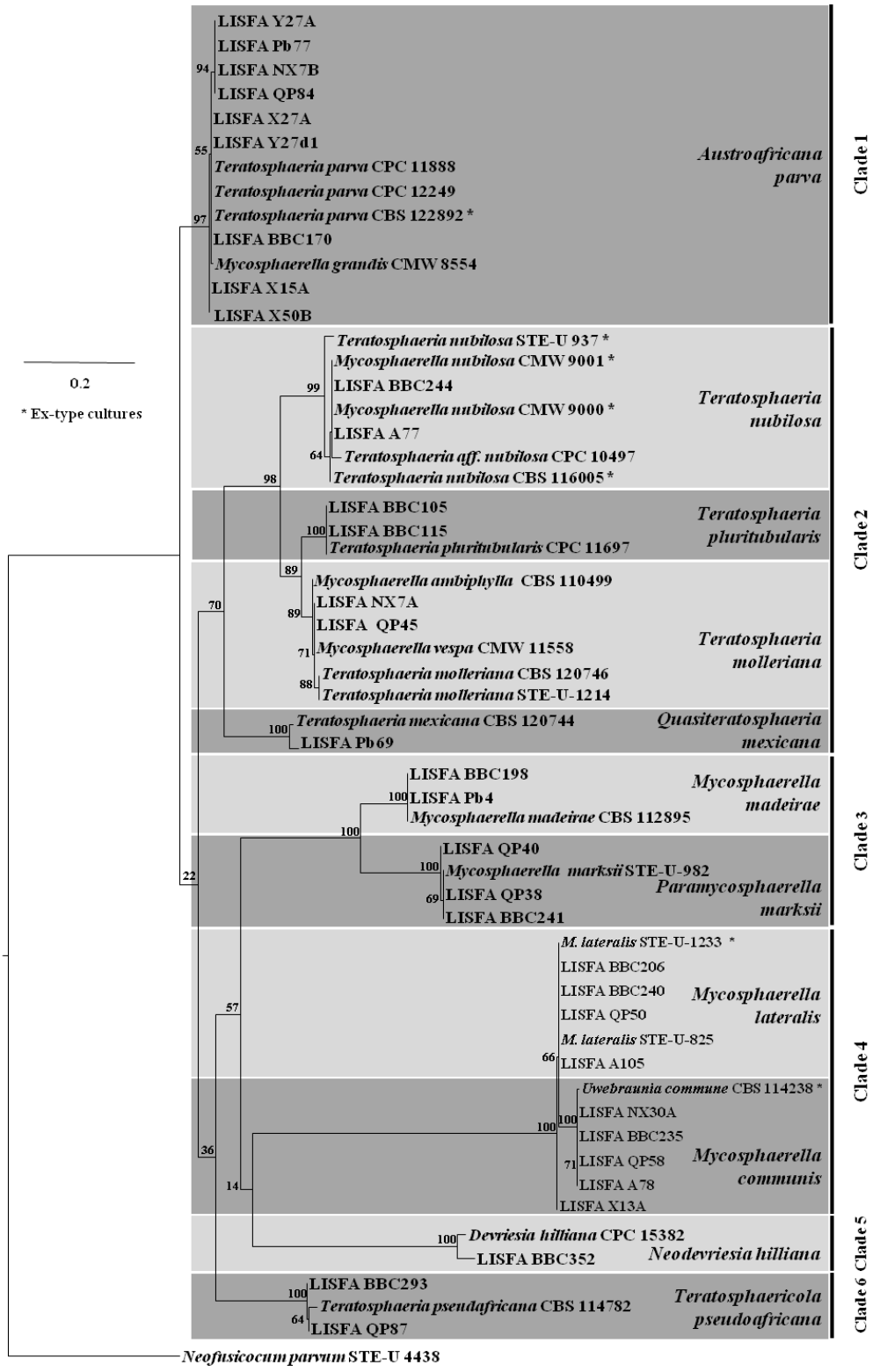

Fig. 4.1 Maximum likelihood (ML) tree constructed from ITS sequence data with RAxML selecting the gamma model of rate heterogeneity for containing isolates related with *Austroafricana parva*, *Mycosphaerella communis*, *M. lateralis*, *M. madeirae*, *Neodevriesia hilliana*, *Paramycosphaerella marksii*, *Quasiteratosphaeria mexicana*, *T. molleriana*, *T. nubilosa*, *T. pluritubularis* and *Teratosphaericola pseudoafricana* species associated with the Eucalyptus Leaf Disease Complex (ELDC). Maximum Likelihood bootstrap values are given at the nodes. The scale bar shows 0.2 changes. Ex-type strains are indicated with a (*).

The species show three types of colonization through the year in their combinations occurrence in the lesion (1) Species occurrence is higher when it is alone in the lesion than in other combinations (e.g. *T. nubilosa*); (2) Species occur more or less equally in cases of one species per lesion or in the sum of other occurrences (e.g. *A. parva*); (3) Species occurrence is low in lesions with one species and high in the other types (e.g. *M. communis, M. lateralis*). Also noticed that *A. parva* is the species that was grouped in more combinations with other species with 27 different ones, followed by *M. lateralis* with 21 (Table 4.4).

The occurrence of species varied throughout the year, showing significant differences among the time of collection, in autumn, winter, early spring and spring. The occurrence of *Teratosphaeria nubilosa* differed significantly between seasons with most occurrences in autumn when others species were present at a low level. During winter, after this species *M. communis* and *M. lateralis* had their high occurrence and *M. lateralis* maintained the level of occurrence during early spring. On the other hand, *A. parva* showed low occurrence in autumn, medium values in winter and early spring and reached a maximum level in spring. *Paramycosphaerella marksii* and *T. molleriana* reached the peak of occurrence in early spring and *P. marksii* also maintained it during spring, while *T. molleriana* decreased in spring and both were practically absent during the rest of the year (Table 4.5).

The comparison of means for severity level (Necrotic leaf surface area %) differed significantly between seasons, according to the Kruskal–Wallis test ($H (3, N=439) = 108.2076$ $p=0$). Values for mean disease severity level varied between 3.0 in autumn and 4.6 in winter (Table 4.5). The mean of number of species per lesion differed significantly between seasons ($H (3, N=439) = 20.65676$ $p=0.0001$). Values of the mean of number of species per lesion varied between 1.2 in autumn and 2.0 in winter (Table 4.5).

Table 4.4 Total of occurrences of species and combinations among them in symptomatic leaf lesions.

			N	P	L	Mk	C	Mll	Clad	Plu	Mx	Md	Paf	Fus	Lus	Ven	Neo	Total ocurrences	Total Lesions
Only one sp.		No.	137	99	19	34	7	21	9	0	2	1	1	0	2	0	1	333	333
		%	23.3	16.9	3.2	5.8	1.2	3.6	1.5	0.0	0.3	0.2	0.2	0.0	0.3	0.0	0.2	**56.73**	
Combinations of 2 spp.	**N**				10	8	2	2	1										
	P					11	10	5	4	1	2			1	1				
	L						1	4	3								1		
	Mk							2	2			1	1						
		No.	23	45	28	19	13	10	1	2	1	1	1	1	0	1	0	146	73
		%	3.9	7.7	4.8	3.2	2.2	1.7	0.2	0.3	0.2	0.2	0.2	0.2	0.0	0.2	0.0	**24.87**	
Combinations of 3 spp.	**N**	**P**				2	2	1											
	N	**L**						4											
	P	**L**					4	2	1			1							
	P	**C**					2		1										
	P	**Mk**								1									
	L	**C**					1												
	L	**Mk**							1										
	C	**Mk**							1				1						
		No.	9	17	16	13	13	4	0	1	1	1	0	0	0	0	0	75	25
		%	1.5	2.9	2.7	2.2	2.2	0.7	0.0	0.2	0.2	0.2	0.0	0.0	0.0	0.0	0.0	**12.78**	
Comb. 4 spp.	**P**	**L**	**N**				1	1		1									
	P	**L**	**C**						1		1				1				
	P	**L**	**Mk**						1										
		No.	3	7	7	2	4	2	1	1	0	0	0	1	0	0	0	28	7
		%	0.5	1.2	1.2	0.3	0.7	0.3	0.2	0.2	0.0	0.0	0.0	0.2	0.0	0.0	0.0	**4.77**	
5 spp.	**N**	**P**	**C**	**Mk**				1											
		No.	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	5	1
		%	0.2	0.2	0.0	0.2	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**0.85**	
Total ocurrences by sp.		No.	173	169	70	69	38	38	11	4	4	3	2	2	2	1	1	587	439
		%	29.5	28.8	11.9	11.8	6.5	6.5	1.9	0.7	0.7	0.5	0.3	0.3	0.3	0.2	0.2	**100**	

(N) *Teratosphaeria nubilosa*
(P) *Austroafricana parva*
(L) *Mycosphaerella lateralis*
(Mk) *Paramycosphaerella marksii*
(C) *Mycosphaerella communis*
(Mll) *Teratosphaeria molleriana*
(Clad) *Cladosporium cladosporioides*
(Plu) *Teratosphaeria pluritubularis*
(Mx) *Quasiteratosphaeria mexicana*
(Md) *Mycosphaerella madeirae*
(Paf) *Teratosphaericola pseudoafricana*
(Fus) *Fusicladium eucalypti*
(Lus) *Teratosphaeria lusitânica*
(Ven) *Venturiaceae* sp.
(Neo) *Neodevriesia hilliana*

Table 4.5 Number of lesions, Severity level (SL), number of species (NSpp) per lesion, count of species (Spp) and species frequency among the seasons.

Seasons/ Stands	No. Lesions	SL (± (*)		NSpp/ Lesion (*)		Count Spp	Species Frequency (*)							
		Mean	SE	Mean	SE		N	P	L	C	Mk	Mll	Clad	Others
Autumn	139	3.04 ±0.92d		1.18 ±0.47d		5	86.5 c	1.9 b	6.7 a	4.6 a	0.0 a	0.4 a	0.0 b	0.0
Winter	15	4.60±1.55a		2.00±1.13a		7	25.0 b	33.3 a	13.3 ab	16.1 b	0.0 a	0.0 a	8.3 a	3.9
Early Spring	188	3.71±0.90c		1.44 ±0.76b		11	11.2 ab	34.7 a	13.2 b	4.1 a	18.1 b	12.9 b	3.5 a	2.4
Spring	97	4.37±0.75b		1.27 ±0.59c		14	7.4 a	57.9 c	4.1 a	2.6 a	18.1 b	3.3 a	2.1 ab	4.5
Total	**439**	**3.67±1.04**		**1.34±0.68**		15								

***** P<0.01, values followed by different letters in columns are significantly different according to Kruskal-Wallis test (n=439)

(N) *Teratosphaeria nubilosa* **(P)** *Austroafricana parva* **(L)** *Mycosphaerella lateralis* **(C)** *Mycosphaerella communis* **(Mk)** *Paramycosphaerella marksii* **(Mll)** *Teratosphaeria molleriana* **(Clad)** *Cladosporium cladosporioides* **Others:** Represented by *Fusicladium eucalypti*, *M. madeirae*, *Neodevriesia hilliana*, *Quasiteratosphaeria mexicana*, *Teratosphaeria lusitanica*, *Teratosphaeria pluritubularis*, *Teratosphaericola pseudoafricana* and *Venturiaceae* sp.

Discussion

In this study 15 species were identified (Table 4.2) on lesions of *E. globulus*. The species belong to several genera namely *Austroafricana, Cladosporium, Fusicladium, Mycosphaerella, Neodevriesia, Paramycosphaerella, Quasiteratosphaeria, Teratosphaeria, Teratosphaericola* and *Venturiaceae.* However the nuclear group of species in the complex remains practically the same, constituted by 6 species, which represent 94.9% in total of occurrences (*A. parva*, *M. communis*, *M. lateralis*, *P. marksii*, *T. molleriana* and *T. nubilosa*) as stated in previous works (Silva *et al*., 2008, 2009, 2012).

The reports of the most commonly foliar fungi in the field for several countries like Australia, Brazil, Portugal, South Africa and Spain have some differences. The highest similarities are between Portugal and Spain. Nevertheless, Australia has many in common with Portugal however there is a better match with Spain rather than Portugal (Park and Keane 1982a, Purnell and Lundquist, 1986, Crous *et al.*, 1989, Park *et al.*, 2000, Maxwell, 2004, Alfenas *et al.*, 2004; Tejedor, 2007; Silva *et al.*, 2008, 2009, 2012; Perez G. *et al.*, 2009; Teodoro, 2010; Carnegie *et al.*, 2011; Teodoro *et al.*, 2012; Aguin *et al.*, 2013; Manzilla *et al.*, 2013).

In more than half of the samples examined only one species was found on each lesion, with *T. nubilosa* as the dominant species. Nevertheless, the importance of *A. parva* cannot be overlooked since it was found as the sole species on 30% of the lesions studied. However when comparing *T. nubilosa* and *A. parva* in all occurrences, i.e. in all types of combinations, the difference between them is less than 1%. Thus, it is concluded that *A. parva* is equally important in the disease as *T. nubilosa*. It is important not to devalue the high occurrence of *A. parva* alone in lesions, indicating that *A. parva* can be compared to *T. nubilosa* in the field, showing two peaks in different seasons of the year, with *A. parva* most prevalent in spring and *T. nubilosa* in autumn.

On the other hand, it is important to highlight that approximately 43% of all occurrences are in lesions with more than one species and *A. parva* is the most represented followed by *M. lateralis*, also with *T. nubilosa*, *P. marksii* and *M. communis* demonstrating an important role in the disease of compose lesions. Despite the sample robustness it is important to state that there are difficulties to isolate all the species present in the complex because some species could hide others with a weak growth in the culture (Hunter *et al.*, 2006). For this reason we would suggest that species in the complex may have a greater role in the disease development together than alone.

Márquez *et al*., (2011) observed several species in leaf litter, such as *Austroafricana parva*, *Cladosporium* spp., *Fusicladium amoenum*, *Mycosphaerella fori*, *Paramycosphaerella marksii*, *Teratosphaeria molleriana* and *T. nubilosa* also observed in symptomatic leaves. Thus leaf litter could be an important source of inoculum for ELDC.

Regarding the cycle of colonization throughout the year, it is possible to predict that *T. nubilosa* is the first species to cause lesions in autumn when it occurs at a high incidence after a period of absence of symptoms in the field during summer. During the winter months *M. communis* and *M. lateralis* were the most frequently encountered species. As these species are frequent in lesions with combinations of *A. parva* and other species, it is predictable that *M. communis* and *M. lateralis* first colonize the lesions and are then followed by *A. parva*. On the other hand it is possible that *A. parva* is the first colonizer. Irrespective of the species that colonize first, the occurrence of *A. parva* is always higher than *M. communis* and *M. lateralis*.

The analysis of severity levels could compliment the prediction about species cycles throughout the year and clarify which species could be more involved in the necrotic leaf area and make the greatest contribution to disease severity. All seasons demonstrate significantly differences in the severity level between them. The species that was more related with low severity level was *T. nubilosa* in autumn. In winter, when *A. parva* reaches medium level of occurrence and *M. communis* and *M. lateralis* reached the highest level of occurrence, is when the severity level has the highest value, thus it is possible to predict that these three species are responsible for high levels of disease severity in the field. The capability to form necroses was demonstrated in leaves of *E. globulus* and revealed that *M. communis* and *M. lateralis* caused larger lesions than *T. nubilosa* (Silva *et al*., *submitted*).

Austroafricana parva could also be more involved in the disease than *T. nubilosa* because the other peak of severity level is when *A. parva* reaches the highest occurrence in the lesion and *T. nubilosa* has the lowest occurrence. Contradicting that *T. nubilosa* is considered to be the main primary pathogen responsible for ELDC in Australia (e.g. Hunter *et al.*, 2009).

The number of species per lesion changed significantly during the course of the seasons. The number of species per lesion was lowest in autumn when *T. nubilosa* reached its highest levels. The greatest number of species per lesion was in winter when *M. communis* and *M. lateralis* reached their highest levels and disease severity level was greatest. Thus can complement with what was previously said about which species are more responsible in the complex for the ELDC. Thus, it is acceptable that *M. lateralis*, *M. communis* and *A. parva* have more impact on disease severity than *T. nubilosa*. However this last species is also important in

the complex because is the one that is most frequent in the biological material analysed and thus very similar to *A. parva*.

It is expected that the severity of this disease will increase if the genetic material is swapped by the species that colonize the same lesion and when others species less representative increase the amount of inoculum becoming potential virulent species. The contribution of this work is to highlight the diversity of fungal species and to better understand the complex of the species interacting to cause leaf lesions in *E. globulus* during the year. Nevertheless, a continuous systematic survey throughout the year of the fungi involve to ELDC, over the course of several years, which will also allow a survey of new species emergence in the field, is important.

It could be suggested that new stands of *E. globulus* should be established in order to avoid the peak of juvenile leaves between winter and early spring, i.e. the best time to establish clones should be chosen so that the adult stage of leaves could coincide with winter or early spring. Consequently, the selection of clones resistant to ELDC should form the basis for future control strategies to prevent economic losses in commercial eucalypt plantations.

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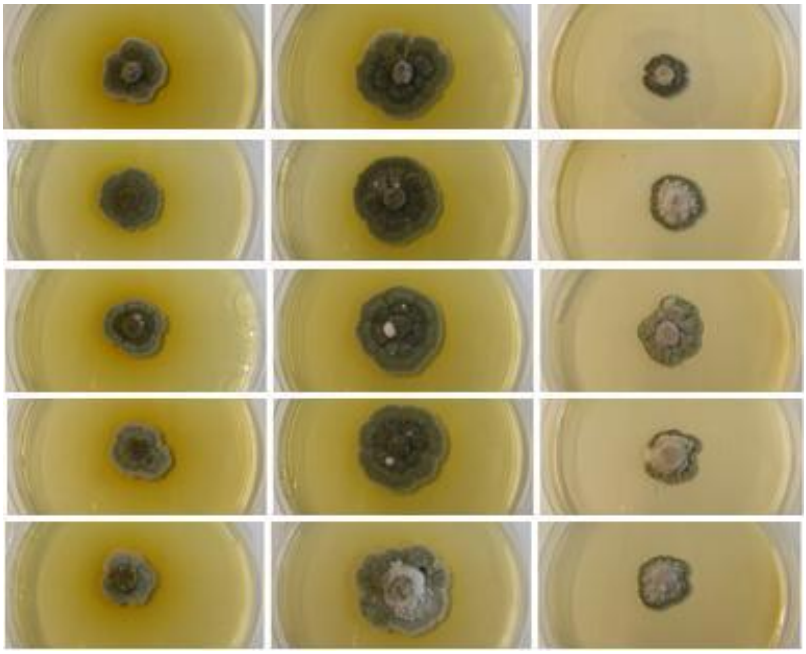
IV

Other Teratosphaeria diseases

CHAPTER 5

Teratosphaeria gauchensis on *Eucalyptus* in Portugal



Chapter 5

RESEARCH ARTICLE

Teratosphaeria gauchensis associated with trunk, stem and foliar lesions of *Eucalyptus globulus* in Portugal

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Summary

Teratosphaeria gauchensis and *T. zuluensis* both cause a stem canker of eucalypts that leads to serious damage in various parts of the world. Until recently, this disease was unknown in Portugal. Nevertheless, severe damage to *Eucalyptus globulus* has been observed in Portugal since 2006 when symptoms appeared as necrotic spots on young green stems and leaves, twig lesions, and dark oval-shaped lesions on stems and trunks. The isolates from affected *E. globulus* tree plantations were identified using sequence data of the ITS1-5.8S-ITS2 and EF-1α clusters, together with morphological characteristics. Based on these results, the causal organism was identified as *T. gauchensis*, which represents the first report of this pathogen from Portugal.

Introduction

Canker caused by *Teratosphaeria gauchensis* and *T. zuluensis* is an extremely damaging disease of Eucalyptus species that has serious effects on tree growth (Cortinas *et al*., 2010). Early symptoms are discrete necrotic spots on young green stems at the top of the trees. The lesions on twigs develop into internal cankers, which then extend and coalesce to form large, dark, oval-shaped cankers on the stems and trunks. A transverse cut through the trunk reveals concentric kino pockets (Cortinas *et al*., 2006b, 2011). Subsequently, soft tissues become deformed, and there is a disruption of water transport to terminal shoots resulting in retarded tree growth as a consequence of the infection and tissue damage (Cortinas *et al*., 2011).

