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PREFACE

At the time of commencing this study, there were only five papers published on Coniothyrium canker disease of *Eucalyptus.* These studies included the formal description of the fungus causing the disease and some aspects of its biology and physiology were characterized. The fungus was described, at that time, as *Coniothyrium zuluense,* which had a very simple morphology, lacked sexual reproductive structures, had small nondescript conidia and it was slow growing in culture. Nevertheless, the taxonomic status of the Coniothyrium canker pathogens changed in several occasions during this study including placement in genera such as *Colletogloeopsis*, *Kirramyces* and *Teratosphaeria.*

After the first appearance of Coniothyrium canker in South Africa, the disease was found in many other parts of the world. DNA sequences from cultures of *C. zuluense* became easier to obtain and this made it possible to undertake phylogenetic comparisons of isolates from various areas. Such studies also showed that *C. zuluense* was closely related to *Mycosphaerella* species. The common appearance of Coniothyrium canker in new areas motivated further studies of this disease and it causal agent, particularly applying newly available rDNA-based techniques. This also provided the motivation for studies presented in this thesis.

The thesis is introduced by means of a literature review that treats Coniothyrium canker on *Eucalyptus.* Briefly, the general characteristics of the host species, *Eucalyptus,* are described. Furthermore, trends relating to emerging diseases in plantations of *Eucalyptus* during the past two decades are treated with particular focus being placed on *Mycosphaerella* diseases. The phylogenetic relationships between *Coniothyrium*, *Mycosphaerella* and its anamorphs are considered together with the population biology of related pathogens.

In chapter two of this thesis, DNA sequence comparisons were used to determine the phylogenetic position of *C. zuluense* related to other fungi. In particular, the question as to whether *C. zuluense* was correctly placed in the genus *Coniothyrium* and its relatedness to *Mycosphaerella* was considered. Comparisons with the type species of *Coniothyrium, C. palmarum* and a collection of sequences of *Mycosphaerella* species were also conducted. In addition, the identity of isolates

obtained from China with similarities in colony morphology to *C. zuluense* was considered.

The objective of the study presented in chapter three was to investigate whether all the available isolates in the FABI collection from different countries and associated with Coniothyrium canker represented a single phylogenetic species. An additional methodological objective of this chapter was to select the best DNA regions for phylogenetic studies on this fungus and its relatives. Four DNA regions were selected based on the informative content as well as ease and reproducibility for Polymerase Chain Reaction (PCR) amplification.

The studies presented in Chapter 3 of this thesis showed that two species cause Coniothyrium canker and these are now known as *Teratosphaeria zuluensis* and *Teratosphaeria gauchensis*. Therefore, the objectives of the studies presented in chapters four and five were to develop highly variable markers to study the genetic variability and population parameters of populations of both species. This included the development of a robust protocol to isolate microsatellites on both fungi and that would also be informative for related genera. The protocol finally developed and used is presented in Appendix 2 of this thesis.

In chapters six and seven, the microsatellite markers developed in the previous chapters were applied. The genetic structure of populations of *T. zuluensis* and *T. gauchensis* was thus studied. Analyses of the amplified alleles and their frequencies were used to determine the levels of genetic diversity, clonality and to draw preliminary conclusions regarding the origin and global movement of the pathogens.

Chapter 1

Literature review: Diseases of *Eucalyptus* with particular reference to the taxonomy and population biology of pathogens in the *Teratosphaeriaceae*

CHAPTER 1

Diseases of *Eucalyptus* with particular reference to the taxonomy and population biology of pathogens in the *Teratosphaeriaceae*

INTRODUCTION

Eucalypt trees are endemic to Australia (including Tasmania), Papua-New Guinea and the Indonesian islands of Timor, Wetar, Flores and the Lesser Sunda Islands (Ladiges, 1997). The name *Eucalyptus* comes from the Greek word, “ευκάλυπτος” meaning "well covered". The trees were named by the botanist Charles Louis L’Hetelier in 1788, probably based on a specimen brought back by Captain James Cook from the Bruny Island in Tasmania on his third expedition in 1777.

In their natural range, eucalypts are adapted to a wide variety of environmental conditions. They occur from 40 degrees north to 45 degrees south covering tropical, subtropical and temperate latitudes (Eldridge *et al.,* 1994). They occur at altitudes from sea level to 1800 m and are found in areas with perennial rainfall or seasonal rains and in areas with more than 3000 mm rainfall a year to semi-desert regions with 300mm a year (Eldridge *et al.,* 1994). The wood produced by different species varies in physical and mechanical properties resulting in a considerable versatility of uses for these trees. The wood can be dense and hard in some species or light and soft in others (Ladiges 1997).

Ancestors of eucalypts came to the Australian region of Gondwana from the Antartica in the late Cretaceous Period, 90-65 million year ago. A rapid species radiation followed in the Tertiary Period (Ladiges 1997). However, there are reports of macrofossils similar to eucalypts in Patagonia, South America from the Miocene or Eocene epochs, (Ladiges 1997) and in New Zealand from the early Miocene (Ladiges 1997). The most recent radiation occurred 200 000 years ago and seems to be associated with an increase of the frequency of fire due to the arrival of humans and the increased aridity of land masses.

Taxonomy of eucalypts

Recent views on the phylogenetic history and the classification of eucalypts are based on both DNA sequencing analysis and morphology. Three major lineages have

been distinguished; *Angophora* (7 species)*, Corymbia* (125 species) and *Eucalyptus* (> 600 spp.) (Ladiges 1997). The majority of species used in forestry are included in one subgenus of *Eucalyptus*, viz. *Symphyomyrtus* (>300 spp.). In 2000, Brooker introduced a new classification of the eucalypts, defending the monophyly of *Angophora*, *Corymbia* and *Eucalyptus* into one genus. However, Ladiges & Frank (2000) rejected this view in support of the currently accepted separation of *Angophora*, *Corymbia* and *Eucalyptus,* based on sequence data of various regions of nuclear and chloroplast DNA (5S rDNA spacer region, ITS1, ITS2, *trn*L intron, *trn*L-F spacer and *psb*A-*trn*H spacer), Restriction Fragment Length Polymorphisms (RFLPs) as well as morphology.

The taxonomy of the eucalypts is continually being updated and a regular surveillance of the literature is needed to remain abreast of the current views. *Eucalyptus* is a large genus comprising more than 700 species. A similar number of sub-species, varieties and natural hybrids (Ladiges, 1997) have also been reported. There is a trend to increase the current number of species and sub-species within the genus. The list of examples in the literature has consistently been growing in the last decade. Reconsideration of the taxonomic status of established species (using both, morphology and DNA sequence analyses) and new discoveries at different taxonomic levels have contributed to the debate on the real number of natural species (Potts & Pederick, 2000). For instance, DNA sequence analyses have helped to improve resolution in difficult areas of the phylogenetic analyses. This approach has been used successfully to clarify the higher level relationships among eucalypts (Steane *et al.,* 2002) and to assess the phylogenetic position of anomalous eucalypts species (Steane *et al.,* 2007). For example DNA sequence data have recently been applied to address infra-generic questions within *Corymbia* (Parra-O *et al.,* 2009) and there has been a recent taxonomic revision of *E. camaldulensis* Dehnh (McDonald *et al*., 2009).

At the specific and sub-specific level, the literature regarding the taxonomy of Eucalyptus also grows steadily. Some recent examples include new subspecies described by Nicolle & Brooker (2005) within the *Eucalyptus spathulata* Hook. complex and new subspecies *within Eucalyptus sargentii* Maiden. *Eucalyptus sargentii* subsp. *onesia* D. Nicolle was separated from subsp. *sargentii* based on the capability to tolerate highly saline soils and a higher propensity to regenerate after fires. Other examples are the new subspecies of *E. jutsonii* Maiden (Nicolle & French, 2007) and a *new subspecies* of Corymbia, C. cadophora *subsp. polychrome* R.L. Barret (Myrtaceae), described in the east Kimberley region of Western Australia (Barrett, 2007). *At the species level,* 14 new species were described in the book by Hill *et al.,* (2001). In South Western Australia the Diamond Gum tree (*Eucalyptus rhomboidea* Hopper & D. Nicolle) was described (Hooper & Nicolle, 2007), along with four other new species viz. E. sinuosa *D. Nicolle, M.E. French & McQuoid*, *E. retusa D. Nicolle, M.E. French & McQuoid ,* E. lehmannii (Schauer) Benth. subsp. *parallela* D. Nicolle & M.E. French and E. conferruminata D. Carr & S. Carr subsp. *recherche* D. Nicolle & M.E. French *(Nicolle* et al., *2008). The natural occurrence of hybrid eucalypt species adds another level of complexity to the taxonomic discussions of the group (McKinnon* et al., *2004; Nicolle* et al*., 2008; Walker* et al., *2009).* For further information, a compilation of 569 papers beginning in 1725 can be found at the Flora Base Botanical Library following the link: *http://florabase.calm.wa.gov.au/search/library?authors=&id=&publdate=&publisher=&series=&source=&subjects=&title=eucalyptus&type=sum&page=1*

Domestication of eucalypt trees for forestry

Eucalypts makes up the second most important tree resource after pines used for plantation forestry worldwide. Estimates included in the Food and Agriculture Organization (FAO) forestry reports (Food and Agriculture Organization of the United Nations 2006 *http://www.fao.org/docrep/008/a0400e/a0400e00.htm*; 2009 *http://www.fao.org/docrep/011/i0640e/i0640e00.htm*) are that there are over 19.6 million hectares of these trees planted worldwide covering 8% of the productive cultivated forests areas. These plantations are a source of wood and wood products in areas with remarkably different climates. They are planted as exotics in more than 60 countries in North Africa, the Middle East, Central and East Asia, Southern Europe, North and South America (Eldridge *et al.,* 1994). It has also been predicted that by 2010, the total area plated to eucalypts would be over 20 million ha (Turnbull 2000).

It was only in the latter part of the last Century that industries based on fast-growing eucalypts developed worldwide. In Australia, 60 out of 400 species are considered to be of economic importance. Of these, 10 to 15 species are commonly cultivated worldwide (Ladiges, 1997). Around 100 species are planted worldwide, including hybrids. *Eucalyptus globulus* Labill, *E. pellita* F. Muell., *E. urophylla* S.T. Blake*, E. camaldulensis, E. nitens* (Deane & Maiden), *E. grandis* Hill: Maiden, and *E. tereticornis* Sm. are the most important species currently in plantations (Turnbull 2000). Reliable and updated information about the status of plantations per species and areas under which they are cultivated in different countries is difficult to collect. Currently available private and public information is scattered. At present, this kind of information is not well captured in global reference reports. Internet sources are useful in this regard and they show the current dynamism of the sector in different countries. A summary list of internet sites including relevant general information on *Eucalyptus* and per species is provided in Table 1.

The initial choice of species for forestry has varied in different countries according to climatic and edaphic factors, and the objective of planting (Eldridge *et al.,* 1994; Florence 1996; Poynton 1979). The most extensive plantations of *Eucalyptus* in the world are found in India (8 million hectares) in relatively low productivity plantations, and Brazil (4 million hectares), where plantations are of hybrid-clones, intensively managed and of high productivity (Stape *et al.* 2010). A

detailed world map of *Eucalyptus* planted areas, compiled from information from the FAO, Department of Forestry, 35 organizations and individual experts worldwide is available at:

http://git-forestry.com/download_git_eucalyptus_map.htm.

There is increasing demand for wood products worldwide. Forestry companies can fulfil these requirements either by increasing cultivated areas or by increasing productivity. Available land for forestry purposes, however, is a limited resource. In countries such as South Africa where expansion of area for planting is not possible, technology will play a fundamental role. In this regard, it has been estimated that the productivity of *Eucalyptus* plantations could potentially be increased by 40% (Little *et al.,* 2003).

Both the health of trees and stress factors are tightly associated with increased productivities of plantations (Keane *et al.,* 2000). Healthy plantations are better able to naturally resist some pathogens and pests. Research is important to understand the stress factors plantations are exposed to and how to avoid or eliminate them. For example, correct nutrition can help to prevent or eliminate stress factors in plantations (Carnegie 2000; Stape *et al.,* 2004). Another important means to avoid stress problems is to achieve a correct site-species matching of trees by choosing tolerant genotypes in high-risk areas for disease (Carnegie 2007). This is an area of concern that is currently strongly supported by multidisciplinary studies including disciplines such as soil science, microclimate modelling and monitoring of climate change (Kirilenko & Sedjo 2007). A general area of recent interest aims to achieve “induced resistance” to pests and pathogens. There is broad experience on how to trigger these mechanisms in herbaceous plants. This is an area currently under investigation for woody plants (Eyles *et al.,* 2010). This approach could lead to important tools in the management of plantations as it could be used to overcome the economic and environment restrictions of pesticides.

Biotechnology relating to *Eucalyptus* plays an important role in increasing productivity. All these technologies rely on the natural variability of *Eucalyptus* to adapt to a large range of bioclimatic conditions and the ability to produce natural hybrids (Eldridge 1994). The level of natural variation within populations is high. It is

common to find variation within provenances that allows for selection of a wide variety of special traits. Some examples are frost tolerance (Byrne *et al.,* 1997; Fernández *et al*., 2006; Moraga *et al.,* 2006; Volker *et al.,* 1994), salinity tolerance and waterlogging (Mahmood *et al*., 2003), adaptation to arid conditions (Merchant *et al.,* 2007), pulpwood quality (Miranda & Pereira 2002) flowering times (Mora *et al*., 2007) and "Mycosphaerella" leaf disease (MLD) disease resistance (Eiles *et al.,* 2010; Milgate *et al.,* 2005).

Vegetative propagation of *Eucalyptus* has made possible the propagation of trees with exceptional characteristics in clonal plantations. Hybrid propagation has been important in fighting disease. One of the first successes was the production of hybrids resistant against Chryphonectria canker caused by *Chryphonectria cubensis* (Bruner) Hodges (= *Chrysoporthe cubensis* (Bruner) Gryzenhout & M.J. Wingf.) in Brazil (Wingfield 2003). Since then, producing and planting hybrids has become a common practice to find resistance in many countries (Denison & Kietzka 1993). There have, nevertheless, been some exceptions. For example, *E. globulus* x *E. nitens* hybrids developed for tolerance to MLD resulted in higher susceptibility than any of the parental trees species to MLD (Carnegie & Ades 2002; Dungey *et al.,* 1997).

Biotechnological developments in particular based on molecular biology have been increasingly incorporated into breeding programmes. The strength of these technologies relies in their power to unravel the basic mechanisms of adaptation and physiology and the ability to determine the genetic basis of desirable characteristics (e.g. disease resistance, quality attributes of the wood, oil production and fragrances).Ultimately, these technologies will allow the direct manipulation of characteristics based on gene transferring approaches. It is certainly expected that there will be a new boost of technological improvements in these research and application areas as a result of the completion of the *Eucalyptus* genome project (DOE Joint Genome Institute, http://www.jgi.doe.gov/ and EUCAGEN http://web.up.ac.za/eucagen/viewnews.aspx?id=28. Currently, a preliminary 8X assembly produced by JGI of the ~600 Mbp *E. grandis* genome (690Mb in 6043 scaffolds) is available at *Eucalyptus*DB, http://eucalyptusdb.bi.up.ac.za/.

EMERGENT FUNGAL PATHOGENS AND PEST IN FOREST PLANTATION

Forestry specialists and organizations around the world recognize that there are increasing numbers of pests and pathogens affecting the health status of forests worldwide (FAO 2006 http://www.fao.org/docrep/008/a0400e/a0400e00.htm; FAO 2009 http://www.fao.org/docrep/011/i0640e/i0640e00.htm; McDonald 2010; Wingfield 2003; Wingfield *et al.,* 2001, 2008). However, it is difficult to source precise data regarding global evaluations of the problem. The FAO forestry assessment reports (produced approximately every 5 years) provide the most comprehensive source of data on the topic. In this section, I examine and introduce some comments on the information included in the latest FAO global forestry report (2009, http://www.fao.org/docrep/011/i0640e/i0640e00.htm) on the general status of diseases in forest and plantations on a global scale.

The 2009 global review of forest pest and diseases by the FAO included information from 25 countries, http://www.fao.org/docrep/011/i0640e/i0640e00.htm. As mentioned in the report, the quality of the information is not homogeneous. Only 13 of the 25 participant countries provided quantitative data. The remaining countries were able to provide only qualitative and fragmented data. The information was not easily accessible for various reasons (e.g. no presence on public databases and presence of manual records only, monitoring programs not implemented due to lack of specialized people in the field and lack of resources). In general terms, more information was gathered from the private sector groups than from the public sectors. The information provided is, in many cases "the best guess" of the researchers and the actual sources and origin of particular species remain unknown. The *Eucalyptus* stem canker pathogen *Teratosphaeria zuluensis* M.J. Wingf., Crous & T.A. Cout.) M.J. Wingf. & Crous provides a good example. It is classified in the FAO study as introduced, although there is actually no proof supporting this status for any of the countries from which the fungus has been reported.

The most relevant global conclusions included in the report are summarized in the following points:

- Seventy seven percent of the reported diseases are caused by insect pests, mainly Coleoptera and Lepidoptera.

- Twenty three percent are reported as caused by other pests or pathogens, mainly from Ascomycota.

- Fifty four percent of pests and pathogens were recorded in cultivated forests.

- In all participating regions, more pests and pathogens were reported in cultivated forests than in regenerated or natural forests.

-Introduced pathogens and pests were found most prevalently in cultivated forests.

- In all geographical regions considered, more pests and pathogens were recorded on broad-leaf trees (62% broad leaf, 30% conifers, 8 % on both). In cultivated forests the same trend was observed; most commonly affected trees were broadleaf trees.

As a further exercise, the numerical information contained in the 2009 FAO report (http://www.fao.org/docrep/011/i0640e/i0640e00.htm) was used to evaluate global trends relating to pests and diseases. The information was compiled by continent and plotted (Fig 1). The graph shows the abundance of pathogen and pests diseases (endemic + introduced diseases) per continent. Interestingly, the diseases caused by pathogens were relatively more abundant than damage caused by pests on the African and Asian continents. The opposite relationship between pathogens and pests was shown for Europe and America.

Specifically relating pests and diseases to eucalypts, the total planted area of *Eucalyptus* per continent was plotted together with the abundance of pests and pathogens. It is not possible to suggest a direct relationship between the abundance of pests and pathogens and planted areas of *Eucalyptus* trees. Nevertheless, it is interesting that the continent with the most extensive areas of cultivated *Eucalyptus* is the continent with highest abundance of pest and pathogens. This might be explained by the fact that *Eucalyptus* provides opportunities on that the continent for pathogens and pests to encounter new niches on susceptible trees. In fact, recent work has shown that the diversity of pathogens in the *Mycosphaerellaceae* and *Teratosphaeriaceae* on *Eucalyptus* in Asia is higher than

previously thought and new species (Burgess *et al.,* 2007b; Crous *et al.,* 2009b; Zhou *et al.,* 2008) as well as host shifts to *Eucalyptus* have been documented (Burgess *et al.,* 2007b).

Emergent fungal pathogens in *Eucalyptus* plantations

In their natural range, eucalypts (*Eucalyptus* and *Corymbia*) are damaged by a wide variety of pests and diseases (Keane *et al.,* 2000). During the first years where eucalypts were established in plantations in new and non-native locations, the trees showed improved development in comparison to that achieved in their natural environments (Wingfield 2003). The explanation for this response is thought to be due to the "enemy and escape hypothesis" originally by Jeffries & Lawton (1984). This hypothesis has subsequently been supported by other authors (Keane & Crawley 2002; Mitchell & Power 2003). The hypothesis suggests that trees in the absence of natural enemies grow more vigorously than in their original geographical range as they grow relatively free of problems. The favourable conditions persist in plantations until the local pests and diseases adapt to the new- comer trees or until their natural enemies are also introduced into the exotic locations.

Unfortunately, this favourable period of *Eucalyptus* forestry has come to its end. There is a constant trend of increasing numbers of pests and diseases in plantations worldwide (Old *et al.,* 2000; Old 2003b; Sankaran *et al.,* 1995; Wingfield *et al.,* 2008). This is not a completely unexpected as it has happened before to more traditional crops (Anderson *et al*., 2004).

A number of factors have contributed to the end of the favourable period for *Eucalyptus* plantations where they were largely free of pests and pathogens. At one side of the spectrum, the initial success of exotic plantations led to clonal forestry and monoculture plantations. Such plantations are characterized by high levels of genetic uniformity. Although appropriate to optimize productivity, uniform monocultures have introduced high levels of risk to establish pests and diseases (Burgess and Wingfield 2004; FAO 2009, http://www.fao.org/docrep/011/i0640e/i0640e00.htm; Jactel *et al.,* 2002; Old *et al.,* 2003b; Wingfield *et al.,* 2008; Zhu *et al.,* 2000). Planted in large areas, monocultures provide the opportunity for pest and pathogens to reach populations of large size in

a short period of time. Large populations become a threat to future attempts to manage and keep the populations of pathogens under control (Keane *et al.,* 2000; Wingfield *et al.*, 1995; Old *et al.,* 2003b).

Original sources of pathogens causing disease in *Eucalyptus* trees plantations

There are few examples of well documented situations regarding the determination of the origins of diseases of *Eucalyptus* in exotic plantations. Many species of pathogens are completely new to forestry and in the majority of the cases there is little knowledge on the biology and geographical ranges of these organisms. In general, the movement and spread of the pathogens does not follow a clear route or pattern of distribution (Wingfield *et al.,* 2008). Recent studies, particularly population genetics studies are making an important contribution to understanding epidemiological aspects of *Eucalyptus* diseases as well as to explain the origins of the major pathogens of these trees.

At a global scale, the problem of the origin of these species gets more complicated as the globalization contributes to the dispersion of pathogens. Transportation of germplasm in the form of seeds has been recognized as an important medium allowing pathogens of *Eucalyptus* to spread globally (Old *et al.,* 2003b). The pathogens can also be accidentally transported and spread between regions or countries by exchanges of infected plant material. For example, they can be carried on machinery, tools and even introduced by humans through the frequent exchange of forestry personal among companies (Wingfield *et al.,* 2008). In many parts of the world, particularly in regions of South-East Asia, non-registered exchange of plant materials between companies is a common practice and the movement of large amounts of seed between many different countries of the world adds to the threats. Analyses of the movement of germplasm based on clear records of exchange could help in the future to understand the movement of diseases around the globe. This would also contribute to more effective risk assessment (Wingfield *et al.,* 2001).

Many pathogens of *Eucalyptus* have spead to new locations, substantially extending their geographical areas of occurrence. For example, native pathogens from Australia have been encountered *Eucalyptus* in non-native locations. This is for

example the case for *Teratosphaeria nubilosa* (Cooke) Crous & U. Braun (Hunter *et al.,* 2004), previously treated as *Mycosphaerella nubilosa* (Cooke) Hansford and *Eucalyptus globulus* in South Africa. *Eucalyptus globulus,* known as the "blue gum" tree, was selected as the main species to initiate a hard-wood forestry industry in South Africa due to the notable growing characteristics of the species (Poynton 1979). Shortly after the establishment of the tree, a devastating leaf blotch disease, thought to be caused by *Mycosphaerella molleriana* (Thuemen) Lindau) (Crous 1998; Crous & Wingfield 1997b; Doidge *et al*., 1953; Lundquist & Purnell 1987) seriously impacted the plantations *E. globulus.* The plantations had to be permanently replaced by new resistant and later, hybrids developed in breeding programs. Population genetic data confirmed that *T. nubilosa* originated from Australia (Hunter *et al.,* 2008) and was subsequently spread to other countries from this source population. Today, the fungus remains a problem and it is the most important species of *Teratosphaeria* causing Mycosphaerella leaf disease (MLD) in South Africa where it affects the growth of Victoria provenances of *E. nitens*.

Fungal pathogens have also found a way to infect *Eucalyptus* trees by host jumping from other plants (Antonovics *et al.,* 2002; Slippers *et al.*, 2005). Cryphonectria canker disease provides a good example. The disease is caused by various species of *Chrysoporthe* (previously *Cryphonectria*) including Chrysoporthe cubensis in plantations of South-east Asia, South America and Africa (Greyzenhout *et al.,* 2004; Wingfield 2003). The sibling species *Chrysoporthe austroafricana* Gryzenhout & M.J. Wingfield has jumped from native myrtaceaous hosts in southern Africa to the exotic *Eucalyptus* in plantations (Heath *et al.,* 2006; Nakabonge *et al.,* 2006; 2007). Other species of *Chrysoporthe* have been found as natives on native Melastomataceae and have also jumped to infect *Eucalyptus* species in South America and South –east Asia (Hodges *et al.,* 1986; Rodas *et al.,* 2005).The impact of Chryphonectria canker was so important in Brazil that resistant hybrids clones *E. grandis* x *E. urophylla* were developed to substitute the widely cultivated and highly susceptible *Eucalyptus grandis* (Wingfield 2003).

There are other examples of host shifts from native trees to newly introduced *Eucalyptus* trees. The *Eucalyptus* disease caused by the fungus *Puccina psidii* Winter (*Eucalyptus* rust) has jumped from native hosts (Myrtaceae) in South

America to the exotic *Eucalyptus* (Coutinho *et al.,* 1998; Glen *et al.,* 2007). The fungus has expanded its geographical range becoming one of the most feared eucalypt pathogens in plantations. It is also of concern due to the possibility of the fungus reaching the natural forests of *Eucalyptus* (Glen *et al.,* 2007), which has recently been heightened by the appearance of the pathogen in Australia (Carnegie *et al.,* 2010). More recently, *Mycosphaerella citri* Whiteside, a serious pathogen of *Citrus* has been shown to have undergone a host-jump from citrus plantations in South-East Asia to *E. camaldulensis* in Vietnam (Burgess *et al.,* 2007b).

On indigenous *Eucalyptus* plantations, the most important infections are caused by native leaf pathogens. These pathogens belong mainly to *Teratosphaeria* spp. and its anamorphs such as *Teratosphaeria destructans* (M.J. Wingf. & Crous) M.J.Wingf. & Crous (Andjic 2007a, b; Burgess *et al.,* 2007a; Crous *et al.,* 2006). There is, however, an increasing concern that fungi that are expanding their geographical ranges such as with *P. psidii* that they will eventually reach the natural forests of *Eucalyptus* trees.

THE GENUS *MYCOSPHAERELLA*

A number of recent comprehensive reviews have examined *Mycosphaerella* Johanson and its anamorphs. This section presents a concise summary of the work and the current taxonomic status of the genus. The second goal is to provide an overview of the phylogenetic context of the causal agent of Coniothyrium canker, as it has emerged as related to *Mycosphaerella* through DNA sequencing comparisons.

Taxonomy of *Mycosphaerella*

Mycosphaerella spp. are Coelomycetes in the *Mycospharellaceae*. Schoch *et al.,* (2006) showed that the *Mycospharellaceae* resides in Capnodiales. In a morphological sense, *Mycosphaerella* includes more than 3000 species (Aptroot 2006) with thousands of additional anamorph species (Arzanlou *et al.,* 2007, 2008; Crous & Brown 2003; Crous *et al*., 2001a, 2004, 2006, 2007). Yet the establishment of links between anamorphs and teleomorphs cannot be made in many cases considering *Mycosphaerella* spp. in the broad sense. The number of links is likely considerably greater than has previously been suggested. At the present time, 30

anamorph genera are linked to *Mycosphaerella* sensu lato (Crous & Braun 2003, Crous *et al.,* 2007).