The pathogen was first reported as *Coniothyrium zuluensis* on *E. grandis* trees in a subtropical forest in South Africa (Wingfield *et al*., 1996), and the disease was given the common name of Coniothyrium canker. Since it was first described, the pathogen has undergone a number of name changes. In a phylogenetic study based on ITS sequence data, Cortinas *et al.,* (2006a) considered that it was more closely related to *Mycosphaerella* than to *Coniothyrium palmarum* or *Paraconiothyrium*, and they placed it in *Colletogloeopsis*. In 2007, two parallel studies presented new information on the phylogeny of Colletogloeopsis. One demonstrated that *Colletogloeopsis* and *Phaeophleospora* represent the same genus and they should be treated as *Kirramyces* (Andjic *et al*., 2007). The other regarded *Colletogloeopsis* as a synonym of *Readeriella* (Crous *et al*., 2007). Finally, the causal pathogen of 'Coniothyrium canker' was consigned to *Teratosphaeria* as *T. zuluensis* (Crous *et al*., 2009a,b). As a consequence of the name changes of the pathogen, the disease was renamed Teratosphaeria canker.

Teratosphaeria zuluensis has been reported on a wide variety of Eucalyptus species and hybrids (Cortinas *et al.*, 2006b). For example, it has been reported from Thailand on *E. camaldulensis*, Mexico and Malawi on *E. grandis*, Vietnam and China on *E. urophylla*, Zambia on *E. cloeziana* and *E. grandis* and Uganda on *E. grandis* (Roux *et al.*, 2002; Van Zyl *et al.*, 2002; Gezahgne *et al.*, 2003; Cortinas *et al.*, 2006a,b; Chungu *et al.*, 2010; Jimu *et al.*, 2014).

Another species that causes similar canker symptoms on eucalypts was described as *T. gauchensis* by Cortinas *et al.*, (2006b). Morphologically, *T. gauchensis* has longer conidia than *T. zuluensis*, and sympodial polyphialidic conidiogenous cells, while conidiogenous cells of *T. zuluensis* are monophialidic (Cortinas *et al.*, 2006b). Both species show optimal growth between 20 and 25°C, although maximum colony diameters of *T. gauchensis* isolates are smaller. The most obvious difference between these species was observed at 35°C, at which temperature *T. gauchensis* isolates barely grow, whereas *T. zuluensis* isolates reach diameters of between 10 and 20 mm after 6 weeks (Cortinas *et al.*, 2006b). Phylogenetically, they differ by 26 fixed nucleotide positions in ITS and partial sequences of β-tubulin, EF1-α and ATPase 6 genes (Cortinas *et al.*, 2006b). *Teratosphaeria gauchensis* has been reported from Uganda on *E. grandis*, from Ethiopia on *E. camaldulensis*, from Argentina, Uruguay and Hawaii, USA on *E. grandis* (Gezahgne *et al.*, 2003, 2005; Cortinas *et al.*, 2004, 2006b) and in Uruguay on other species such as *E. maidenii* and *E. tereticornis* (Pérez *et al.*, 2009).

In South Africa, Teratosphaeria canker disease appeared quite suddenly and soon became recognized as a serious threat to productivity in the forest industry (Wingfield *et al.*, 1996; Cortinas *et al.*, 2006b). In the first years after, it was detected this disease caused severe damage on the most susceptible hosts, but has almost disappeared after resistant clones of *E. grandis* were introduced (Cortinas *et al.*, 2010). In South America, where the eucalyptus plantations are rapidly expanding, *T. gauchensis* is the cause of a serious disease and currently poses a growing threat to the industry (Cortinas *et al.*, 2011).

Teratosphaeria canker has never been found on any host genus other than *Eucalyptus* (Cortinas *et al.*, 2011), and the actual distribution of the two species shows that in most countries only a single species can be found. Thus, *Teratosphaeria gauchensis* is restricted to Africa (Uganda and Ethiopia), South America (Argentina and Uruguay) and USA (Hawaii) (Gezahgne *et al.*, 2003, 2005; Cortinas *et al.*, 2004, 2006b). On the other hand, *T. zuluensis* is limited to Africa (Malawi, Uganda, Zambia), Asia (China, Thailand, Vietnam) and Central America (Mexico) (Roux *et al.*, 2002; Van Zyl *et al.*, 2002; Gezahgne *et al.*, 2003; Cortinas *et al.*, 2006a,b; Chungu *et al.*, 2010). Thus, Uganda (Africa) is the only country where both species have been found (Cortinas *et al.*, 2006b; Jimu *et al.*, 2014).

Until recently, eucalypts were relatively free of diseases in Portugal. However, since the 1990s, *Botryosphaeriaceae* and *Mycosphaerella* s.l. were referred to as having some importance in plantations (Valente *et al*., 2008; Silva *et al*., 2009). Since 2006, severe damage on *E. globulus* trunks and stems has been observed associated with necrotic spots on young green stems and leaves, twig lesions and dark oval shapes on stems and trunk lesions, similar to Teratosphaeria canker.

The aim of this study was to determine which species is associated with the disease in Portugal by analysing isolates collected during the course of *Eucalyptus* plantation surveys. The relationship of these species to other phylogenetically closely related species is also evaluated.

Materials and methods

Isolation and morphology

Typical symptoms of Teratosphaeria canker were observed on *E. globulus* in several Portuguese regions including Algarve, Alto Alentejo, Baixo Alentejo, Beira Litoral and Douro Litoral (Table 5.1), and material was collected for characterization of the causal agent. Single conidial isolates were established from mature pycnidia formed on lesions on stems and leaves of *E. globulus* trees showing typical symptoms of Teratosphaeria canker (Fig. 5.1). When necessary, the samples were incubated in moist chambers to induce sporulation. Conidia exuding from single pycnidia were collected with a sterile needle and spread on the surface of 2% (w/v) malt extract agar (MEA) (Difco) plates. After 24–48 h of incubation, single germinating conidia were transferred to fresh MEA plates and incubated for 30 days at 25°C in the dark. Cultures were incubated under continuous near-ultraviolet light at room temperature to promote sporulation. Observations of micromorphological features were made with an Olympus BX41TF microscope (Olympus Corporation, Hamburg, Germany) with bright field illumination. Digital images were recorded with a ProgRes Speed XT Core 5 camera (JENOPTIK Optical Systems GmbH, Jena, Germany). Measurements were made with the ProgRes Capture Pro 2.8.8 JENOPTIK Optical Systems GmbH. Descriptions were based on sporulation on the host. Wherever possible, 30 measurements (9600 magnification) were made of structures mounted in autoclaved distilled water. Isolates were deposited in the culture collection of LISFA (Instituto Nacional de Investigação Agrária e Veterinária, I.P., Oeiras, Portugal).

Plugs (5 mm diameter) of colonized agar were cut from actively growing cultures and positioned at the centre of petri dishes containing MEA. Three plates were prepared for each

isolate and incubated at 20°C, 25°C or 35°C for 4 weeks after which time colony diameters were measured (Fig. 5.2; Table 5.2). Analysis of variance (ANOVA) was carried out to test the effect of temperature on the colony diameter of isolates. Multiple mean comparisons were made with Fisher's least significant difference (LSD) test (Table 5.3).

DNA isolation and phylogeny

Molecular characterization of single-spore isolates was based on sequence analysis of the internal transcribed spacer regions and intervening 5.8S rDNA (ITS) and partial translation elongation factor 1-alpha gene (EF-1α). 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 and ITS4 (White *et al.*, 1990). The cycling conditions were 95°C for 10 min for the initial denaturation and cycles of 95°C, 1 min, 55°C, 1 min, 72°C, 30 s repeated 35 times with a final elongation step of 10 min at 72°C. PCRs were prepared in a total volume of 25 µl including 1 µl of genomic 1/10 dilution DNA, 1.25 U of Taq DNA polymerase, 2x reaction buffer, 1 µM of each primer, 0.4 mM of each dNTP and 6 mM $MgCl_2$.

The EF-1α cluster was amplified with primers EF1-728F and EF1-986R (Carbone and Kohn 1999). The cycling conditions were 94°C for 5 min for the initial denaturation and cycles of 94°C, 45 s, 52°C, 30 s, 72°C, 90 s repeated 40 times with a final elongation step of 7 min at 72°C. PCRs were prepared in a total volume of 25 µl including 1 µl of genomic 1/10 dilution DNA, 1.5 U of Taq DNA polymerase, 1x reaction buffer, 1.2 µM of each primer, 0.6 mM of each dNTP and 2.5 mM $MgCl_2$. PCR products (ITS and EF-1α) were separated by electrophoresis on 1% agarose gels and visualized with UV light after staining. PCR amplification products were purified using JETquick spin columns (Genomed GmbH) and sequenced in both directions.

Sequences of closely related species were retrieved in BLAST searches in GenBank (http://www.ncbi.nlm.nih.gov/genbank). Sequences for the ex-type strain of the closest matching species were downloaded, along with representative sequences of *T. gauchensis* and *T. zuluensis* (Fig. 5.3) reported on *Eucalyptus* from studies of Roux *et al.*, (2002), Cortinas *et al.*, (2006a,b), Crous *et al.*, (2007), Pérez *et al.*, (2009) and Chungu *et al.*, (2010). Sequences of other phylogenetically closely related species including *Teratosphaeria callophylla*, *T.*

corymbiae, *T. considenianae*, *T. foliensis*, *T. majorizuluensis*, *T. ovata* and *T. stellenboschiana*, available in GenBank for both loci (ITS/EF-1α), were also retrieved (Fig. 5.4).

Table 5.1 Collection details and GenBank accession numbers of isolates included in this study.

Species	Strain no.[1]	Host	Date	Collector	Substrate	Origin[2]	GenBank accession no.[3]	
							ITS	EF-1α
Pseud. secundaria	CBS 118507	*Eucalyptus* sp.	–	A.C. Alfenas	–	Brazil	KF901621	KF903236
M. colombiensis	CBS 110969	*E. urophylla*	1995	M.J. Wingfield	–	Colombia	AY725534	AY752183
T. callophylla	CBS 124584[T]	*Corymbia* sp.	–	K. Taylor	–	Australia	KF901566	KF903289
T. considenianae	109D1	*E. grandis x E. camaldulensis clone*	–	M. Gryzenhout	–	South Africa	JQ732898	JQ732996
	CMW 37671	*E. grandis x E. camaldulensis clone*	–	M. Gryzenhout	–	South Africa	JQ732893	JQ732990
	CMW 37674	*E. grandis x E. camaldulensis clone*	–	M. Gryzenhout	–	South Africa	JQ732896	JQ732994
	CMW 37675	*E. grandis x E. camaldulensis clone*	–	M. Gryzenhout	–	South Africa	JQ732897	JQ732995
	CMW 37678	*E. grandis x E. camaldulensis clone*	2009	M. Gryzenhout	–	South Africa	JQ732901	JQ732999
	CMW 37680	*E. grandis x E. camaldulensis clone*	2009	M. Gryzenhout	–	South Africa	JQ732903	JQ733001
	CPC 13032	*Eucalyptus* sp.	–	B.A. Summerell	–	Australia	KF901567	KF903291
	CPC 14057	*E. stellulata*	–	B.A. Summerell	–	Australia	KF901568	KF903292
T. corymbiae	CBS 124988	*Corymbia henryi*	–	A.J. Carnegie	–	Australia	KF901569	KF903293
T. foliensis	CBS 124581[T]	*E. globulus*	–	S. Collins	–	Australia	KF901580	KF903311
T. gauchensis	CBS 119465	*E. grandis*	–	M.J. Wingfield	–	Uruguay	KF901787	KF903312
	CBS 119468	*E. grandis*	–	M.J. Wingfield	–	Uruguay	KF901788	KF903313
	CBS 120304[T]	*E. grandis*	2002	M.J. Wingfield	–	Uruguay	KF901789	KF903314
	CMW 10893	*E. grandis*	2002	–	–	Hawaii	DQ240193	DQ240135
	CMW 10894	*E. grandis*	2002	–	–	Hawaii	DQ240194	DQ240136
	CMW 10895	*E. grandis*	2002	M.J. Wingfield	–	Hawaii	DQ240192	DQ240134
	CMW 14336	*E. grandis*	2003	–	–	Argentina	DQ240198	DQ240140
	CMW 15835	*E. grandis*	1999	J. Roux	–	Uganda	DQ240200	DQ240142
	CMW 17328	*E. grandis*	2005	M.N. Cortinas	–	Uruguay	DQ240190	DQ240132
	CMW 17330	*E. grandis*	2005	–	–	Uruguay	DQ240191	DQ240133
	CMW 40384	*E. grandis*	2012	L. Jimu	–	Uganda	KF891414	KF891419
	CMW 40386	*E. grandis*	2012	L. Jimu	–	Uganda	KF891416	KF891421
	CMW 40387	*E. grandis*	2012	L. Jimu	–	Uganda	KF891417	KF891422
	CMW 40388	*E. grandis*	2012	L. Jimu	–	Uganda	KF891418	KF891423
	CMW 7137	*E. grandis*	2001	–	–	Uganda	DQ240199	DQ240141
	CMW 7274	*E. grandis*	2001	–	–	Uruguay	DQ240187	DQ240129
	CMW 7294	*E. grandis*	2001	–	–	Uruguay	DQ240188	DQ240130
	CMW 7300	*E. grandis*	2001	–	–	Uruguay	DQ240189	DQ240131
	CMW 7302	*E. grandis*	2001	–	–	Uruguay	DQ240186	DQ240128
	CMW 7331	–	–	–	–	Argentina	DQ240195	DQ240137
	CMW 7342	*E. grandis*	2001	–	–	Argentina	DQ240196	DQ240138
	CMW 7378	*E. grandis*	2001	–	–	Argentina	DQ240197	DQ240139
	CMW 8978	*E. camaldulensis*	2001	–	–	Ethiopia	DQ240202	DQ240144
	CMW 8991[T]	*E. camaldulensis*	2001	–	–	Ethiopia	DQ240201	DQ240143
	CPC 120303[T]	*E. grandis*	–	M.J. Wingfield	–	Uruguay	KF901790	KF903315
	LISFA PE19	*E. globulus*	2011	H. Bragança & E. Diogo	Green stems	Portugal (AA)	**KJ410730**	**KM276830**
	LISFA PE22	*E. globulus*	2011	H. Bragança & E. Diogo	Twig lesions	Portugal (AA)	**KJ410731**	**KM276832**
	LISFA PE24	*E. globulus*	2011	H. Bragança & E. Diogo	Oval trunk lesions	Portugal (AA)	**KJ410732**	**KM276835**
	LISFA PE56	*E. globulus*	2012	H. Bragança & E. Diogo	Green stems	Portugal (BL)	**KJ410733**	**KM276828**
	LISFA PE57	*E. globulus*	2012	H. Bragança & E. Diogo	Oval trunk lesions	Portugal (BL)	**KJ410734**	**KM276833**
	LISFA PE78	*E. globulus*	2012	H. Bragança & E. Diogo	Green stems	Portugal (BA)	**KJ410735**	**KM276829**
	LISFA PE91	*E. globulus*	2012	H. Bragança & E. Diogo	Twig lesions	Portugal (A)	**KJ410736**	**KM276834**
	LISFA PF2	*E. globulus*	2006	M.R.C.Silva & H. Machado	Leave lesions	Portugal (DL)	**KJ410728**	**KM276836**
	LISFA PT1	*E. globulus*	2006	M.R.C.Silva & H. Machado	Oval trunk lesions	Portugal (DL)	**KJ410729**	**KM276831**

Table 5.1 Continued

Species	Strain no.[1]	Host	Date	Collector	Substrate	Origin[2]	GenBank accession no.[3]	
							ITS	EF-1α
	UY23	*E. grandis*	–	C. A. Perez	–	Uruguay	EU851910	–
T. majorizuluensis	CBS 120040[T]	*E. botryoides*	–	B.A. Summerell	–	Australia	KF901581	KF903319
T. ovata	CBS 124052	*E. phoenicea*	–	B.A. Summerell	–	Australia	KF901591	KF903345
T. stellenboschiana	CBS 124989	*E. punctata*	–	P.W. Crous	–	South Africa	GQ852823	GU384364
	CBS 124989	*E. punctata*	–	P.W. Crous	–	South Africa	KF901732	KF903356
	CPC 12283	*Eucalyptus* sp.	–	J. Dijksterhuis	–	France	KF901646	KF903354
	CPC 13764	*E. punctata*	–	P.W. Crous	–	South Africa	GQ852825	GQ852717
T. zuluensis	CMW 13324	*E. grandis*	–	–	–	South Africa	AY738214	DQ240173
	CMW 13328	*E. grandis*	1989	M.J. Wingfield	–	South Africa	DQ239974	DQ240172
	CMW 15078	*Eucalyptus* sp.	–	–	–	China	DQ239966	–
	CMW 15080	*E. urophylla*	2004	–	–	China	DQ240209	DQ240151
	CMW 15833	*E. grandis*	2000	–	–	Mexico	DQ239988	DQ240162
	CMW 15833	*E. grandis*	2000	M.J. Wingfield	–	Mexico	AF385610	DQ240162
	CMW 15834	*E. grandis*	2000	–	–	Mexico	DQ239987	DQ240161
	CMW 15834	*E. grandis*	2000	M.J. Wingfield	–	Mexico	AF385611	DQ240161
	CMW 15964	*E. urophylla*	2004	–	–	China	DQ240210	DQ240152
	CMW 15971	*E. urophylla*	2004	–	–	China	DQ240208	DQ240150
	CMW 15971	*E. urophylla*	2004	–	–	China	DQ240208	DQ240150
	CMW 17314	*E. grandis*	2005	–	–	South Africa	DQ240204	DQ240146
	CMW 17314	*E. grandis*	2005	M.J. Wingfield	–	South Africa	DQ240204	DQ240146
	CMW 17316	*E. grandis*	2005	–	–	South Africa	DQ240205	DQ240147
	CMW 17318	*E. grandis*	2005	–	–	South Africa	DQ240213	DQ240174
	CMW 17320	*E. grandis*	2005	–	–	South Africa	DQ240206	DQ240148
	CMW 17321[T]	*E. grandis*	2005	M.J. Wingfield	–	South Africa	DQ240207	DQ240149
	CMW 17322	*E. grandis*	2005	–	–	South Africa	DQ240214	DQ240175
	CMW 17438	*E. grandis*	2004	–	–	Malawi	DQ240212	DQ240154
	CMW 1772	*E. grandis*	1989	–	–	South Africa	DQ240203	DQ240145
	CMW 1772	*E. grandis*	1989	–	–	South Africa	DQ240203	DQ240145
	CMW 28714	*E. cloeziana*	2008	J. Roux & D. Chungu	–	Zambia	FJ617253	–
	CMW 37687	*E. grandis* x *E. camaldulensis clone*	2009	M. Gryzenhout	–	South Africa	JQ732912	JQ733009
	CMW 5235	*E. camaldulensis*	1997	–	–	Thailand	DQ239990	DQ240163
	CMW 5235	*E. camaldulensis*	1997	M.J. Wingfield	–	Thailand	AF376828	DQ240163
	CMW 5236	*E. camaldulensis*	1997	–	–	Thailand	DQ239989	DQ240164
	CMW 5236	*E. camaldulensis*	1997	M.J. Wingfield	–	Thailand	DQ239989	DQ240164
	CMW 6857	*E. urophylla*	2000	–	–	Vietnam	DQ239986	DQ240171
	CMW 6857	*E. urophylla*	2000	M.J. Wingfield	–	Vietnam	DQ239986	DQ240171
	CMW 6859	*E. urophylla*	2000	M.J. Wingfield	–	Vietnam	DQ239984	DQ240159
	CMW 6860	*E. urophylla*	2000	–	–	Vietnam	DQ239985	DQ240160
	CMW 7426	*E. grandis*	1997	–	–	South Africa	DQ239979	DQ240182
	CMW 7442	*E. grandis*	1997	–	–	South Africa	DQ239978	DQ240157
	CMW 7449	*E. grandis*	1997	–	–	South Africa	DQ239976	DQ240155
	CMW 7452	*E. grandis*	1997	–	–	South Africa	DQ239977	DQ240156
	CMW 7452	*E. grandis*	1997	M.J. Wingfield	–	South Africa	DQ239977	DQ240156
	CMW 7459	*E. grandis*	1997	–	–	South Africa	DQ239981	DQ240183
	CMW 7468	*E. grandis*	1997	–	–	South Africa	DQ239983	DQ240158
	CMW 7488	*E. grandis*	1997	–	–	South Africa	DQ239975	DQ240184
	CMW 7489	*E. grandis*	1997	–	–	South Africa	DQ239980	DQ240185

Table 5.1 Continued

Species	Strain no.[1]	Host	Date	Collector	Substrate	Origin[2]	GenBank accession no.[3]	
							ITS	EF-1α
	157B2	*E. grandis x E. camaldulensis clone*	2009	M. Gryzenhout	Leaves	South Africa	JQ732917	JQ733014
	CBS 120301[T]	*E. grandis*	–	M.J. Wingfield	–	South Africa	KF901735	KF903368
	CBS 120302	*E. grandis*	–	M.J. Wingfield	–	South Africa	KF901736	KF903369
	CMW 37672	*E. grandis x E. camaldulensis clone*	2009	M. Gryzenhout	Leaves	South Africa	JQ732894	JQ732992
	CMW 37679	*E. grandis x E. camaldulensis clone*	2009	M. Gryzenhout	Leaves	South Africa	JQ732902	JQ733000
	CMW 37688	*E. grandis x E. camaldulensis clone*	2009	M. Gryzenhout	Leaves	South Africa	JQ732913	JQ733010
	CMW 37690	*E. grandis x E. camaldulensis clone*	2009	M. Gryzenhout	Leaves	South Africa	JQ732915	JQ733012
	CMW 37693	*E. grandis x E. camaldulensis clone*	2010	M. Gryzenhout	Leaves	South Africa	JQ732920	JQ733016

GenBank entries generated in this study **on bold.**
[T]Ex-type cultures.
[1]**CBS** Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, The Netherlands; **CMW** culture collection of M.J. Wingfield, housed at Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; **LISFA** Herbarium Code of collection of UEIS-SAFSV (Sistemas Agrários e Florestais e Sanidade Vegetal of Unidade Estratégica de Investigação e Serviços of Instituto Nacional de Investigação Agrária e Veterinária, I.P.) Oeiras, Portugal.
[2]Portuguese region - **A**: Algarve; **AA**: Alto Alentejo; **BA**: Baixo Alentejo; **BL**: Beira Litoral; **DL**: Douro Litoral.
[3]**ITS**: internal transcribed spacer regions of the nrDNA operon; **EF-1α**: Translation elongation factor 1-alpha.

Sequences from nine Portuguese isolates obtained in this study (Table 5.1) together with 25 sequences of *T. gauchensis* and *T. zuluensis* (Fig. 5.3) and 80 sequences of other phylogenetically closely related species (Fig. 5.4) retrieved from GenBank were aligned with Clustal X v. 2.0 (Thompson *et al.,* 1997) and manually adjusted where necessary with BIOEDIT version 7.0.5.3 (Hall, 1999). All sequences generated in this study were deposited in GenBank (Table 5.1) and the alignments in Tree-BASE (www.treebase.org). *Mycosphaerella colombiensis* (CBS 110969) served as an out-group for ITS analysis (Fig. 5.3) while *Pseudoteratosphaeria secundaria* (CBS118507) was used as out-group for the combined ITS/EF-1α dataset (Fig. 5.4).

PAUP version 4.0b10 (Swofford, 2002) was used for the maximum parsimony and neighbour-joining distance analyses. For maximum parsimony, the heuristic search option with 1000 random taxon addition and TBR (tree bisection and reconnection) was used as the branch-swapping algorithm. All characters were unordered and of equal weight, and alignment gaps were treated as a fifth character state. 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.

Maximum-likelihood analyses (ML) were conducted in CIPRES Science Gateway V. 3.2 using RAXML-HPC BLACKBOX v.7.3.2 (Stamatakis, 2006; Stamatakis *et al.,* 2008; Miller *et al.,* 2010), selecting the gamma model of rate heterogeneity. Bayesian analyses were performed with MRBAYES v. 3.2.1 (Ronquist *et al.,* 2011) on the two loci (ITS, EF1-α) using the GTR model with gamma-distributed rate variation across sites and a proportion of invariable sites.

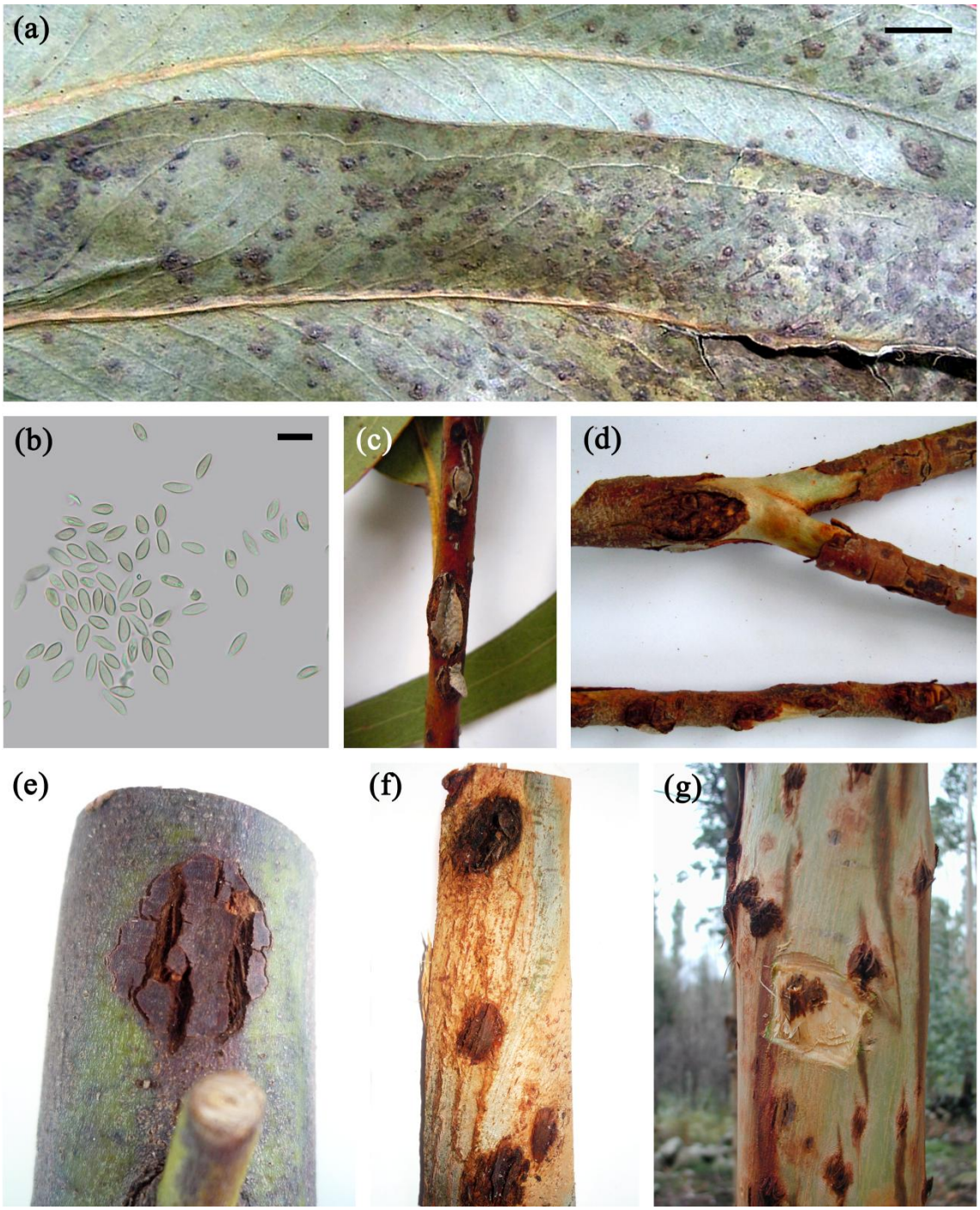

Fig. 5.1 Symptoms of Teratosphaeria canker disease. **(a)** Infected leaf. **(b)** Conidia of *Teratosphaeria gauchensis*. **(c)** Infected shoot with necrosis. **(d)** Cankered shoot. **(e)** Canker stem. **(f, g)** Cankers on trunk. Scale bars: **(a)** = 1 cm, **(b)** = 10 lm.

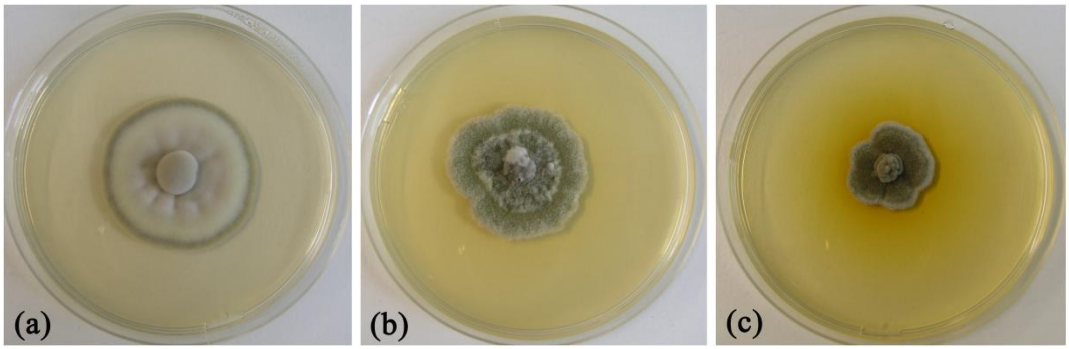

Fig. 5.2 Characteristics of Portuguese isolates of *Teratosphaeria gauchensis*. (a) LISFA PF2 (type A) at 20°C. (b) LISFA PE78 (type B) at 25°C. (c) LISFA PE19 (type C) at 20°C.

Table 5.2 Comparison with others studies of the culture growth at different temperatures and conidial dimensions.

	Culture growth (mm) (min. – max.)				
	20ºC	**25ºC**	**35ºC**	**Conidial (µm)**	**References**
T. zuluensis (South African) (6wk)	± 50	45–49	25–28[(a)] 10[(b)]	(4–)4.5–5(–6)×2–2.5(–3.5)	**(c)**
T. gauchensis (Uruguayan) (6wk)	36 –45	38 –41	0 –2	(4–)5–6(–7.5)×(2–)2.5(–3)	**(c)**
T. gauchensis (Portuguese) (4wk)	18–43	30–53	12–21	(6.1–)6.8–9.2(–10.2)×(2.7–)3.0–3.9(–4.9)	**(d)**

CMW culture collection of M.J. Wingfield, housed at Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa **(a)** CMW 7479 **(b)** CMW 7442 **(c)** Cortinas *et al.*, 2006b **(d)** This study

Table 5.3 Mean of culture growth (mm) of Portuguese isolates of *Teratosphaeria gauchensis* at different temperatures after 4 weeks on MEA. Means comparison were made with Fishers Least Significant Difference (LSD) test. Within each column means with different letters are significantly different (p <0.05).

Portuguese Isolates	**20ºC**		**25ºC**		**35ºC**	
LISFA PE19	21.17	abc	34.00	ab	14.17	bc
LISFA PE22	22.17	bc	31.67	a	20.17	a
LISFA PE24	20.33	abc	31.67	a	21.00	a
LISFA PE56	18.83	ab	38.17	b	19.33	a
LISFA PE57	18.17	a	31.00	a	18.50	a
LISFA PE78	22.83	c	34.67	ab	17.83	ac
LISFA PE91	20.33	abc	33.33	a	17.83	ac
LISFA PF2	42.83	e	53.33	c	11.83	b
LISFA PT1	37.83	d	30.00	a	12.17	b

LISFA Herbarium Code of collection of UEIS-SAFSV (Sistemas Agrários e Florestais e Sanidade Vegetal of Unidade Estratégica de Investigação e Serviços of Instituto Nacional de Investigação Agrária e Veterinária, I.P.) Oeiras, Portugal.

Results

Morphology

In 2006, cankers caused by *Teratosphaeria* were first identified in Douro Litoral (LISFA PT1 and LISFA PF2). Six years later, the disease was detected in several places around the country (Table 5.1). A total of nine isolates of *Teratosphaeria* were obtained from *E. globulus* from different regions of Portugal (Table 5.1). Characteristic symptoms of the canker disease were observed (Fig. 5.1). The strain from the most severely affected trees with characteristic dark oval lesions on the trunk was LISFA PT1 and from the least severely affected trees was LISFA PE91.

Morphological characteristics of the isolates largely agreed with the descriptions given by Cortinas *et al.,* (2006b) except for differences in conidial dimensions. The range of conidial lengths of the Portuguese isolates on host material was (6.1–) 6.8–9.2(–10.2) 9 (2.7)3.0–3.9(–4.9) µm. Conidia of Portuguese isolates were larger than the Uruguayan *T. gauchensis* isolates examined by Cortinas *et al.,* (2006b) (Table 5.2).

The average colony diameter of Portuguese isolates after 4 weeks on MEA at different temperatures (20, 25 and 35°C) showed optimal growth at 25°C. Two isolates (LISFA PF2 and LISFA PT1) from the north coastal region (Douro Litoral) grew faster at 20 and 25°C than all other isolates while growth at 35°C was considerably less than all the other isolates (Table 5.3).The characteristics of the Portuguese isolates are shown in Fig. 5.2.

Phylogenetic analyses

ITS loci

The ITS alignment comprised 34 taxa (including the out-group) and 435 characters including alignment gaps. The ML tree resolved two main clades, one representing *T. gauchensis* (87% bootstrap support) and the other comprised of *T. zuluensis* isolates (94% bootstrap support) (Fig. 5.3). Two subclades were resolved within *T. zuluensis*, but there was no clear correlation between isolate origin and the subclade within which it clustered. The *T. gauchensis* clade could also be resolved into two subclades supported by moderate to high bootstrap values. The first subclade (87% bootstrap) is represented by isolates from Argentina, Ethiopia, Hawaii, Portugal and Uganda while the second subclade (99% bootstrap) comprised isolates from Uruguay, including the ex-type isolate of *T. gauchensis*. The two lineages in *T.*

gauchensis were separated by three fixed polymorphisms in ITS. These were one indel (gap/C) at position 16 (except for Portuguese isolate LISFA PF2), and two transitions occurring at position 22 (T/C) and position 91 (C/T). There were also some differences between the Portuguese isolates. A group of isolates from Alto Alentejo and UY23 from Uruguay have an indel at position three (gap/A), which differs from all other isolates. An indel at position seven separates isolate CMW7302 from all the others, a transition at position 15 separates LISFA PF2 from all others, and a transition at position 216 separates LISFA PE56 from all others.

ITS/EF1-α

Alignments of the 89 isolates listed in Table 5.1 (including the out-group) were 426 bp (ITS) and 202 bp (EF1-α). The EF1-α sequences of the Portuguese isolates were all identical. Nine fixed position differences, including a 20-bp indel, were found between *T. zuluensis* and *T. gauchensis*, as reported by Cortinas *et al.,* (2006b), plus a C/T transition at position 202. No differences in EF1-α were found between the isolates of *T. gauchensis* from the various geographic regions.

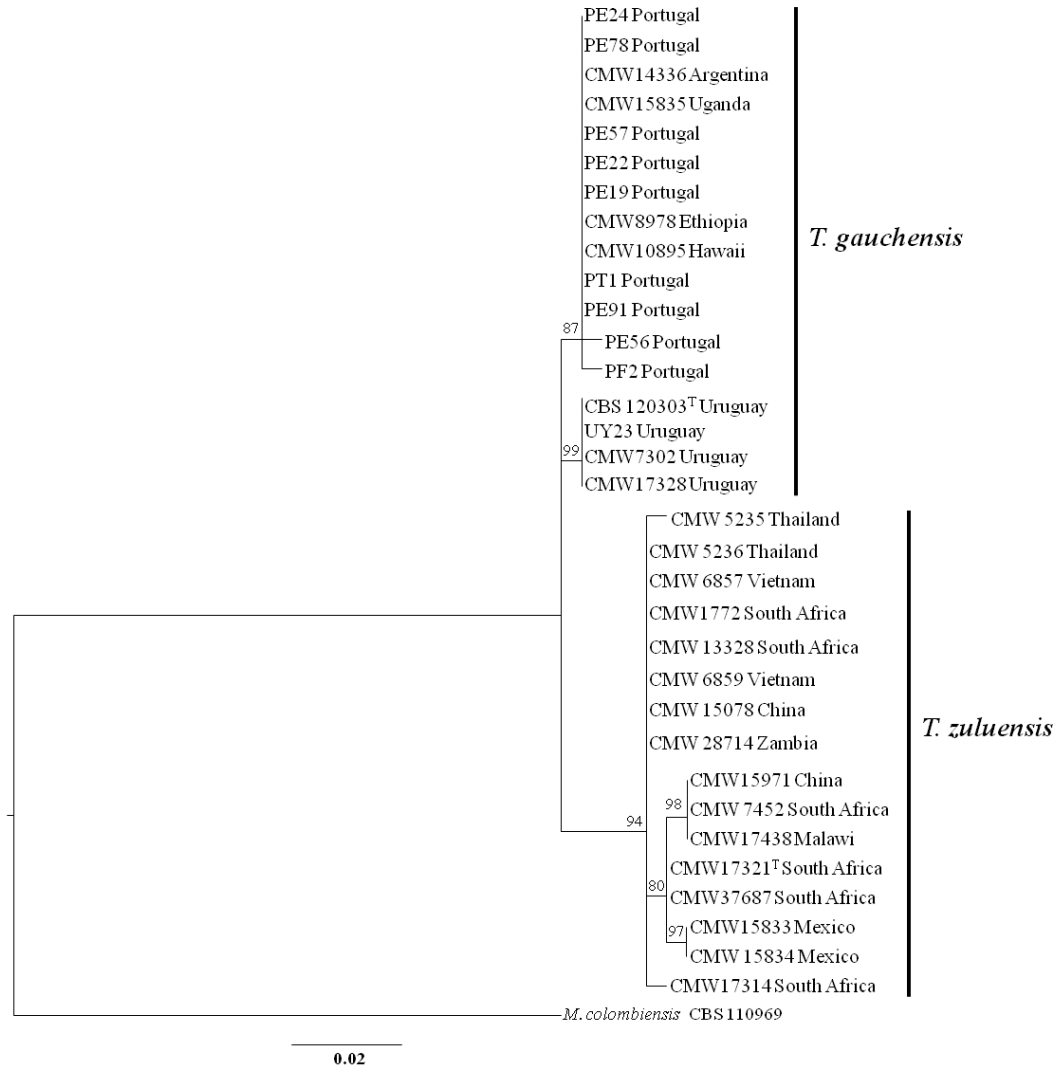

Fig. 5.3 Maximum-likelihood (ML) tree constructed from ITS sequence data with RAxML selecting the gamma model of rate heterogeneity for *Teratosphaeria gauchensis* and *T. zuluensis*. Maximum-likelihood bootstrap values are given at the nodes. The scale bar shows 0.02 changes. Ex-type strains are indicated with a (T).