Approximately 100 species of *Mycosphaerella* are known to cause leaf and stem diseases of *Eucalyptus* trees (Crous 1998; Crous *et al*., 2004, 2006). This number might appear high but considering there are more than 700 species of *Eucalyptus* (Potts & Pederick, 2000), it is possible that there are many other species yet to be described. Indeed there has been a steady flow of new species of *Mycosphaerella* being described from *Eucalyptus* during the course of the last decade. Some species can be found on the same tree or even co-occurring in the same lesion (Crous & Braun 2003; Crous & Mourichon 2002; Taylor & Fisher 2003).

The phylogeny of *Mycosphaerella sensu lato* and its anamorphs represents a complex taxonomic challenge that is far from resolved. The number of species has increased significantly in recent years and as mentioned above, there are reasons to believe that this trend will continue. The trend of increasing numbers of *Mycosphaerella* spp. and its anamorphs being described over the last 35 years is illustrated in Fig 2 and this is likewise captured in research papers and in data bases (Crous 1998; Crous *et al.,* 2004, 2006, 2009a, b; Maxwell *et al*., 2003; Mycobank at http://www.mycobank.org/).

The use of DNA sequence comparisons with which to define species has inevitably revealed that identifications based solely on morphological characters has underestimated species boundaries. Many *Mycosphaerella* spp. resulting in the same or similar symptoms, the same morphological characteristics and the same germination patterns have thus been shown to represent distinct taxa. As a result, there is a consensus of opinion that DNA sequence analyses and phylogenetic inference is required to circumscribe species in this group (Crous *et al*., 2004, 2006).

Contribution of DNA sequence studies to the taxonomy of *Mycosphaerella*

The first universally used DNA region to study the phylogenetic relationship of this fungus (Crous *et al.,* 2001a, b, 2004) was the internal transcribed spacer region ITS 1 and ITS2 region, including the 5.8S gene of the ribosomal RNA operon. This region is commonly referred to as the ITS region for simplification. ITS DNA sequence comparisons offered more discriminatory power than morphological studies to

identify species and to establish species boundaries within *Mycosphaerella* (Crous *et al.,* 2004, 2006; Hunter *et al.,* 2006). Thus, cryptic species sharing symptoms and morphological characteristics were frequently found within *Mycosphaerella.* Recent examples are the identification of "complexes" of species within *M. nubilosa*, *M. parkii* Crous, M.J. Wingfield, F.A. Ferreira & Alfenas*, M. africana* Crous & M.J. Wingfield*, M. suberosa* Crous, F.A. Ferreira, Alfenas and M.J. Wingfield*, M. cryptica* (Cooke) Hansford*, M. endophytica* Crous and H. Smith to name but a few (Crous *et al.,* 2006). In other cases, ITS sequence comparisons made it possible to show that different species of *Mycosphaerella* reported on *Eucalyptus* can co-occur on the same tree, and even in the same lesion. This is the case for *M. cryptica, M. nubilosa* and *M. lateralis* Crous & M.J. Wingfield (Jackson *et al.,* 2004) or *M. secundaria* Crous & A.C. Alfenas*,* found in leaf lesions caused by *M. suberosa* (Crous *et al*., 2006).

For some *Mycosphaerellaceae* phylogeny based on the ITS region has proved to be of limited value (Crous *et al.,* 2004, 2006; Hunter *et al.,* 2006). It is clear for instance, that the ITS region is not able to provide sufficient information in the deep branches of the phylogenies and is not suitable to distinguish species in all species complexes (Crous *et al.,* 2004; Hunter *et al.,* 2006). Differences in rhythm of the "molecular clock" of the ITS region of different species explain the failures to identify and separate species. Nevertheless, the ITS region seems to provide sufficient phylogenetic information to separate species when restricted to local regions of the phylogenetic trees reviewed in (Andjic *et al*., 2007a; Cortinas *et al.,* 2006a; Crous *et al*., 2006).

Current alternatives to the ITS region for phylogenetic studies on *Mycosphaerella* and related fungi include other DNA regions and thus, reveal significant information at different time frames of the phylogeny. A common approach is to utilise multilocus DNA sequencing analyses such as those of Hunter *et al.,* (2006) and Cortinas *et al.,* (2006c) Using this approach, some *Mycosphaerella* spp. were found to represent complexes of cryptic species. In other cases, the multilocus approach allowed candidate species to be reduced to synonymy when their DNA sequences were identical across several DNA regions (*M. grandis* Carnegie & Keane – *parva* R.F. Park & Keane */ M. flexuosa* Crous & M.J. Wingfield –

M. ohnowa Crous & M.J. Wingfield */ M. amphybila* A. Maxwell*, M. molleriana, M. marksii* Carnegie & Keane *and M. intermedia* M.A. Dick & K. Dobbie) (Hunter *et al.,* 2006).

A major assumption, based on ITS data and that has been supported for years, was that *Mycosphaerella* was monophyletic (Crous *et al.,* 2001a; Crous *et al.,* 2004, 2006 Goodwin *et al.,* 2001). DNA sequence analyses using the large subunit of the RNA operon (28S or LSU) have been used recently to study deep branches in the phylogeny of *Mycosphaerella* (Hunter *et al*., 2006; Batzer *et al.,* 2008). The results have suggested that *Mycosphaerella* is not monophyletic as was previously believed.

Analyses by Crous *et al.,* (2007) concluded that *Mycosphaerella* is polyphyletic. In this study, the family *Mycosphaerellaceae* was divided into five major clades. The name *Mycosphaerellaceae* was retained for one clade including *Mycosphaerella* spp. and four new families were delimited. According to this new arrangement, the fungal diseases of *Eucalyptus* are included in a resurrected genus, *Teratosphaeria,* within the new family *Teratosphaeriaceae*. Thus, all fungal species noted thus far in this review from *Eucalyptus* have names in *Teratosphaeria.*

Mycosphaerella anamorphs

Traditionally, morphological characters have been used to separate anamorph genera associated with *Mycosphaerella*. More than 30 anamorph genera have been described and considered linked to this genus (Crous & Brown 2003; Crous *et al.,* 2006, 2007). DNA studies have rejected some of these links, included some anamorphs from other genera (e.g *Coniothyrium*) and they have led to the recognition of new genera and species.

Initial work using ITS sequence comparisons of anamorph forms suggested monophyly in *Mycosphaerella*. In addition, these studies provided sufficient grounds to support the fact that *Mycosphaerella* could be split according the anamorph genera (Sutton & Hennebert 1994; Crous 1998). The same view was supported by Crous *et al*., (2001a, b) although it was shown that some phenotypic characters evolved more than once and thus, some anamorph genera did not form clear groups. More recently, different phylogenetic analyses (Hunter *et al*., 2004,

2006; Crous *et al.,* 2007) analysing different DNA regions showed that the notion that it would be possible to predict the taxonomic location using anamorph characteristics should be discarded. This is because many anamorphs in *Mycosphaerella* are polyphyletic (Crous *et al.,* 2006). Examples of such morphological polyphyletic evolution are found in the anamorph genera *Passalora, Pseudocercospora, Phaeophleospora* and *Stenella, Colletogloeopsis* and *Kirramyces*. In a major taxonomic treatment of *Mycosphaerella* by Crous *et al.,* (2007), the mitotic genera linked to *Mycosphaerella* were considered polyphyletic and treated in *Readeriella* (*Teratosphaerellaceae*).

Crous *et al.,* (2007) introduced major controversy regarding the taxonomic treatment of the mitotic fungi on *Eucalyptus* residing in the new clades. The proposal to consider *Readeriella* as a polyphyletic group was not widely accepted. For example, the majority of the most important pathogenic species of *Eucalyptus,* including *Kirramyces* formed a strongly supported monophyletic group in previous analyses considering *Mycosphaerella* (Andjic *et al.,* 2007a; Cortinas *et al.,* 2006a; Crous *et al.,* 2006; Hunter *et al.,* 2006). Thus, the proposal of Crous *et al.,* (2007) had considerable merit, but *Readeriella* is polyphyletic and thus the monophyletic group defined for the pathogens of *Eucalyptus* was not logical.

The decision to reduce *Kirramyces* to synonymy with *Readeriella* would have serious consequences. The fact that *Kirramyces* spp. on *Eucalyptus* reside in a monophyletic group has important biological and ecological relevance as all of these fungi are important pathogens of *Eucalyptus*. This fact indicates common ancestral characteristics that allow members of the group to be pathogens of *Eucalyptus* plantations in many parts the world. Formally, there are also problems arising from the inclusion of *Kirramyces* in *Readeriella* as mentioned by Andjic *et al.,* (2007a). These authors showed that *Readeriella* is similar to *Kirramyces* but clearly different as they have phialidic conidiogenesis. Following to these morphological observations, *Readeriella* could include *Kirramyces* only if the description of the former genus were emended.

Recently, Crous *et al*., (2009c) have made an effort to alleviate the discomfort caused among the scientific community, by attempting to revise the genera in the *Mycosphaerellaceae* and *Teratosphaeriaceae* based on clear rules.

The approach here was to achieve a classification that respects the genealogical "natural" relationships, as resolved by DNA sequence LSU comparisons as well as morphological information. The proposed rules to define the genera were 1) One generic name per clade 2) DNA sequence similarity accepted over anamorph and teleomorph characteristics and they are considered equally relevant for taxonomic purposes 3) In case there are already names for anamorphs and teleomorphs, the preference is given to the oldest published name. As a result of this study, 12 genera were defined in *Mycosphaeriaceae* and nine in *Teratosphaeriaceae.*

Crous *et al.,* (2009a) published an additional study to bring taxonomic stability at the specific level to the *Teratosphaeriaceae.* LSU DNA sequences were used to study *Teratosphaeriaceae* and four main clades were defined (Fig 3). It is difficult to judge if the proposals contained in this work will result in consensus within the research community interested in this group of fungi. The analysis includes some nomenclatural inconsistencies compared to previous work (Crous *et al.,* 2007). To mention some controversial examples, the polyphyletic nature of *Readeriella* species in Crous *et al.,* (2007) re-appear in this 2009 study. *Readeriella* is together with *Teratosphaeria, Cibiessia* and *Mycosphaerella* within Clade 1 and close to *Davidiellaceae*. Formally, *Teratosphaeria zuluensis* and *T. gauchensis* (M.N. Cortinas, Crous & M.J. Wingf.) M.J. Wingf. & Crous, previously treated as *Kirramyces, Colletogloeopsis and Coniothyrium,* are proposed as *Teratosphaeria* for the first time in this paper within Clade 4. *Teratosphaeria* as well as *Batcherolomyces* remain polyphyletic among the *Teratosphaeriaceae* clades and *Readeriella, Teratosphaeria, Colletogloeopsis* and *Kirramyces* are polyphyletic within the clades. Only *Cibiessia* and *Catenulostroma* are not polyphyletic in the analyses. However, these two groups do not appear to have sufficient support to be considered as "natural" clades by themselves which challenges their "standing alone" status within the phylogeny (Fig 3).

There is a general consensus regarding the need to treat *Mycosphaerella* in more natural groups that describe genealogical relationships. The separation between *Mycospharellaceae* and *Teratosphaeriaceae* families is currently accepted and supported. However, controversy remains at the generic and species levels. Further attempts to improve taxonomic stability in these groups of fungi should

include refinements of theoretical criteria to define genera and species. On the technical side, the refinement of the phylogenies is also necessary. A first step could be achieved by including a study of several DNA regions (Crous *et al.,* 2009b, c; Hunter *et al.,* 2007). These future studies will hopefully improve the resolution of existing phylogenies by discovering new natural groups and by increasing the support of those that already exist.

Teratosphaeria (previously _Mycosphaerella_) diseases of _Eucalyptus_

The first Mycosphaerella leaf deseases (MLD) outbreaks, also referred to as Mycosphaerella Leaf Blotch (MLB) diseases, were associated with *T. cryptica* and *T. nubilosa* species (Cheah 1977; Crous & Wingfield 1996; Wingfield *et al.,* 1996; Dungey *et al.,* 1997, Park *et al.,* 2000a, b). Later, it became clear that there are more species of *Mycosphaerella* involved in causing foliar diseases (*et al.,* 1998; Crous *et al*., 2004, 2006, 2008, 2009a, b).

From 100 species reported as pathogens, only a sub-group are considered to be serious agents of disease (Crous 1998; Crous *et al.,* 2004*.,* 2006, 2008). This group includes teleomorph and anamorph species of fungi. The most important economic impacts have been caused by outbreaks from *T. cryptica*, *T. nubilosa* and more recently by the mitotic species *Teratosphaeria destructans* (Cooke and Massee) J. Walker, B. Sutton and Pascoe in South-east Asia (Barber 2004; Burgess *et al*., 2007a; Burgess & Wingfield 2004; Carnegie 1991; Carnegie *et al.,* 1998; Carnegie & Ades, 2002; Crous & Wingfield 1996; Crous *et al.,* 1989; Park 1988a; Park *et al.,* 2000b; Park & Keane 1982; Hunter *et al*., 2008, 2009; Wingfield *et al*., 1996, 2008).

Symptoms of _Teratosphaeria_ diseases (former _Mycosphaerella_ diseases)

Teratosphaeria spp. on *Eucalyptus* cause spots on the leaves of trees of different sizes and shapes. Depending on the severity of the infection and extension of the lesions, MLD can be present in a variety of forms, from mild spotting, to leaf blotches, leaf blight, leaf withering, tip die back, to growth stunting and necrosis (Crous 1998; Crous *et al.,* 1989; Park *et al.,* 2000a,b; Wingfield *et al.,*1996, 1997). In severe cases, the lesions increase in size covering extended areas of the leaves. The

photosynthetic surfaces of the plants can be seriously reduced causing premature defoliation. In extreme cases, infections can interfere with the normal growth and alter the tree structure and form (Carnegie *et al*., 1998; Lunquist and Purnell 1987) and premature defoliation can cause the trees to die (Carnegie 1991; Carnegie 2000; Park & Kane 1982).

In general, different fungal species produce characteristic lesion types. The lesions have been classified according to differences in their colour, colour of their margins and texture as well as their occurrence on the abaxial or adaxial leaf surfaces. Nevertheless, these lesion characteristics cannot be used as absolute parameters for classification and identification of fungal species. For example, *Teratosphaeria epicoccoides* M.J. Wingfield & Crous can present a variation of symptoms depending on the host species and stage of infection (Walker *et al.,* 1992*)* and can be confused with infections caused by other species such as *T. destructans* (Burgess *et al.,* 2007a). In these cases DNA sequencing studies are recommended to confirm the initial diagnoses (Crous *et al.,* 2004, 2006; Hunter *et al.,* 2004).

The severity of the symptoms is dependent on the susceptibility of the trees. This susceptibility varies according to species (Carnegie *et al*., 1998: Hood *et al*., 2002), provenances (Carnegie *et al.,* 1998; Dungey *et al*., 1997) and families (Dungey *et al.,* 1997; Carnegie & Johnson 2004). In addition, outbreaks can be caused by a group of species or a disease complex (Carnegie 1991, 2000; Park & Keane 1982) modifying the “pure” symptoms of the species involved.

Important MLD diseases caused by *Teratosphaeria*

The first species identified to cause MLD, *T. cryptica* and *T. nubilosa,* are also the best studied species of *Teratosphaeria*. Numerous studies have been undertaken to consider on the biology, disease cycle, host range, distribution and epidemiology and more recently population genetics of these species (Beresford 1978, Carnegie 2000, Carnegie *et al.,* 1998; Cheah 1977; Cheah & Hartill 1987; Crous & Wingfield 1996; Dungey *et al.,* 1997; Hunter *et al.,* 2002; 2008; Park 1988a, b; Park & Keane 1982; Wingfield *et al.,* 1996). This is consistent with the fact that they are the two

most important species causing MLD in Australia (Carnegie 2000; Carnegie *et al.,* 1998; Park 1988a; Park *et al.,* 2000a; Park and Keane 1982).

Outside Australia, *T. cryptica,* together with *T. nubilosa* are also serious pathogens in *Eucalyptus* plantations. They cause MLD in New Zealand (Carnegie 2000; Carnegie *et al.,* 1998; Park 1988a; Park *et al.,* 2000 a,b; Park & Keane 1982) and *T. nubilosa* was reported early in the history of plantations in South Africa (Crous 1998; Lundquist 1987; Lundquist & Prunell 1987). Infections caused by *T. nubilosa*, originally reported as *T. molleriana,* were important as early as 1930 in South Africa. The infections were so important that *E. globulus* could not continue to be grown in the country (Park *et al.,* 2000a, b). Currently, *T. nubilosa* has become the most widespread species in this country (Hunter *et al.,* 2004, 2008) where it causes a serious disease on *E. nitens*.

There are areas in which *T. cryptica* and *T. nubilosa* can co-occur. Co-occurring species have been shown to have different biology to infections caused by a single species. For instance, *T. cryptica* can penetrate juvenile and adult leaves and can infect either leaf surface, whereas *T. nubilosa* only infects juvenile leaves (Park 1988a, b). *T. cryptica* produces ascocarps and acervulli on both surfaces of the leaves whereas *T. nubilosa* produces ascospores predominantly on the abaxial surface. *Teratosphaeria nubilosa* can be monocyclic or bicyclic whereas *T. cryptica* is polycyclic, at least, in South-East Australia (Park 1988a). The anamorph of *T. nubilosa* remains unknown (Park & Keane 1982) while the anamorph of *T. cryptica* has been identified as *Colletogloeopsis nubilosum* (Ganap. & Corbin) Crous & MJ. Wingf. This mitotic form is also important as it can cause cankers on young branches and shoots of *E. obliqua* L' Herit and *E. globulus* subsp. *globulus* (Dick 1982; Park and Keane 1982).

Host ranges of *Teratosphaeria* diseases

As the areas where *Eucalyptus* spp. are planted have expanded, the incidence of *Teratosphaeria* diseases has also steadily increased (Burgess *et al.,* 2007b; Maxwell *et al.,* 2003; Park *et al*., 2000a, b; Wingfield *et al.,* 2008). From 100 pathogenic *Teratosphaeria* species currently described on *Eucalyptus*, nearly half have been reported outside Australia (Crous *et al.,* 2004, 2006, 2008, 2009b; Hunter *et al.,*

2004). It is thus likely that in the future, more *Teratosphaeria* species endemic to Australia will be found outside the country. *Teratosphaeria destructans* was first reported in Indonesia, found in other South-east Asian countries (Burgess *et al.,* 2006; Old *et al.,* 2003a, b) and later reported causing disease in Australia (Jackson *et al.,* 2005; Whyte *et al.,* 2005). This is an interesting situation where species in non-native environments are clearly exposed to large, uniform areas of susceptible trees and their occurrence is noticed much more readily than it would be in native situations.

Some of the important *Teratosphaeria* species causing diseases to *Eucalyptus* are *T. epicoccoides* (Andjic *et al*., 2007a; Crous 1998), *T. destructans* (Andjic *et al*., 2007b; Old *et al.,* 2003a), *T. nubilosa* (Hunter *et al.,* 2009; Pérez *et al.,* 2009; Pérez *et al.,* 2009) and *T. cryptica* (Carnegie 2000). These species have broadened their original geographical distribution ranges. Other species, however, have remained limited within narrow geographic ranges as for example in the case of *T. ohnowa* Crous & M.J. Wingfield in South Africa (Crous *et al.,* 2004). The previously *Mycosphaerella* spp. from *Eucalyptus* now included in *Terathosphaeria* (Crous *et al.,* 2008) are considered eucalypt specific and to have, in general, narrow host ranges. But there are exceptions as it has been found with *T. epicoccoides* that occurs on a very wide range of *Eucalyptus* species (Sankaran *et al.,* 1995). Similarly, *T. cryptica* has been reported on more than 50 different species of *Eucalyptus* but it has never been reported from *Corymbia* (Crous 1988; Dick 1982; Ganapathi & Corbin 1979, Park *et al.,* 2000a, b; Park & Keane 1982; Wingfield *et al.,* 1995). More recently, *T. nubilosa* has been found on substantially greater numbers of *Eucalyptus* spp. and this appears to be linked to its spread to new geographic areas. *T. nubilosa* was initially best-known on *E. globulus* in plantations in Australia, New Zealand and South Africa. Currently, it is reported from many countries and numerous *Eucalyptus* species and hybrids (Hunter *et al*., 2009). Nevertheless, *E. globulus* and its close relatives remain the most susceptible species to *T. nubilosa* (Carnegie & Kane 1994; Crous *et al*., 2004; Hunter *et al.,* 2004, 2009; Jackson *et al.,* 2005; Park & Kane 1982); and this emphasises a relatively high level of host specificity within *Eucalyptus.*

Important diseases caused by mitotic *Teratosphaeria* species

Kirramyces spp.as re-defined by Andjic *et al*. (2007a) and now treated as *Teratosphaeria*, includes some of the most serious pathogens of *Eucalyptus*. They occur in plantations as foliar and stem diseases worldwide. Approximately ten of these species affect *Eucalyptus* leaves (Andjic *et al.,* 2007a, b). Of these, only a small number are considered to have an important impact on plantations and the majority are known from the native range of *Eucalyptus*.

The most important species on *Eucalyptus* leaves are *T. eucalypti* (Cooke & Massee) J. Walker, B. Sutton and Pascoe*, T. epicoccoides, T. nubilosum* Ganap. & Corbin) Andjic (anamorph of *M. cryptica*) *T. destructans* (Wingfield *et al.,* 1996; Crous *et al.,* 2006, 2007a) and recently, *T. viscidus* Andjic, Barber & T.I. Burgess (Andjic *et al.,* 2007b). All these species and the diseases they cause have been thoroughly reviewed (Barber 2004; Carnegie *et al.,* 2007; Park *et al.,* 2000b). Of these species *T. eppicoccoides* is the most widely studied and *T. destructans* is the most serious species in terms of the damage caused to plantation forestry.

T. eppicocoides has a broad geographical distribution, occurring worldwide in plantations of the tropics and subtropics (Crous 1998; Crous & Wingfield 1997b). Typically the infections are found on mature leaves on trees under stress conditions (Knipscheer *et al.,* 1990). Prolonged infections lead to the infection of young leaves. The teleomorph of the species, *T. suttoniae,* Crous & M.J. Wingfield (Crous *et al*., 1997) produces ascospores that can be wind dispersed. Nevertheless, the distribution of the teleomorph is narrower than the distribution of the anamorph. *Teratosphaeria suttoniae* has only been reported from Brazil, Indonesia and North-East Australia.

Amongst the leaf pathogens in the previous genus *Kirramyces*, *T. destructans* is considered to be the most serious (Burgess *et al.,* 2006; Carnegie 2007; Wingfield *et al.,* 1996). It was first described in Sumatra and Indonesia causing a devastating disease in plantations of one to three- year-old trees resulting in extensive and premature defoliation (Wingfield *et al.,* 1996). It was later reported from nurseries and young trees in Thailand and Vietnam on *E. camaldulensis* and hybrids. It has been also reported from native *E. urophylla* in East Timor (Old *et al.,* 2003b). In 2006, *T. destructans* was reported from China (Burgess *et al.,* 2006).

DNA sequence comparisons using six gene regions have shown that isolates of *T. destructans* from China, Indonesia, Thailand and Vietnam are genetically identical (Andjic *et al*., 2007c). In 2006, infected leaves collected from a clonal taxa trial on Melville Island, 50 km off the coast from Darwin, Northern Territory, Australia (Andjic *et al*., 2007b). Although the symptoms were atypical to *T. destructans*, they were found to belong to this species. Greater variability was found in Australia than the previously observed in South-East Asia and China, suggesting that the species is endemic to this region of Australia (Burgess *et al.,* 2007a).

Another devastating outbreak of a leaf disease linked to *Teratosphaeria* was reported in Northern Australia during 2006 (Burgess *et al.,* 2007a). It was thought to be caused by *T. destructans.* However, when DNA sequence regions were compared to the Asian isolates, fixed polymorphisms were found in the three gene regions studied. Based on these results, a new species *T. viscidus* was described (Andjic *et al,* 2007b).

CONIOTHYRIUM CANKER DISEASE

Species involved

When Coniothyrium canker of *Eucalyptus* was first discovered, the taxonomy of the causal agent was poorly understood. Based on morphology, the fungus was best placed in *Coniothyrium*. At that time, *Coniothyrium* represented a large genus of mitosporic fungi that produce conidia in pycnidia. It is one of the oldest genera of Coelomycetes and has included more than 800 species. Recognition of species has been mostly based on the morphology of the single-celled conidia including wall ornamentation, pigmentation and size (Crous 2001a, b; Taylor *et al.,* 1999).

Sutton (1980) clarified the generic concepts for *Coniothyrium*, limiting the genus to species in which conidia arise via the percurrent proliferation of the conidiogenous cells. Thus, *Coniothyrium* is characterized by having unilocular, immersed, ostiolate, thin-walled and dark brown pycnidia. Conidia are brown, ellipsoidal to cylindrical, formed on annellidic conidiogenous cells. The mentioned characteristics have proven not to be taxonomically meaningful. As time has passed, a high degree of morphological overlap has been observed between *Coniothyrium*

and other taxa. Thus, in the strict sense, *Coniothyrium* should represent anamorphs of *Leptosphaeria* that are morphologically and phylogenetically similar to *Coniothyrium palmarum* (Corda), the type species of *Coniothyrium* (Crous, 1998).

Recent phylogenetic studies based on DNA sequence comparisons have shown that *Coniothyrium* is polyphyletic, encompassing many groups of unrelated species. *Coniothyrium*-like anamorphs can be linked to many Ascomycete genera other than *Leptosphaeria*. For example, *Coniothyrium*- like anamorphs have been accommodated in genera such as *Prosopidicola* (Lennox *et al*., 2004), *Paraconiothyrium* (Verkley *et al.,* 2004), *Colletogloeopsis* (Crous & Wingfield, 1997a), *Phaeophleospora* (Crous *et al.,* 1997) and *Kirramyces* (Andjic *et al.,* 2007a). The latter three genera are anamorphs of *Mycosphaerella* and *Teratosphaeria* and thus relevant to this review.

The morphology of cultures obtained from Coniothyrium canker symptoms resembled those typically of the description of *Coniothyrium* at the time of the description of *Coniothyrium zuluense* M.J. Wingf., Crous & T.A. Cout, (Van Zyl 1999; Wingfield *et al.,* 1997). Nevertheless, some doubt arose as these cultures were highly variable in texture, colour and growth characteristics (Fig 4E), and they also varied markedly in their pathogenicity to clones of *Eucalyptus* (Van Zyl 1999; Wingfield *et al.,* 1997*).* In the case of *C. zuluense* it was clear that DNA sequence comparisons were required to identify this fungus with certainty. The first of these DNA sequencing studies determined that all isolates taken from canker symptoms in South Africa represent the same species. This was despite the phenotypic variability of cultures but did not test the taxonomic relationships with *Coniothyrium* and *Leptosphaeria* (Van Zyl 1999; Van Zyl *et al.,* 2002b).