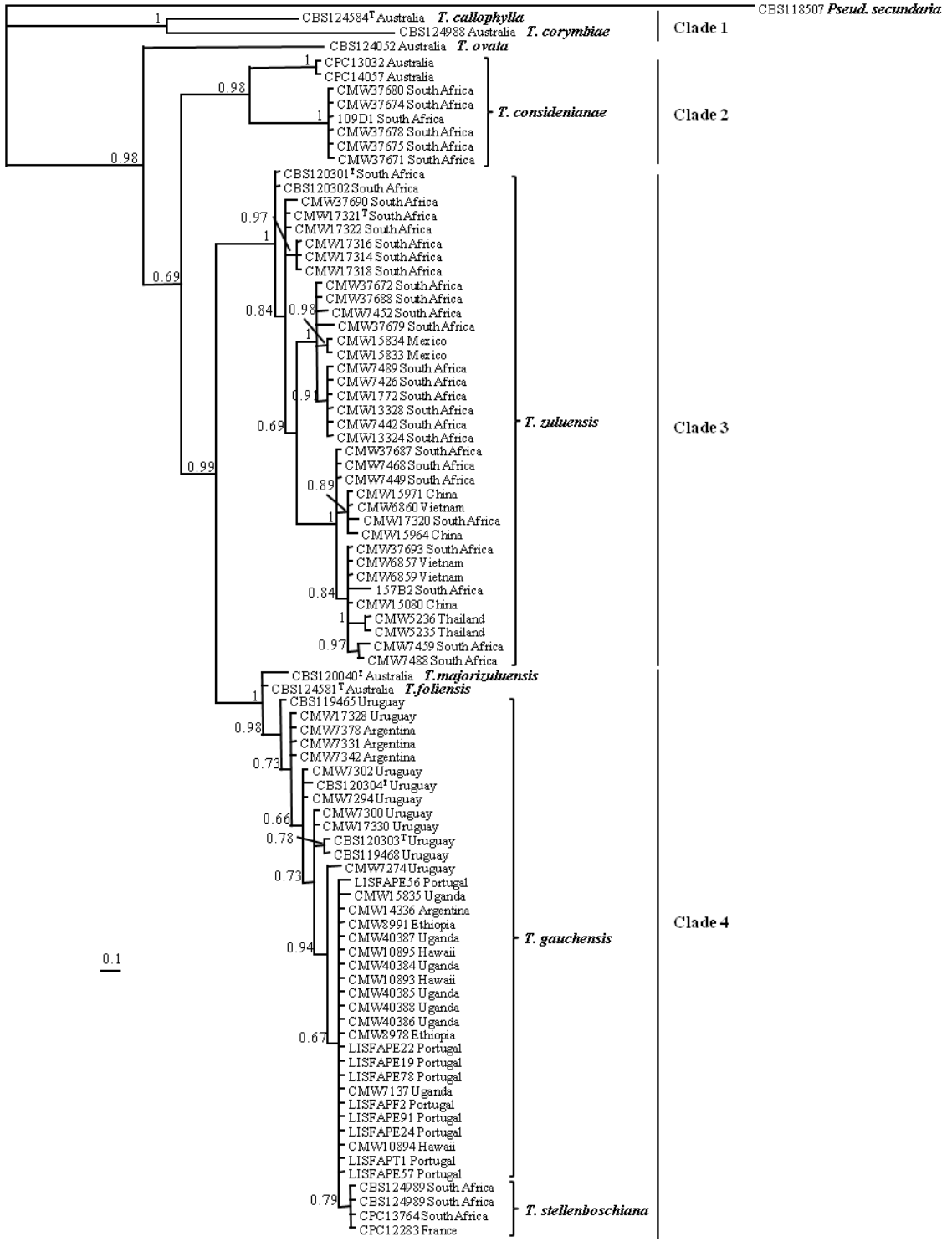

Fig. 5.4 A Bayesian 50% majority rule consensus tree based on a combined ITS, EF-1α alignment, containing all isolates associated with *Teratosphaeria callophylla*, *T. corymbiae*, *T. considenianae*, *T. foliensis*, *T. gauchensis*, *T. majorizuluensis*, *T. ovata*, *T. stellenboschiana* and *T. zuluensis* species available at GenBank with both two genes. Bayesian posterior probabilities support values for the respective nodes are displayed in the tree. The tree was rooted to *Pseudoteratosphaeria secundaria* (CBS118507). The scale bar indicates 0.1 expected changes per site. Ex-type strains are indicated with a (T).

The tree topologies generated by NJ, MP, ML and Bayesian analyses for the combined ITS/EF1-α sequences were similar. Bayesian analyses (Fig. 5.3) resolved four main clades with moderate to high Bayesian posterior probabilities scores; Clade 1 (probability support value = 1.00) representing *T. callophylla* and *T. corymbiae*; Clade 2 (0.98) with *T. considenianae*; Clade 3 (0.99) representing *T. zuluensis* and Clade 4 (1.00) comprising *T. majorizuluensis* and *T. foliensis* subclade (1.00), and a *T. gauchensis* and *T. stellenboschiana* subclade (0.98). The *T. stellenboschiana* clade was separated from *T. gauchensis* with a Bayesian posterior probability score of 0.79.

Discussion

In this study, *T. gauchensis* was determined to be the causal agent of Teratosphaeria canker in Portugal. The two species of *Teratosphaeria* that cause cankers have a non-overlapping geographic distribution, except in Uganda, and are treated as sibling species since they are morphologically and ecologically closely related and cause the same symptoms. The only way to differentiate them is by comparing DNA sequences. Nevertheless, *T. gauchensis* is characterized morphologically by slighter longer conidia than *T. zuluensis*, and the presence of sympodial polyphialidic conidiogenous cells, whereas conidiogenous cells of *T. zuluensis* are monophialidic.

A few morphological differences were found regarding isolates from Portugal and the description of *T. gauchensis* based on the Uruguayan isolates as presented by Cortinas *et al.*, (2006b). The conidia of Portuguese isolates were longer than those of typical isolates of *T. gauchensis* and *T. zuluensis*. The isolates from Portugal showed maximal diameters in culture at 25°C, and the average growth rate was consistent with the values for *T. gauchensis* (Cortinas *et al.*, 2006b). However, the reduction of growth at 35°C was not so marked in the Portuguese isolates, with colony diameters of 12–21 mm after 4 weeks at this temperature. Among Portuguese isolates, two from the north coastal region (Douro Litoral) showed a contrasting behaviour, with faster growth at 20 and 25°C and a more marked reduction of growth at 35°C. This possibly suggests some adaptation of isolates from cooler regions.

Cortinas *et al.*, (2006b) showed that multigene analysis can discriminate between isolates of *T. gauchensis* and *T. zuluensis*. These two species could be separated by 26 fixed nucleotide positions in four loci. These differences were five fixed positions in the ITS region, 11 fixed positions in exons 3 to 6 and the respective introns of the b-tubulin gene, nine positions in the

intron of the EF1-α gene, and one fixed position in intron 2 and exon 3 of the ATP6 gene (ATP6) (Cortinas *et al.*, 2006b). However, as shown in the present study, *T. gauchensis* and *T. zuluensis* can be separated solely on ITS sequence data confirming the report by Crous *et al.*, (2009a) and with EF1-a gene as reported by Cortinas *et al.*, (2006b).

The phylogenetic analyses presented here showed that all Portuguese isolates were *T. gauchensis*, although ITS sequences of some isolates from Alto Alentejo were closer to an isolate from Uruguay. Furthermore, one isolate collected from a leaf in Portugal was closer to Uruguayan isolates at one position in the ITS region than to the other Portuguese isolates.

The only reported study of genetic diversity on *T. gauchensis* was conducted on two South America populations, one in Uruguay and the other in Argentina (Cortinas *et al.*, 2011). The diversity was higher than previously supposed and was inconsistent, indicating that the genetic structure of the populations was not as expected for a recently introduced pathogen. It was also demonstrated that both populations showed levels of moderate to high genetic variation, had a homogeneous distribution of haplotypes, that recombination had occurred, and that there was no differentiation between populations (Cortinas *et al.*, 2010). Cortinas *et al.*, (2011) also compared levels of genetic diversity of *T. gauchensis* with other *Mycosphaerella* and *Teratosphaeria* species from native ranges and noticed that all species (*Pseudocercospora musae*, *P. fijiensis*, *T. nubilosa*) except *T. gauchensis* had sexual states. Although a sexual state has never been observed in *T. gauchensis*, it is expected that it has both asexual and sexual states as in *Mycosphaerella* s.l. (Cortinas *et al.*, 2011; Crous *et al.*, 2013).

The two species of *Teratosphaeria* causing Teratosphaeria canker disease have never been observed in the native range of *Eucalyptus* in Australia, nor had they previously been recorded in Europe. The disease is considered to be more serious in forests in regions with a typically subtropical climate than in those with a temperate climate (Wingfield *et al.*, 1996). This could explain why the disease has not become a serious problem in the Iberian Peninsula. The emergence in other countries could be related to introduction of the pathogen on plant material and trade of seeds (Cortinas *et al.*, 2006b). The origin of *T. gauchensis* remains unclear. Cortinas *et al.*, (2011) made two suppositions to corroborate the idea that *T. gauchensis* is not a recently introduced pathogen in Argentina and Uruguay. They suggested that the fungus may have either originated from native host stock in Australasia, or that there was a host jump from other species native to these countries. Therefore, as noted by them, to answer the question about the origin of this species, it will be necessary to reassess the studies made so far, supplemented with new representative isolates from Africa (Uganda and Ethiopia), Hawaii and Europe (Portugal), as well as a survey in Australia.

Dissimilar morphology was found in *T. gauchensis* and *T. stellenboschiana* species (Crous *et al.*, 2006, 2009a; Andjic *et al.*, 2010). Nevertheless, in the study by Quaedvlieg *et al.*, (2014), the phylogenetic species concept was uncertain for *T. gauchensis* (four isolates that revealed a wide genetic drift) and *T. stellenboschiana* (isolates were relatively conserved). On the other hand, by applying the genealogical concordance phylogenetic species recognition concept, they concluded that there was no support that these isolates belong to the same species, as there was no significant recombination between *T. gauchensis* and *T. stellenboschiana*. Thus, these isolates actually belong to two distinct taxa (Quaedvlieg *et al.*, 2014). The present study found similar topology to Quaedvlieg *et al.*, (2014); however, higher expected changes per site were obtained and higher posterior probabilities support values on Bayesian analysis that could give more certainty to the phylogenetic species concept of these species.

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V

Species Behaviour in the Complex

CHAPTER 6

Pathogenicity of species in the Eucalyptus Leaf Disease complex

Chapter 6

RESEARCH ARTICLE

Pathogenicity of species in the Eucalyptus Leaf Disease Complex

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Summary

Almost 700 species of fungi have been recorded on *E. globulus*. Several species of *Mycosphaerella* and *Teratosphaeria* are known to cause diseases that threaten *Eucalyptus* plantations worldwide, causing leaf spots while others behave as saprobe. Frequently, several leaf pathogens are present in a single lesion but principally *Teratosphaeria cryptica* and *T. nubilosa* have been reported as being responsible for major damage. There is a need to evaluate the contribution of individual species present in the leaf disease complex to understand this disease. In this paper the individual behaviour of several species found associated with diseased leaves of *E. globulus* were studied to compare their ability to cause leaf disease. *Cladosporium cladosporioides, Mycosphaerella communis* and *M. lateralis* had a more aggressive behaviour than *T. nubilosa*, which has been considered the major damage agent on eucalypts foliage.

Introduction

Eucalypts are native to Australia but since the late 18th century they have been planted worldwide. They are well-known for their fast growth, straight form, their capacity to adapt to soils and a wide range of climates like high rainfall, semi-arid, sea level and alpine tree line zones (Potts, 2004). Commercially they are important for their special wood properties and pulp production. In Portugal, the plantations are concentrated in the wetter coastal regions where productivity is higher and attack by pathogens is generally low. *Eucalyptus* spp. are susceptible to a large number of foliar pathogens. Pests and diseases are known to seriously threaten *Eucalyptus* plantations throughout the world (Wingfield *et al*., 2008).

About 696 species of fungi have been recorded on *E. globulus*, a large part of them specific to *Eucalyptus* (Hyde *et al.*, 2007). Several species of *Mycosphaerella* and *Teratosphaeria* (Crous, 1998; Crous *et al*., 2007) cause one of the diseases threatening *Eucalyptus* plantations worldwide (Wingfield *et al*., 2008). Eucalyptus leaf disease complex reduces the photosynthetic capacity of leaves causing premature defoliation, shoot dieback and decreasing growth resulting in significant losses (Carnegie, 2007b; Hunter *et al*., 2009; Lundquist and Purnell, 1987).

Mycosphaerella s. l. species can be found in different ecosystems as hyperparasites, phytopathogens (primary and secondary) and saprobes. Some species grow as endophytes and become apparent only when the host becomes senescent or is exposed to stress (Crous *et al.*, 2000; Ganley *et al.*, 2004; Crous *et al.*, 2007; Márquez *et al.*, 2011). Yet others form mutualistic

associations in lichens (Crous *et al.*, 2000, 2006). Normally, when they appear on living hosts, they are phytopathogenic, frequently causing leaf spots. They are rarely hyperparasitic or saprobic (Crous 1998, Crous *et al.*, 2000, 2004b).

Up to five *Mycosphaerella* and *Teratosphaeria* species could take place mutually in a small leaf lesion (Mohammed *et al*., 2003; Crous *et al*., 2004a). On the other hand, as an example, several species of the complex, were not only found on juveniles leaves but could also be observed on adult foliage such as *Amycosphaerella africana*, *M. lateralis*, *M. madeirae*, *Pallidocercospora heimii, Paramycosphaerella marksii*, *Sonderhenia eucalypticola* (≡ *M. walkeri*), *T. cryptica*, *T. molleriana*, *T. nubilosa* (Carnegie and Keane, 1994; Maxwell *et al*., 2000; Milgate *et al*., 2001; Barber *et al*., 2008; Pérez, 2008; Pérez *et al*., 2009; Pérez *et al*., 2009a, 2009b; Marquez *et al*., 2011). In general, several leaf pathogens could be found in a lesion but mostly *T. nubilosa* and *T. cryptica* appear to be responsible for major damage (Park and Keane 1982a, 1982b; Park and Kean, 1984; Carnegie *et al*., 1994; Park *et al*., 2000; Milgate *et al*., 2001; Mohammed *et al*., 2003; Hunter *et al*., 2011). As a result, the epidemiology and pathology research was practically made from these species and using a mix of ascospore suspension (Cheah, 1977; Beresford, 1978; Ganapathi, 1979; Park and Kean, 1982b; Cheah and Hartill, 1987; Park and Keane, 1987; Park, 1988a, 1988b; Carnegie, 2000).

On a eucalypt leaf lesion the mechanisms that permit several species to occupy the same niche are not known and it is necessary a comparison of individual contribution of species of the complex to fully understand this disease (Maxwell *et al*., 2003; Crous *et al*., 2009). Thus the aim of this research was to access the individual capacity of the species found in the leaf disease complex to develop lesions on *E. globulus* and to distinguish different individual behaviours.

Materials and Methods

Plant materials and fungal strains

Four-month-old cuttings from one *Eucalyptus globulus* clone were used in the pathogenicity tests. The plants were maintained in a controlled environment chamber and irrigated twice a week. During the experiments air temperature ranged from 18 to 22ºC, relative humidity from 65 to 75% with a photoperiod of 16h/8h. At the time of inoculation the mean plant size was 19 cm with an average of 9 leaves per plant.

The strains used in the experiments were isolated from juvenile leaves of *E. globulus* from 7 plantations during a survey between 2004 and 2010 in Portugal (Table 6.1). All strains

were maintained in malt extract agar (MEA) McCartney flasks at 8ºC before use. Previous to the inoculation, the isolates were recovered by plating a mycelia plug in MEA plates and incubated at 24ºC for 30 days.

Leaf inoculation

Inoculations were made with single spore cultures. A completely randomized experimental design was set up in which seedlings were inoculated in groups of 10 plants with the isolates listed in Table 6.1 Two apparently healthy leaves from the top of each plant were inoculated: the first expanded leaf was non-wounded (nW) and the second expanded leaf was wounded with a needle (W). This leaf was cleaned with sterile distilled water and the cuticle damaged with a sterile needle (lesion length of approximately 0.05 mm diameter). A mycelial plug (1 mm in diameter) from the margin of a fungal colony was then placed on each leaf and covered with transparent adhesive tape. Adhesive tape was removed 4 weeks later. Fifteen control plants were inoculated with sterile MEA plugs using the same procedure.

Monitoring and data analysis

Plants were maintained for 102 days. Observation of foliar necrosis was done weekly during the first month and measured every 3 weeks after. Necrosis diameter (mm) on adaxial and abaxial surface was measured and mean diameter of the lesion (mm) calculated when the lesion was not circular. The relation between adaxial and abaxial necrosis length leaves was analysed with a linear regression. Data from the assays were analysed using IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp. Data were checked for normality and equal variance distributions and analysed by one-way ANOVA. The variation between necrosis length mean values of non-wounded and wounded leaves [Δ(nW-W)] was calculated to estimate species aggressiveness.

Table 6.1 List of taxa and strains isolated from juvenile leaves of *Eucalyptus globulus* plantations surveyed in Portugal between 2004 and 2010 and inoculated on this study.

Taxa	Strain no.[1]	Isolation date
Austroafricana parva (≡*T. parva*)	LISFA BBC155	Feb 2010
	LISFA BBC1B	Jan 2009
	LISFA X53A	Mar 2005
	LISFA Y2B	Mar 2005
Cladosporium cladosporioides	LISFA Pb15	Feb 2010
Fusicladium eucalypti	LISFA BBC264	May 2010
Mycosphaerella communis	LISFA A107	Nov 2009
	LISFA BBC37	Feb 2010
	LISFA Nx30B	Mar 2005
M. lateralis	LISFA BBC76	Feb 2010
	LISFA QP50	Feb 2010
	LISFA X13A	Mar 2005
M. madeirae	LISFA Pb4	Feb 2010
Neodevriesia hilliana	LISFA BBC352	May 2010
Paramycosphaerella marksii	LISFA Pb51	May 2010
	LISFA Pb7	Feb 2010
	LISFA QP38	Feb 2010
Teratosphaeria gauchensis	LISFA PF2	Nov 2006
T. lusitanica	LISFA BBC239	Feb 2010
T. molleriana	LISFA BBC324	May 2010
	LISFA QP46	Feb 2010
	LISFA Y16F	Mar 2005
T. nubilosa	LISFA A11	Feb 2008
	LISFA BBC332	May 2010
	LISFA Y8B	Mar 2005
T. pluritubularis	LISFA BBC317	May 2010
Teratosphaericola pseudoafricana (≡*T. pseudafricana*)	LISFA BB293	May 2010
Quasicercospora mexicana (≡*T. mexicana*)	LISFA QP13	Jan 2009
Venturiaceae sp.	LISFA BBC191	Feb 2010

([1]) All strains were deposited at **LISFA** Herbarium Code of collection of UEIS-SAFSV (Unidade Estratégica de Investigação e Serviços de Sistemas Agrários e Florestais e Sanidade Vegetal), Instituto Nacional de Investigação Agrária e Veterinária, I.P., Oeiras, Portugal.

Results

Control plants grew adequately and produced asymptomatic leaves during the trial, non-wounded control leaves did not show any lesions, although wounded control leaves showed a few lesions with a maximum diameter of 1.35 mm. These were considered to be the marks caused by the needle wound. According to the results obtained, plants did not show secondary fungal colonization and did not die due to interaction host/pathogen. Foliar lesions caused by inoculated fungi did not extend to others parts of the plant during the assay. They regularly consisted of circular, necrotic, brown coloured areas.

The relation between adaxial and abaxial necrosis length on non-wounded leaves was analysed with a linear regression model ($y=0.9582x + 0.1199$; $R2 = 0.9054$) and on wounded leaf ($y=0.9145x + 0.1163$; $R2 = 0.7853$). Thus both analyses showed a close relationship between adaxial and abaxial necrosis length and therefore abaxial necrosis length was selected for further statistical analysis. Foliar lesions on non-wounded and wounded leaves at the end of experiment were expressed in diameter and grouped by species in Table 6.2 (abaxial necrosis length). A previous analysis of variance of the means showed no significant differences ($p\leq 0.05$) between isolates of the same species.

Diameters of lesions on non-wounded leaves varied from 4.64±0.33 mm (*M. communis*) to 1.93±0.24 mm (*M. madeirae*). On wounded leaves extension of lesions ranged between 3.97±0.54 mm (*Cladosporium cladosporioides*) and 2.14±0.26 mm (*Teratosphaeria lusitanica*). The largest lesions were caused by *M. communis* on non-wounded leaves and also developed large lesions (3.52mm) on wounded leaves leading to a value of Δ(nW-W) (variation between mean values of nW and W) of 1.12. This value is the higher positive value, i.e lesions on non-wounded leave are larger than lesions on wounded leaves on day 102. A similar behaviour is observed with *M. lateralis*, *T. nubilosa*, *C. cladosporioides*, *T. lusitanica*, *T. molleriana* and *N. hilliana*. Thus, all these species presented larger lesions on non-wounded than on wounded leaves.

All the other tested species presented negative values of Δ(nW-W) varying between *Quasiteratosphaeria mexicana* (-0.24Δ) and *Fusicladium* sp. (-1.71Δ) (Table 6.2). Thus, all these species caused larger lesions on wounded leaves than on non-wounded ones.

Table 6.2 Mean values and standard error of lesion diameter (mm) on abaxial surface of non-wounded (nW) and wounded (W) leaves, 102 days after inoculation with different species. For each column, values with different letters differ significantly according with the Duncan's test. Values are sorted by Δ(nW-W) representing the variation between mean values of lesions on nW and W leaves.

Species	N	nW			W			Δ(nW-W)
		Mean	Std. Error		Mean	Std. Error		
Mycosphaerella communis	30	4,64	0,33	a	3,52	0,21	ab	1,12
Mycosphaerella lateralis	30	3,50	0,41	abcd	2,72	0,24	abc	0,78
Teratosphaeria nubilosa	30	2,85	0,25	defg	2,30	0,20	abc	0,55
Cladosporium cladosporioides	10	4,49	0,66	ab	3,97	0,54	a	0,52
Teratosphaeria lusitanica	10	2,57	0,22	defg	2,14	0,26	bc	0,43
Teratosphaeria molleriana	30	3,65	0,33	abc	3,40	0,22	ab	0,25
Neodevriesia hilliana	10	3,23	0,41	defg	3,09	0,30	ab	0,14
Quasiteratosphaeria mexicana	10	3,31	0,49	bcde	3,55	0,47	ab	-0,24
Austroafricana parva	40	2,70	0,16	defg	2,97	0,60	ab	-0,28
Teratosphaeria gauchensis	10	2,18	0,25	fg	2,74	0,29	abc	-0,56
Paramycosphaerella marksii	30	2,33	0,18	efg	2,98	0,38	ab	-0,66
Teratosphaericola pseudoafricana	10	2,36	0,26	defg	3,06	0,26	ab	-0,70
Teratosphaeria pluritubularis	10	2,86	0,27	defg	3,75	0,31	ab	-0,89
Venturiaceae sp.	10	2,82	0,27	defg	3,86	0,33	ab	-1,04
Control	**10**	**0,00**	**0,00**		**1,35**	**0,14**	**c**	**-1,35**
Mycosphaerella madeirae	10	1,93	0,24	g	3,46	0,22	ab	-1,53
Fusicladium eucalypti	10	2,05	0,11	fg	3,75	0,38	ab	-1,71

To analyse the patterns of necrosis development along the time of the experiment three species were selected based on its predominance on Eucalyptus leaf disease complex detected during field survey (Silva *et al.*, unpublished). Mean values of lesion development on abaxial surface of non-wounded (nW) and wounded (W) leaves during 102 days after inoculation are compared for *Mycosphaerella communis* (Cm), *Teratosphaeria nubilosa* (N) and *Austroafricana parva* (P) (Figure 6.1).

The species *M. communis* caused necrosis more quickly on wounded inoculated leaves however on non-wounded leaves the necroses are larger on 102 days of the assay. The species *T. nubilosa* and *A. parva* need more time to start necrosis than *M. communis* and both attained similar development on non-wounded leaves. In wounded leaves *A. parva* showed higher values of necrosis development than *T. nubilosa*. Furthermore, after 60 days of inoculations on non-wounded leaf lesion, *M. communis* showed lesions on 93%, *A. parva* on 75% and *T. nubilosa* on 63 % of all leaves inoculated.

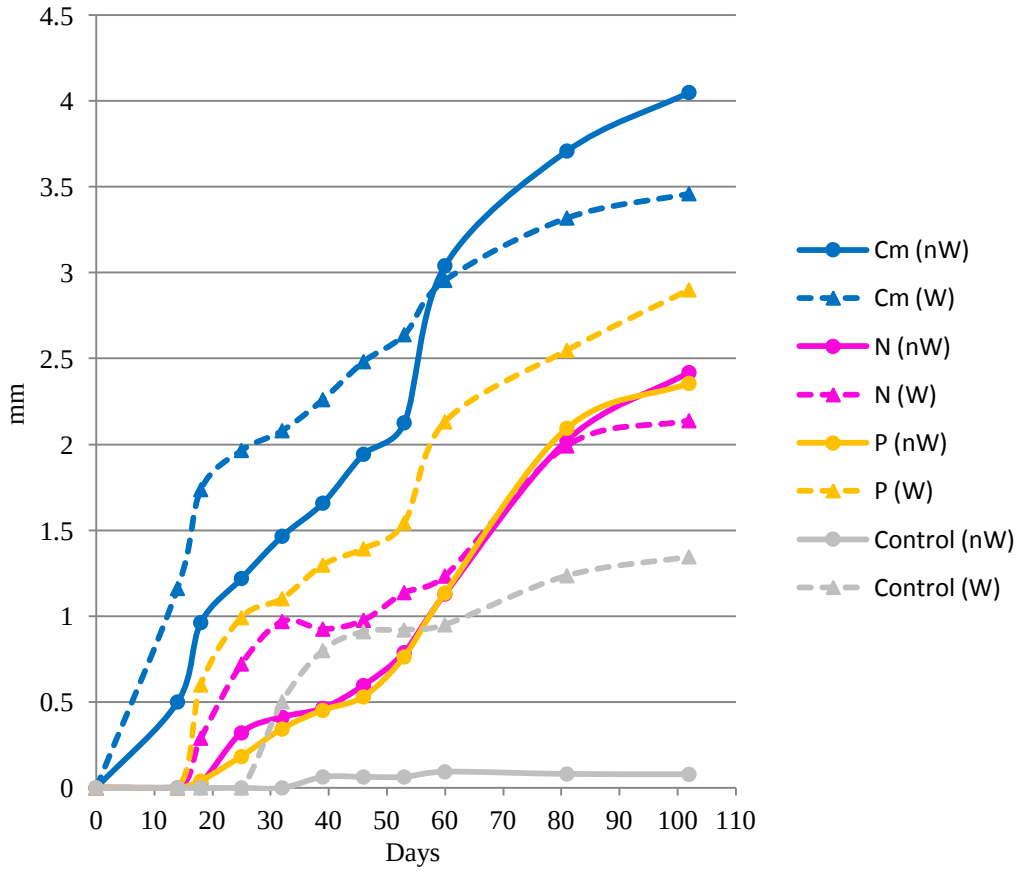

Fig. 6.1 Mean values of lesion diameter (mm) on abaxial surface of *Mycosphaerella communis* (Cm), *Teratosphaeria nubilosa* (N) and *Austroafricana parva* (P) during 102 days after inoculation on non-wounded (nW) and wounded (W) leaves.

Discussion

Leaf characteristics of *Eucalyptus globulus* diverge in response to plant genotype, ontogenetic position and environmental conditions. Adult foliage of *E. globulus* has lower stomatal density and thicker cuticles than juvenile leaves (James and Bell, 2001). On the other hand, no variation was recorded in concentration of total phenolics, only tannin activity is significantly higher on juvenile leaves (Gras *et al.*, 2005). Regarding constitutive anatomy, cellular and histochemical changes on *Eucalyptus* juvenile leaves after infections by *Mycosphaerella* species suggested that characteristics such as cuticle thickness, palisade mesophyll density and wax coverage of stomata, are associated with resistance (Smith *et al.*, 2006, 2007). This might clarify why Eucalyptus leaf disease complex incidence and severity on several *Eucalyptus* species is generally more severe on juvenile-phase foliage (Pinkard and Mohammed, 2006; Hunter *et al.*, 2009) and most of *Mycosphaerella* s.l. occur on juvenile

leaves and not in adult ones, once juvenile leaves are more disposed to infection by pathogens and endophytes (Marquez *et al*., 2011).

Species occurring in eucalyptus leaf disease complex can be found living as plant pathogenic fungi, as opportunistic or secondary colonists of lesions or as endophytes, hyperparasite, mutualistic and saprobic on plants growing on rocks (De Hoog *et al*., 1983, 1991; Ganley *et al*., 2004; Crous *et al*., 2006, 2007).

The most common species on eucalyptus leaf disease complex are considered to be the primary pathogen *T. nubilosa* based on severe defoliation events and dominance in frequency of isolation (Crous *et al*., 1989; Park and Keane, 1982b; Park and Keane, 1984; Maxwell *et al*., 2003; Carnegie, 2007a; Hunter *et al*., 2009). Others species are also considered primary pathogens as they can damage about 70% of susceptible trees foliage like *M. lateralis*, *T. molleriana* and also *T. nubilosa*, previously mentioned (Whyte *et al*., 2005; Jackson *et al*., 2005). The results of this study confirmed this behaviour for those species with the addition of *M. communis*, *C. cladosporioides*, *T. lusitanica* and *Neodevriesia hilliana* as probable primary pathogens.

Comparison of leaf lesions developing on wounded and non-wounded leaves was used to evaluate the aggressiveness of each species permitting to distinguish different behaviours, from primary to secondary pathogens. During the assay the species *M. communis* showed high level of aggressiveness, not only on the size of lesions but also on the infection's efficiency, causing necrosis more rapidly than others species like *T. nubilosa*. In addition, this species produced the larger lesions on non-wounded leaves. Indeed *T. nubilosa* with lesion of small dimension presented the same curve type than *M. communis*. It was considered that species that show higher lesions on non-wounded leaves correspond to a primary pathogen behaviour that has an easier way to go through the plant defences and do not have to deal with plant defences reactions.

The greater pathogenicity of *M. communis* was confirmed in the experiments reported here, not only in the size of lesions but also in terms of infection efficiency with 93% of non-wounded inoculated leaves showing lesions after 60 days of inoculation. The species *Austroafricana parva* had 75% of inoculation success and *T. nubilosa* had 63 %.

The species *A. parva* caused more severe necrosis on wounded leaves than *T. nubilosa.* This behaviour could be supported by field observations when this species is associated with aged juvenile leaves lesions and with more species on same lesion. Rather *T. nubilosa* is associated with young juvenile leaves lesions and most of the times present alone on the lesion

(Park and Keane, 1982b; Silva *et al*., unpublished). Furthermore, on wounded leaves the higher values of lesions length represent secondary opportunistic pathogen behaviour, especially if the corresponding non-wounded leaves lesions were lower than wounded lesion, represented on this study by *A. parva* behaviour.

The *Cladosporium* spp. and *A. parva* are suggested to behave like saprobes (Marquez *et al*., 2011; Park and Keane, 1982a; Crous *et al*., 1989). However in this study the behaviour of *Cladosporium cladosporioides* was more similar to *M. communis*, which had a more aggressive behaviour in this study than *T. nubilosa*, a primary pathogen.

Also in a leaf litter, a saprobic environment, were find *A. parva*, *P. marksii*, *T. molleriana* and *T. nubilosa* which are considered leaf pathogens. It is possible that these species could have a saprobic behaviour and represent an inoculum pool in their cycle of life (Marquez *et al*., 2011). On the contrary *P. marksii* has been suggested as minor pathogen (Park *et al.*, 2000) seemed to be prevalent in *Eucalyptus* plantations in Uruguay and there is some evidence that it contributes to disease (Pérez, 2008). However in view of our results *P. marksii* should be considered as a secondary pathogen.

It was also suggested that *M. lateralis* could act as a hyperparasite of others species with highest incidence on eucalypts like *T. cryptica* and *T. nubilosa*, in the same way as *Dissoconium aciculare* was isolated as hyperparasite from *Erysiphe* on *Medicago lupulina* and *D. dekkeri* (*M. lateralis* anamorph) hyperparasite *Tilletiopsis* on *Junipero chinensis* (De Hoog *et al*., 1983, 1991). However, Jackson *et al*. (2004) indicated that *M. lateralis* is not a hyperparasite as previously suggested but may be a foliar pathogen. In this study, *M. lateralis* behaviour was more comparable to *M. communis* than to *T. nubilosa*, i.e with higher lesions than *T. nubilosa*.

The tested species that develop small lesions in both wounded and non-wounded leaves and present negative values of Δ(nW-W) could be considered as secondary or opportunistic pathogens. In this group values varied between, for example, *Quasiteratosphaeria mexicana* (Δ(nW-W) = -0.24) and *Fusicladium* sp. (Δ(nW-W) = -1.71). However, all these species produced larger lesions than *T. nubilosa* on wounded inoculation, which could indicate that these species can act as parasites after others start the lesion or on a weakened host.

It is probable that mycelium of several *Mycosphaerella* and *Teratosphaeria* species colonizing the same lesion could swap genetic material among themselves and form hybrids (Hunter *et al*., 2009) that could increase virulence of these genera. Thus it becomes difficult to attribute the contribution of individual species to disease development. On other hand, this study only observed the individual behaviour of each species on the lesion contrasting with field

behaviour that likely involves a complex of the species acting together resulting in an additive effect of species in the same lesion. Indeed, perhaps the most studied foliar pathogen on *Eucalyptus* (*T. nubilosa*) does not appear to be the most damaging when it acts individually. However, other species are also involved in the damage, which is caused by a conjugation of species.

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VI

General Conclusions

General Conclusions

The Eucalyptus Leaf Disease Complex (ELDC) was recently reviewed resulting in a re-evaluation of the genera. Species were re-distributed into several genera providing an update on the disease. The species present on symptomatic leaves from *Eucalyptus globulus* were characterised in terms of morphology and phylogeny based on the ITS1-5.8S-ITS2 cluster. Several new records of *Teratosphaeria* species and their close relatives were reported from the Iberian Peninsula, new anamorphic structures were described, new combinations were made, and a new species was introduced. New symptoms on eucalypt leaves and cankers implicated have been taken into care, with a new canker report. Finally, an evaluation of the individual behaviour of species previously detected in the complex was made.

Synthesis of the main findings

The main species occurring in Portuguese plantations were found to be *Austroafricana parva*, *Mycosphaerella communis, M. lateralis, Paramycosphaerella marksii, Teratosphaeria molleriana* and *T. nubilosa*. These species were distributed throughout the coastal regions. Species diversity was generally lower in the north of the country compared to the south. The following species were reported for the first time from *E. globulus* plantations in Portugal: *Neodevriesia hilliana* (new host record), *Quasiteratosphaeria mexicana* (new combination), *Teratosphaeria pluritubularis*, *Teratosphaericola pseudoafricana*, (first reported from the Iberian Peninsula), *T. lusitanica* (new species), *Cladosporium cladosporioides, Fusicladium eucalypti*, *M. madeirae* (first report from continental Portugal) and a unidentified *Ventureaceae*.

New anamorph structures were described for *A. parva, Q. mexicana*, *T. pluritubularis* and *T. pseudoafricana* and their descriptions were emended. Based on phylogenetic placement *Mycosphaerella quasicercospora* was transferred to *Amycosphaerella* as *Amycosphaerella quasicercospora* and *Mycosphaerella mexicana* was transferred to *Quasiteratosphaeria* as *Quasiteratosphaeria mexicana*.

Besides species causing leaf disease *Teratosphaeria gauchensis* was detected for the first time as causal agent of Teratosphaeria canker in Portugal. A few morphological differences were found between Portuguese and Uruguayan isolates, namely, conidia of Portuguese isolates were longer, showed maximal colony diameters in culture at 25°C while a reduction of growth at 35°C was not so discernible in the Portuguese isolates.

During an evaluation of the contribution of individual species to the development ELDC *M. communis, T. molleriana* and *M. lateralis* were considered primary pathogens since they caused larger lesions than *T. nubilosa* in both types of inoculation (on non-wounded and wounded leaves) and they caused larger lesions on non-wounded than on wounded leaves. Species that produced small lesions in both types of inoculation and presented negative values of variation between mean values of non-wounded and wounded were considered as secondary pathogens, e.g. *Quasiteratosphaeria mexicana* and *Fusicladium eucalypti*. In species like *A. parva* and *P. marksii* their saprobic behavior was confirmed with the development of larger lesions on wounded than on non-wounded leaves. In contrast, species usually considered saprobe like *C. cladosporioides* developed larger necrosis in both types of inoculation (non-wounded and wounded) a characteristic of primary pathogen.

The species associated with ELDC were studied in Portuguese eucalypt plantations. Isolates were characterized in terms of morphology and phylogenetic relationships inferred from ITS sequence data. Morphological characteristics of the species identification agree with the previous descriptions (Park and Keane, 1982; Carnegie and Keane, 1994, 1997; Crous and Wingfield, 1996, 1997; Crous *et al.,* 2004; Hunter *et al.,* 2006; Silva *et al.,* 2009). In the phylogenetic analyses tree topologies were similar to those reported by Crous *et al.* (2007) and Quaedvlieg *et al*. (2014) for all the species represented.

The present study updated the number of species reported in Portuguese *E. globulus* plantations with new records in several families. The species composition in ELDC was similar to that recorded in the last reports (e.g. Silva *et al.,* 2009) but a greater diversity was observed in the disease complex. Although several new reports have been identified, they appear outnumbered than those that were previously reported with a great representativeness in the complex. Furthermore, species distribution was less diverse in northern rather than in southern locations. Disease severity was higher in coastal plantations where fog and moisture conditions would like favour infection. Thus more research is needed to understand and identify the factors implicated in the differences within the composition of the species on the complex.

Although *T. molleriana* was reported from Portugal in 1881, the first record of *Mycosphaerella s. l.* outside of Australia, it did not spread out or cause problems during all these years as *T. nubilosa* did in the last years since the first detection. All *T. molleriana* reports were mostly on locations in the centre of the country where were found more species diversity. Consequently it is possible that along the years the new species introduced into Portugal overshadowed the impact of *T. molleriana* in the field. It is important to highlight that despite examining several hundreds of isolates Silva *et al.* (2009, 2012, submitted) did not find any isolate of *T. cryptica*, although it is known from Spain (De Blas *et al.,* 2009).

Outbreaks of Teratosphaeria canker were investigated and the causal agent was determined to be *Teratosphaeria gauchensis*. Two species of *Teratosphaeria* have been associated with this disease (*T. gauchensis* and *T. zuluensis*). These two species have a non-overlapping geographic distribution, except in Uganda, and are treated as sibling species since they are morphologically, phylogenetically and ecologically closely related and cause the same symptoms. Some Portuguese isolates of *T. gauchensis* from the north coastal regions of Portugal showed a reduction of growth at higher temperatures, suggesting some adaptation to cooler regions.

Frequently more than one species is found in a single leaf lesion, making necessary the evaluation of the individual contribution of species in ELDC. In order to understand their individual behavior the ability of the species to colonize and produce leaf necrosis on non-wounded and wounded leaves was evaluated and some considerations were drawn and allow distinguishing primary from secondary pathogens. Species with primary pathogen behaviour in our study, like *T. molleriana* or *M. lateralis*, have already been considered to be responsible for 70% of the damage on susceptible tree foliage (Jackson *et al.*, 2005). Other primary pathogens highlighted in our study include *C. cladosporioides*, which is in contrast to what has been said about *Cladosporium* spp. acting as saprobes (e.g. Marquez *et al.*, 2011). Secondary pathogen behaviour, e.g. by *A. parva*, can be supported by field observations when is usually associated with more species in aged lesions (Park and Keane, 1982). Species with secondary pathogen behavior could cause disease after colonizing lesions started with other primary pathogen species, especially in the presence of susceptible hosts.

Based on field observations *T. nubilosa* has been considered the main primary pathogen responsible for ELDC (e.g. Hunter *et al.*, 2009) and little importance has been given to other species in the complex. Our field observations also suggested the same conclusion, however also including *A. parva*. Nevertheless, when confronting the results of species frequency in the field to results of individual species inoculation tests it seems that other species in the complex have a more aggressive behaviour than *T. nubilosa*. When comparing individual species effects on *E. globulus* leaves it is possible to extrapolate that the severity of attack in the field could be correlated with species frequency and their combination in the complex. Therefore when *T. nubilosa* is observed with other more aggressive species in the complex greater severity of disease would be expected in the field.