During the early stage of the studies presented in this thesis, a pilot phylogenetic analysis using DNA sequences showed that *C. zuluense* was not related to *Leptosphaeria* but rather to *Mycosphaerella.* The study also confirmed the earlier association between a *Coniothyrium* sp. and *Mycosphaerella* by Milgate *et al.,* (2001). The latter study based on traditional morphological investigation, linked *Coniothyrium ovatum* Swart as the anamorph of the leaf *Eucalyptus* pathogen, *Mycosphaerella vespa* Carnegie & Keane (Carnegie & Kane 1998). This result has however, never been confirmed using genetic analyses. This group of preliminary

results showed that a more comprehensive study was required and this led to the chapters that follow this review (Cortinas *et al.,* 2006b).

Symptoms, distribution and general characteristics of the disease

Symptoms of the disease known as Coniothyrium canker caused by the pathogen first known as *C. zuluense* were first observed in 1988 in plantations of *E. grandis* trees in the Kwa-Zulu Natal province of South Africa (Wingfield *et al.,* 1997). The causal agent was identified only a decade later based on classical morphological studies and pathogenicity tests (Van zyl *et al*., 2002a; Wingfield *et al.,* 1997). In South Africa and all other countries where Coniothyrium canker occurs, the symptoms are similar, irrespective of the *Eucalyptus* species on which the disease occurs.

Coniothyrium canker first appears as discrete necrotic spots on the young green stems at the tops of the trees (Wingfield *et al.*, 1997). Later, the lesions extend and coalesce to form larger cankers and these interrupt water transport to terminal shoots (Fig 4A, B). These infections result in the production of epicormic shoots on the stems and ultimately dead tops (Fig 5A, B). This in turn leads to dead tops on trees and reduced wood quality due to the formation of Kino pockets in the wood (Fig 4A, B). In transverse sections of the trunks, the distribution of Kino pockets follows concentric rings indicating that infections occur seasonally (Fig 4D).

The severity of Coniothyrium canker varies depending on the susceptibility of the affected trees. In South Africa, *E. grandis* trees are particularly susceptible but hybrids produced through crossing *E. gandis* with other species such as *E. camaldulensis, E. urophylla* and *E. tereticornis* can also be severely affected. Infections on the stems make it difficult to peel the bark from the stems prior to pulping and this leads to increased production costs (Van Zyl *et al.,* 1997, 2002a; Wingfield *et al.,* 1997).

After its first appearance in South Africa, Coniothyrium canker was found in various other countries (Fig 6). These included Thailand (Van Zyl, 1999; Van Zyl *et al.,* 2002b), Mexico (Roux *et al.,* 2002), and during the course of producing this thesis, in Vietnam (Gezahgne 2004; Old *et al.,* 2003b), Ethiopia and Uganda (Gezahgne *et al.,* 2003, 2005), Hawaii (Cortinas *et al.,* 2004), Argentina (Gezahgne

et al., 2004) and Uruguay, (Cortinas *et al*., 2006c) (see Chapter 3) and China (Cortinas *et al.* , 2006b) (see Chapter 2). It also emerged during this time that Coniothyrium canker is caused by two different species named as *K. zuluensis* (M.J. Wingf., Crous and T.A. Cout.) Andjic & M.J. Wingf. and *K. gauchensis* (M.N. Cortinas, Crous and M.J. Wingf.) Andjic, M.N. Cortinas & M.J. Wingf. (Cortinas *et al*., 2006c) and now in the genus *Teratosphaeria*; (see Chapter 3). The taxonomic discoveries and dates of new records of these fungi are presented in a time line in Fig 7 and Fig 8.

Despite various surveys during the course of the two decades and subsequent to the first discovery of Coniothyrium canker in South Africa, this disease has not been found in Australia where *Eucalyptus* spp. are native. This supports the view that the pathogen might represent a host shift from some other plant, possibly species of Myrtales, as has been found with Cryphonectria canker (Heath *et al.,* 2006; Nakabonge *et al*., 2006; Roux *et al.,* 2003). Nevertheless, there remains a possibility that Australia is the true source of the pathogen (Gryzenhout *et al.,* 2004; Wingfield 2003; Seixas *et al.*, 2004).

Recently, a new species phylogenetically closely related to *T. zuluensis* has been found in Australia. The new fungus was found to cause leaf spots lesions on *Eucalyptus botryoides* Smith leaves instead of stem cankers as *T. zuluensis*. This fact and the finding of minor morphological differences, led the researchers to consider the fungus a new species, *Teratosphaeria majorizuluensis* Crous and Summerell (Crous *et al.,* 2009b). Nevertheless, the relatedness of the two species will require further evaluation.

Pathogenicity studies have been suggested (Crous *et al.,* 2009b) to test the hypothesis that *T. zuluensis* is in reality a mixed group of cryptic taxa (Cortinas *et al.,* 2006c) that have the ability to cause canker and leaf spot lesions. Comparisons including a collection of *T. zuluensis* sequences will be necessary to further evaluate the genetic relationships within *T. zuluensis*. A study using microsatellite markers could also be helpful. Microsatellites have been shown to discriminate between species. For instance, *T. zuluensis* microsatellites give no amplification with DNA samples representing *T. gauchensis* and *vice versa* (Cortinas *et al.,* 2006a, 2008).

POPULATION BIOLOGY OF *MYCOSPHAERELLA* AND *TERATOSPHAERIA* SPECIES

Relatively little is known regarding the origin, biology, life cycles, genetics, epidemiology and population structure of *Mycosphaerella* and *Teratosphaeria* pathogens. Population genetic studies have been carried out for only six species in these genera. With the exception of *M. graminicola* (Fuckel) J. Schröter studies, the outcome is still fragmented and incomplete for the other species as the sampling scales considered are different. Furthermore, the distribution ranges are not always complete. It is the purpose of this section to briefly summarize the contents of population level studies on *M. graminicola*. This information will be fundamental in assisting the interpretation of the population genetics results obtained for the *T. zuluensis* and *T. gauchensis* presented in the last two chapters of this study.

Population biology studies of *M. graminicola*

The best studied *Mycosphaerella* spp. is the wheat pathogen *Mycosphaerella graminicola*. The population genetics of this species has been studied for 20 years. It has consequently become the iconic species of the group. *Mycosphaerella graminicola* (anamorph: *Septoria tritici* Roberge) is a serious pathogen occurring in wheat fields worldwide (Baearchell *et al.,* 2005). It is the cause of the *Septoria tritici* blotch in its mitotic form. The fungus is haploid, heterothallic, with both sexual and asexual reproduction (McDermot & McDonald 1993; Sanderson 1976; Stukenbrock *et al.*, 2007; Van Ginkel *et al.,* 1999 at: http://libcatalog.cimmyt.org/download/cim/68090.pdf).

Recently, *M. graminicola* has been selected for genome sequencing (Department of Energy of the United States (DOE) through the Joint Genome Institute (JGI). Details of the projects and results can be followed on the internet website for the project: http://genome.jgi-psf.org/Mycgr3/Mycgr3.download.html. Phylogenetic studies have indicated that the fungus is distantly related to other ascomycete fungi already sequenced. Thus, data arising from the *M. graminicola* genome project is expanding the genetic knowledge of these fungi beyond the currently studied phylogenetic groups. The project status is “in progress” and can be monitored at: http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genomeprj&cmd=ShowDetailView&TermToSearch=13707.

The most important information regarding the population biology of *M. graminicola* is summarized in Table 2. A selection of studies covering 20 years of investigations have evaluated and included in this table Results indicate that the fungus has a high degree of diversity across all tested spatial and temporal scales, including intercontinental studies and 20 different countries. The measurement of genetic diversity was the main focus in a group of these papers. High levels of diversity in *M. graminicola* in the majority of the populations was confirmed by using different types of markers (RFLPs, Microstellites, RAPDs, electrophoretic karyotypes) (Banke & McDonald 2005; Brunner *et al.,* 2008; Linde *et al.,* 2002; McDonald & Martinez 1990, 1991a, b; Zhan & McDonald 2004;). One of the most interesting results in this regard was to find lower variability in the mitochondrial genome compared to the nuclear genome (Torriani *et al.,* 2008). These data support the hypothesis of "selective sweep" (Zhan *et al.,* 2004). Following this hypothesis, mitochondrial haplotypes having more rapid metabolic rates are favoured and selected in *M. graminicola*.

The neutral variability is correlated with the variation in quantitative traits in *M. graminicola* (Jürgens *et al.,* 2006; Zhan *et al.*, 2005.). Countries in which the pathogen has more genetic diversity have greater additive genetic variance for most quantitative characters (Zhan *et al.*, 2005). These results suggest that the Australian *M. graminicola* population has been recently introduced as it has all the characteristics of a founder effect population (Zhan *et al.*, 2005) showing the lowest genetic diversity (Zhan *et al.,* 2003) and lowest additive variance (Zhan *et al.*, 2005.)

Estimation of other population parameters including population size, historical gene flow and recombination can explain the high levels of genetic diversity found in *M. graminicola* populations. Gene flow is extensive and global (Zhan *et al.,* 2003). The main mechanism of dispersion appears to be the dispersion by seeds, as ascospores are only important for dispersal at a regional level (Zhan *et al.,* 1998, 2000). Population size calculations have shown that populations of *M. graminicola* are large even at the scale of a single wheat field (N_e > 24.000). Under these conditions, extensive gene flow is expected and the genetic drift is not important allowing the accumulation of mutations (Zhan & McDonald 2004; Zhan *et al.,* 2001). Atypically for eukaryotic populations, *M. graminicola* populations are in

drift/migration equilibrium (Zhan & McDonald 2004). This implies that regardless of population size, new alleles that arrive in a population by migration are balanced by the loss of alleles through genetic drift.

There are clear signs of panmixia in the *M. graminicola* populations. Thus mating types occur at equal frequency at all spatial scales (Zhan *et al.,* 2002b) and there is random association among alleles at unlinked loci (Chen & McDonald 1996). Nevertheless, strains representing the MATI-1 gene are more virulent than the MATI-2 gene (Zhan *et al.,* 2007b). The presence of clones in the populations has been described as "ephemeral" as replicates of individual clones have only be found few meters apart and identical clones were never found in different fields in different years (Chen *et al.,* 1994; Zhan *et al.,* 2001). These observations suggest high degrees of recombination (Zhan *et al.,* 2007a). In fact, some papers show that new alleles are produced by intrageneic recombination during each growing season (Banke & McDonald 2005; Brunner *et al.,* 2008; Zhan *et al.,*1998, 2000).

Questions relating to the age and origin of the populations of *M. graminicola* have also been addressed for *M. graminicola*. This pathogen has been postulated to have emerged and evolved at the time that wheat was domesticated. It was thus calculated that *M. graminicola* has been evolving for >10.000 years, which is consistent with the length of time that wheat has been domesticated. An important factor that adds support to these assumptions was to discover that the main source of migrants was from the Fertile Crescent and Old World (Banke & McDonald 2005). In addition, the discovery of relatives of the fungus living on wild grasses in the Fertile Crescent of Iran is consistent with the view that *M. graminicola* populations have been evolving alongside the movement and domestication of wheat (Stukenbrock *et al.,* 2007).

The main driving force of evolution in *M. graminicola* appears to be natural selection (McDonald *et al.,* 1996). Given the very large sizes of *M. graminicola* populations, resistant mutants will be generated, selection will raise their frequency and recombination will rapidly homogenize the resistant or virulent genes (McDonald & Linde 2002). The competition among strains of the fungus was found to be high (Zhan *et al.,* 2002a) and it has been shown that adaptation can occur within a growing season (Cowger *et al.,* 2000; Zhan *et al.,* 2002a). Populations can

become resistant to fungicides rapidly; one generation was enough to find azole resistance new alleles at the CYP 5I locus, one of the genes in charge of metabolizing of the toxic substance (Brunner *et al.,* 2008; Torriani *et al.,* 2008). Nevertheless, disruptive evolution occurs if host mixtures co-exist (Zhan *et al.,* 2002a).

A positive association between virulence and fungicide resistance has been detected (Zhan *et al.,* 2005). More resistant strains also tend to be more virulent. The virulence and fungicide resistance characters were found to be mainly quantitative characters (Zhan *et al.,* 2005). As a consequence, to achieve an effective management and control of this pathogen, it would be necessary to build up resistance on crops based on quantitative resistance or R-gene Pyramids (McDonald & Linde 2002; Zhan *et al.,* 2005). Also, the application of chemical fungicides as mixtures to avoid generating rapid resistance should be considered.

Population biology of *Mycosphaerella* and *Teratosphaeria* spp. causing tree diseases

An attempt to compare the population genetic studies conducted on the other five species of *Mycosphaerella* spp. causing diseases on trees is presented in Table 3. The compared species are *M. populorum* G.E. Thompson (anamorph *Septoria musiva* Peck that infects poplar trees) (Feau *et al.,* 2005), *M. musicola* R. Leach ex J.L. Mulder (anamorph *Cercospora musa* Massee, a pathogen of banana) (Hayden *et al.,* 2003b, 2005; Zandjanakou-Tachin *et al.,* 2009) and *M. fijiensis* M. Morelet on banana. (Carlier 2004; Hayden *et al,.* 2003a), *T. cryptica* (anamorph *Colletogloeopsis nubilosum* Ganap. and Corbin on *Eucalyptus*) (Milgate *et al.,* 2005) and *T. nubilosa* that infects *Eucalyptus* (Hunter *et al.,* 2008).The last two species, *T. cryptica* and *T. nubilosa,* are genetically closely related to *T. zuluensis* and *T. gauchensis*, both also occurring on *Eucalyptus* trees.

Studies considering the population biology of *Mycosphaerella* spp. and *Teratosphaeria* spp. in tree crops have not been nearly as comprehensive as those on *M. graminicola*. There is clearly a gap of knowledge on the biology of these species regarding the history of the occurrence in the areas where they have been found. These gaps in knowledge complicate the interpretation and comparison of

population genetic information. For instance, sampling scales are different between studies, the number of isolates used is different and frequently low, the molecular markers used are different and the geographical distributions ranges are only partially covered. Nevertheless, the evidence provided in this group of studies (Table 3) is sufficient to outline the basic general population structure of the species concerned.

Globally, moderate to high levels of genetic diversity were found for *M. musicola, M. fijiensis, M. populorum, T. cryptica* and *T. nubilosa*. The distribution of the variability at different scales was different for different species. The majority of the diversity was found at the plant and plantation levels for *T. nubilosa* (Hunter *et al.,* 2008) and *M. fijiensis* (Carlier 2004; Hayden *et al*., 2003a; Rivas *et al*., 2004). This is comparable to the diversity of *M. graminicola,* where the majority of diversity can be found within wheat plots (Boeger *et al.,* 1993; Zhan *et al.,* 2003). *Mycosphaerella populorum* (Feau *et al.,* 2005) and *M. musicola* (Hayden *et al.,* 2003b; 2005) displayed the majority of diversity within a single tree and within a lesion or at the plant level respectively. No comparable information is available for *T. cryptica*.

Population Biology studies of *T. zuluensis*

A pilot population study was carried out on *T. zuluensis* (under the name *C. zuluense*) by Van Zyl *et al.,* (1997, 2002a). In those studies, considerable variability in colony colour and pathogenicity among cultures of *T. zuluensis* from South African plantations was found. Accordingly, it was expected to find high levels of genetic variation. Nevertheless, low levels of genetic variation were found using Amplified Fragment Length Polymorphisms (AFLPs) (Van Zyl *et al.*, 2002b). Unfortunately, these AFLP studies could not be continued at the time in order to arrive to sound conclusions on the genetic variability of the fungus.

For the purposes of the studies conducted as part of this thesis, microsatellites or Simple Sequence Repeats (SSRs) were chosen over continuing with AFLPs studies to determine the level of genetic diversity in populations. In contrast to AFLP data, microsatellite results are easily reproducible and allow comparisons across different studies. They consist of repeating units of 1-6 base pairs in length. They are co-dominant, typically neutral (Jarne & Lagoda 1996) with

the capability of revealing polymorphisms at a given locus and showing high levels of polymorphism in relatedness studies (Tautz & Renz 1984; Tenzer *et al.*,1999). A considerable effort to establish a protocol that would be robust and effective to discover and develop microsatellites for fungi was made in this study. This led to a combination of two protocols, (Hamilton *et al.,* 1999, Zane *et al.,* 2002) with some modifications needed for optimization. The final protocol chosen to select and develop microsatellites for population biology studies on *T. zuluensis* and *T. gauchensis* is presented in Appendix II of this thesis.

CONCLUSIONS

The taxonomy of eucalypts remains dynamic after 300 years of study. More than 700 species have been recognized and a similar number of subspecies and natural hybrids within its natural range in Australasia. Eucalypts harbour tremendous natural variation that has allowed these trees to adapt to very wide climatic and soil conditions.

Only a relatively small number of *Eucalyptus* and *Corymbia* species have been exploited during the domestication of these trees. The domestication process, as for other crops has been linked to productivity needs. There is an increased demand for *Eucalyptus* products worldwide and this is linked increased pressure to increase productivity. As a result, new agricultural and production technologies are constantly being developed and applied. There are enormous possibilities to extend the use of the genetic potential using these new technologies. For example, biotechnological initiatives including the *Eucalyptus* genome project will extend the way genetic variation can be used. It will also introduce into the industry technological possibilities that will enable greater control over favourable phenotypic characteristics.

Concomitant with the increase of plantation areas worldwide, there is an increase in emergent pests and pathogens. There is a repeated pattern emerging from new tree plantations. After a short period of healthy and vigorous growth, pest and pathogens begin to impart damage. Currently, they represent one of the most substantial challenges to the forestry industry worldwide. Current knowledge

regarding the identity and biology of these pest and pathogens remains very limited. Studies are thus needed to better understand the interaction of these pests and pathogens and their eucalypt hosts and further, to incorporate such information into planting and breeding programs.

Ascomycetes, in particular belonging to *Teratosphaeria* represent one of the largest group of pathogenic fungi on *Eucalyptus*. The taxonomy of this group is complex and frustrated by morphological characteristics that are reduced, variable and can be redundant (polyphyletic) across genera. DNA studies have thus become essential to achieve reliable identifications. The most recent phylogenetic studies have shown that the *Eucalyptus* pathogens previously in *Mycosphaerella* are best treated in *Teratosphaeria*.

The taxonomy of the pathogens causing Coniothyrium canker disease of *Eucalyptus* has been heavily affected by the contemporary phylogenetic studies on *Mycosphaerella*. These studies have shown that the species causing this disease reside in *Teratosphaeria* as *Teratospaheria zuluensis* and *Teratosphaeria gauchensis* (Fig 8, 9). For the present *Teratosphaeria* is the most useful genus to accommodate these fungi. Nevertheless, we might expect in the future further taxonomic changes as there are additional ongoing studies on the treatment of *Teratosphaeria*.

Little is known regarding the biology and population structure of species of *T. zuluensis* and *T. gauchensis* causing leaf and stem diseases of *Eucalyptus* trees (Fig 8). There are various intriguing questions at the population level concerning the origin, genetic variation, reproduction, and spread of these species. It is, therefore, important to consider that other phylogenetically closely related pathogenic species probably occur in the natural range of eucalypts and these might appear as pathogens in plantations in the future.

This thesis includes studies on the so-called Coniothyrium canker pathogens, *T. zuluensis* and *T. gauchensis*. The aims of the studies were to resolve various taxonomic questions relating to the pathogens and various new geographic reports are included for them. Furthermore, a suite of studies consider, for the fist time, the population genetics of these two fungi that are emerging as amongst the most important constraints to *Eucalyptus* plantation forestry in the world.

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Zhan J, Kema GHJ, McDonald BA (2004). Evidence for natural selection in the mitochondrial genome of *Mycosphaerella graminicola. Phytopathology* **94**, 26–267.

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Zhan J, McDonald BA (2004). The interaction among evolutionary forces in the pathogenic fungus *Mycosphaerella graminicola. Fungal Genetics and Biology* **41,** 590–599.

Zhan J, Mundt CC, Hoffer ME, McDonald BA (2002a). Local adaptation and effect of host genotype on the rate of pathogen evolution: an experimental test in a plant pathosystem. *Journal of Evolutionary Biology* **15,** 634–647.

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Zhan J, Mundt CC, McDonald BA (2000). Estimation of rates of recombination and migration in populations of plant pathogens - A reply. *Phytopathology* **90,** 324–326.

Zhan J, Mundt CC, McDonald BA (2001). Using restriction fragment length polymorphisms to assess temporal variation and estimate the number of ascospores that initiate epidemics in field populations of *Mycosphaerella graminicola. Phytopathology* **91,** 1011–1017.

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Zhan J, Torriani SFF, McDonald B (2007b). Significant difference in pathogenicity between MAT1-1 and MAT1-2 isolates in the wheat pathogen *Mycosphaerella graminicola*. **Fungal Genetics and Biology *44, 339–346.***

Zhou XD, Xie YJ, Chen SF, Wingfield MJ (2008). Diseases of eucalypt plantations in China: challenges and opportunities. *Fungal Diversity* **32,** 1–7.

Zhu Y, Chen H, Fan J, Wang Y, Li Y, Chen J, Fan J, Yang S, Hu L, Leung H, Mew TW, Teng PS, Wang Z, Mundt CC (2000). Genetic diversity and disease control in rice. *Nature* **406,** 718–722.

Table 1 Selection of search results of web sites containing useful information regarding forestry and eucalypts species. Examples of results using string of words within“ ” are shown, examples of result searching for books are shown, and different kind of sites containing general information on plantations or species specific information. Links are functional and can be followed using Ctrl+click.

Examples of searches using strings of words (string within “ ”)
„*Eucalyptus* diseases“ the search retrieved 2880 direct links http://books.google.ch/books?ei=18szSo-WAsausAbHv7zMCQ&ct=result&lr=&q=eucalyptus+diseases&sa=N&start=0 **„*Eucalyptus* transgenic“** the search retrieved 600 direct links http://books.google.ch/books?ei=18szSo-WAsausAbHv7zMCQ&ct=result&q=eucalyptus+transgenic&lr=&sa=N&start=0
search results of web pages on *Eucalyptus* species
General information, maps, statistics http://www.git-forestry.com/ http://www.git-forestry.com/downloads/GIT_Forestry_Global_Eucalyptus_Map_2009_Marketing_Campaign_ENG.pdf General information http://florabase.calm.wa.gov.au/browse/profile/21824 General information http://trees.stanford.edu/ENCYC/EUCdiv.htm General information http://www.eucalyptus.com.br/index_eng.html General information http://en.wikipedia.org/wiki/Eucalyptus General information http://www.worldagroforestrycentre.org/SEA/Products/AFDbases/AF/asp/SearchList.asp?txtSearch=Eucalyptus&Submit2=Search&intCat=1 General information http://www.angelfire.com/bc/eucalyptus/
Examples of search results for Books
K. Eldridge, J. Davidson, C. Harwood, Garrit Eucalypt Domestication and Breeding http://books.google.ch/books?id=XrKmcLpu1DsC&pg=PA139&lpg=PA139&dq=E+tereticornis&source=bl&ots=VcwKfPaNqq&sig=0jTNk9Ub1nszndNcK1o6JktSEi8&hl=de&ei=NtIzSo4l08r-BvGY3bUK&sa=X&oi=book_result&ct=result&resnum=6 PJ. Keane, GA. Kile, FD. Podger Diseases and pathogens of eucalypts http://books.google.ch/books?id=8ZCnv-ClKvAC&pg=PA223&lpg=PA223&dq=E+citridora+diseases&source=bl&ots=9P84ACHpaQ&sig=j_U_8ZFlDiCCN1FvSsrIY4XPhSU&hl=de&ei=18szSo-WAsausAbHv7zMCQ&sa=X&oi=book_result&ct=result&resnum=1

JJW. Coppen Eucalyptus
http://books.google.ch/books?id=sovmINZsxdEC&pg=PA208&lpg=PA208&dq=E+citridora+diseases&source=bl&ots=-cyfFMvvp9&sig=tihajCNI0IBAiMb1HEt29xKcp6g&hl=de&ei=18szSo-WAsausAbHv7zMCQ&sa=X&oi=book_result&ct=result&resnum=4#PPA19,M1

R-P. Wei, D. Xu Eucalyptus plantations
http://books.google.ch/books?id=qtjcvNSMupUC&pg=PA88&dq=eucalyptus+transgenic&lr=&ei=3OA0SrWpLIS8yQSD9_SKCQ

Examples of search results as per species

E. globulus
General characteristics
http://www.herbs2000.com/herbs/herbs_eucalyptus.htm
General characteristics
http://www.git-forestry.com/

E. camaldulensis
General characteristics
http://en.wikipedia.org/wiki/Eucalyptus_camaldulensis
Transformation techniques
http://jxb.oxfordjournals.org/cgi/reprint/47/2/285

E. grandis
General characteristics
http://www.na.fs.fed.us/pubs/silvics_manual/Volume_2/eucalyptus/grandis.htm
General characteristics
http://www.australiaplants.com/Eucalyptus_grandis.htm
E. grandis in Argentina
http://www.iufrodurban.org.za/Presentations/Thursday/JuanPaul_GermanRaute.pdf

E. nitens
General characteristics
http://git-forestry.com/EucalyptHighlandForests01.htm
General characteristics
http://www.australiaplants.com/Eucalyptus_nitens.htm

E. urophylla
Soil preparation and weed control
http://www.inta.gov.ar/bellavista/info/documentos/forestales/respuestas.pdf
General characteristics
http://www.worldagroforestry.org/sea/Products/AFDbases/af/asp/SpeciesInfo.asp?SpID=821
FAO corporate document repository:
Linkage maps
http://www.pubmedcentral.nih.gov/picrender.fcgi?artid=1206059&blobtype=pdf
Information on Patent of transformation method
http://www.wipo.int/pctdb/en/wo.jsp?wo=2006052554

E. pellita
Productivity comparison
http://www.springerlink.com/content/wj4j58p218g81p11/
Productivity of monocultures vs. mixed plantations
http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6T6X-4K7WJ67-

1&_user=10&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_acct=C000050221&_version=1&_urlVersion=0&_userid=10&md5=4281ff63a4c4e488a147c2d136117cb7

Mixed plantations vs. monoculture
http://espace.library.uq.edu.au/eserv/UQ:8212/R104_Bristow_pp.pdf
Diseases
http://www2.dpi.qld.gov.au/hardwoodsqld/1819.html

E. tereticornis
General characteristics
http://en.wikipedia.org/wiki/Eucalyptus_tereticornis
General characteristics
http://plantnet.rbgsyd.nsw.gov.au/cgi-bin/NSWfl.pl?page=nswfl&lvl=sp&name=Eucalyptus~tereticornis
Wood quality in India http://209.85.129.132/search?q=cache:rzlK6ZReeJcJ:bio.kuleuven.be/sys/iawa/IAWA%2520J%2520pdf%27s/26.no.1.2005/137-147.pdf+Eucalyptus+tereticornis+India&cd=12&hl=de&ct=clnk&gl=ch
General characteristics
http://www.worldagroforestry.org/sea/Products/AFDbases/af/asp/SpeciesInfo.asp?SpID=817

Table 2 Summary on population genetic studies of *Mycosphaerella graminicola*. Population parametes, major findings for such parameters and main references.