The attribution of the eucalypts leaf disease to a single species has to be taken with some caution especially when field observations are simply based on morphological data. This study suggests that this disease involves a complex of species and disease severity depends of the number and combination of aggressive species and the edaphoclimatic conditions in the field.

Thus the subject is deepest that we could assume as a simple evaluation of the symptoms and lesions types.

Future Perspectives

The centre of origin of *T. gauchensis* remains uncertain. Cortinas *et al*. (2011) advanced two possibilities to corroborate the idea that *T. gauchensis* was not a recently introduced pathogen in Argentina and Uruguay. They suggested that the fungus may have either originated from a native host stock in Australasia, or that there was a host jump from other native species to these countries. It will be essential to re-examine the studies made so far, supplemented with new representative isolates from Africa (Uganda and Ethiopia), Hawaii and Europe (Portugal), as well as a survey in Australia, using for example microsatellite markers. The correct understand of origin of some species of ELDC populations can also benefit with this tool especially in a global scenario of biological material exchanging.

In the future an increasing of ELDC attacks and their aggressiveness can be expected due to climate changes and likely a swap of genetic material amongst themselves when several species mycelium colonize the same lesion (Lundquist and Purnell, 1987; Crous *et al.*, 2004; Hunter *et al.*, 2009). It is predictable that incidence and severity of this disease and also cankers will increase especially in favorable climate conditions.

Although more knowledge was acquired about species life cycles along the year, with an decrease of species on the complex in autumn when *T. nubilosa* has the highest occurrence, fungal spore traps could be used to monitor the populations involved in the disease levels and discern more about their life cycle.

There are no approved fungicides for application in the Portuguese eucalypts plantations. This measure is expensive, environmentally unacceptable and impractical for tree plantations. Therefore, it is important to find solutions to maintain eucalypts plantations healthy. Breeding programs should include the search for resistance genes and the production of vegetative material could take into account other promising characteristics, e.g. clones with premature transition to adult foliage. An adequate selection of sites for installation of new plantations and good forestry practices, as weeds control and fertilization, are also important aspects to better manage threatened stands.

Portuguese forest is an essential resource normally underestimated and often forgotten, especially their health. It is more seriously endangered today than ever before, in particular the

eucalypts, a non-native tree, which during many years have been kept apart from their natural enemies. Due to worldwide exchange of biological material it is important that eucalypts monoculture stands are periodically monitored for the occurrence of new potentially threatening species and the most aggressive ones should be better studied.

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APPENDIX A

Complementary Studies and Conference Posters

In vitro Pathogenicity Tests with *Mycosphaerella* Species Isolated from Portuguese Eucalyptus Plantations

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Summary

Recent surveys showed a high number of *Mycosphaerella sensu lato* species in Portuguese eucalyptus plantations with more than one species occurring in the same lesion. This work intends to develop an in vitro pathogenicity to clarify the pathogenic role of each of the coexisting species. Micropropagated plantlets of a susceptible Eucalyptus globulus clone with three pairs of completely expanded leaves were used in two inoculation series. First, plantlets were inoculated with pure cultures of the species previously identified based on morphologic and molecular characteristics. In a second series, plantlets were inoculated with ascospores collected from fresh symptomatic leaves. Drops of ascospore suspensions were applied to the leaves. Additional drops were used to quantify ascospores and obtain single spore isolates. Both inoculation methods resulted in leaf lesions similar to those observed in the field. All isolates were reisolated at the end of the experiment.

Rapid pathogenicity tests can be useful to determine which species are the most aggressive with regard to eucalyptus production. Commercial E. globulus clones showed significant differences in disease severity in field trials. Quantifying infection under in vitro conditions will be essential to devise future screening tests for vegetative material resistance in areas where MLD is a problem.

Keywords: Pathogenicity; *Mycosphaerella*; *Eucalyptus globulus*; Leaf Disease

Introduction

Mycosphaerella sensu lato is one of the largest genera of ascomycetes. *Mycosphaerella* leaf disease (MLD) on eucalypts have been associated with 78 species of *Mycosphaerella* and *Teratosphaeria* and more than 30 anamorphs. In 1999 the first cases of severe damage on eucalypts in Portugal were reported. Until now, a total of 11 species of *Mycosphaerella* have been recorded on Portuguese eucalypts (*M. communis*, *M. heimii*, *M. lateralis*, *M. madeirae*, *M. marksi*, *M. walkeri*, *M. vespa*, *T. africana*, *T. molleriana*, *T. nubilosa*, *T. parva* (=*M. grandis*)).

Recent surveys showed a high number of *Mycosphaerella sensu lato* species in Portuguese eucalyptus plantations with more than one species occurring in the same lesion. This work intends to develop an *in vitro* pathogenicity test to clarify the pathogenic role of each of the coexisting species, i.e. mainly to determine species agressiveness. Micropropagated plantlets of a susceptible *Eucalyptus globulus* clone were used in two inoculation series. First, plantlets were inoculated with pure cultures of the species previously identified based on morphological and molecular characteristics (Crous, 1998 and Crous *et al*., 2004). In a second series they were inoculated with ascospores collected from fresh symptomatic leaves.

Methods

Micropropagated plantlets were grown in autoclavated Murashige and Skoog Medium (Cód. Duchefa MO236) supplemented with vitamins (100 mgL^{-1} Myo-Inositol; 0.1 mgL^{-1} Pyridoxine-HCl; 0.5 mgL^{-1} Thiamine-HCl; 0.1 mgL^{-1} Niacin), micro-nutrients (0.03mgL^{-1} $CoCl_26H_2O$; 0.03 mgL^{-1} $CuSO_45H_2O$; 10 mgL^{-1} $FeC_6H_5O_7.3H_2O$; 6.20 mgL^{-1} H_3BO_3; 16.90 mgL^{-1} $MnSO_4.H_2O$; 0.25 mgL^{-1} $Na_2MoO_4.2H_2O$; 8.60 mgL^{-1} $ZnSO_4.7H_2O$), 5 mgL^{-1} dithiothreitol; 0.2 mgL^{-1} 6-benzilaminopurina; 7 gL^{-1} agar and 20 gL^{-1} sucrose in a FitoclimaS600PLH growth chamber with a light-dark cycle of 16:8h and 22:20ºC at 3400 lux.

Results

Most of the times *T. parva* and *M. grandis* germinate in less than 24h (except in lesion 3). Showing the best germination time and rate of isolation efficiency. *T. parva* and *M. lateralis* were present in all the lesions (except *M. lateralis* in lesion 6). The species that produced lesions and necroses on the stem (not usually observed in the field) were *T. nubilosa* (the most aggressive), *T. parva* and *M. grandis*. The others just produced small lesion. Both inoculation methods resulted in leaf lesions similar to those observed in the field. However, it was not possible to make histological sections or reisolation with ascospores. All isolates were reisolated at the end of the experiment, but this was only possible with mycelial inoculum.

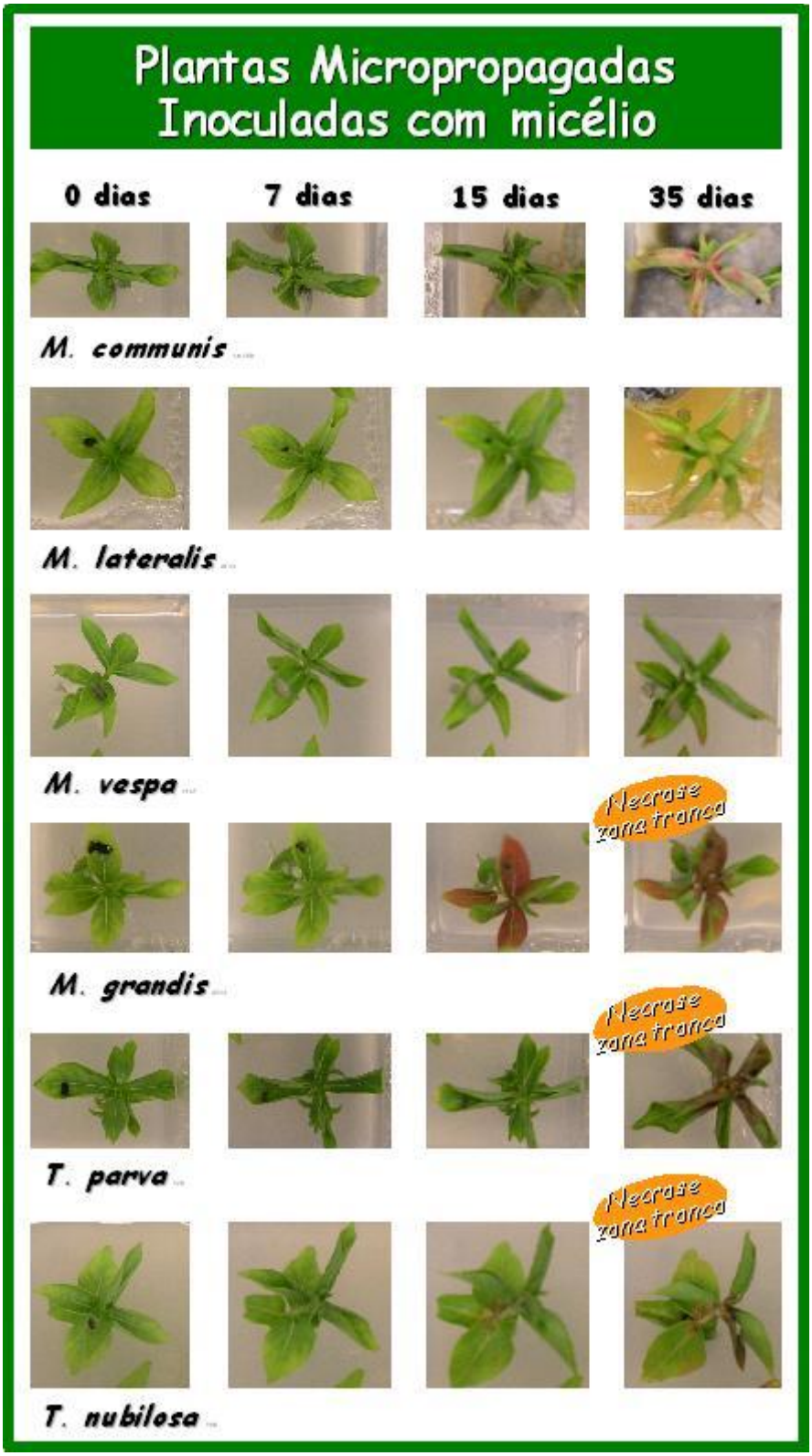

Fig. A.1 Pathogenicity test *in vitro* on *E. globulus at* 0, 7, 15 and 35 days, after inoculation with mycelia of *Mycosphaerella communis*, *M. lateralis*, *Teratosphaeria molleriana* (≡*M. vespa*), *Austroafricana parva* (≡*M. grandis*), *A. parva* and *T. nubilosa*.

Conclusions

The most effective means to control MLD is through the use of disease tolerant or resistant clones. Rapid pathogenicity tests can be useful to find out which species are the most aggressive with regard to eucalyptus production. Commercial *E. globulus* clones showed significant differences in disease severity in field trials. Quantifying infection under in vitro conditions will be essential to devise future screening tests for vegetative material resistance in areas where MLD is a problem. Thus, it is imperative to have a complete knowledge of the variations in virulence within and between the species.

Acknowledgements

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In vitro pathogenicity tests with *Mycosphaerella* species isolated from Portuguese eucalyptus plantations

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Introduction

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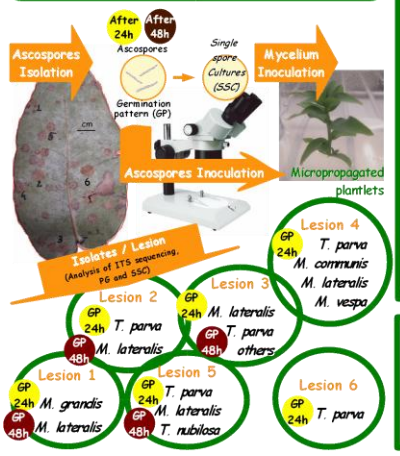
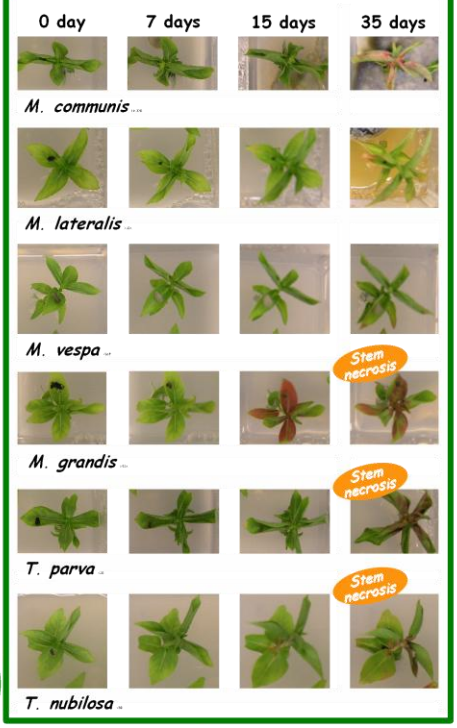
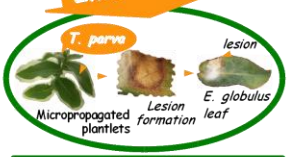


Micropropagated plantlets inoculated with mycelium



Mycelium Inoculations



Methods

Micropropagated plantlets were grown in autoclavated Murashige and Skoog Medium (Cód. Duchefa MO236) supplemented with vitamins (100 mgL^{-1} Myo-Inositol; 0.1 mgL^{-1} Pyridoxine-HCl; 0.5 mgL^{-1} Thiamine-HCl; 0.1 mgL^{-1} Niacin), micro-nutrients (0.03mgL^{-1} $CoCl26H2O$; 0.03 mgL^{-1} $Cu5O45H2O$; 10 mgL^{-1} $FeC6H5O7.3H2O$; 6.20 mgL^{-1} $H3BO3$; 16.90 mgL^{-1} $MnSO4.H2O$; 0.25 mgL^{-1} $Na2MoO4.2H2O$; 8.60 mgL^{-1} $ZnSO4.7H2O$), 5 mgL^{-1} dithiothreitol; 0.2 mgL^{-1} 6-benzilaminopurina; 7 gL^{-1} agar and 20 gL^{-1} sucrose in a Fitoclima5600PLH growth chamber with a light-dark cycle of 16:8h and 22:20ºC at 3400 lux.

Results

Most of the times *T. parva* and *M. grandis* germinate in less than 24h (except in lesion 3). Showing the best germination time and rate of isolation efficiency. *T. parva* and *M. lateralis* were present in all the lesions (except *M. lateralis* in lesion 6). The species that produced lesions and necroses on the stem (not usually observed in the field) were *T. nubilosa* (the most aggressive); *T. parva* and *M. grandis*. The others just produced small lesion. Both inoculation methods resulted in leaf lesions similar to those observed in the field. However, it was not possible to make histological sections or reisolation with ascospores. All isolates were reisolated at the end of the experiment, but this was only possible with mycelial inoculum.

Conclusion

The most effective means to control MLD is through the use of disease tolerant or resistant clones. Rapid pathogenicity tests can be useful to find out which species are the most aggressive with regard to eucalyptus production. Commercial *E. globulus* clones showed significant differences in disease severity in field trials. Quantifying infection under in vitro conditions will be essential to devise future screening tests for vegetative material resistance in areas where MLD is a problem. Thus, it is imperative to have a complete knowledge of the variations in virulence within and between the species.

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 the material. Experimental plantations were supported by Technical assistance protocolo of Instituto RAIZ/Silvicaima/INRB and the PhD grant funded by the Fundação para a Ciência e a Tecnologia.

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Survey of *Mycosphaerella* species complex on *Eucalyptus globulus* in Portugal

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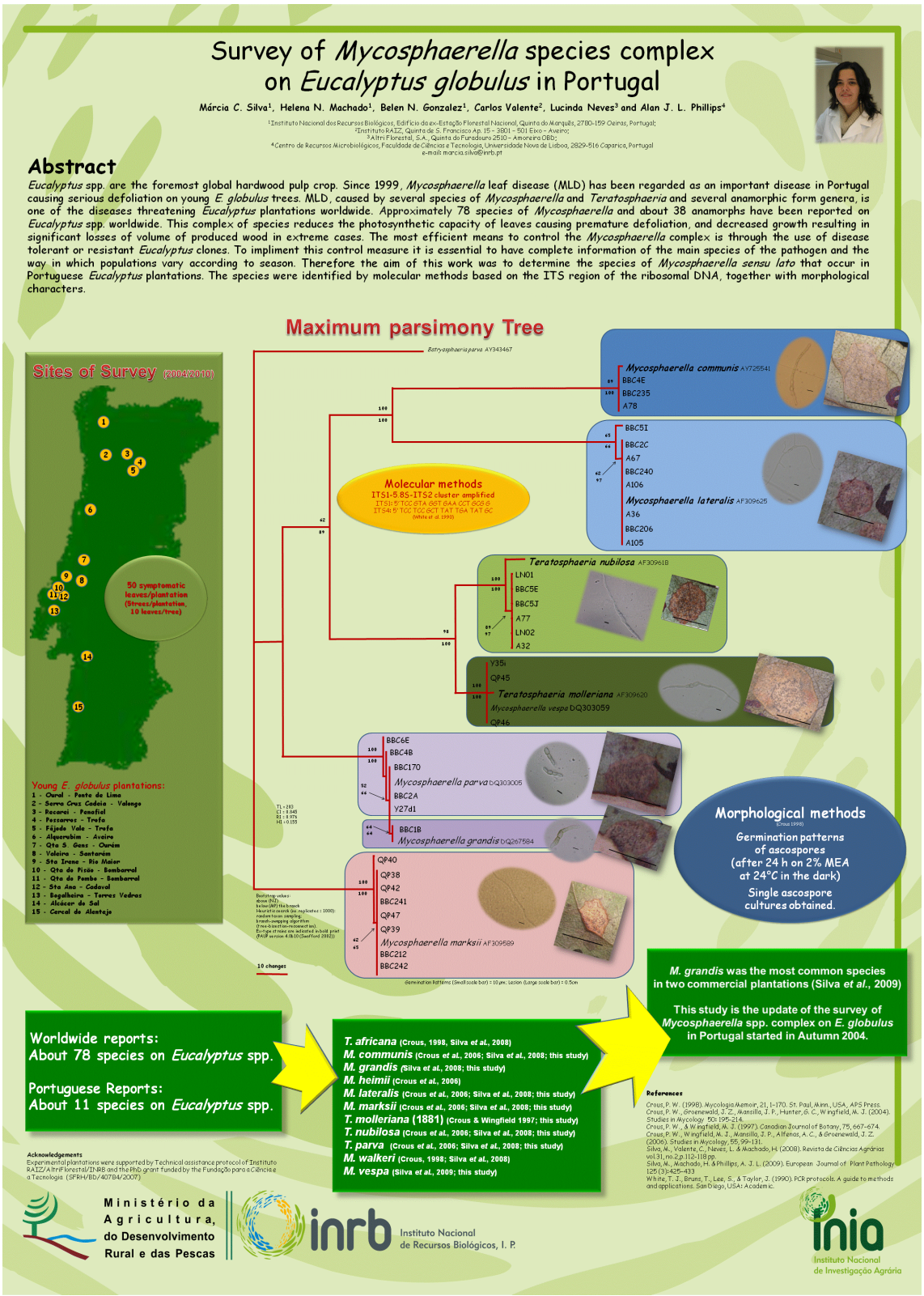
Abstract

Eucalyptus spp. are the foremost global hardwood pulp crop. Since 1999, *Mycosphaerella* leaf disease has been regarded as an important disease in Portugal causing serious defoliation on young *E. globulus* trees. MLD, caused by several species of *Mycosphaerella* and *Teratosphaeria* and several anamorphic form genera, is one of the diseases threatening *Eucalyptus* plantations worldwide. Approximately 78 species of *Mycosphaerella* and about 38 anamorphs have been reported on *Eucalyptus* spp. worldwide. This complex of species reduces the photosynthetic capacity of leaves causing premature defoliation, and decreased growth resulting in significant losses of volume of produced wood in extreme cases. The most efficient means to control the *Mycosphaerella* complex is through the use of disease tolerant or resistant *Eucalyptus* clones. To impliment this control measure it is essential to have complete information of the main species of the pathogen and the way in which populations vary according to season. Therefore the aim of this work was to determine the species of *Mycosphaerella sensu* lato that occur in Portuguese *Eucalyptus* plantations. The species were identified by molecular methods based on the ITS region of the ribosomal DNA, together with morphological characters.