Main Topic	Findings	References
***Mycosphaerella graminicola* populations are high variable**	High diversity across all tested spatial and temporal scales (more than 20 countries in 5 continents). Consistent results across different nuclear markers.	Linde *et al*., 2002 McDonald and Martinez, 1990; 1991. Zhan *et al.,*2002b Zhan and McDonald 2004. Banke and McDonald 2005. Brunner *et al*., 2008.
Lower variability in the mitochondrial genome	Diversity tested using RFLPs. Mt DNA lower diversity is hypothesized as selective sweep where haplotypes with faster metabolic rate are favored.	Torriani *et al*., 2008a. Zhan *et al*.,2004.
Variation in neutral markers and variation in quantitative traits	They are correlated. Australia with the lowest genetic diversity (neutral markers) had the lowest additive genetic variance for most quantitative characters.	Zhan *et al.,* 2005.
Population size	>24.000 per field. Few mutations are lost by genetic drift.	Zhan and McDonald 2004. Zhan *et al*., 2001.
Gene flow	Very extensive and global. Major source of migrants was from the Fertile Crescent and "Old world". Global populations are at drift/migration equilibrium. Clear founder effect in Australia. Seeds are proposed to be the most likely mechanism of historical intercontinental gene flow. Ascospores are important at the regional level.	Zhan *et al.,* 2003. Banke and McDonald 2005. Zhan and McDonald 2004.
Recombination and "sex signature"	Ascospores are primary ans secondary inoculum during growing season. High degree to generate new alleles through recombination. Mating types occurring at equal frequency at all spatial scales. Random association among alleles at unlinked loci. Clones are "ephemeral".Individual clones found in a very few meters scale.Identical clones never found in different fields across years.	Zhan *et al*., 1998; 2000. Banke and McDonald 2005. Bruner *et al*., 2008. Zhan *et al*., 2002b. Chen and McDonald 1996. Chen *et al*.,1994 Zhan *et al*., 2001.
Origin, Age	>10.000 years. Relatively old for a crop disease. Timeframe to accumulate mutations. *M. graminicola* emerged during the same time as the domestication of wheat. Close relatives are still present on wild grasses I the Fertile Crescent in Iran.	Stukenbrock *et a*l., 2007.
Evolution	Selection seems to be a main driver of evolution: Competition among strains is very high. Adaptation for higher virulence can occur over short periods of time. MATI-1 is more virulent than MATI-2. Local adaptation can occur in a single growing season in field experiments. Sexual recombination enables faster evolution of the pathogen. Disruptive evolution occurred in host mixtures. Populations rapidly can become resistant to fungicides.	Cowger *et al*., 2000. Zhan *et al*., 2002. Zhan *et al*., 2007. Brunner *et al*., 2008. Torriani *et al*., 2008b. Zhan *et al.,* 2006.

	Possible association between virulence and fungicide resistance.	
Genetics of Virulence and Resistance	Virulence an fungicide resistance are mainly quantitative characters. From theoretical point of view, Search for breeding resistance should be based on quantitative resistance or R-gene pyramids.	Zhan *et al.,* 2005. McDonald and Linde 2002.

Table 3 Summary of the population genetic studies on pathogenic *Mycosphaerella and Teratosphaeria* spp.

Teleomorph	***M. populorum***	***M. musicola***	***M. fijiensis***	***T. cryptica***	***T. nubilosa***
Anamorph	*Septoria musiva*	*Cercospora musae*	Paracercospora fijiensis	*Colletogloeopsis nubilosum*	*(Uwebraunia)* In nature only the sexual state is found.
Host	Poplar trees	Banana trees	Banana trees	*Eucalyptus* trees	*Eucalyptus* trees
Studied Geographic range.	North America	Africa, Latin America, Caribbean, Australia, Indonesia	Philippines, Papua new Guinea, Africa, Latin America, Pacific Ilands, Australia	Australia	Spain, Portugal, Tanzania, South Africa, Australia
Used Molecular markers.	RAPDs	RFLPs; SNPs	RFLPs; SNPs		Microsatellites
Global range genetic diversity.	Moderate (Isolation by distance)	Moderate to High	Moderate	No data	Moderate.
Distribution of diversity: Sampling level containing higher genetic diversity.	90 % diversity within a single tree	Lesion and Plant level in Australia	Plant and plantation	No data	Plant and plantation in South Africa
Linkage disequilibrium (Evidence of recombination)	Yes Gamet eq. at pop. level	Yes Gamet eq. at pop. level Gamet deseq. at plant level	Yes Gamet eq. at pop. level	No	Yes.
Level of differentiation among populations	High	High Low within Australia: (Founder effect)	Low. Lack of significant differentiation among populations of Aus, Papua, Pacific Ilands	No Data	Low. Lack of significant differentiation among populations

Source hypothesis	North America	South-East Asia	South-East Asia	Australia	East Australia
References	Feau *et al.,* 2005	Hyden *et al.,* 2003b; 2005 ; Zandjanakou-Tachin *et al.,* 2009	Carlier2004; Hyden *et al*., 2003a ; Rivas *et al*., 2004 ; Zandjanakou-Tachin *et al.,* 2009;	Milgate *et al.,* 2005	Hunter *et al.,* 2008

RFLP = Restriction Fragment Lenght Polymorphism; RAPD ; = Random Amplified Polymorphic DNA ; SNP = Single Nucleotide Polymorphism ; H = Nei gene distance ; G= Genotypic Diversity.

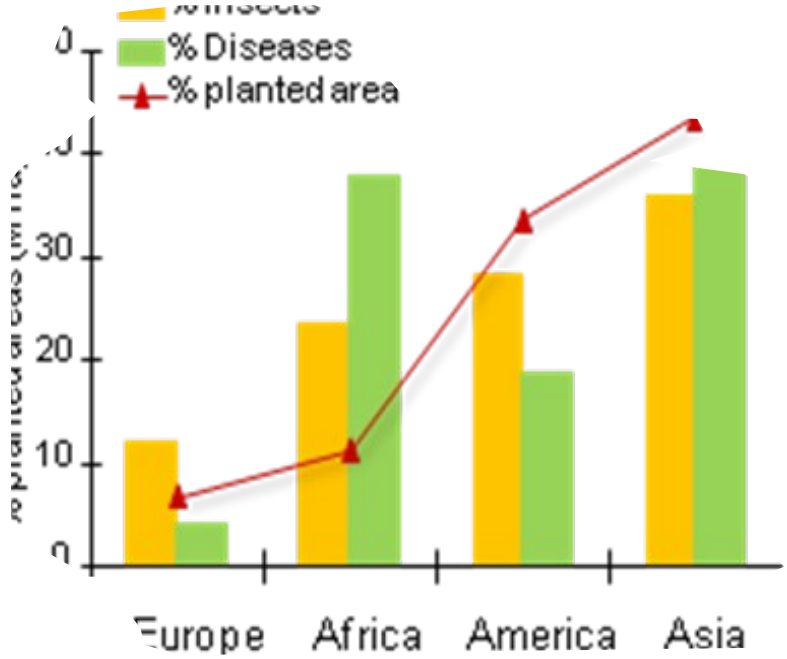

Fig 1 Plot showing the abundance in percentage of pests (orange), diseases (light green) of the world forests as per continent (source FAO, 2009) and *Eucalyptus* planted areas of as per continent (purple line).

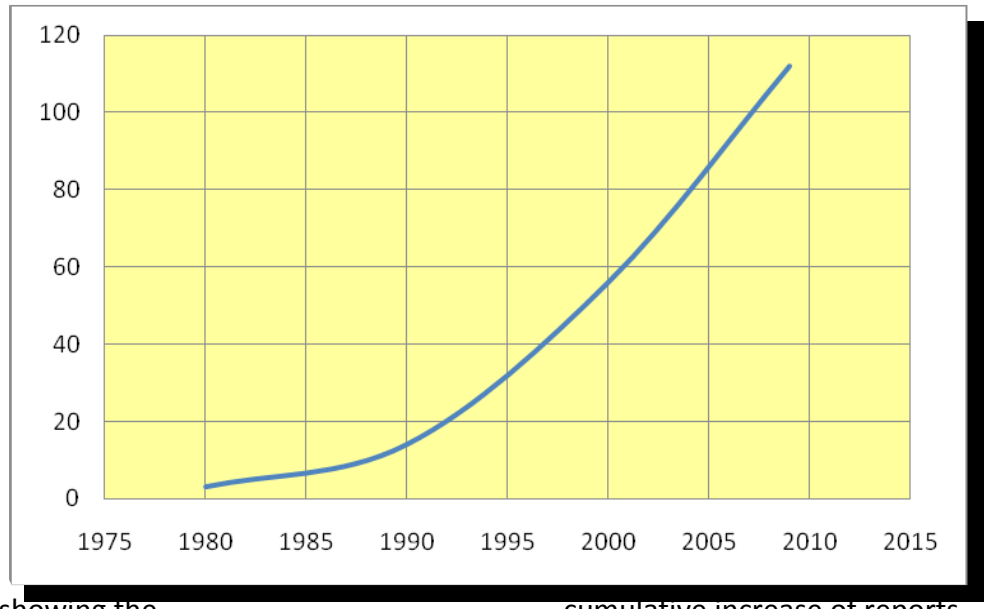

Fig 2 Graph showing the cumulative increase of reports (axis y) on *Mycosphaerella* species of *Eucalyptus* during the last 35 years (axis x) of research on this genus.

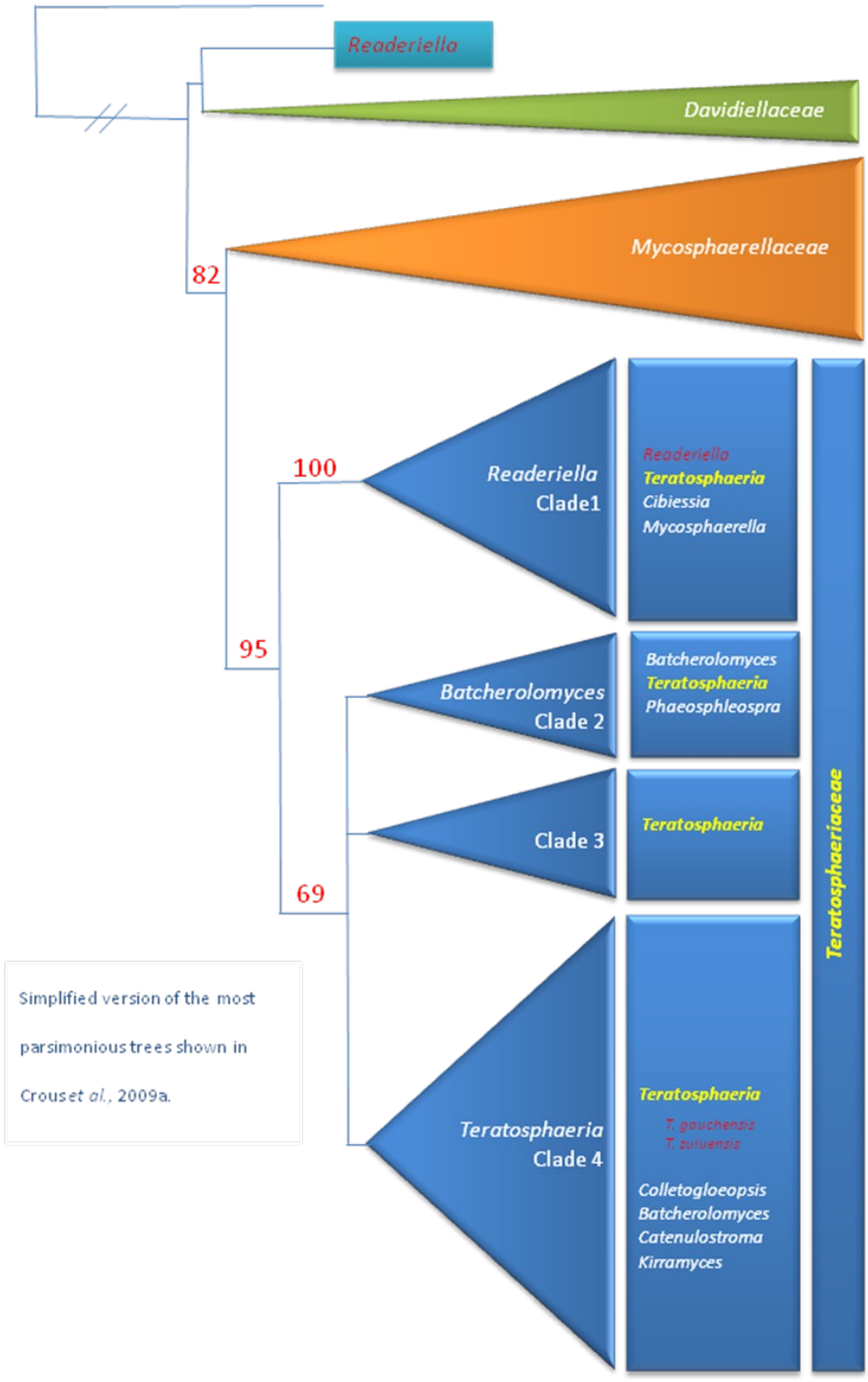

Fig 3 Simplified maximum parsimony tree as in Crous *et al.,* 2009. The support values separating *Davidiellaceae, Mycospharellaceae, Teratosphaeriaceae* and the Clades within *Teratosphaeriaceae* are indicated with red numbers on the corresponding nodes.

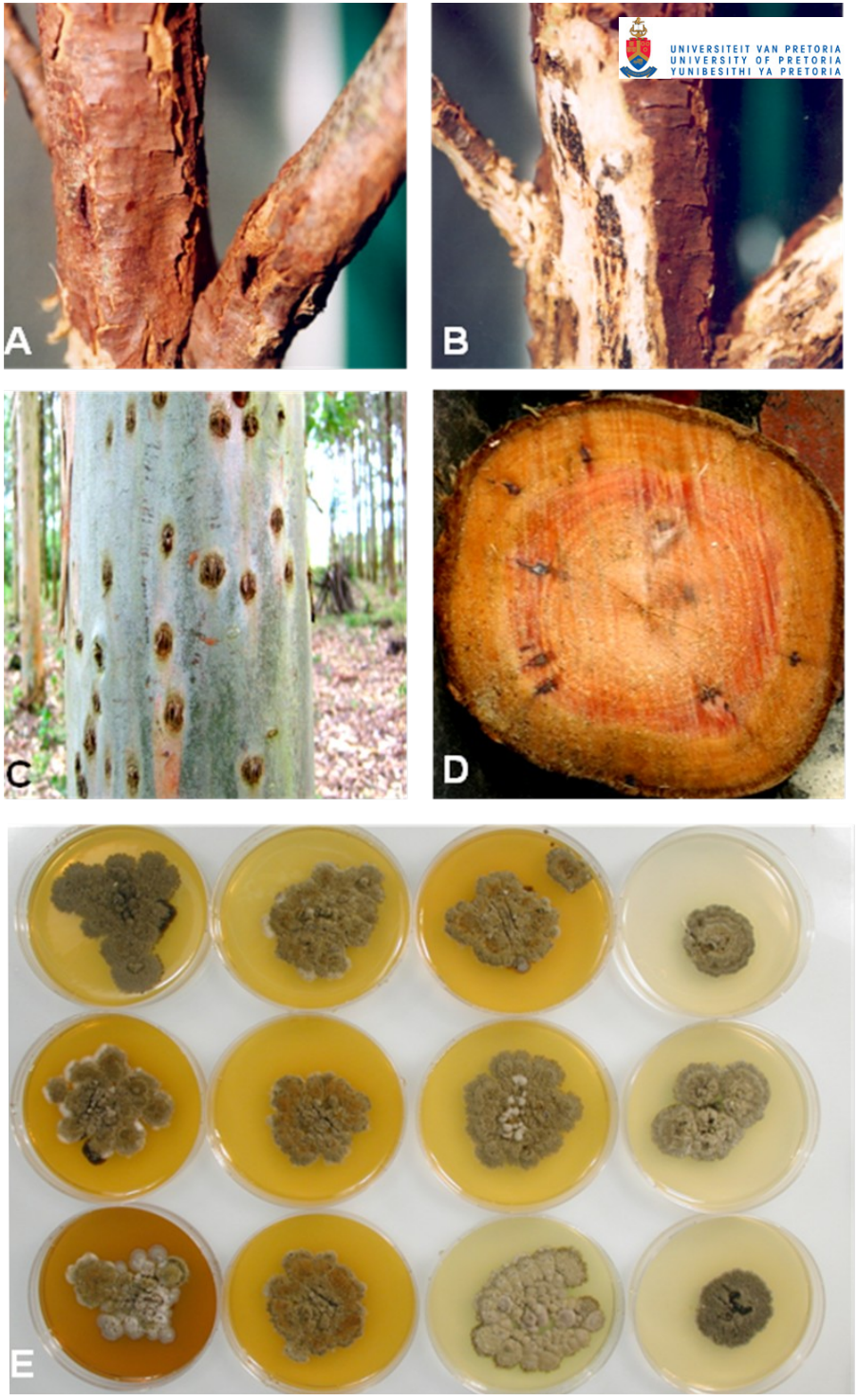

Fig 4 Symptoms and culture morphological characteristics of Coniothyrium canker. A) Lesions on twig of *Eucalyptus* B) the same twig, peeled, showing the internal cankers C) typical lesions on the trunk D) transversal cut of a trunk showing concentric kino pockets. E) Variability of morphology in culture. In this picture is possible to appreciate differences in colour as well as the texture, rate of growth in some cases and staining of the growing media.

Fig 5 Example of two infected plantations. Severe cases in the locations of A. Venters and B. Mtubatuba, both in Kwa-Zulu Natal.

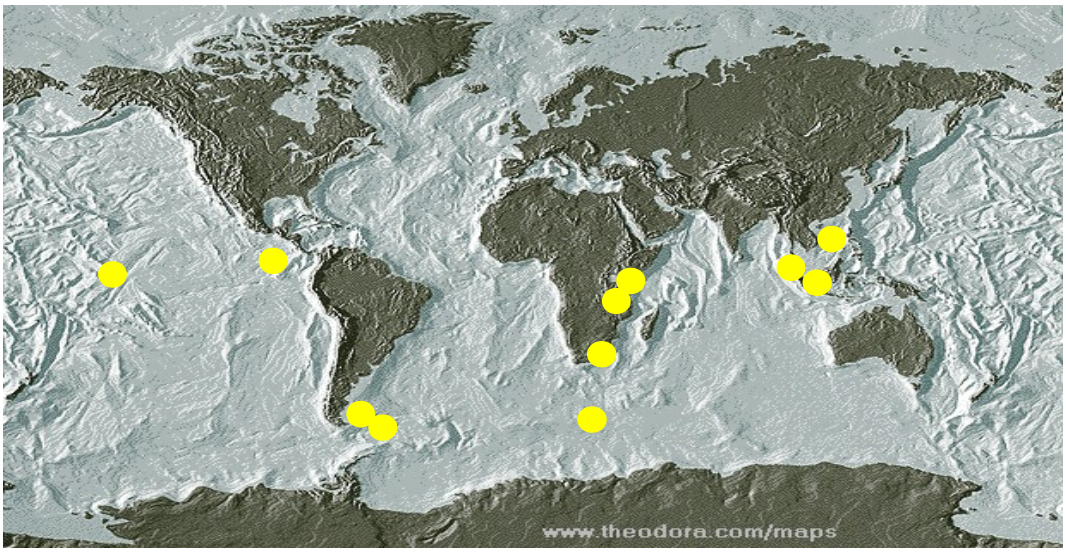

Fig 6 Countries where *Coniothyrium zuluensis* has been reported. Countries are indicated with yellow dots (South Africa, Malawi, Uganda, Ethiopia, China, Thailand, Vietnam, Hawaii-US, Mexico, Uruguay, Argentina). Map by www.theodora.com.

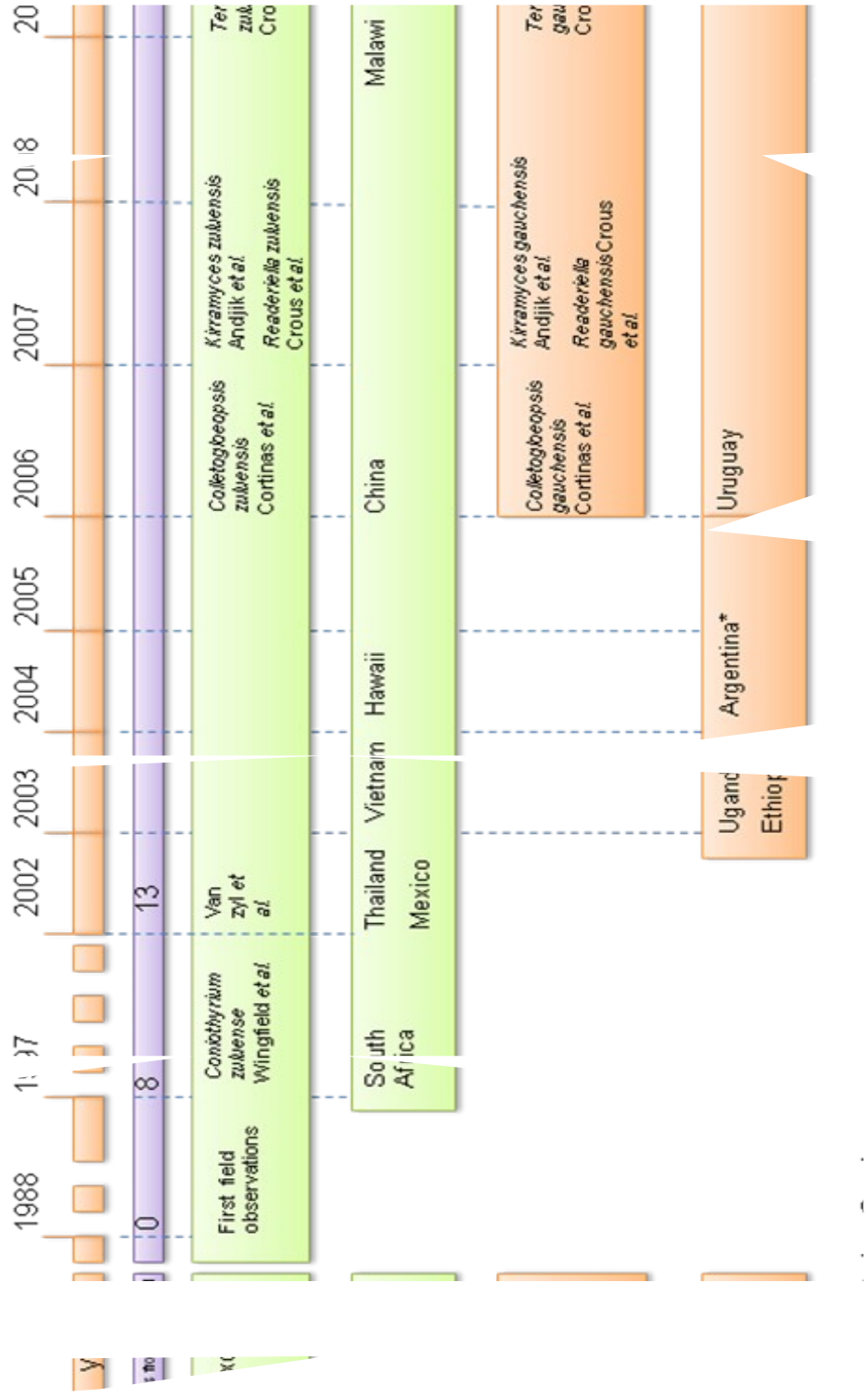

Fig 7 Timeline 1 of Coniothyrium canker disease showing the dates the fungus has been reported in different countries and taxonomic changes since its first description in 1997.

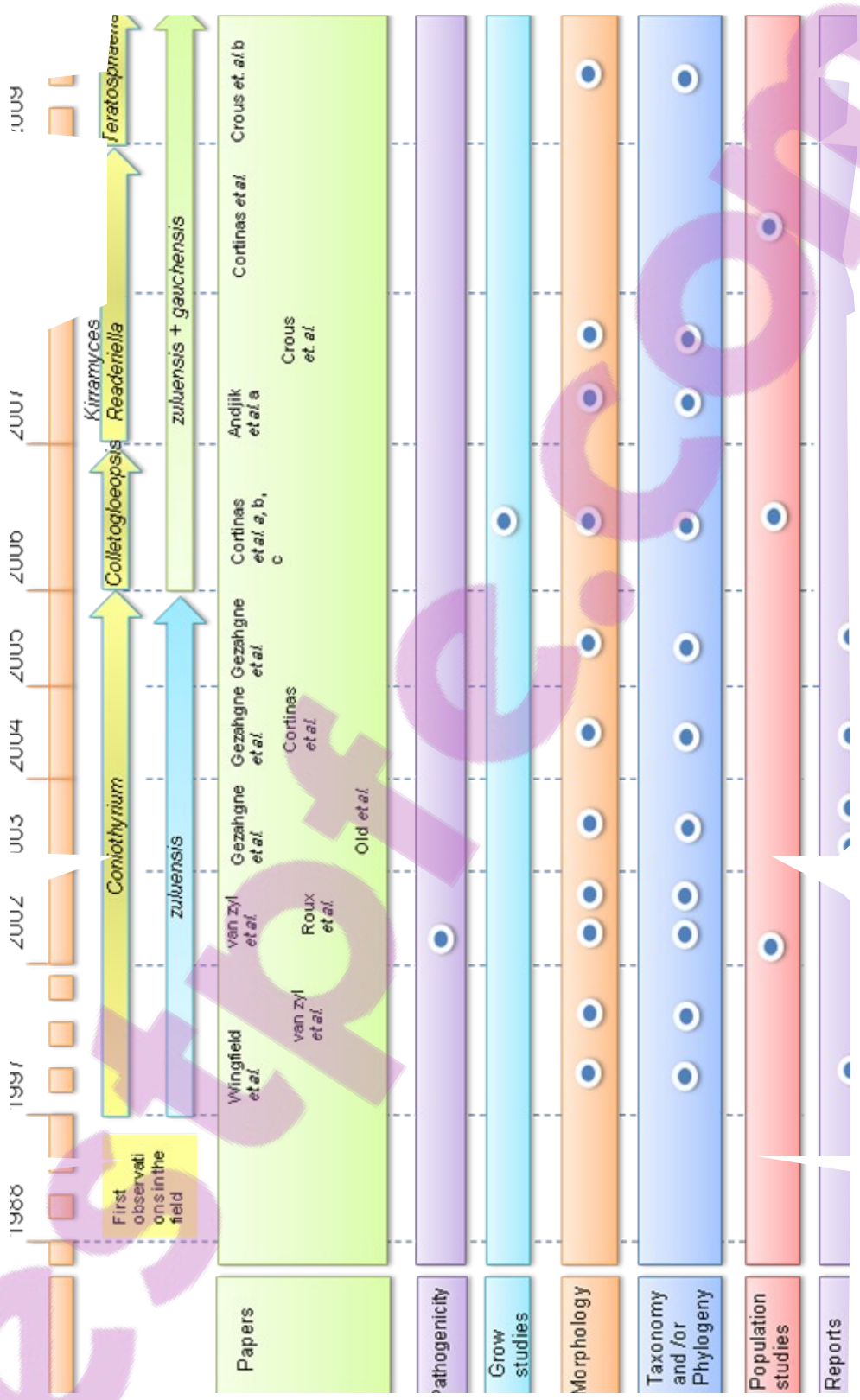

Fig 8 Timeline 2 Coniothyrium canker disease: evolution of taxonomic changes, publications and summary of topics included in the publications.

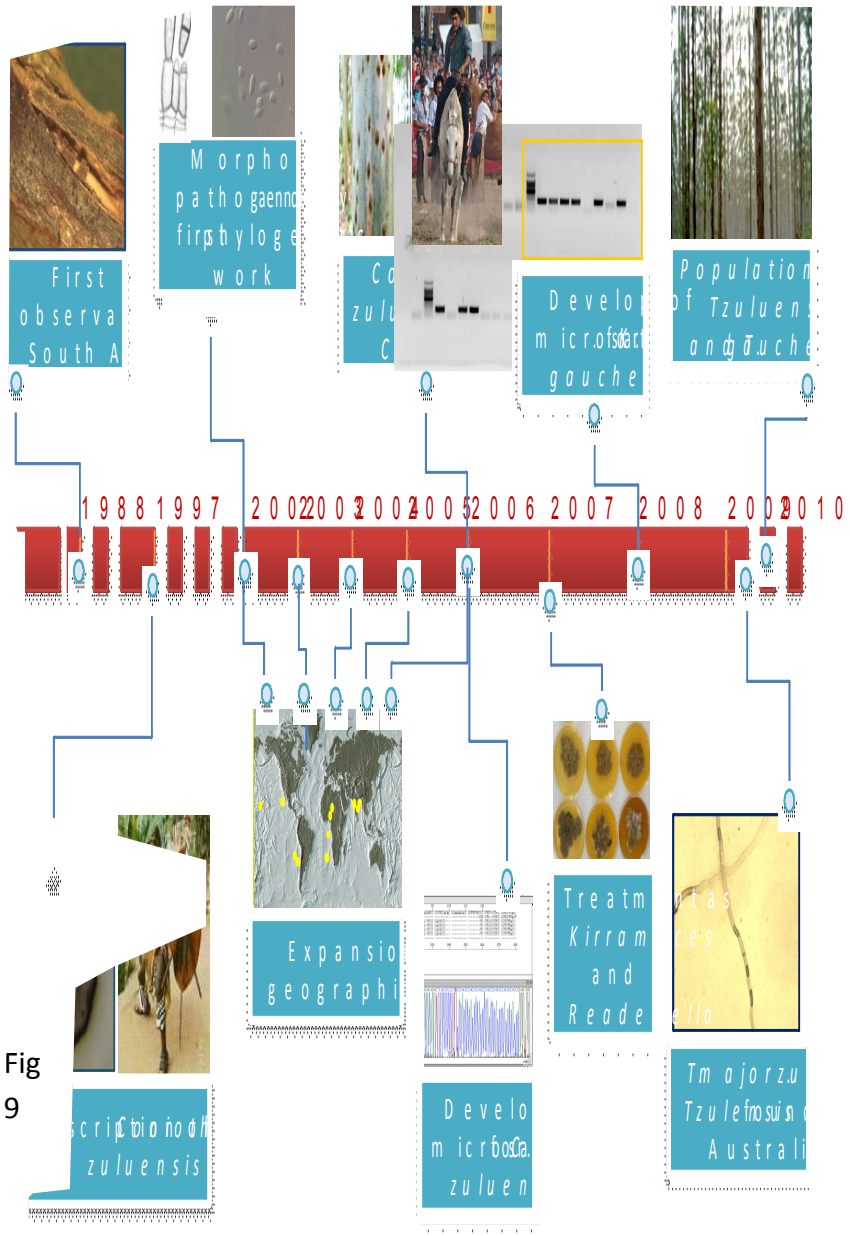

Fig 9 Summary of the Coniothyrium canker story. Important discoveries along time are highlighted.

Chapter 2

First record of *Colletogloeopsis zuluense* comb. nov., causing a stem canker of *Eucalyptus* spp. in China

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ABSTRACT

Coniothyrium zuluense causes a serious canker disease of *Eucalyptus* in various parts of the world. Very little is known regarding the taxonomy of this asexual fungus, which was provided with a name based solely on morphological characteristics. In this study we consider the phylogenetic position of *C. zuluense* using DNA-based techniques. Distance analysis using 18S and ITS regions revealed extensive sequence divergence relative to the type species of *Coniothyrium, C. palmarum* and species of *Paraconiothyrium. Coniothyrium* zuluense was shown to be an anamorph species of *Mycosphaerella*, a genus that includes a wide range of Eucalyptus leaf and stem pathogens. Within *Mycosphaerella* it clustered with taxa having pigmented, verruculose, aseptate conidia that proliferate percurrently and sympodially from pigmented conidiogenous cells arranged in conidiomata that vary from being pycnidial to acervular. The genus *Colletogloeopsis* is emended to include species with pycnidial conidiomata, and the new combination *Colletogloeopsis*

zuluense is proposed. This is also the first report of the pathogen from China where it is associated with stem cankers on *Eucalyptus urophylla*.

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INTRODUCTION

Coniothyrium Corda 1840 represents a large genus of asexual fungi that produce conidia in pycnidia. It is one of the oldest genera of coelomycetes and includes more than 800 species, with *C. palmarum* representing the type (Corda 1840). Sutton (1980) clarified the generic concepts for *Coniothyrium*, limiting it to species in which conidia arise from the percurrent proliferation of conidiogenous cells. Thus, *Coniothyrium* is characterized by having unilocular, immersed, ostiolate, thin-walled and dark brown pycnidia. Conidia are brown, ellipsoidal to cylindrical, formed on percurrently proliferating conidiogenous cells.

In the strict sense, *Coniothyrium* should represent anamorphs of *Leptosphaeria* that are morphologically and phylogenetically similar to *C. palmarum*, the type species of *Coniothyrium* (Crous 1998). *Coniothyrium zuluense* would thus be expected to represent a member of this group. In contrast, a recent study in which ITS sequence data were used to confirm a record of *C. zuluense* from Ethiopia, has suggested that this fungus is related to species of *Mycosphaerella* (Gezahgne *et al.*, 2005). This, together with the importance of the disease has led us to re-evaluate the taxonomic status of *C. zuluense*.

Coniothyrium zuluense causes a very serious stem canker disease on *Eucalyptus* in South Africa, from where it was originally described (Wingfield *et al.,* 1997; Van Zyl 1999). Since then, it has become one of the most serious pathogens of plantation grown *Eucalyptus* spp. in the world. In recent years, Coniothyrium stem canker has been recorded on *Eucalyptus* spp. in Thailand (Van Zyl 1999; Van Zyl *et al.,* 2002), Mexico (Roux *et al.,* 2002), Hawaii (Cortinas *et al.,* 2004) Vietnam (Old *et al.,* 2003), Ethiopia and Uganda (Gezahgne *et al.,* 2003), Argentina (Gezahgne *et al.,* 2004) and Uruguay, (M.J. Wingfield, unpubl.). It is thus intriguing that the fungus is not known from Australia, the area of origin of *Eucalyptus*. While *C. zuluense* might be present on *Eucalyptus* spp. where they are native, but sufficiently unimportant to be noted, it could also have originated on trees related to *Eucalyptus* elsewhere in the world. This would be similar to the case of the pathogens causing the important Cryphonectria canker of *Eucalyptus* (Burgess & Wingfield 2002; Wingfield 2003).

Coniothyrium species have very few useful morphological characteristics of taxonomic relevance. Recognition of species has been based on the morphology of the single-celled conidia including wall ornamentation, pigmentation and size (Taylor & Crous 2001). These characteristics have been shown to be insufficient to differentiate between species where various features overlap. This has been especially problematic in the case of *C. zuluense*, in which cultures are highly variable in texture, colour and growth and they also vary markedly in their pathogenicity to clones of *Eucalyptus* (Wingfield *et al.,* 1997; Van Zyl 1999). These apparent differences led Van Zyl (1999) to believe that *C. zuluense* might encompass more than one taxon. Thus, isolates from South Africa and Thailand were compared based on sequences of the ITS region, but these were found to represent a single phylogenetic species despite their extensive phenotypic variation (Van Zyl *et al.,* 1997).

During the course of surveys of *Eucalyptus* plantations in Africa, South and Central America, and South-East Asia, a large collection of *C. zuluense* cultures have become available to us. These also include a recent collection of isolates from lesions resembling those of Coniothyrium canker on the stems of *Eucalyptus urophylla* trees in China. The aim of this study was primarily to reconsider the taxonomic position of *C. zuluense* as a member of the genus *Coniothyrium,* based ona large global collection of isolates. A secondary objective was to identify the fungus suspected to represent *C. zuluense*, collected from lesions on *Eucalyptus* stems in China.

MATERIALS AND METHODS

Isolates and DNA extraction

Single conidial cultures were established from pycnidia of *Coniothyrium zuluense* collected from host material. The contents of single pycnidia were diluted in sterile distilled water and spread on the surface of 2 % malt extract agar (MEA) plates. After 24 h, germinating conidia were transferred to new MEA plates and these were incubated for 25 d at 25 °. All cultures used in this study are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (CMW),

University of Pretoria, South Africa, and a representative set has been deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, (Table 1).

After 25 d, mycelium was scrapped from the Petri dishes, freeze dried, frozen in liquid nitrogen and ground to a fine powder. DNA was then extracted using a phenol-chlorophorm protocol for which details are described by Cortinas *et al.,* (2004).

PCR and sequencing

A list of isolates and DNA sequences considered in this study are presented in Table 1. Two regions of the ribosomal DNA operon were amplified by PCR for 27 isolates. The partial small nuclear ribosomal subunit (18S) was amplified with the primers NS3: 5' GCA AGT CTG GTG CCA GCA GCC and NS4: 5' CTT CCG TCA ATT CCT TTA AG (White *et al.,* 1990). Partial amplification of the internal transcribed spacer 1, the 5.8S ribosomal RNA gene and the complete internal transcribed spacer 2 (ITS1, 5.8S, ITS 2) was achieved using the primers ITS1: 5' TCC GTA GGT GAA CCT GCG G and ITS4: 5' GCT GCG TTC TTC ATC GAT GC (White *et al.,* 1990). All the PCR reactions were performed in 25 µl total volume including 1µl of genomic DNA from 1/50 dilutions, 1 U Taq polymerase, 10 pmol of each primer, 0.8 mM of each dNTPs, 1 × Taq buffer and 2 mM $MgCl_2$. Cycling conditions were as follows: initial denaturation at 96 ° for 2min, followed by 10 cycles of 30 s at 95 °, 30 s at 54 °, 1 min at 72 ° and 25 cycles of 30 s at 95 °, 30 s at 56 °, 1min at 72 °, with 5 s extension after each cycle. A final elongation step was carried out for 7min at 72 °. PCR amplicons were visualized under UV light on a 1 % agarose gel and then purified by gel filtration through Sephadex G-50 (Sigma S5897) followed by vacuum drying.

Sequencing reactions were performed in 10 µl with 2 µl of purified PCR product, 10 pmol of the same primers used in the PCR, 2 µl 5 × dilution buffer and using the ABI Prism Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA). PCR conditions: were: 25 cycles of 10 s at 96 °C; 4 s at 50 °C; 4 min at 60 °C. Sequencing products were purified by gel filtration through Sephadex G-50 (Sigma S5897) followed by vacuum drying and electrophoresis using an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA).

Phylogenetic analyses

In addition to the sequence data derived in this study, sequences were extracted from GenBank (Table 1). Alignments were carried out using Clustal under MEGA 3 (Kumar, Tamura & Nei 2004). Where necessary, alignments were adjusted manually. All sequences generated in this study have been deposited in GenBank and the accession numbers are shown in Table 1 (marked with *).

Distance analyses were conducted using MEGA 3.0 (Kumar *et al.,* 2004). Pairwise distances were estimated using the Kimura with two parameters model (Kimura 1980). Neighbour-joining was used as grouping algorithm (Saitou & Nei 1987) to reconstruct the trees. Gaps generated in the alignment were treated as missing data. One thousand bootstrap replicates were done in each case to assess the statistical support of nodes in the phylogenetic trees (values indicated on the branches).

The most parsimonious (MP) trees were generated using PAUP v. 4.0b10 (Swofford 2002). For parsimony analyses, heuristic searches were used with the steepest descent option and the TBR swapping algorithm. The characters were equally weighted and treated as unordered. Statistical support of the nodes in the trees was tested with 1000 bootstrap replicates. GenBank AY351901 and AY351899 sequences of *Ophiostama quercus*, (Ophiostomatales) were included as outgroups for 18S and ITS analyses respectively.

Morphology

Growth characteristics of the *Coniothyrium*-like isolates from *Eucalyptus* in China were observed after 25 d. Colours were described following the notations of Rayner

(1970). General morphological features were examined microscopically. Pycnidia-like masses from cultures were mounted on slides in 5 % lactic acid.

RESULTS

Phylogenetic analyses

SSU sequences

A total of 565 bp characters of the 18S ribosomal gene were compared amongst 43 taxa corresponding to *Mycospharellaceae*, *Leptosphaeriaceae* and *Ophiostoma quercus* used as outgroup. The reconstructed distance tree (Fig 1) showed that the type species of *Coniothyrium*, *C. palmarum*, grouped with members of *Leptosphaeria* (*Leotosphaeriaceae*, *Pleosporales*). Isolates of *C. zuluense* from South Africa and China grouped distant from *C. palmarum* with species of *Mycosphaerella*. Furthermore, isolates of *C. zuluense* clustered to a sub-clade of *Mycosphaerella* including the leaf pathogenic species of *Eucalyptus; M. molleriana, M. vespa, M. ambyphilla, Phaeophleospora eucalypti, M. nubilosa*, *M. cryptica and M. suttoniae.*

ITS sequences

After alignment of the ITS region, 535 characters were compared corresponding to 56 taxa. The range of taxa comprised *Mycosphaerellaceae* and *Leptophaeriaceae* and *O. quercus* included as outgroup. Additionally, the number of representatives of *C. zuluense* was increased. The reconstructed tree (Fig 2) showed *C. palmarum* grouping with other *Coniothyrium* species belonging in *Leptosphaeria*. The sub-grouping of *C. zuluense* in the ITS tree had high statistical support. The sequences of *C. zuluense* were located within a *Mycosphaerella* cluster including *M. molleriana, M. vespa, M. ambiphylla, P. eucalypti, M. cryptica, M. nubilosa* and *M. suttoniae.* The topology of the most parsimonious trees and consensus trees was equivalent to the topology obtained by distance-reconstructed trees (data not shown). The DNA sequences of newly acquired isolates from China clustered within the *C. zuluense* cluster.

Characteristics of cultures from China

Cultures of *Coniothyrium zuluense* from China have a variety of surface colony colours ranging from olive-grey, greenish glaucous to a greyish olive (Rayner 1970) with feathery margins. Cultures varied from greenish to brownish in reverse, to darkly so, with dark brown submerged mycelium. Some of the cultures developed white mycelial rings close to the margins. Aerial mycelium was moderate, and varied from white to pinkish in colour.

Morphology

The pathogen causing stem lesions on *Eucalyptus* was originally described as a new species of *Coniothyrium* based on its pigmented conidia that arose from percurrently proliferating conidiogenous cells that were formed in pycnidia. From the present as well as other phylogenetic studies (Crous *et al.,* 2004; Lennox *et al.,* 2004), it is clear that *C. zuluense* clusters with a complex of species that have fusoid to ellipsoidal pigmented conidia, that develop percurrently and (or) sympodially from pigmented conidiogenous cells, arranged in conidiomata that vary from being more pycnidioid to acervuloid. In previous studies, species of *Mycosphaerella* forming acervuli were placed in the anamorph genus *Colletogloeopsis* (Crous & Wingfield 1997), while those that were formed in pycnidia, have been placed in *Phaeophleospora* (Crous *et al.,* 2004).

In phylogenetic studies focusing on *Mycosphaerella* and its anamorphs (Crous *et al.,* 2000, 2001a, 2004; Crous; Kang & Braun 2001b), it became clear that many of the anamorph morphologies have evolved more than once in *Mycosphaerella*, and that anamorph morphology is phylogenetically less informative in *Mycosphaerella* than previously suspected (Crous 1998). From the present study it is clear that *Coniothyrium zuluense* is not congeneric with the *Leptosphaeriaceae*, and thus needs to be accommodated in an anamorph genus of *Mycosphaerella*. Previous *Coniothyrium*-like anamorphs of *Mycosphaerella* have been accommodated in *Phaeophleospora* (Crous *et al.,* 2004). However, the type species of *Phaeophleospora, P. eugeniae*, has scolecosporous, multiseptate conidia, and clusters distant from the *C. zuluense* subcluster (P. W. Crous, unpubl.). In contrast, *C. zuluense* always clusters in the same clade as *Colletogloeopsis*

nubilosum and *C. molleriana*, which are morphologically similar to *Coniothyrium zuluense* except that they tend to form acervuloid conidiomata and not pycnidia. Within *Mycosphaerella*, conidiomatal structure has been observed to vary, and to be less important in generic circumscription (Crous *et al.,* 2001a, b). For this reason, we have chosen to emend the generic circumscription of *Colletogloeopsis* to accommodate species with pycnidia. This is consistent with the observation that the transition between pycnidia and acervuli is rather subtle, and has been seen to frequently develop in the same species, depending on the age of the material (Verkley *et al.,* 2004b). Furthermore, *Colletogloeopsis nubilosum*, which forms acervuli on host tissues, has also been observed to form pycnidia in agar when sporulating in culture (crous unpubl. data). For these reasons we do not introduce a new genus for *Coniothyrium zuluense*, but rather emend the description of *Colletogloeopsis* to accommodate this fungus.

TAXONOMY

Colletogloeopsis Crous & M.J. Wingf., *Can. J. Bot.* **75**: 668 (1997).

Mycelium internal and external, consisting of pale brown, septate, branched hyphae, smooth to finely verruculose. *Conidiomata* acervuloid to pycnidioid, immersed to erumpent, dark brown to black. *Conidiogenous cells* arising from the upper cells of a stroma, or superficial hyphae (when cultivated), doliiform to subcylindrical, or somewhat irregular, subhyaline to pigmented, smooth to verruculose, proliferating sympodially and percurrently. *Conidia* single, aseptate, rarely 1-septate, pigmented, smooth to verruculose, fusoid to subcylindrical to ellipsoidal, straight to slightly curved, apex obtuse, base truncate to subtruncate, frequently with a marginal frill.

Teleomorph: *Mycosphaerella*.

Type species: *C. nubilosum* Crous & M.J. Wingf. 1997.

Colletogloeopsis zuluense (M.J. Wingf., Crous & T.A. Cout.) M.N. Cortinas, M.J. Wingf. & Crous, **comb. nov.**

Basinonym.: *Coniothyrium zuluense* M.J. Wingf., Crous & T.A. Cout., *Mycopathologia* **136**: 142 (1997).

DISCUSSION

By utilising a large number of isolates of the fungal stem pathogen that has been known as *Coniothyrium zuluense,* we have been able to confirm preliminary findings that this fungus is an anamorph of *Mycosphaerella.* This result has emerged not only from a global collection of isolates of the fungus, but also using analysis of both the 18S and ITS regions of the ribosomal DNA operon. Although the fungus is known only in its anamorph state, if its sexual state were to be found, this would clearly be a species of *Mycosphaerella.*

The genus *Coniothyrium* is typified by *Coniothyrium palmarum* that is a member of *Leptosphaeria* (*Leptosphaeriaceae*, *Pleosporales*). Corlett (1991) reported several *Coniothyrium* species as possible anamorphs of *Mycosphaerella*. However, this possibility was not further explored due to the established link between *Coniothyrium* and *Leptosphaeria* (Crous 1998). Nevertheless, Milgate *et al.,* (2001) reported the link between *Mycosphaerella vespa* and an anamorph, which they identified as *Coniothyrium ovatum*. Clearly, several links between probable *Coniothyrium*-like anamorphs and species of *Mycosphaerella* are known from the literature. The recent circumscription of *Coniothyrium* (Lennox *et al.,* 2004; Verkley *et al.,* 2004a) makes this genus unavailable for *Coniothyrium*-like anamorphs residing in *Mycosphaerella*. In the past this situation has been resolved by describing these anamorphs in *Phaeophleospora* (Crous *et al.,* 2004). This situation is no longer tenable, however, as the type species of *Phaeophleospora*, *P. eugeniae*, clusters well apart from the *Coniothyrium*-like anamorphs, which reside in a clade with species of *Colletogloeopsis*. By emending the generic circumscription of the latter genus, we have provided a suitable home for the *Coniothyrium*-like anamorphs of *Mycosphaerella.*

Coniothyrium zuluense constitutes a demonstrated link between *Coniothyrium*-like anamorphs and *Mycosphaerella.* This fact raises the possibility that other *Coniothyrium* species on *Eucalyptus*, such as *C. eucalypticola* Sutton and *C. kallangurense* Sutton & Alcorn are also anamorphs of *Mycosphaerella.* Cultures

of these fungi are currently not available and their transfer to *Colletogloepsis* must await further study.

In addition to re-considering the generic placement of *Coniothyrium zuluense*, this study has provided the first firm evidence that the fungus has entered areas of *Eucalyptus* propagation in China. Plantation forestry in China is rapidly expanding, and now exceeds more than 1.3 million hectares, mostly *Eucalyptus urophylla*, *E. grandis* and their hybrids (Minsheng 2003). Areas such as Guandong Province where *Colletogloeopsis zuluense* was discovered have a hot humid climate that is ideally suited to infections by the fungus. Although the disease has not reached serious levels in China, the occurrence of *C. zuluense* in that country deserves serious consideration.

Records of the stem canker disease caused by *C. zuluense* have rapidly increased in number since its first discovery in South Africa in 1988. The origin of this pathogen remains unknown. After its first discovery, Wingfield *et al.,* (1997) speculated that it might have originated on native *Myrtaceae*. This was primarily based on the fact that the fungus was not known to occur in any other country of the world. *C. zuluense* is now known from many countries where eucalypts are being cultivated (Van Zyl 1999; Roux *et al.,* 2002; Van Zyl *et al.,* 2002; Gezaghne *et al.,* 2003; Old *et al.,* 2003; Cortinas *et al.,* 2004). Thus, *C. zuluense* in China could have originated in any one of these countries, or alternatively it could be native on *Eucalyptus* in the centre of origin of these trees, but not yet discovered there.The significant damage that *C. zuluense* causes to *Eucalyptus* propagation justifies further studies on its biology and population genetics. Such studies would give rise to management options for the canker disease and enhance understanding of its origin, which would also contribute to efforts to breed and select resistant trees.

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Table 1 Fungal isolates and DNA sequences used for SSU and ITS analyses.

Culture numbers	Name	Origin	18S GenBank Acc. number	ITS GenBank Acc. number
Strain AA6	*Alternaria alternata*	Canada	U05194	
CPC 4572	*Alternaria malorum*	USA	AY251131	
CPC 4303	*Cercospora oryzae*		AY251103	
CPC 3955	*Cercospora zebrina*	Canada	AY251104	
CPC 3687	*Cladosporium staurophorum*	Colombia	AY251121	
ATCC 200938	*Cladosporium staurophorum*			AF393723
CBS 67268	*Coniothyrium cereale*			AJ293812
CBS 85971	*Paraconiothyrium minitans*			AJ293810
CMW 5283, CBS 75873	*Coniothyrium palmarum*	Israel	DQ240002[a]	DQ240000[a]
CBS 21868	*Paraconiothyrium sporulosum*			AJ293814
CMW 15833 (CRY 1662)	*Coniothyrium zuluense*	Mexico		AF385610, DQ239988[a]
CMW 15834 (CRY 1664	*Coniothyrium zuluense*	Mexico	DQ240022[a]	AF385611, DQ239987[a]
CMW 4507	*Coniothyrium zuluense*	Thailand	DQ240024[a]	
CMW 5236	*Coniothyrium zuluense*	Thailand		AF376829, DQ239989[a]
CMW 5235	*Coniothyrium zuluense*	Thailand		AF376828, DQ239990[a]
CMW 7449	*Coniothyrium zuluense*	South Africa	DQ240021[a]	DQ239976[a]
CMW 7479	*Coniothyrium zuluense*	South Africa	DQ240020[a]	DQ239982[a]
CMW 7468	*Coniothyrium zuluense*	South Africa		DQ239983[a]
CMW 7442	*Coniothyrium zuluense*	South Africa		AF376819, DQ239978[a]
CMW 7452	*Coniothyrium zuluense*	South Africa		DQ239977[a]
CMW 7488	*Coniothyrium zuluense*	South Africa		DQ239975[a]
CMW 7489	*Coniothyrium zuluense*	South Africa		AF276820, DQ239980[a]
CMW 7426	*Coniothyrium zuluense*	South Africa		DQ239979[a]
CMW7459	*Coniothyrium zuluense*	South Africa		AF376816, DQ239981[a]
CMW 13328	*Coniothyrium zuluense*	South Africa	DQ240018[a]	DQ239974[a]
CMW 13324	*Coniothyrium zuluense*	South Africa	DQ240019[a]	AY738214
CMW 6857	*Coniothyrium zuluense*	Vietnam	DQ240023[a]	DQ239986[a]
CMW 6860	*Coniothyrium zuluense*	Vietnam		DQ239985[a]
CMW 15957	*Coniothyrium zuluense*	China	DQ240017[a]	DQ239962[a]
CMW 15968	*Coniothyrium zuluense*	China		DQ239965[a]
CMW 15961	*Coniothyrium zuluense*	China		DQ239961[a]
CMW 15966	*Coniothyrium zuluense*	China		DQ239963[a]
CMW 15078	*Coniothyrium zuluense*	China	DQ240016[a]	DQ239966[a]

CMW 15958	*Coniothyrium zuluense*	China		DQ239964[a]
CMW 15087	*Coniothyrium zuluense*	China		DQ239967[a]
CBS 17193	*Discosphaerina fagi*	UK	AY016342	
CPC 1535	*Dissoconium dekkeri*	Netherlands	AY251101	
CBS 64286	*Leptosphaeria bellynckii*			AF439458
ATCC 42652	*Leptosphaeria bicolor*		U04202	
CBS 24464	*Leptosphaeria congesta*			AF439460
CBS 59186	*Leptosphaeria typharum*			AF439465
CMW 13704, CBS 110499	*Mycosphaerella ambiphylla*	Australia	DQ240005[a]	AY725530, DQ239970[a]
CMW 11255,	*Mycosphaerella colombiensis*	Colombia	DQ240011[a]	AF309612, DQ239993[a]
CMW 3279, CPC 936	*Mycosphaerella cryptica*	Australia	DQ240003[a]	AF309623, DQ239971[a]
CPC 355	*Mycosphaerella cryptica*	Chile		AF309622
CMW 3042, CPC 801	*Mycosphaerella crystallina*	South Africa	DQ240009[a]	AF309611, DQ239997[a]
CMW 5165, CPC 850	*Mycosphaerella ellipsoidea*		DQ240014[a]	DQ239994[a]
CMW 4942, CPC 760	*Mycosphaerella heimii*	Madagascar		AF309606, DQ239992[a]
CMW 5223, CPC 1362	*Mycosphaerella irregulariramosa*	South Africa	DQ240012[a]	AF309608, DQ239991[a]
CBS 65285	*Mycosphaerella latebrosa*	Netherlands	AY251114	
CMW 5150, CPC 935	*Mycosphaerella marksii*	Australia	DQ240008[a]	AF309588, DQ239998[a]
CMW 4940, CPC 1214	*Mycosphaerella molleriana*	Portugal	DQ240004[a]	AF309619, DQ239969[a]
CPC 4661	*Mycosphaerella nubilosa*	Spain	AY251120	AY725570
CMW 6210	*Mycosphaerella nubilosa*	Australia	DQ240006[a]	AF449095, DQ239999[a]
CMW13333, CBS 113265	*Mycosphaerella punctiformis*	Netherlands	AY490775, DQ240010[a]	AY490763, DQ239996[a]
CPC 3837	*Mycosphaerella sp.*	Venezuela	AY251116	
CMW 5348, CPC 1346	*Mycosphaerella suttoniae*	Indonesia	DQ240007[a]	AF309621, DQ239972[a]
CMW11558, Strain A-1-7	*Mycosphaerella vespa*	Australia		DQ239968[a]
Strain Brun/ 1/ 5	*Mycosphaerella vespa*	Australia	AY110906	AY045497
Strain B/ 3/ 2/ 1	*Mycosphaerella vespa*	Australia		AY045500
CMW 5164, CPC 1232	*Mycosphaerella lateralis*	Zambia		AF309624
CMW5565	*Ophiostoma quercus*	Ecuador	AY351901	AY351899
CBS 102207	*Paraphaeosphaeria pilleata*	USA	AF250821	
CPC 3688	*Passalora fulva*	Netherlands	AY251109	AY251069
CPC 5121	*Phaeoramularia hachijoense*	USA	AY251100	
CMW 11687	*Phaeophleospora eucalypti*	New Zeland	DQ240015[a]	DQ230001[a]
CPC1454	*Phaeophleospora eugeniae*		AF309613	
CPC 4195	*Ramularia sp.*		AY251112	
CPC 658	*Septoria tritici*	South Africa	AY251117	

CPC 1488	*Trimmatostroma macowanii*	South Africa	AY260096

[a] GenBank entries generated in this study CPC= Culture collection of Pedro Crous, housed at CBS (Culture collection of Centraalbureau voor Schimmelcultures) CMW= Culture collection at FABI.