Keywords: Pathology, Dothideomycetes, *Mycosphaerella*, *Teratosphaeria*, Leaf Disease, MLD

Scientific topic: Epidemiology, modeling and forecasting of plant diseases or Molecular variability of pathogens

Silva M.R.C., H. Machado, B.N. Gonzalez, C. Valente, L. Neves and A.J.L. Phillips, 2010. Survey of *Mycosphaerella* species complex on *Eucalyptus globulus* in Portugal. 9th Conference of the European Foundation for Plant Pathology/6th Congress of the Sociedade Portuguesa de Fitopatologia – Integrated Plant Disease Management, 15-18 November, Évora-Portugal. Book of Abstracts P8.3, pp 139.



Distribution of *Mycosphaerella* leaf disease on Eucalyptus in Portugal

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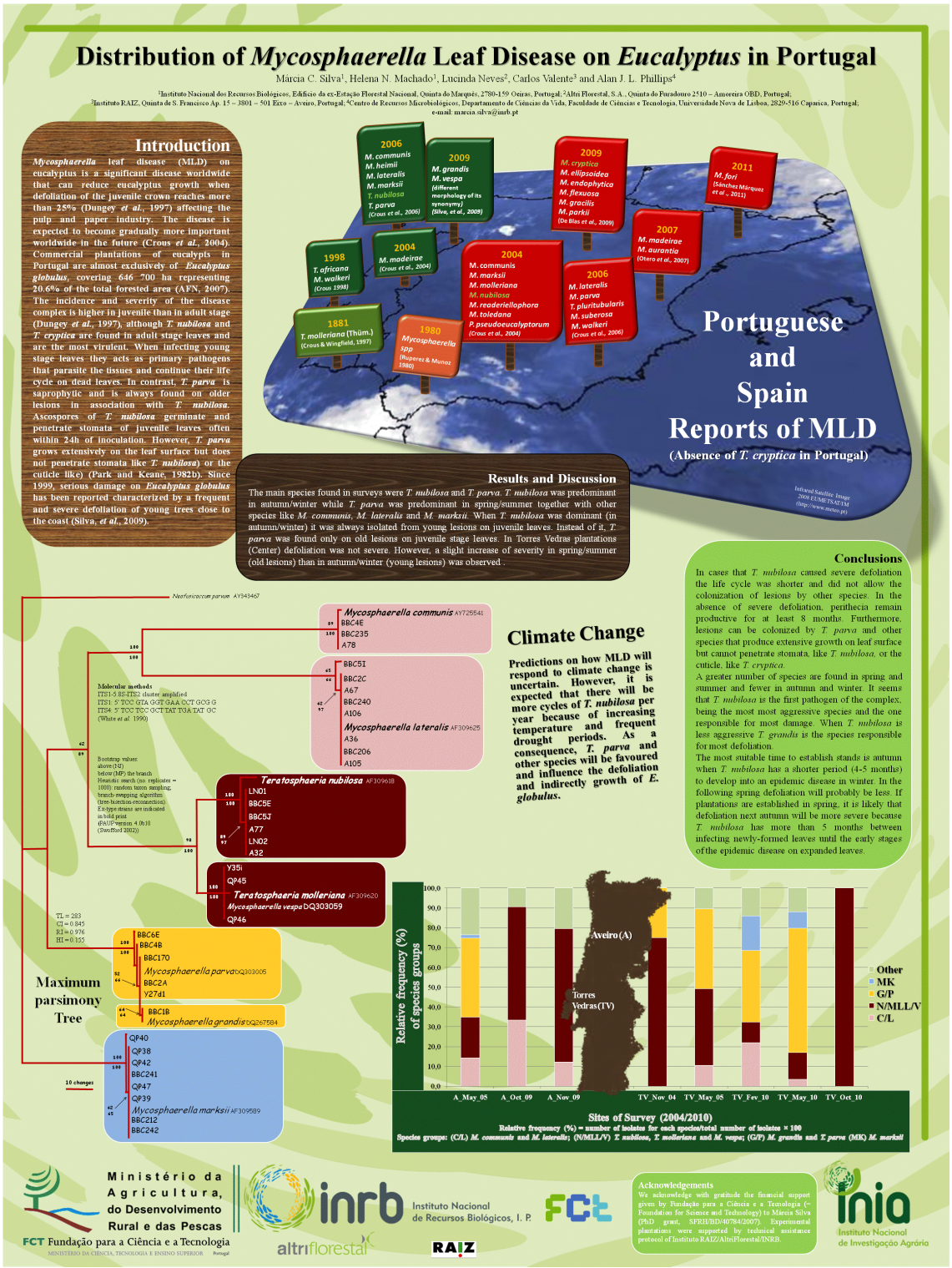
Eucalypt plantations represent the main source of wood for the pulp and paper industry and are affected by an important foliage disease worldwide - the complex of *Mycosphaerella* and *Teratosphaeria* species (*Mycosphaerella* leaf disease). These genera affect mainly young trees with juvenile-phase foliage, causing premature defoliation, decreased growth and wood production. Species of *Mycosphaerella senso lato* reported on eucalypts in Portugal are *Teratosphaeria molleriana*, *Teratosphaeria africana*, *Mycosphaerella walkeri*, *Mycosphaerella madeirae*, *Mycosphaerella communis*, *Mycosphaerella heimii*, *Mycosphaerella lateralis*, *Mycosphaerella marksii*, *Teratosphaeria nubilosa* and *Teratosphaeria parva*. Since 2004, in order to complete the survey, symptomatic leaves were collected from *E. globulus* plantations. Morphological and molecular characterization was used to give a clear indication of the population composition and the main species.

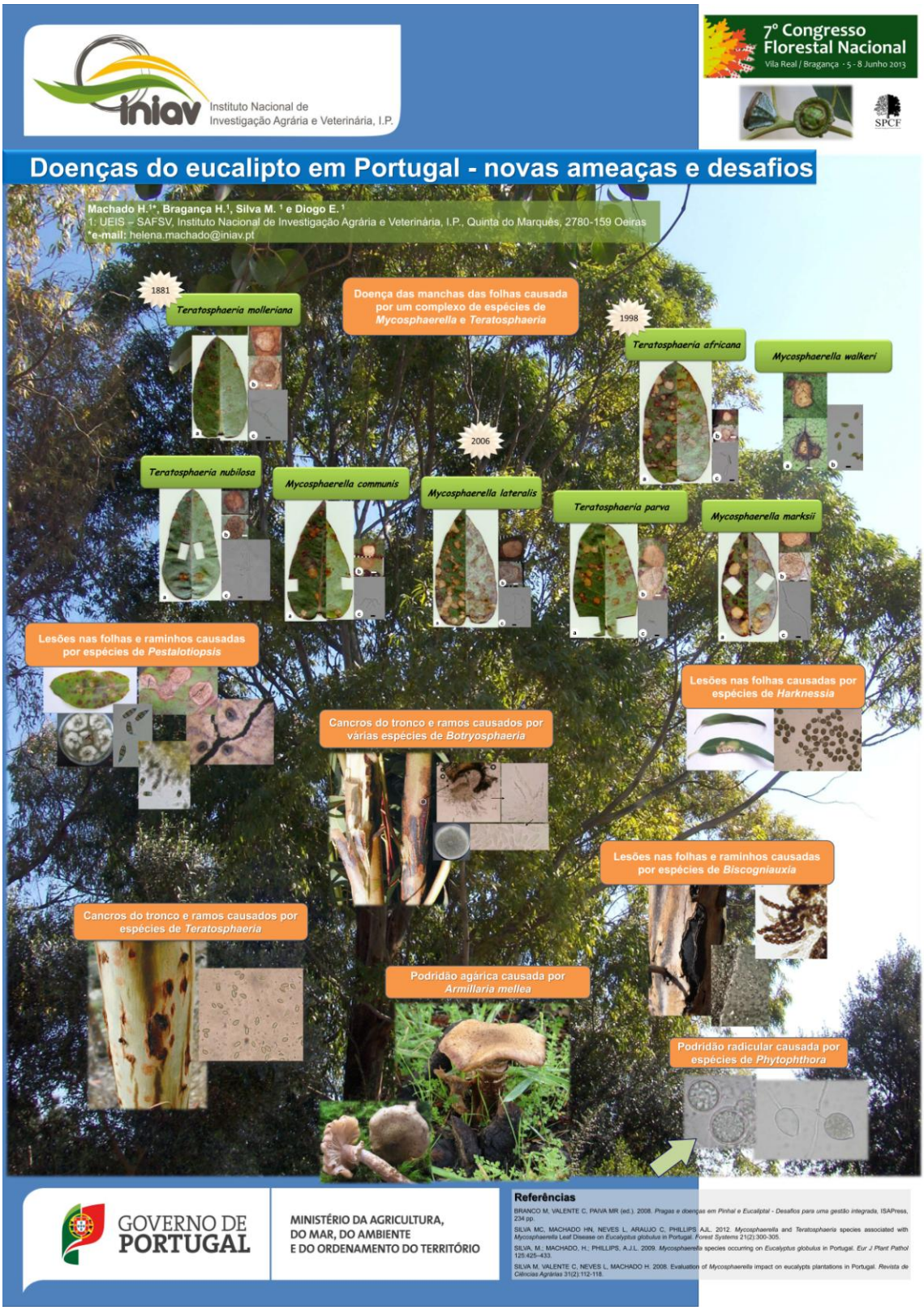
Key words: *Mycosphaerella*, *Teratosphaeria*, Leaf Disease, MLD, *Eucalyptus*.

Silva M.R.C., H. Machado, L. Neves, C. Valente and A.J.L. Phillips, 2012. Distribution of Mycosphaerella leaf disease on *Eucalyptus* in Portugal. IUFRO WP 7.02.02 2011 Global Change and Forest Diseases: New Threats, New Strategies, Montesclaros Monastery in Cantabria (Spain) from 23rd to 28th of May 2011. *Journal of Agricultural Extension and Rural Development*, Conference Proceedings 4, (9) 298.

Silva M.R.C., H. Machado, L. Neves, C. Valente and A.J.L. Phillips, 2012. 2011. Distribution of Mycosphaerella Leaf Disease on Eucalyptus in Portugal. IUFRO WP 7.02.02 2011 Global Change and Forest Diseases: New Threats, New Strategies, Montesclaros Monastery in Cantabria (Spain) from 23rd to 28th of May 2011, Abstracts book pp.116 (poster).



Machado, H.*, H. Bragança, M.R.C. Silva and E. Diogo, 2013. Doenças do eucalipto em Portugal – novas ameaças e desafios. 7º Congresso Florestal Nacional "Florestas – Conhecimento e Inovação", Vila Real/Bragança, 5-8 junho. Livro de Resumos pp. 197.



Silva M.R.C., E. Diogo, H. Bragança, H. Machado and A.J.L. Phillips, 2014. New disease linked with trunk and stem canker of *Eucalyptus* in Portugal. 1º Simpósio SCAP "Novos desafios na Proteção das Plantas e 7ºCongresso da SPF. P03.12. pp.52.

Instituto Nacional de Investigação Agrária e Veterinária, I.P.

New disease linked with trunk and stem canker of *Eucalyptus* in Portugal

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Abstract

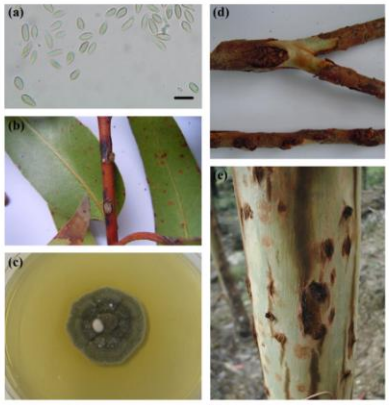
Canker caused by *Teratosphaeria gauchensis* and *T. zuluensis* is a very damaging disease of *Eucalyptus* species that has serious effects on tree growth in worldwide. The symptoms are discrete necrotic spots on young green stems, lesions on twigs that develop into internal cankers. In old stages, canker can coalesce to form large, dark, oval-shaped cankers on the stems and trunks and is able to affect adult eucalypts. The aim of this work is to clarify the identity of the pathogen involved on new symptoms observed in Portuguese *Eucalyptus globulus* plantations.

Introduction

Eucalyptus species are affected by a canker caused by *Teratosphaeria gauchensis* and *T. zuluensis* that cause serious damage and affects the growth of trees (Cortinas *et al.*, 2010. *Australas. Plant Pathol.* 39, 383.). This canker was reported for the first time in South Africa on *E. grandis* trees and the cause attributed to *Coniothyrium zuluensis* (Wingfield *et al.*, 1996. *Mycopathologia* 136, 139–145.). It was subsequently found to affect a wide range of *Eucalyptus* species and hybrids and has never been observed in the native range of *Eucalyptus* in Australia (Cortinas *et al.*, 2006. *Stud. Mycol.* 55, 133–146.). However it does affect eucalypts in several countries (see Table below). In South Africa the disease increased rapidly, with losses to the forest industry and caused damage in most susceptible hosts in the first years after its detection (Cortinas *et al.*, 2006. *Stud. Mycol.* 55, 133–146.). In South America, because of the rapid increase in the number of eucalypt plantations *T. gauchensis* has became a growing threat to the industry (Cortinas *et al.*, 2011. *Australas. Plant Pathol.* 40, 497–503.).

Regions where canker were observed:

Algarve, Alto Alentejo, Baixo Alentejo, Beira Litoral and Douro Litoral.

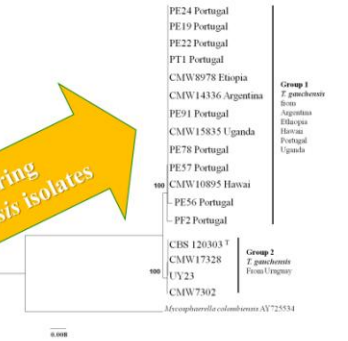


Symptoms of canker disease

(a)Conidia of *Teratosphaeria gauchensis*.
(b)Infected shoot with necrosis.
(c)Culture isolate on MEA (25ºC).
(d)Cankered shoot.
(e)Cankers on trunk.
Scale bars: (a) 10 µm.

Species	Year	Country	Host	Reference
Teratosphaeria zuluensis	1996	South Africa	*E. grandis*	Wingfield *et al.*, 1996. *Mycopathologia* 136, 139–145
	1996	Thailand	*E. camaldulensis*	Van Zyl *et al.*, 2002. *Mycological Research* 106, 51–59.
	2000	Mexico	*E. grandis*	Rous *et al.*, 2002. *Plant Pathology* 51, 382.
	2000	Vietnam	*E. urophylla*	Gezahgne *et al.*, 2003. *South African Journal of Science* 99, 587–588.
	2004	Malawi	*E. grandis*	Cortinas *et al.*, 2006. *Studies Mycology* 55, 133–146.
	2004	China	*E. urophylla*	Cortinas *et al.*, 2006. *Mycological Research* 110, 229–236.
	2010	Zambia	*E. clocziana and E. grandis*	Chungu *et al.*, 2010. *Forestry* 83, 507–515.
	2012	Uganda	*E. grandis*	Jimu *et al.*, 2014. *Forest Pathology* 44, 242–245.
T. gauchensis	2001	Uganda	*E. grandis*	Cortinas *et al.*, 2006. *Studies Mycology* 55, 133–146.
	2001	Ethiopia	*E. camaldulensis*	Gezahgne *et al.*, 2005
	2001	Argentina	*E. grandis*	Gezahgne *et al.*, 2003. *South African Journal of Science* 99, 587–588.
	2001	Uruguay	*E. grandis, E. maidenii and E. tereticornis*	Cortinas *et al.*, 2006. *Studies Mycology* 55, 133–146.; Pérez *et al.*, 2009. *Forest Pathology* 39, 349–360.
	2002	Hawaii	*E. grandis*	Cortinas *et al.*, 2004. *Australasian Plant Pathology* 33, 309.
	2006	Portugal	*E. globulus*	Silva *et al.*, 2015. Forest Pathology. doi: 10.1111/efp.12160.

Comparing *T. gauchensis* isolates



Maximum likelihood analyses (ML) tree using RAxML (Randomized Accelerated Maximum Likelihood) –HPC BlackBox v. 7.3.2 selecting the gamma model of rate heterogeneity for DNA sequence alignment on Internal Transcribed Spacer (ITS) rDNA data of *T. gauchensis*. Maximum likelihood will search for best-scoring tree after the 1000 bootstraps and Maximum Likelihood bootstrap value (MLBP) are given at the nodes. The scale bar shows 0.008 changes. Ex-type strains are indicated by (T).

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Silva M.R.C., H. Machado and A.J.L. Phillips, 2014. Eucalyptus Leaf Disease Complex in Portugal and its recent taxonomy. 1º Simpósio SCAP "Novos desafios na Proteção das Plantas e 7º Congresso da SPF. P03.13. pp.53. 20 e 21 Novembro, Oeiras.

Eucalyptus Leaf Disease Complex in Portugal and its recent taxonomy

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Eucalyptus Leaf Disease Complex

Eucalypts are the second most important forest plantation species grown globally. A complex of species of *Mycosphaerella sensu lato* cause leaf disease on *Eucalyptus* that results in significant economic losses worldwide.

Mycosphaerella is inserted in *Dothideomycetes*, *Capnodiales*, a large genus of ascomycetous fungi, generally leaf infecting fungi that contain about 3000 species. *Eucalyptus* are affected by species included mainly in this genus which was recently re-evaluated and transferred to several new genera. So, several species of *Mycosphaerella s.l.* can occur on the same lesion as a complex of species (Silva *et al.*, 2009).

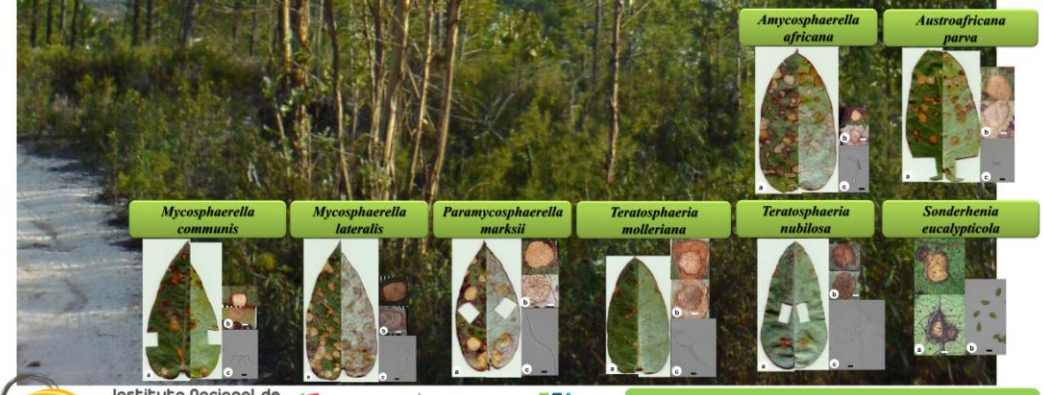
The Leaf Disease Complex has caused widespread damage in Australasia, South America, Western Europe, Southern Africa and South-East Asia (Park *et al.*, 2000) and it is expected to become gradually more important worldwide in the future (Crous *et al.*, 2004).

In Portugal

In Portugal, *Eucalyptus globulus* is the most important forest plantation species, covering about 26% of continental forestry area. *Eucalyptus* plantations are clustered in the coastal regions where pathogen attack is lower.