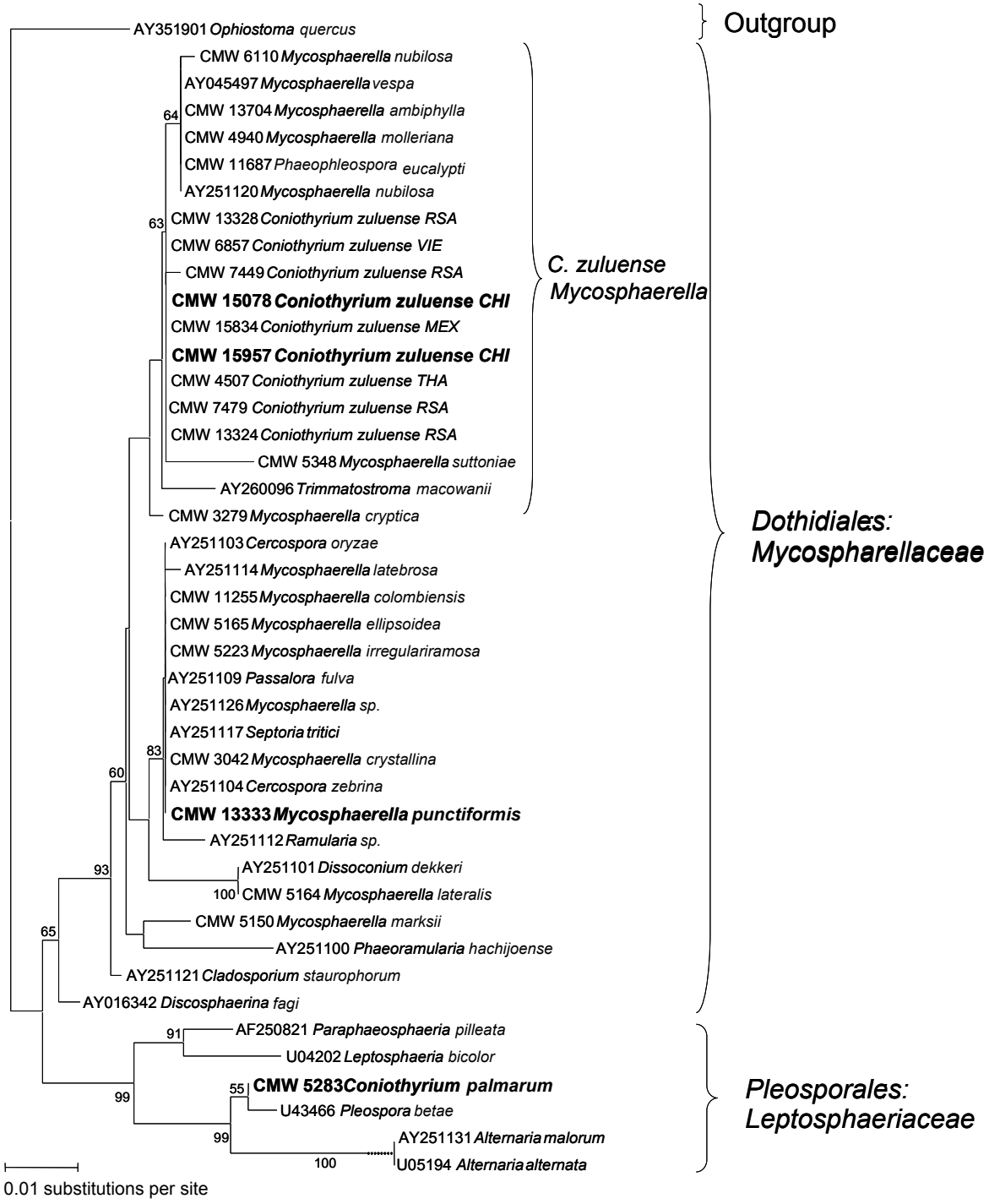


Fig 1 Small subunit 18S rRNA gene phylogram using Kimura with the two parameters nucleotide substitution model and neighbour-joining Bootstrap support values from 1000 replicates are shown at nodes. Only values of 60 % or higher are included and *Ophisotoma quercus* is used as outgroup. RSA=South Africa; VIE=Vietnam; CHI=China; THA=Thailand; MEX=Mexico.

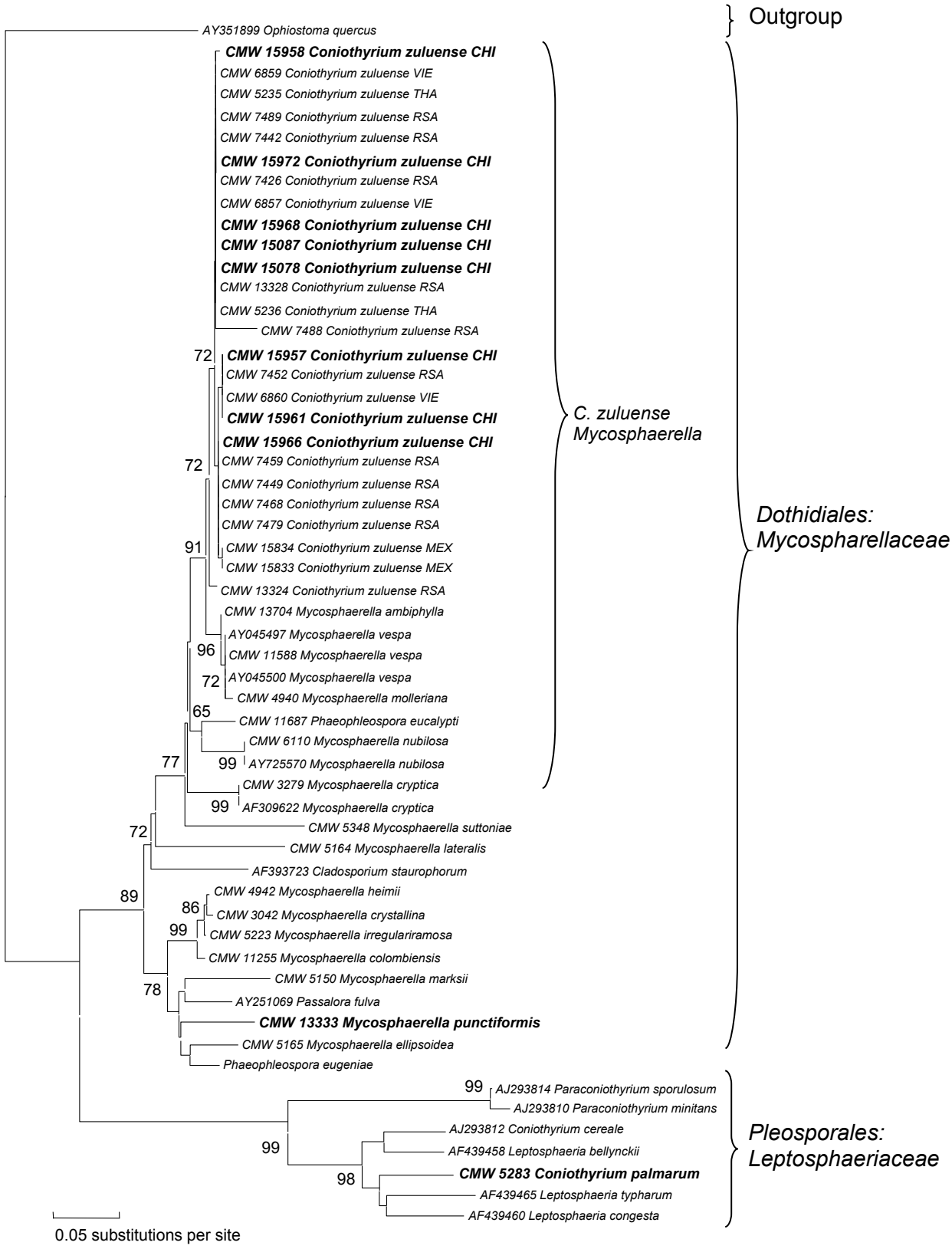
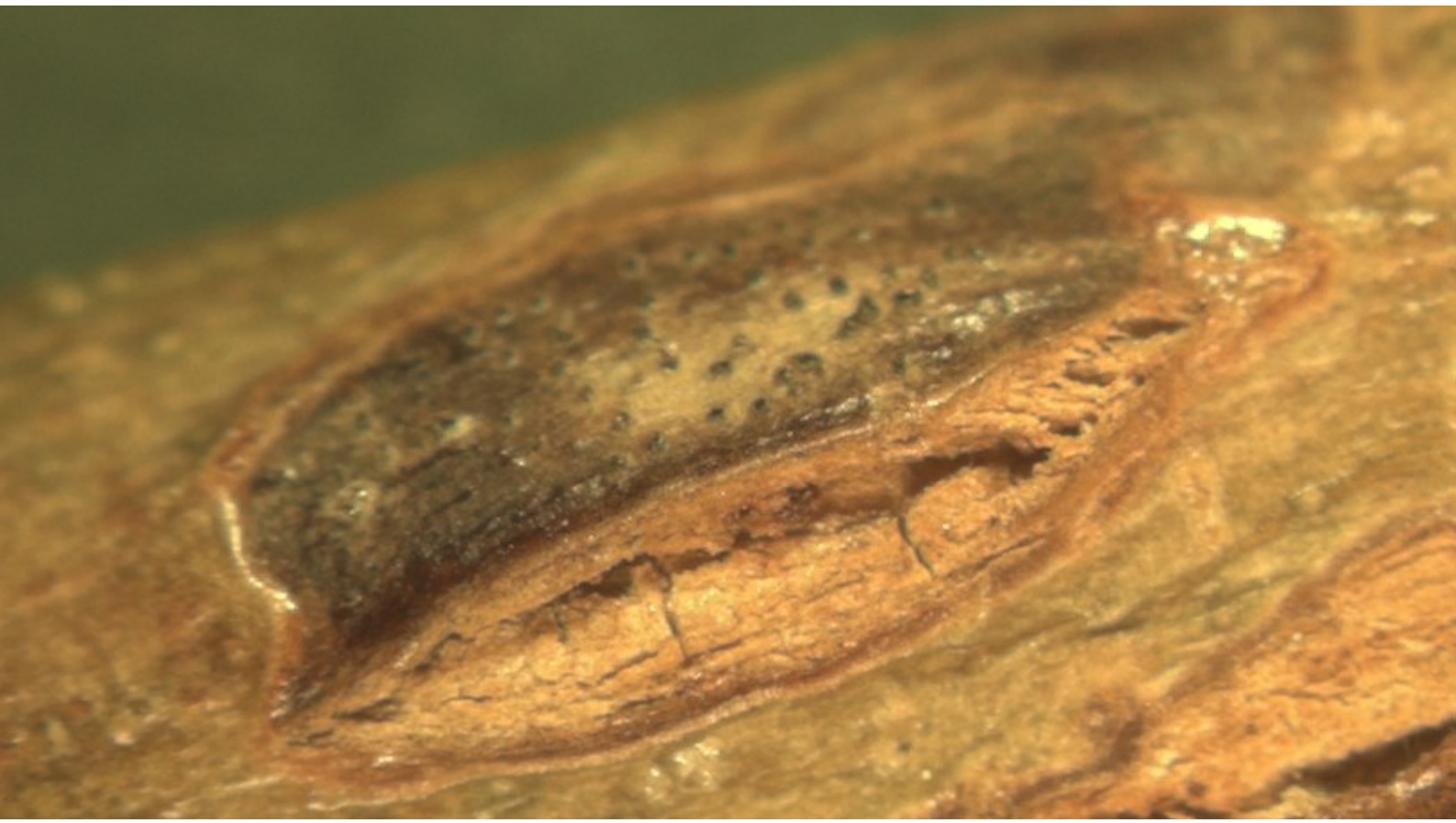


Fig 2 Phylogram obtained from ITS sequencing data gene using the Kimura with two parameters nucleotide substitution model and neighbour-joining Bootstrap support values from 1000 replicates are shown at nodes. Only values of 65 % or higher are included and *Ophisotoma quercus* is used as outgroup. RSA=South Africa; VIE=Vietnam; CHI=China; THA=Thailand; MEX=Mexico.

Chapter 3

Multi-gene gene phylogenies and phenotypic characters distinguish two species within the *Colletogloeopsis zuluensis* complex associated with *Eucalyptus* stem cankers



Chapter 3

Multi-gene gene phylogenies and phenotypic characters distinguish two species within the *Colletogloeopsis zuluensis* complex associated with *Eucalyptus* stem cankers

ABSTRACT

Colletogloeopsis zuluensis, previously known as *Coniothyrium zuluense* causes a serious stem canker disease on *Eucalyptus* spp grown as non-natives in many tropical and sub-tropical countries. This stem canker disease was first reported from South Africa and it has subsequently been found on various species and hybrids of *Eucalyptus* in other African countries as well as in countries of South America and South-East Asia. In previous studies, phylogenetic analyses based on DNA sequence data of the ITS region suggested that all material of *C. zuluensis* was monophyletic. However, the occurrence of the fungus in a greater number of countries, and analyses of DNA sequences with additional isolates has challenged the notion that a single species is involved with Coniothyrium canker. The aim of this study was to consider the phylogenetic relationships amongst *C. zuluensis* isolates from all available locations and to support these analyses with phenotypic and morphological comparisons. Individual and combined phylogenies were constructed using DNA sequences from the ITS region, exons 3 through 6 of the β-tubulin gene, the intron of the translation elongation factor 1-α gene, and a partial sequence of the mitochondrial ATPase 6 gene. Both phylogenetic data and morphological characteristics showed clearly that isolates of *C. zuluensis* represent at least two taxa. One of these is *C. zuluensis* as it was originally described from South Africa, and we provide an epitype for it. The second species occurs in Argentina and Uruguay, and is newly described as *C. gauchensis.* Both fungi are serious pathogens resulting in identical symptoms. Recognising them as different species has important quarantine consequences.

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INTRODUCTION

Colletogloeopsis zuluensis (MJ Wingf., Crous & TA Cout.) MN Cortinas, MJ Wingf & Crous (Cortinas *et al.,* 2006) causes a serious stem canker disease on *Eucalyptus* species.The disease was first reported in 1987 in South Africa, and the pathogen was described as a species of *Coniothyrium*, namely *C. zuluense* MJ Wingf., Crous & TA Cout, (Wingfield *et al.,* 1997). The disease spread very rapidly through the country, initially occurring only on a single *Eucalyptus grandis* clone, but ultimately occurring in all parts of South Africa with a sub-tropical climate, and on a wide variety of *Eucalyptus* species and hybrids. Substantial research has thus been undertaken to better understand the disease and to develop disease-resistant planting stock through breeding and selection programmes (Van Zyl *et al.,* 1997, 2002a).

Symptoms of Colletogloepsis canker are very obvious, at least at the onset of disease. Initial infections include small, circular necrotic lesions on the green stem tissue in the upper parts of trees. These lesions expand, becoming elliptical, and the dead bark covering them typically cracks, giving a “cat-eye” appearance (Fig 1). Lesions coalesce to form large cankers that girdle the stems, giving rise to the production of epicormic shoots and ultimately trees with malformed or dead tops. Infections occur annually on the new green tissue and they penetrate the cambium to form black kino-filled pockets. Thus kino pockets with irregular borders of infected tissue can be seen within the infected wood of trees coincident with the annual rings (Fig 1). Small black pycnidia can be seen on the surface of dead bark tissue (Fig 1), from where black conidial tendrils exude under moist conditions. Conidia are small, aseptate and dematiaceous, appearing black in colour when seen in mass on the host or agar media.

Subsequent to the discovery of Coniothyrium canker in South Africa, the disease has been found in many other countries. Its first discovery outside South Africa was in Thailand where it is associated with typical symptoms on *E. camaldulensis* (Van Zyl *et al.,* 2002b). More recently, the disease has been found in other countries in Africa (Gezahgne *et al.,* 2003, 2005), South and Central America (Roux *et al.,* 2002; Gezahgne *et al.,* 2004), as well as South-East Asia (Old *et al.,* 2003; Cortinas *et al.,* 2004, 2006) (Fig 2). Interestingly, the disease remains

unknown in the areas of origin of *Eucalyptus*, although it might occur there at very low and undetectable levels (Wingfield 2003; Slippers *et al.,* 2005).

The first taxonomic treatment of *C. zuluensis* was based on morphological characteristics of the pathogen. The presence of pycnidia and pigmented aseptate, ellipsoidal conidia arising from percurrently proliferating conidiogenous cells were consistent with species placed in *Coniothyrium* Corda. DNA sequence comparisions have, however, made it possible to recognise that the fungus has a clear phylogenetic position in *Mycosphaerella* Johanson (Gezahgne *et al.,* 2005). It is moreover not related to species of *Coniothyrium s. str.,* which are anamorphs of *Leptosphaeria* spp. This realisation has led to the transfer of *Coniothyrium zuluense* to *Colletogloeopsis* Crous & MJ Wingf. (Cortinas *et al.,* 2006) *Colletogloeopsis* is a well-recognised *Mycosphaerella* anamorph and its circumscription was amended to include species with pycnidioid conidiomata. Within *Mycosphaerella, C. zuluensis* clusters with a group of well-known leaf and stem pathogens of *Eucalyptus* including *M. ambiphylla* A Maxwell*, M. cryptica* (Cooke) Hansf, *M. molleriana* (Thüm) Lindau*, M.. nubilosa* (Cooke) Hansf*, M. vespa* Carnegie & Keane*, M. suttonii* Crous & MJ Wingf., and *Phaeophleospora eucalypti* (Cooke & Massee) Crous, FA Ferreira & B Sutton (Cortinas *et al.,* 2006).

Different isolates of *C. zuluensis* have been found to be highly variable in morphology (Fig 3) and pathogenicity to different *Eucalyptus* clones (Van Zyl 1997; Wingfield *et al* 1997; Van Zyl 2002a). Nonetheless, previous phylogenetic analyses based on the nuclear ribosomal small subunit (18S) and internal transcribed spacer regions and the ribosomal 58 gene (ITS1, 58S, ITS2) had shown that *C. zuluensis* was monophyletic (Van Zyl 2002b; Gezahgne *et al.,* 2005). As additional surveys of *Eucalyptus* plantations are undertaken, an understanding of the geographical range of *C. zuluensis* continues to expand. Additional isolates from new regions have thus become available for DNA sequence comparisons and these have provided the opportunity to re-consider the taxonomic status of *C. zuluensis*, and the variation observed in its morphology and pathogenicity.

The aim of this study was to consider whether the previously recognised *C. zuluensis* can be retained when applying multigene analyses using a large collection of isolates not previously available. To accomplish this objective, individual and

combined phylogenetic analyses using the ITS region, β-tubulin gene (BT2), the elongation factor 1α (EF1α) gene, and the mitochondrial ATPase 6 (ATP6) gene, were carried out. Morphological and other phenotypic characters were also considered.

MATERIALS AND METHODS

Isolates

A collection of 45 isolates was chosen to reflect the geographical distribution of *C. zuluensis* In addition, several species of *Mycosphaerella* known to be closely related to *C. zuluensis* were also included (Table 1). All these isolates were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), Pretoria, South Africa. Single-conidial cultures were established from mature pycnidia isolated from lesions taken from the stems of *Eucalyptus* trees in South Africa and Uruguay. The contents of single pycnidia were diluted in sterile distilled water, and spread on the surface of Petri dishes containing MEA (20 g/L Biolab malt extract, 15 g/L Biolab agar). After 24–36 h, germinating conidia were transferred to fresh MEA plates and incubated for 30 d at 25 °C. Reference strains are preserved in CMW, and have been deposited at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (Table 1). Nomenclature, descriptions and illustrations were deposited in MycoBank.

DNA extraction and amplification

To extract DNA, mycelium was scraped from the surface of cultures grown in Petri dishes, freeze dried, frozen in liquid nitrogen and ground to a fine powder. The protocol followed by Cortinas *et al.,* (2004) was simplified as follows: DBE extraction buffer (200 mM Tris-HCl pH 8, 150 mM NaCl, 25 mM EDTA pH 8, 05 % SDS) was added directly to the ground mycelium and incubated for 2 h at 80 °C (or until pigments changed colour from green to red). In the extraction-DNA enrichment procedure, one volume of phenol was used first and one volume of a 1:1 phenol-chloroform solution thereafter.

Four gene regions were amplified for all isolates included in this study (Fig 4). The ITS region of the ribosomal DNA was targeted using the primers ITS1: 5' TCC GTA GGT GAA CCT GCG G and ITS4: 5' GCT GCG TTC TTC ATC GAT GC (White *et al.,* 1990). Exons 3 to 6 and the respective introns (BT2) of the β-tubulin gene region were amplified using the primers BT2A: 5' GGT AAC CAA ATC GGT GCT GCT TTC and BT2B: 5' AAC CTC AGT GTA GTG ACC CTT GGC (Glass & Donaldson 1995). The intron sequence of the EF1-α gene was amplified using the primers EF1-728F: 5' CAT CGA GAA GTT CGA GAA GG and EF1-986R: 5' TAC TTG AAG GAA CCC TTA CC (Carbone & Kohn 1999) and intron 2 and exon 3 of the ATP6 gene was amplified using the set of primers 5'ATT AAT TSW CCW TTA GAW CAA TT and 5'TAA TTC TAN WGC ATC TTT AAT RTA developed by Kretzer & Bruns (1999).

PCR reactions were prepared in a total volume of 25 µL including 1.5 µL of genomic 1/10 dilution DNA, 1 U of *Taq* polymerase, 10 × *Taq* buffer, 10 pmol of each primer, 0.8 mM of each dNTPs, and 2.0 mM $MgCl_2$ (ITS) or 4.0 mM $MgCl_2$ (BT2, EF1-α, ATP6). PCR amplicons were visualised under UV light on 1 % or 2 % agarose gels. Different cycling conditions were used for the various gene regions. For the ITS region, 96 °C, 3 min initial denaturation and cycles of 95 °C, 30 s, 54 °C, 30 s, 72 °C, 1 min were repeated 10 times followed by 25 cycles of 95 °C, 30 s, 56 °C, 30 s, 72 °C, 1 min with 5 s extension after two cycles. A final elongation step of 7 min at 72 °C was also included. The same cycling conditions were used for ATP6 region changing the annealing temperature to 50 °C. For β-tubulin, 96 °C, 3 min initial denaturation and cycles of 95 °C, 30 s, 57 °C, 45 s, 72 °C, 45 s were repeated 40 times. For EF1- α, 96 °C, 3 min and cycles of 95 °C, 30 s, 54 °C, 45 s, 72 °C, 45 s were repeated 40 times with 5 s extension after two cycles. A final elongation step of 7 min at 72 °C was included.

PCR amplification products were purified using Sephadex G-50 columns (Sigma- Aldrich, Steinheim, Germany) or treated with a mix of Exonuclease III and Shrimp alkaline phosphatase (Exo-Sap); 0.7 U of each enzyme per PCR reaction were incubated at 37 °C for 15 min followed by 80 °C for 15 min before sequencing. Sequencing reactions were prepared in 10 µL with 2 µL of purified PCR product, 10 pmol of the same primers used for the first PCR amplifications, 2 µL 5× dilution

buffer and ABI Prism Big Dye Terminator mix, v. 3.1 (Applied Biosystems Inc., Foster City, California). Sequencing PCR cycles consisted of 25 repetitions at 96 °C, 10 s; 50 °C, 4 s; 60 °C, 4 min. Sequencing reactions were cleaned using Sephadex G-50 or precipitated using EDTA, Sodium Acetate and Ethanol according to the protocol supplied by Applied Biosystems (Applied Biosystems Inc., Foster City, California).

Phylogenetic analyses

Alignments of sequence data were made using Clustal W under MEGA 3.0 (Kumar *et al.,* 2004) and manually adjusted. All sequences generated in this study were deposited in GenBank (Table 1). Alignments were deposited in TreeBASE.

Maximum parsimony and distance analyses were conducted considering the individual and combined partitions. Most parsimonious (MP) trees were generated using PAUP v. 4.0b10 (Swofford 2002). For parsimony analyses, heuristic searches were used with the steepest descent option and the TBR swapping algorithm. The characters were equally weighted and treated as unordered. Statistical support of the nodes in the trees was tested with 1000 bootstrap replicates. Distance analyses were conducted using MEGA 3.0 (Kumar *et al.,* 2004). Pairwise distances were estimated using the Kimura with two parameters model (Kimura 1980). A gamma distribution γ= 0.5 was used to take into account the differences in mutation rate among sites, due to the mix of coding and non-coding sequences present in the analysed fragments. The individual gene reconstructions were performed with Minimum Evolution (Rzhetsky & Nei 1993). Gaps generated in the alignment were treated as missing data. One thousand bootstrap replicates were made to assess the statistical support of the nodes in the phylogenetic trees. Trees were rooted to midpoint.

Partitions were considered together using Bayesian analyses (Ronquist & Huelsenbeck 2003). It has recently been shown that the Bayesian method is more sensitive to under-specification than over-specification of the evolutionary model (Huelsenbeck & Rannala 2004) when calculating the posterior probabilities. Consequently, a time-reversible complex model with gamma-distributed rate variation (GTR + I + G) was selected to combine the data sets. This model of DNA

substitution allows the consideration of different rates of substitutions among sites, different nucleotide frequencies, and differences in the rate of substitutions among nucleotides. Therefore, four sets of analyses were run in MrBayes 3.1.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) calculating marginal posterior probabilities using the selected time reversal GTR + I + G model of nucleotide substitution (Tavaré 1986; Yang 1993, 1994) and default values for the prior settings. Four Monte Carlo Markov chains were run for 3 million generations. Trees and parameters were recorded every 100 generations. Likelihood stability was reached at 30 000 generations. This number of generations was then established as the ''burn-in'' period (represented by 3001 trees). A half compatible consensus tree was recovered from the remaining sampled trees. The Bayesian procedure was repeated four times. The posterior probabilities are indicated close to the respective nodes on the tree and the sequences of *Mycosphaerella colombiensis* Crous & MJ Wingf. and *M. suttonii* were used as outgroups.

Temperature sensitivity studies

Plugs (3 mm diam) of colonised agar were cut from actively growing cultures and placed at the centres of Petri dishes containing MEA. Isolates tested for growth characteristics at different temperatures included those from South Africa (CMW 7442, CMW 7449, CMW 7479, CMW 7488), and others from Uruguay (CMW 7269, CMW 7274, CMW 7279, CMW 7300). Three plates were prepared for each isolate and these were incubated at temperatures between 5 °C and 35 °C at 5 ° intervals, for 6 wk. A second set of isolates from Ethiopia (CMW 8282, CMW 8292) and from China (CMW 15966, CMW 15971) were tested in a similar manner but for an incubation period of 8 wk. Growth was recorded weekly by measuring average colony diameter.

Morphology

Descriptions are based on sporulation *in vivo*. Wherever possible, 30 measurements (× 1000 magnification) were made of structures mounted in lactic acid, the 95% deviation determined, and the extremes of spore measurements given in

parentheses. Colony colours (surface and reverse) were assessed after 25 d on MEA at 25 °C in the dark, using the colour charts of Rayner (1970).

RESULTS

PCR and sequence analyses

Sequenced amplicons obtained from *C. zuluensis* isolates for the four different gene regions were aligned to study fixed polymorphisms. Alignments of 469 bp (ITS), 308 bp (BT2), 254 bp (EF1-α) and 656 bp (ATP6) were generated. The intron between the exons 3 and 4 of the β–tubulin gene was missing in all isolates studied. Visual analyses of the characters defined two groups among the isolates based on the fixed, shared polymophisms. The first group included isolates from South Africa, China, Thailand, Vietnam and Malawi and a second group comprised isolates from Uruguay, Argentina, Hawaii, Uganda and Ethiopia. Positions in base pairs of the different fixed characters in the alignments for the various isolates are shown in Table 2. Five fixed characters were found at the ITS region, eleven were found in the BT2 dataset, eight were found at the EF1-α intron where a 20-base-pair indel was also found (Fig 5). One fixed position was found in the ATP6 region.

Phylogenetic analyses

Individual phylograms were obtained for each gene region and parsimony data produced very similar topologies to those of the distance trees. Therefore, only distance trees are presented (Fig 6). In all cases the Bootstrap cut-off of 70 % was established.

Analyses of sequence data for the ITS region resolved two coherent clusters for the *Colletogloeopsis* isolates considered. These groups represented isolates from South Africa, Malawi, Mexico, Thailand, Vietnam and China (Group1) and those from Uruguay, Argentina, Hawaii, Ethiopia and Uganda (Group 2). The separation of these two groups had 98 % bootstrap support in the ITS tree. In the BT2 and EF-1α trees, these two groups had 99 % and 100 % support, respectively. For the ATP6 tree, three groups could be distinguished although only one of these had strong

support (100 %). The group having reasonable support included isolates from Vietnam, Mexico, Malawi, China and South Africa. Internal sub-clusters could be distinguished within the Group 1 and Group 2 clusters in the ITS, BT2 and EF1-α trees. These sub-clusters had greater than 70 % bootstrap support only in the BT2 tree. The assortment of isolates within the sub-clusters was different in different trees.

The level of polymorphism observed in the datasets was different for each individual analysed region. The β -tubulin data set presented the highest level of variation followed by the EF1-α and ATP6 data sets, respectively. A close inspection of the ATP6 data matrix showed few polymorphisms explaining the poor resolution obtained in the tree.

After the individual analyses, combined parsimony and Bayesian analysis were carried out (Fig 7). The reconstructed trees included the collection of *Colletogloeopsis* isolates together with *Mycosphaerella* spp. A posterior probability of 1 and a 100 % bootstrap value separated the *Colletogloeopsis* isolates from the rest of *Mycosphaerella* spp. The parsimony and Bayesian half-compatible trees showed two major groups representing isolates from South Africa, Malawi, Mexico, Thailand, Vietnam and China (Group1) and those from Uruguay, Argentina, Hawaii, Ethiopia and Uganda (Group 2) supported by posterior probabilities of 1 and 0.95 and 98 % and 100 % bootstrap values, respectively. A rich internal topology was found within these two groups. Numerous sub-clusters were supported with high probabilities and bootstrap values. A number of these subclusters included more than one isolate from the same locality. Nevertheless, location was not sufficient to explain how the sub-clusters were formed.

Temperature sensitivity studies

Average colony diameter for the isolates from South Africa and from Uruguay was different at some of the tested temperatures after 6 wk (Fig 8). No measurable growth was found at 5 °C, optimal growth occurred between 20 and 25 °C, and the diameters of colonies decreased when they were incubated at temperatures of 30 °C and above. Differences between isolates from the two regions were seen at 10 °C where the Uruguayan isolates grew more rapidly than isolates from South Africa.

Between 20 °C and 25 °C both groups of isolates achieved their maximum diameter. Nevertheless, these maximum diameters were smaller for the Uruguayan isolates. The most obvious difference between South African and Uruguayan isolates was observed at 35 °C. At this temperature, the Uruguayan isolates hardly displayed growth whereas South African isolates reached between 10 and 20 mm diam.

The results obtained in a second experiment including isolates from China and Ethiopia, were very similar to those comparing isolates from South Africa and Uruguay. After 8 wk, the differences in growth of the isolates from both origins were obvious at 35 °C (Fig 8). This is consistent with the fact that isolates from China are phylogenetically related to those from South Africa and those from Ethiopia are related to those from Uruguay.

Morphology

Isolates of *Colletogloeposis* included in this study were morphologically variable in culture. Colony characteristics overlapped for isolates from South Africa and Uruguay, but it was possible to recognise some characteristics apparently exclusive to the Uruguayan isolates. Likewise, distinctly different conidial and conidiogenous cell characteristics were found when isolates from Uruguay were compared with those of *C. zuluensis* from South Africa (Fig 9). The range of conidial lengths overlapped almost entirely between *C. zuluensis* [conidia (4–)4.5–5(–6) × 2–2.5(–3.5) µm] and the isolates from Uruguay [conidia (4–)5–6(–7.5) × (2–)2.5(–3) µm]. The Uruguayan conidia, however, had a larger maximum length, reaching 7.5 µm (6 µm for *C. zuluensis*). Conidia of *C. zuluensis* were slightly wider (3.5 µm) as opposed to those from Uruguay, which were an average of 3 µm. Another distinctive characteristic of the fungus from Uruguay is that it has sympodial polyphialidic conidiogenous cells, which is different to *C. zuluensis*, which has percurrently proliferating monophialidic conidiogenous cells.

Taxonomy

Phylogenetic analyses in this study supported two distinct groups of isolates, encompassed within the fungus currently treated as *C. zuluensis*. One of these groups of isolates is from South Africa, Malawi, Thailand, Vietnam, China and

Mexico. The other group includes isolates from Uruguay, Argentina, Hawaii-U.S.A., Ethiopia and Uganda. These fungi can also be separated by characteristics of growth in culture, morphology and growth at different temperatures. Clearly, the South African fungus must retain the name *C. zuluensis*. At the time of describing this fungus, no ex-type cultures were deposited. We have thus provided a suite of isolates for which DNA sequence data are available, and that are tied to herbarium specimens to serve as epitypes. The fungus occurring in Uruguay and other countries represents a distinct taxon that is described below.

Colletogloeopsisgauchensis MN. Cortinas, Crous & MJ. Wingf., **sp. nov.** MycoBank MB500854. Figs 9–10.

Etymology: Named after the gauchos people of South America that live in the same area where this species is distributed and where it was first collected. In the same genus, *C. zuluensis* is named after the KwaZulu-Natal Province and the "Zulu" people of South Africa.

Latin – *Colletogloeopsidi zuluensi* similis, sed conidiis angustioribus, (4-)5- 6(-7.5) x (2-)2.5(-3) µm et phialidibus nonnumquam sympodialiter proliferentibus distincta.

Lesions caulicolous, subcircular to irregular, dark brown, 2–10 mm diam, with a raised, red-brown border. *Conidiomata* pycnidial to somewhat acervular, subepidermal, single, rarely aggregated, occurring in necrotic tissue, globose to slightly depressed, becoming erumpent, up to 120 µm diam, exuding conidia in a long cirrus; conidiomatal walls composed of 2–3 layers of medium brown *textura angularis*; opening by a central ostiole or irregular rupture; ostiolar region lined with thick-walled, brown, smooth, septate hyphae that are sometimes branched below, 3–4 µm wide, with obtuse ends that flare apart (upper 1–6 cells). *Conidiophores* subcylindrical, subhyaline to medium brown, smooth to finely verruculose, 0–3-septate, unbranched or branched below, 10–20 × 3–6 µm. *Conidiogenous cells* subhyaline to medium brown, dolilform to subcylindrical, smooth to finely verruculose, mono- to polyphialidic, proliferating percurrently,

with several percurrent proliferations near the apex. *Conidia* medium brown, thick-walled, finely verruculose, broadly ellipsoidal, apex obtuse to subobtuse, base subtruncate to bluntly rounded, (4–)5–6(–7.5) × (2–)2.5(–3) µm; base frequently with a minute marginal frill.

Specimens examined: **Uruguay**, El Tarugo, bark of 1-yr-old *E. grandis* tree, Feb. 2005, M.J. Wingfield, CBS H-19724 **holotype**, cultures ex-holotype CMW 17331–17332; La Herradura, CBS H-19722, cultures CBS 119467–119466 = CMW 17542–17543; ibid., CBS H-19723, cultures CBS 119465 = CMW 17545, CMW 17544; La Juanita, CBS H-19725, cultures CBS 119468 = CMW 17558, CMW 17559; ibid., CBS H-19726, cultures = CMW 17560–17561.

Cultural characteristics: Colony characteristics on MEA at 25°C are variable. Colony colours were similar to those of *C. zuluensis* (Van Zyl *et al.,* 1997, 2002). Surface colours range from greyish yellow-green, dull green, isabelline, greenish olivaceous to grey-olivaceous; colonies in reverse range from dark grey, dark olive-grey to dark green (Rayner 1970); margins are smooth, regular or irregular. Some cultures develop a characteristic white outer zone of aerial mycelium (Fig. 3). Paler colonies develop smoother surfaces with white aerial mycelium; some strains produce a diffuse yellow pigment in MEA.

Notes: *Colletogloeopsis gauchensis* [conidia (4–)5–6(–7.5) × (2–)2.5(–3) µm] can readily be distinguished from *C. zuluensis* [conidia (4–)4.5–5(–6) × 2–2.5(–3.5) µm] by its slightly longer conidia, and the presence of sympodial polyphialidic conidiogenous cells (Figs 9–10). Furthermore, it grows readily at 10 °C, with hardly any to no growth at 35 °C. In contrast, *C. zuluensis* grows more slowly at 10 °C, and faster at 35 °C than *C. gauchensis*, and strains of *C. gauchensis* do not form conidiomata in culture.

Colletogloeopsis zuluensis (MJ. Wingf., Crous & TA. Cout.) MN. Cortinas, MJ. Wingf. & Crous, Mycol. Res. 110: 235. 2006. Figs 9-10 [as *zuluense*].
Basionym: *Coniothyrium zuluense* MJ. Wingf., Crous & TA. Cout., *Mycopathologia* 136, 142. 1997.

Specimens examined: South Africa, KwaZulu-Natal, Kwambonambi, Teza nursery, bark of 1-yr-old *E. grandis* tree, Jan. 1996, M.J. Wingfield, IMI 370886 **holotype**; KwaZulu-Natal, Kwambonambi, *E. grandis*, Feb. 2005, M.J. Wingfield, CBS H-19721 **epitype here designated**, culture ex-epitype CMW 17321–17322; CBS H-19717, culture CBS 119427 = CMW 17531, CMW 17530; CBS H-19720, culture CBS 119471 = CMW 17528, CMW 17529; CBS H-19719, culture CBS 119470 = CMW 17320, CMW 17319; CBS H-19718, culture CBS 119469 = CMW 17526, CMW 17527.

DISCUSSION

Phylogenetic analyses for a large number of *C. zuluensis* isolates from different parts of the world and based on multiple gene regions have shown clearly that this material represents at least two discrete taxa. These species are described based on material from South Africa and Uruguay, but both taxa include collections from many different countries. Thus *C. zuluensis* is now known from South Africa, Malawi, Thailand, Vietnam, China and Mexico. Likewise, *C. gauchensis* described in this study occurs not only in Uruguay but also in Argentina, Hawaii-U.S.A., Ethiopia and Uganda. The two fungi thus represent distinct phylogenetic species but they can clearly be distinguished from each other based on morphological characteristics and growth characteristics in culture.

Twenty-six fixed nucleotide positions allowed us to separate the collection of *C. zuluensis s. lat.* isolates used in this study into two distinctive groups. One of these fixed polymorphisms found in the EF1-α intron can easily be used to discriminate between *C. zuluensis* and *C. gauchensis*. This 20 bp fragment between positions 153 to 172 in *C. zuluensis* is absent in *C. gauchensis.* The p-distance among the *Colletogloeopsis* isolates considered in this study displayed a range of 0 to 1 % divergence in ITS sequences, 0–8 % for BT2 sequences, 0–24 % for EF1-α sequences and 0–4 % for ATP6 data-matrices respectively. These ranges showed that there

was sufficient variation within *Colletogloeopsis* to suspect that more than one taxon was represented in the collection of isolates. The distances are also consistent with values used in previous studies (Couch & Kohn 2002; Barnes *et al.,* 2005) to separate taxa.

Very few morphological differences were found between isolates of *C. zuluensis* from South Africa and isolates of *C. gauchensis* from Uruguay. These differences include the fact that Uruguayan isolates have polyphialidic, sympodially and percurrently proliferating conidiogenous cells as opposed to the monophialidic, percurrently proliferating conidiogenous cells in *C. zuluensis*. The conidia of *C. gauchensis* are also consistently longer than those of *C. zuluensis* (Figs 9-10). Furthermore, *C. gauchensis* is adapted to cooler climates than *C. zuluensis.* On the contrary, isolates of *C. zuluensis* grow well at 35 °C, whereas those of *C. gauchensis* barely grow at this temperature.

Results of this study provide added support for the view that *C. zuluensis* and *C. gauchensis* are anamorphs *of Mycosphaerella*. They have an allopatric distribution and are considered sibling species only in terms of the fact that they are ecologically and morphologically very similar. The extent to which cryptic and sibling species occur in taxonomic groups varies depending on the group of fungi studied. However, the discovery of cryptic species such as *C. gauchensis* in this study is becoming a commonplace when DNA studies are implemented (see Crous *et al.,* 2006). Results of such studies reveal that these species reflect collections of morphologically similar taxa that can only be discriminated based on minute morphological details or characteristics in pure culture. A further example of such a species complex in *Mycosphaerella* concerns "*Coniothyrium*" *ovatum* H.J. Swart (Crous *et al.,* 2004a, b, 2006).

Intraspecific variation detected amongst isolates of *C. zuluensis* and to a lesser extent *C. gauchensis* showed internal structure in the individual and combined trees. Such intraspecific structure was only well-supported in the BT2, ATP6 and combined trees. Based solely upon the phylogenetic species concept, it would be possible to recognise additional species especially in this complex. For the present, however, we choose to not provide additional names before robust population biology studies are available.

Coniothyrium canker is one of the most important diseases of *Eucalyptus* worldwide (Old *et al.,* 2003). In South Africa, it appeared relatively suddenly in a very limited location and spread rapidly, resulting in very substantial losses to the local forestry industry. The disease has also caused substantial damage to plantations in other countries such as Argentina and Uruguay. It is thus intriguing that there are two distinct fungi associated with indistinguishable symptoms. The origin of the fungus is unknown and it is not known to occur in the native range of *Eucalyptus.* The evidence from this study shows that the two fungi are closely related and have differently adapted based on some ecological factor. Like most *Mycosphaerella* spp. they are highly host-specific to certain species of *Eucalyptus*, grow poorly in culture, and thus it seems reasonable to expect that their origin would be on *Eucalyptus* or a host closely related to it. A similar situation has emerged for species of *Chrysoporthe* Gryzenh. & MJ. Wing. (Gryzenhout *et al.,* 2004). that are well-known pathogens of *Eucalyptus* but that appear to have originated on a wide variety of woody plants in the order *Myrtales* (Wingfield 2003; Gryzenhout *et al.,* 2004; Seixas *et al.,* 2004).

Recognition of two species within a collection of isolates that have previously been recognised as belonging to the single taxon has important consequences for disease control and quarantine. In the past, it has been suggested that the fungus originated in South Africa, and that it was restricted to that country (Wingfield *et al.,* 1997). Thus, the appearance of the disease in other countries has often been linked to the movement of plant material and particularly seed to other countries. Although it has not been shown experimentally that *C. zuluensis* is moved on seed, this appears to be a likely mode of global distribution. There is a large international trade in *Eucalyptus* seed, which is variably controlled and monitored. Both *C. zuluensis* and *C. gauchensis* have now wide geographic distributions and this implies that they have been spread from one or a number of sources. Every effort should now be made to restrict them from further movement to new countries and areas.

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Table 1 Isolates of *Colletogloeopsis* and related species used in the phylogenetic studies.

Species	Strain numbers		Country	Host	Date	GenBank no.			
						ITS	BT2	EF1-α	ATP6
Colletogloeopsis gauchensis	CMW7302		Uruguay	*E. grandis*	2001	DQ240186	DQ240075	DQ240128	DQ240025
	CMW7274;	CBS117830	Uruguay	*E.grandis*	2001	DQ240187	DQ240076	DQ240129	DQ240026
	CMW7294;	CBS117832	Uruguay	*E. grandis*	2001	DQ240188	DQ240077	DQ240130	DQ240027
	CMW7300;	CBS117831	Uruguay	*E. grandis*	2001	DQ240189	DQ240078	DQ240131	DQ240028
	CMW7270		Uruguay	*E. grandis*	2001	-	-	-	DQ240068
	CMW17328		Uruguay	*E. grandis*	2005	DQ240190	DQ240079	DQ240132	DQ240029
	CMW17330		Uruguay	*E. grandis*	2005	DQ240191	DQ240080	DQ240133	DQ240030
	CMW17323		Uruguay	*E. grandis*	2005	DQ240215	DQ240122	-	DQ240069
	CMW17324		Uruguay	*E. grandis*	2005	DQ240216	DQ240123	-	DQ240070
	CMW17326		Uruguay	*E. grandis*	2005	DQ240217	DQ240124	-	DQ240071
	CMW17332;		Uruguay	*E. grandis*	2005	DQ240218	DQ240125	-	DQ240072
	CMW10895;	CBS117260	Hawaii-US	*E. grandis*	2002	DQ240192	DQ240081	DQ240134	DQ240031
	CMW10893;	CBS117834	Hawaii-US	*E. grandis*	2002	DQ240193	DQ240082	DQ240135	DQ240032
	CMW10894		Hawaii-US	*E. grandis*	2002	DQ240194	DQ240083	DQ240136	DQ240033
	CMW7331;	CBS117256	Argentina	*E. grandis*	2001	DQ240195	DQ240084	DQ240137	DQ240034
	CMW7342		Argentina	*E. grandis*	2001	DQ240196	DQ240085	DQ240138	DQ240035
	CMW7378		Argentina	*E. grandis*	2001	DQ240197	DQ240086	DQ240139	DQ240036
	CMW14336;	CBS117257	Argentina	*E. grandis*	2003	DQ240198	DQ240087	DQ240140	DQ240037
	CMW7137		Uganda	*E. grandis*	2001	DQ240199	DQ240088	DQ240141	DQ240038
	CMW15835;	CBS117261	Uganda	*E. grandis*	1999	DQ240200	DQ240089	DQ240142	DQ240039
	CMW8991;	CBS117833	Ethiopia	*E. camaldulensis*	2001	DQ240201	DQ240090	DQ240143	DQ240040
	CMW8978		Ethiopia	*E. camaldulensis*	2001	DQ240202	DQ240091	DQ240144	DQ240041
	CMW19356		Ethiopia	*E. camaldulensis*	2000	-	-	DQ240181	-
Colletogloeopsis zuluensis	CMW1772		South Africa	*E. grandis*	1989	DQ240203	DQ240092	DQ240145	DQ240042
	CMW7426		South Africa	*E. grandis*	1997	DQ239979	-	DQ240182	-
	CMW7459		South Africa	*E. grandis*	1997	DQ239981	-	DQ240183	-
	CMW7488;	CBS117829	South Africa	*E. grandis*	1997	DQ239975	-	DQ240184	-
	CMW7489		South Africa	*E. grandis*	1997	DQ239980	-	DQ240185	-

	CMW17314		South Africa	*E. grandis*	2005	DQ240204	DQ240093	DQ240146	DQ240043
	CMW17316		South Africa	*E. grandis*	2005	DQ240205	DQ240094	DQ240147	DQ240044
	CMW17320		South Africa	*E. grandis*	2005	DQ240206	DQ240095	DQ240148	DQ240045
	CMW17321		South Africa	*E. grandis*	2005	DQ240207	DQ240096	DQ240149	DQ240046
	CMW13328;	CBS113399	South Africa	*E. grandis*	-	DQ239974	-	DQ240172	-
	CMW13324;	CBS111125	South Africa	*E. grandis*	-	AY738214	-	DQ240173	-
	CMW17318		South Africa	*E. grandis*	2005	DQ240213	DQ240126	DQ240174	DQ240073
	CMW17322		South Africa	*E. grandis*	2005	DQ240214	DQ240127	DQ240175	DQ240074
	CMW7449;	CBS117262	South Africa	*E. grandis*	1997	DQ239976	DQ240102	DQ240155	DQ240052
	CMW7452		South Africa	*E. grandis*	1997	DQ239977	DQ240103	DQ240156	DQ240053
	CMW7442		South Africa	*E. grandis*	1997	DQ239978	DQ240104	DQ240157	DQ240054
	CMW7468		South Africa	*E. grandis*	1997	DQ239983	DQ240105	DQ240158	DQ240055
	CMW15971		China	*E. urophylla*	2004	DQ240208	DQ240097	DQ240150	DQ240047
	CMW15080		China	*E. urophylla*	2004	DQ240209	DQ240098	DQ240151	DQ240048
	CMW15964		China	*E. urophylla*	2004	DQ240210	DQ240099	DQ240152	DQ240049
	CMW17425		Malawi	*E. grandis*	2004	DQ240211	DQ240100	DQ240153	DQ240050
	CMW17438		Malawi	*E. grandis*	2004	DQ240212	DQ240101	DQ240154	DQ240051
	CMW17356		Malawi	*E. grandis*	2004	DQ240219	-	-	-
	CMW6859		Vietnam	*E. urophylla*	2000	-	DQ240106	DQ240159	DQ240056
	CMW6860		Vietnam	*E. urophylla*	2000	DQ239985	DQ240107	DQ240160	DQ240057
	CMW6857;	CBS118125	Vietnam	*E. urophylla*	2000	DQ239986	-	DQ240171	-
	CMW15834;	CBS117835	Mexico	*E. grandis*	2000	DQ239987	DQ240108	DQ240161	DQ240058
	CMW15833;	CBS118149	Mexico	*E. grandis*	2000	DQ239988	DQ240109	DQ240162	DQ240059
	CMW5235;	CBS117263	Thailand	*E. camaldulensis*	1997	DQ239990	DQ240110	DQ240163	DQ240060
	CMW5236		Thailand	*E. camaldulensis*	1997	DQ239989	DQ240111	DQ240164	DQ240061
Mycosphaerella ambiphylla	CMW13704;	CBS110499	Australia	*Eucalyptus*	-	DQ239970	DQ240116	DQ240169	DQ240066
Mycosphaerella colombiensis	CMW4944;	CPC1106	Colombia	*Eucalyptus* sp.	-	DQ239993	DQ240112	DQ240165	DQ240062
Mycosphaerella molleriana	CMW4940;	CPC1214	Portugal	*Eucalyptus*	-	DQ239969	DQ240115	DQ240168	DQ240065
Mycosphaerella nubilosa	CMW6210;	CBS114706	Australia	*Eucalyptus*	-	DQ239999	DQ240113	DQ240166	DQ240063
Mycosphaerella suttonii	CMW5348,	CPC1346	Indonesia	*Eucalyptus*	-	DQ239972	DQ240117	DQ240170	DQ240067
M.ycosphaerella vespa	CMW11588		Australia	*Eucalyptus*	-	DQ239968	DQ240114	DQ240167	DQ240064

CMW= Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
CBS= Culture collection of the Centraalbureau voor Schimmelcultures, Uppsalalaan, Utrecht, The Netherlands. CPC= Culture collection of Pedro Crous housed at CBS.

Table 2 Summary of the shared fixed positions found in the DNA regions of ITS, BT2, EF1-α and ATP6 among *Colletogloeopsis* isolates associated with *Eucalyptus* stem cankers. The total number of fixed shared positions between the two groups is given in the last column.

Locus	Locations according to the alignments* and the nucleotide fixed state found in group 1[a] and group 2[b]											No of fixed positions[c]
ITS	89* T[a]/C[b]	107 T/C	116 T or C/del	396 C/T	436 C/T	-	-	-	-	-	-	5
BT2	8 T/C	28 A/G	29 G/A	35 G/A	38 G/A	41 T/G	46 T or G/A	50 A/G	174 T/C	261 G/C	300 T/C	11
EF1- α	114 C/T	122 del/ C	137 C/A	143 C/T	153 to172 in /del[c]	175 G/A	183 C/T	195 G/A	196 A/G	-	-	9
ATP6	644 A/G	-	-	-	-	-	-	-	-	-	-	1

* Location of the fixed shared polymorphisms. The number in this cell and in all the other cells represent the location of fixed shared polymorphisms. They are defined in base pairs counting from the beginning of the alignment.

[a] The first letter before the slash bar represents the state character shared by isolates of the group 1, *C. zuluensis.*

[b] Character state shared by isolates of the group 2, *C gauchensis.*

[c] The grey box in the EF1-α line indicates the position of the 20 bp in/del that could be used for diagnostic purposes.

Fig 1 External symptoms of the stem canker disease on *E. grandis* in Uruguay caused by *C. gauchensis*. A, B. Mature clones showing the typical lesions on the surface of the trunk. C. Distinctive black circular lesions on green twigs. D. Stem with typical cracked lesions. E. Stem showing internal symptoms below the bark lesions. F. Kino-pockets of infected tissue within the wood. G. Pycnidia on cracked lesions.