The first report of *Mycosphaerella* s. l. on eucalypts outside of Australia was in 1881 when *Teratosphaeria molleriana* was described in Portugal (Crous and Wingfield, 1997). Until 1999 leaf disease complex on eucalypts was virtually unknown in Portugal when serious damage on *Eucalyptus globulus* characterized by a frequent and severe defoliation of young trees close to the coast was reported (Silva *et al.*, 2009). In 2008, Silva *et al.* (2008) reported the presence of *Amycosphaerella africana*, *Austroafricana parva*, *M. communis*, *M. lateralis*, *Paramycosphaerella marksii*, *Sonderhenia eucalypticola*, *T. molleriana*, *T. nubilosa* using morphological characteristics. Silva *et al.* (2009) analysed more isolates by morphological characteristics and by molecular methods based on the ITS cluster to confirm the species previously recorded.

In recent years, following a survey throughout the country, more species were reported including *M. madeirae* (in the mainland) *Cladosporium* sp., new reports of *Teratosphaerias*, *Mycosphaerellas*, new combinations and new species (Silva *et al.*, *unpublished*).



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A.J.L. Phillips thanks Fundação para a Ciência e a Tecnologia for financial support through grant PEst-OE/BIA/UI0457/2011.

APPENDIX B

Plantations, Symptomatic leaves, Pattern of Ascospore Germination and Cultures

Table B.1 Number of *Eucalyptus globulus* plantations selected to evaluate ELDC, collections, trees, symptomatic leaves, lesions, isolates obtained and sequences analysed.

Plantations	Collections	Trees	Symptomatic Leaves	Lesions	Isolates	Sequences
15	37	77	602	1443	764	289

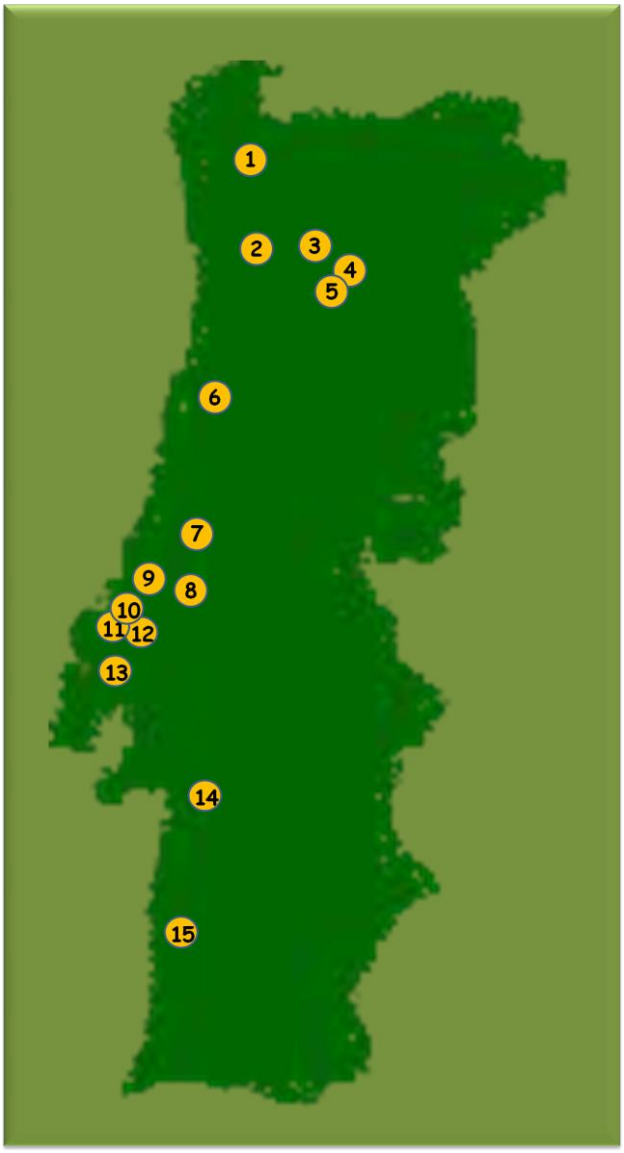

Fig. B.1 Plantations sites of *Eucalyptus globulus* selected to evaluate ELDC species. **(1)** Oural - Ponte de Lima; **(2)** Serra Cruz Cadeia – Valongo; **(3)** Recarei – Penafiel; **(4)** Possarros – Trofa; **(5)** Fôjodo Vale – Trofa; **(6)** Alquerubim – Aveiro; **(7)** Qta S. Gens – Ourém; **(8)** Valeira – Santarém; **(9)** Sta Irene – Rio Maior; **(10)** Qta do Pisão – Bombarral; **(11)** Qta do Pombo – Bombarral; **(12)** Sta Ana – Cadaval; **(13)** Bogalheira – Torres Vedras; **(14)** Alcácer do Sal; **(15)** Cercal do Alentejo.

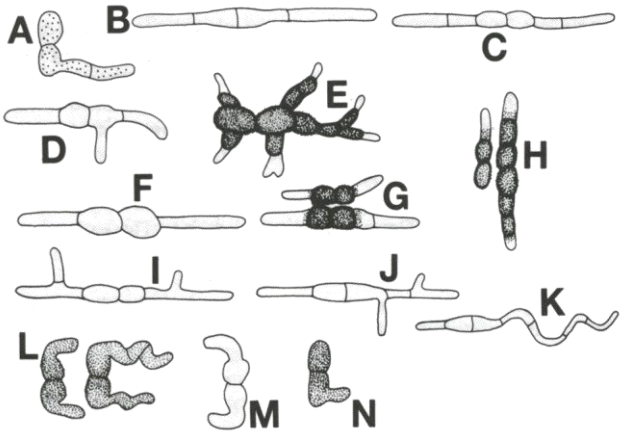

Fig. B.2 Patterns of ascospore germination of several *Mycosphaerella* s.l. (**A)** *T. cryptica;* (**B)** *Pseudocercospora gracilis* (≡*M. gracilis*), *Paramycosphaerella marksii*; (**C)** *Pallidocercospora heimii* (≡*M. heimii*), *Phaeophleospora gregaria* (≡*M. gregaria*), *Teratosphaeria molleriana; T. nubilosa, Sonderhenia eucalypticola* (≡*M. walkeri*); (**D)** *Zasmidium parkii* (≡*M. parkii*)*;* (**E)** *Suberoteratosphaeria suberosa* (≡*M. suberosa*)*;* (**F)** *M. juvenis* (≡*T. nubilosa*)*;* (**G)** *Amycosphaerella africana* (≡*T. africana*)*;* (**H)** *Q. mexicana* (≡*T. mexicana*); (**I)** *Pallidocercospora crystallina* (≡*M. crystallina*)*, M. ellipsoidea (now≡Am. africana*), *M. endophytica, M. lateralis, Pallidocercospora irregulariramosa* (≡*M. irregulariramosa*), *Parapenidiella* (≡*M. tasmaniensis*); (**J)** *Pallidocercospora colombiensis* (≡*M. colombiensis*, *M. keniensis*; (**K)** *Pseudoteratosphaeria flexuosa* (≡*M. flexuosa*)*;* (**L)** *T. suttonii* (≡*M. suttoniae*); (**M)** *Pallidocercospora heimioides* (≡*M. heimioides*); (**N)** *Austroafricana* (≡*T. parva*) (adaptado de Crous, 1998).

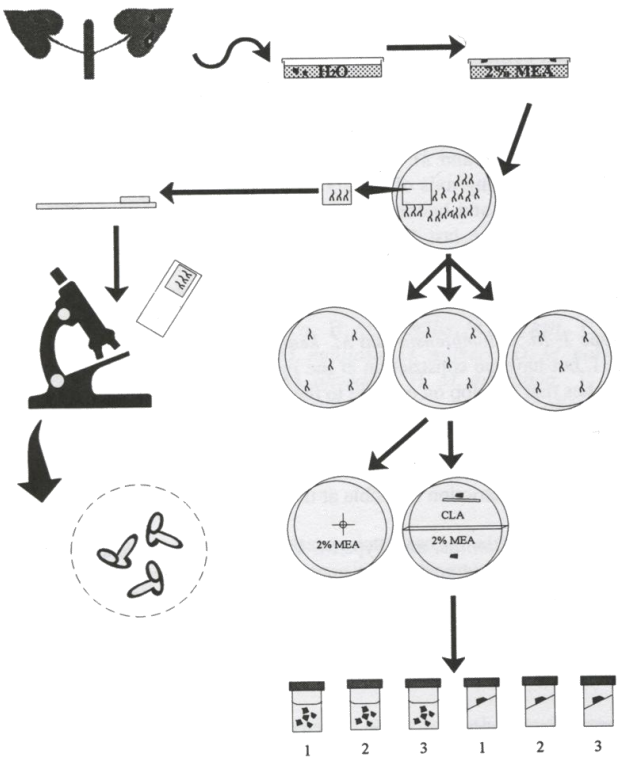

Fig. B.3 Isolations of symptomatic leaves of *Eucalyptus Leaf Disease Complex* (ELDC) (Crous, 1998).

Fig. B.4 Adult symptomatic leaf with a amplified lesion of *Teratosphaeria nubilosa*.

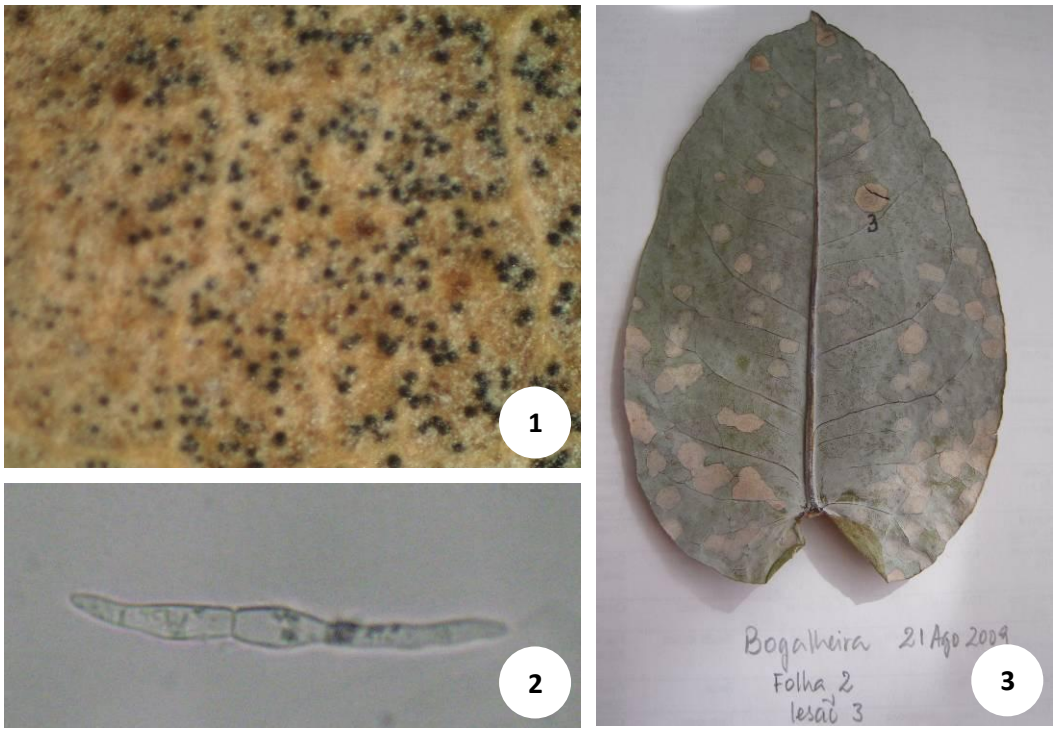

Fig. B.5 Isolations of *Teratosphaeria nubilosa* in juvenile leaves of *Eucalyptus globulus*. **(1**) The fruiting bodies (pseudothecias); **(2)** Ascospore germination; **(3)** Juvenil leaf with ELDC.

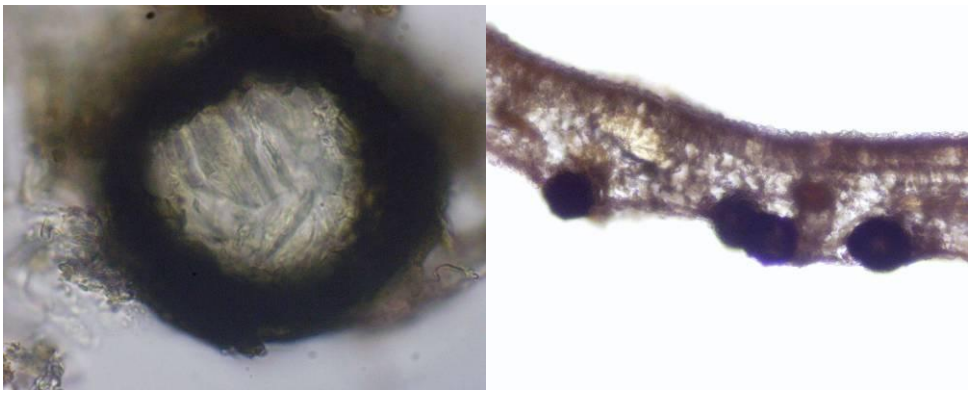

Fig. B.6 Fungi fruiting bodies (pseudothecias) on *Eucalyptus globulus.*

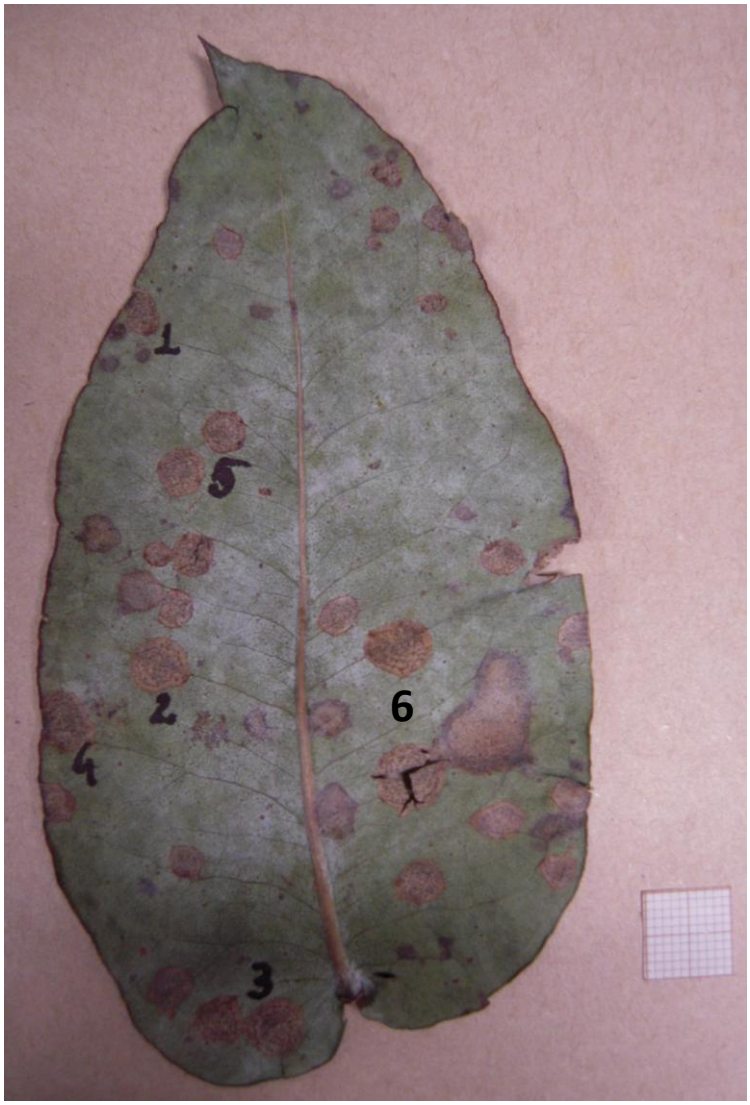

Fig. B.7 Symptomatic juvenile leaf of ELDC from *Eucalyptus globulus* with six lesions **(1)** *M. communis* and *A. parva*; **(2)** *M. lateralis* and *A. parva*; **(3)** *M. communis* and *A. parva*; **(4)** *M. communis* and *A. parva*; **(5)** *M. lateralis, T. nubilosa*; **(6)** *A. parva*; Bar = 10 mm.

Fig. B.8 Adult leaf with ELDC with *Austroafricana parva*, *Paramycosphaerella marksii*, *Teratosphaeria molleriana*, on the right = abaxial leaf surface, on the left = abaxial leaf surface; Bar = 10 mm.

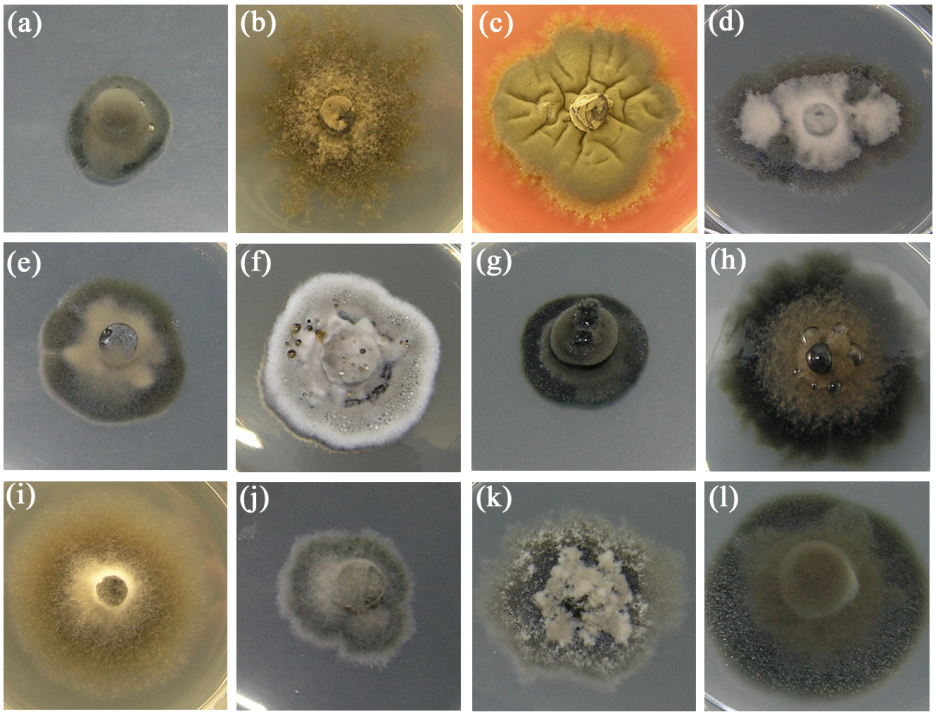

Fig. B.9 Single spore cultures of *Mycosphaerella* s.l. used in this work (after 1 month on MEA at 25°C in the dark) **(a)** *Austroafricana parva* **(b)** *Mycosphaerella communis* **(c)** *M. lateralis* **(d)** *M. madeirae* **(e)** *Neodevriesia hilliana* **(f)** *Paramycosphaerella marksii* **(g)** *Quasiteratosphaeria mexicana* **(h)** *Teratosphaeria lusitanica* **(i)** *T. molleriana* **(j)** *T. nubilosa* **(k)** *T. pluritubularis* **(l)** *Teratosphaericola pseudoafricana.*

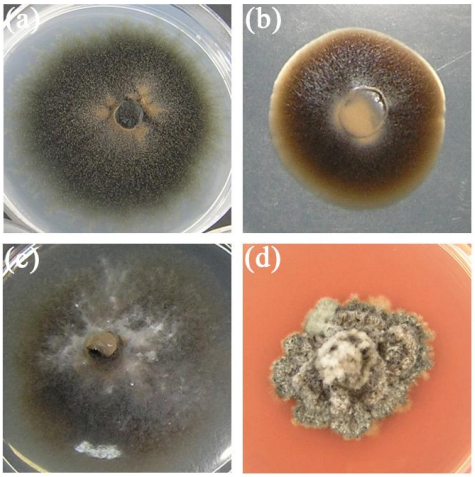

Fig. B.10 Single spore cultures of others species used in this work (after 1 month on MEA at 25°C in the dark) **(a)** *Cladosporium* sp. **(b)** *Fusicladium eucalyptorum* **(c)** unidentified *Venturaceae* **(d)** unidentified *Teratosphaeria.*