Fig 2 Geographic range of the collection of isolates used in this study. The map includes isolates from South Africa, Malawi, Vietnam, Thailand and China, indicated with white dots (Group 1) and isolates from Uruguay, Argentina, Hawaii-U.S.A., Ethiopia and Uganda, indicated with black dots (Group 2).

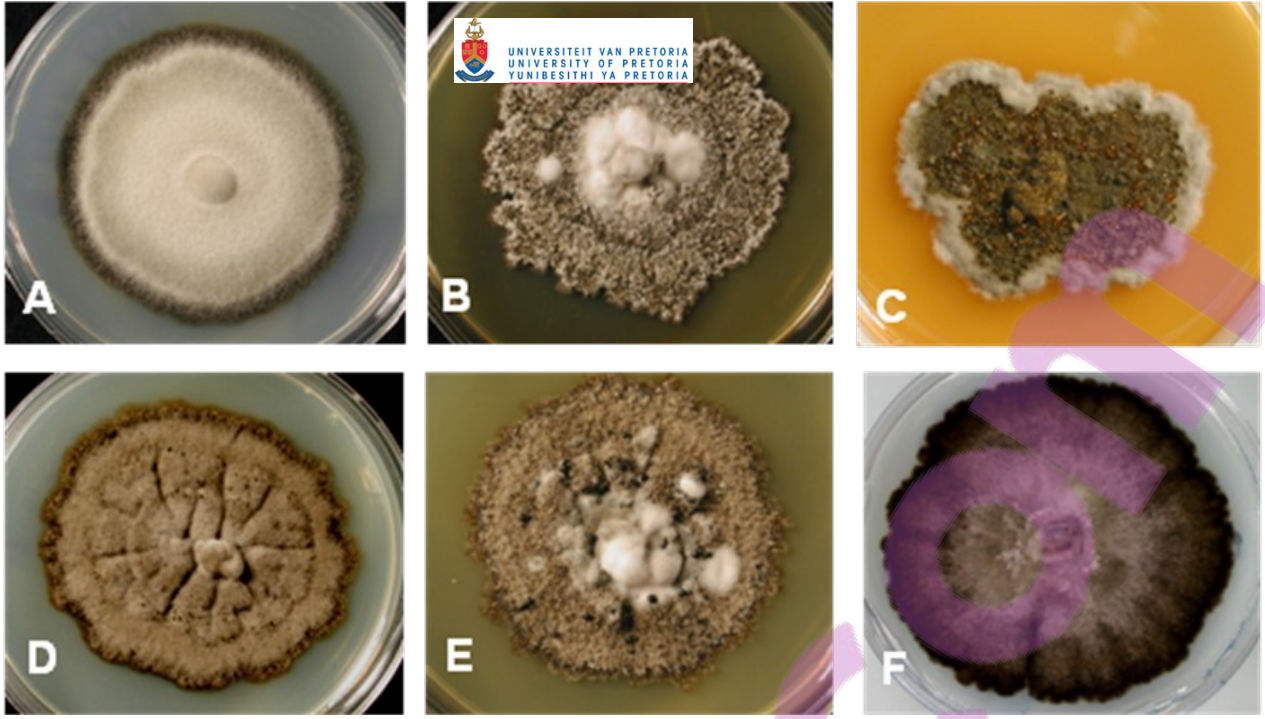

Fig 3 Characteristics of isolates of Group 1 (*C. zuluensis*), and isolates of Group 2 (*C. gauchensis*). Columns A–C show three different colony morphologies belonging to the Group 2 isolates: CMW 7272, CMW 7269, CMW 7293. Columns D–F show three different colony morphologies that belong to the Group 1 isolates: CMW 7488, CMW 5236, CMW 7479.

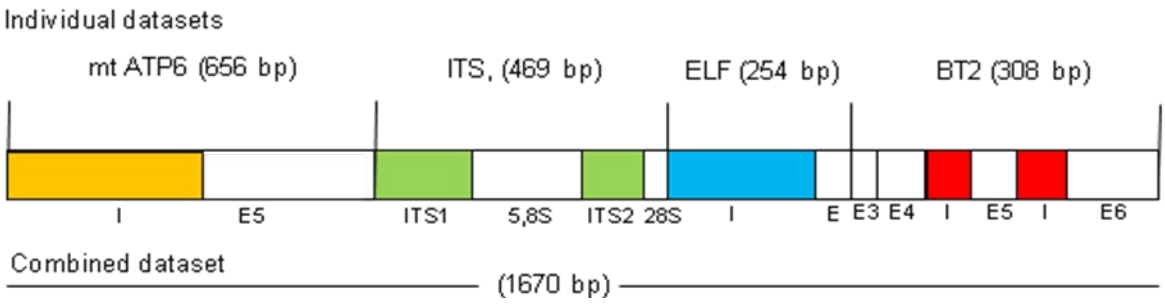

Fig 4 Schematic structural organization of the genomic regions used in this study. ITS regions and intron sequences are represented in solid black. Letters “I” indicate introns and letters “E” indicate exons. Sizes of the individual and combined partition alignments are given in brackets. Note that intron between E3 and E4 in the BT2 region is not present.

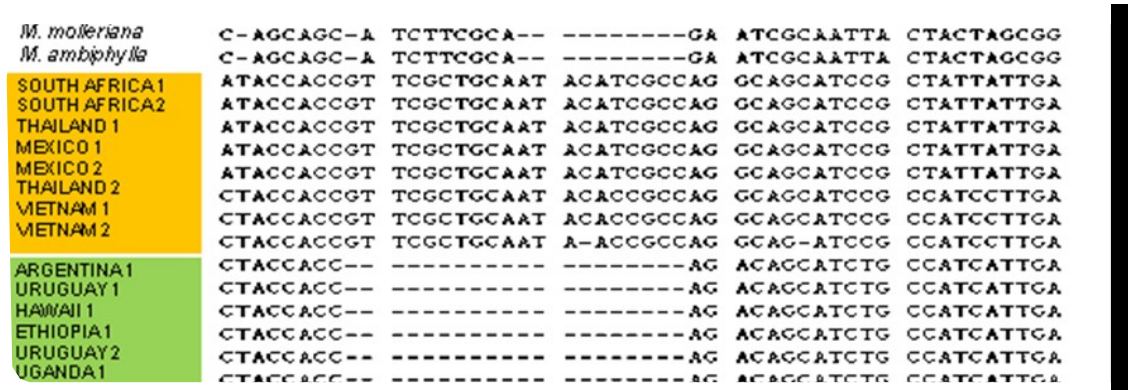

Fig 5

Partial alignment of isolates showing the characteristic 20 bp elongation factor 1-α in del. The presence of the in/del identifies the Group 1 isolates (light grey) from *Group 2* (dark grey) isolates. All isolates in Table 1 can be assigned correctly into Groups 1 or 2 according to the presence/absence of this fragment.

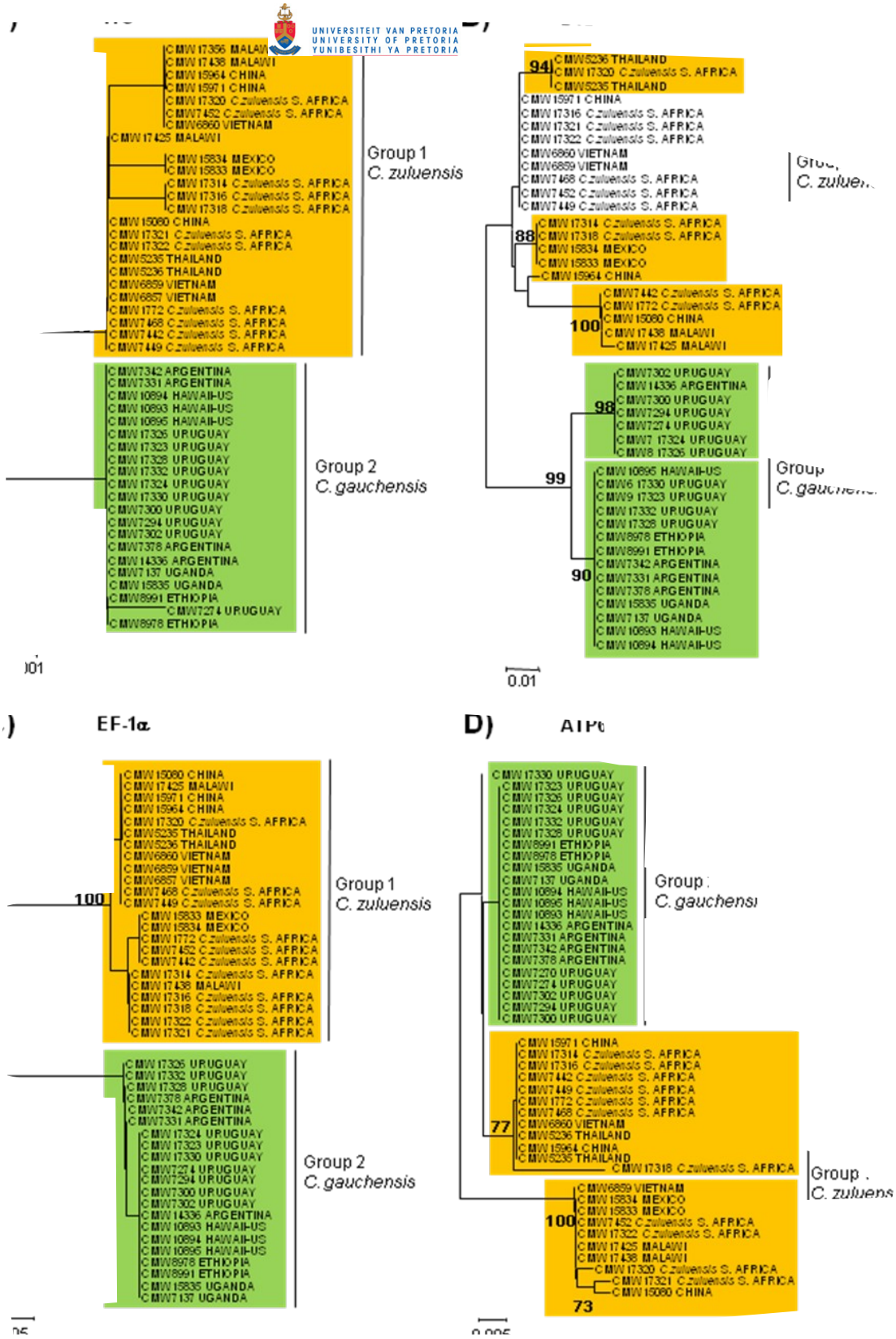

Fig 6 Phylograms generated using Minimum Evolution and K2P with gamma distribution, γ= 1. A. ITS. B. β-tubulin. C. EF1-α. D. ATP6. Values on branches are bootstrap support (1000 replicas).

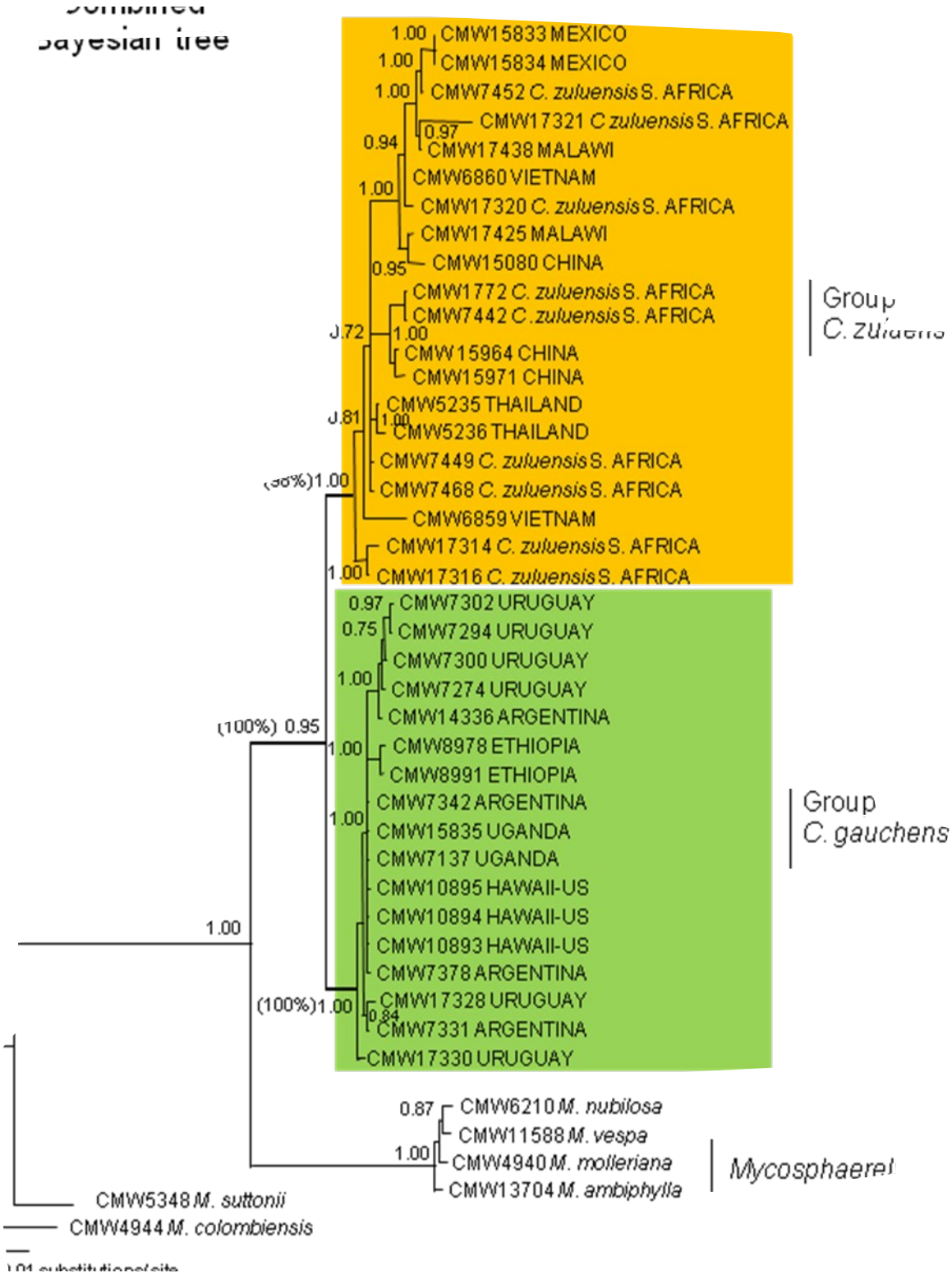

Fig 7 Bayesian Bayesian combined tree using a GTR+G+I model of substitutions. Posterior probabilities are shown on the branches. Parsimony bootstrap values are shown in brackets.

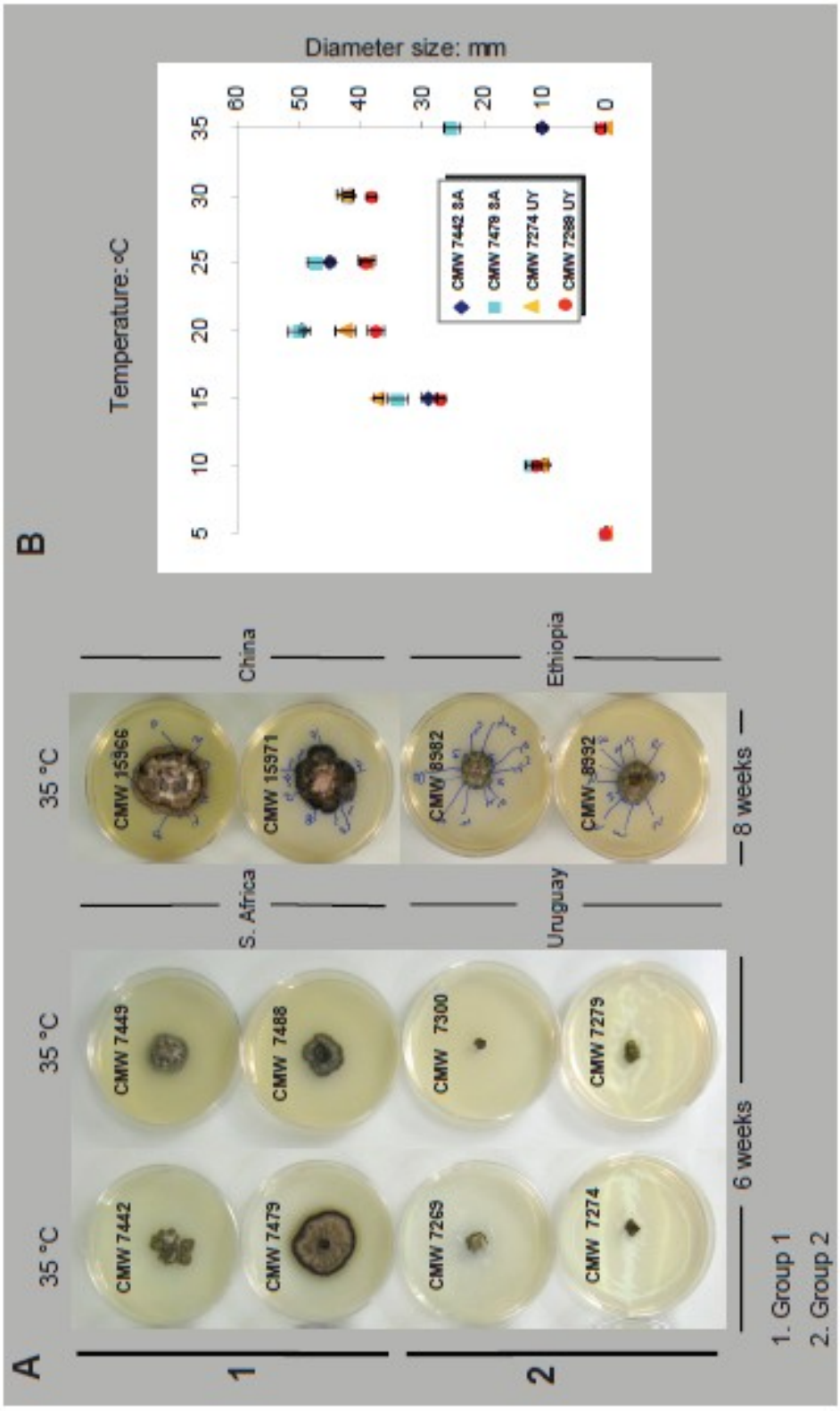

Fig 8 Results of culture growth studies at different temperatures. A. Isolates from South Africa and Uruguay were tested for a period of 6 weeks and those from China and Ethiopia for a period of 8 weeks. Each point on the graph represents the average of 6 measurements taken at each temperature.

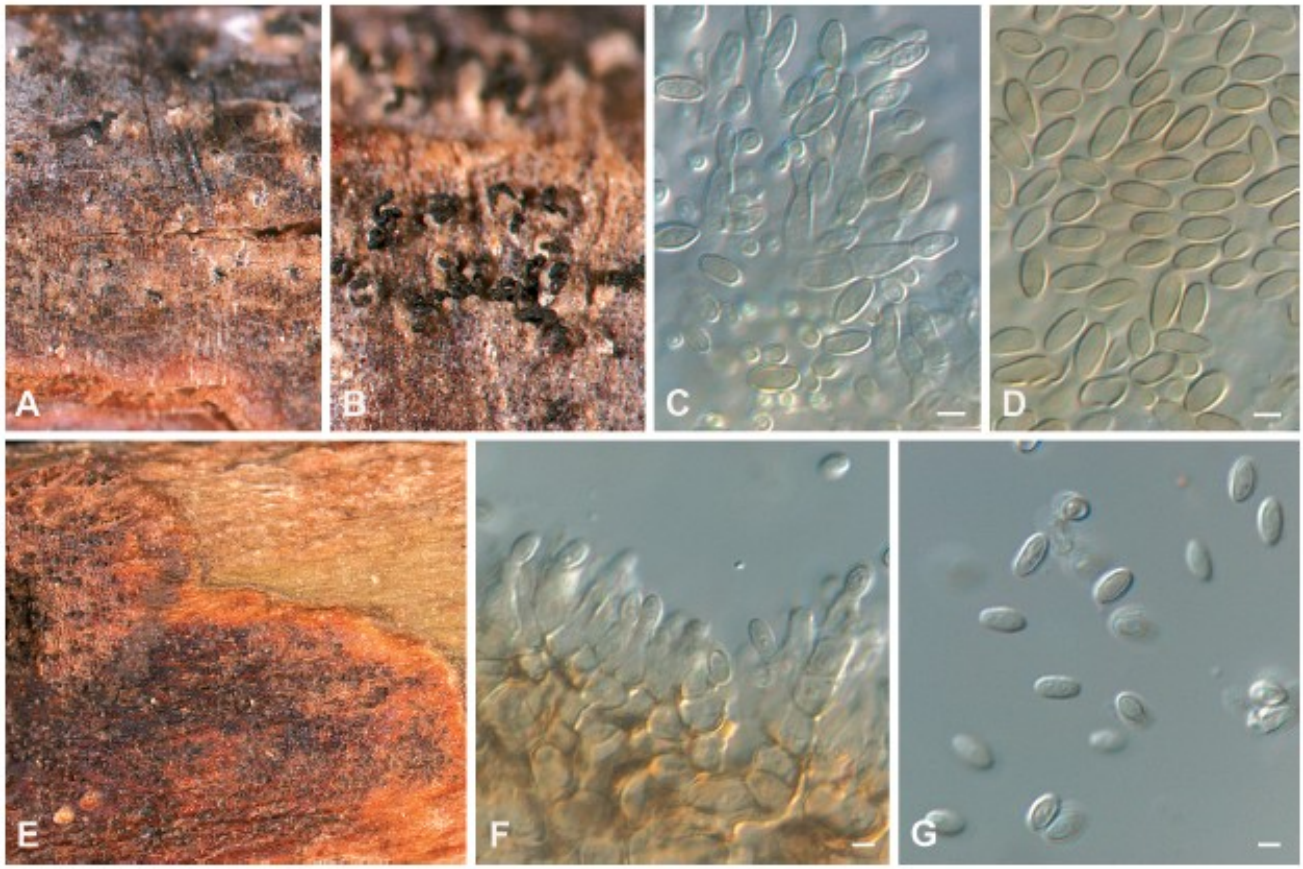

Fig 9 Colletogloeopsis spp. sporulating on *E. grandis* stems. A–D. *Colletogloeopsis gauchensis* (holotype). A–B. Pycnidia with black cirri. C. Conidiogenous cells. D. Conidia. E–G. *Colletogloeopsis zuluensis* (epitype). E. Pycnidia. F. Conidiogenous cells. G. Conidia. Scale bars = 2.5 µm.

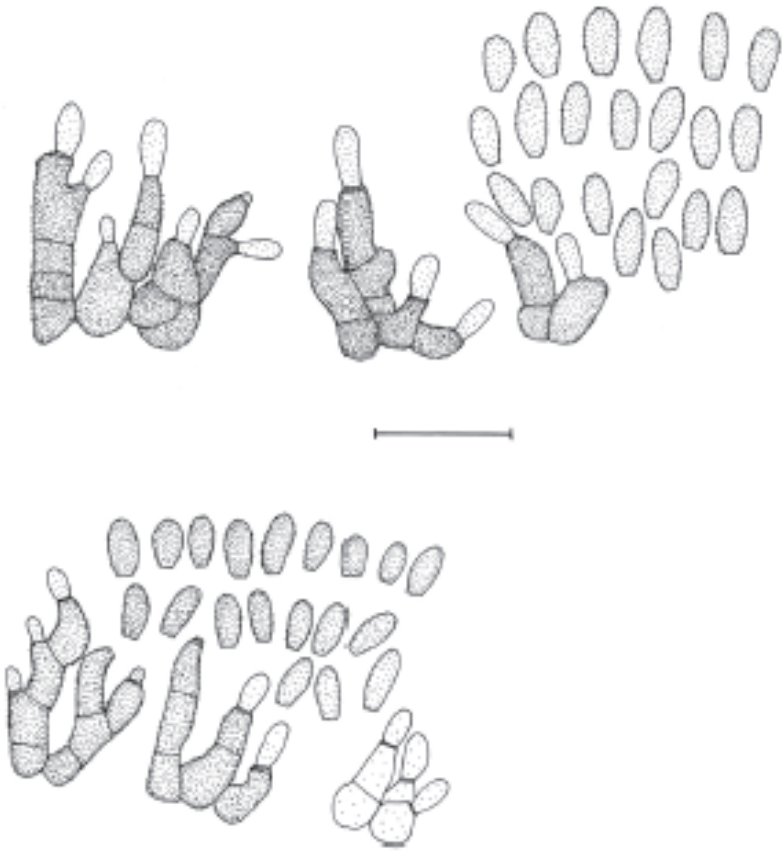

Fig 10 *Colletogloeopsis* spp. sporulating on *E. grandis* stems. Conidiogenous cells and conidia of *Colletogloeopsis gauchensis* (holotype) (top). Conidiogenous cells and conidia of *Colletogloeopsis zuluensis* (epitype) (bottom). Scale bar = 10 µm.

Chapter 4

Polymorphic microsatellite markers for the *Eucalyptus* fungal pathogen *Colletogloeopsis zuluensis*

Chapter 4

Polymorphic microsatellite markers for the *Eucalyptus* fungal pathogen *Colletogloeopsis zuluensis*

ABSTRACT

Nine polymorphic microsatellite markers for the phytopathogenic fungus *Colletogloeopsis zuluensis*, the causal agent of an important stem canker disease of *Eucalyptus*, were isolated and characterised. Two methods, (RAMS) and fast isolation by AFLP of sequence containing repeats with modifications (M-FIASCO) were used to isolate the microsatellites. Primers for 28 prospective microsatellite regions were designed and nine of these were polymorphic for *C. zuluensis*. Allelic diversity ranged from 0.12 to 0.80 with a total of 37 alleles. These markers will be used in future to determine the population genetic structure of *C. zuluensis* isolates and to monitor their global movement.

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INTRODUCTION

Colletogloeopsis zuluensise causes a serious stem canker disease on *Eucalyptus* (Wingfield *et al.,* 1997; Cortinas *et al.*). The fungus was first discovered in South African plantations and has subsequently been found in many other tropical and sub-tropical countries. *Colletogloepsis zuluensis* is an ascomycete closely related to *Mycosphaerella* Johansson, a genus of more than 800 species, approximately 60 of which have been identified as the causal agents of *Eucalyptus* leaf diseases (Crous *et al.,* 2004). Interestingly, this pathogen occurs only on stems of trees and never infects leaves. Sexual structures have never been reported and in contrast to many other *Mycosphaerella* spp., it has never been observed in the native range of *Eucalyptus*. The aim of this study was to isolate polymorphic microsatellite markers for *C. zuluensis* to be used in future studies considering the genetic structure, mode of reproduction and relationships among individuals emerging from disease outbreaks in many parts of the world.

MATERIALS AND METHODS

Two methods were used to screen for microsatellite sequences in *C. zuluensis*. Random Amplified Microsatellite Sequences (RAMS) (Hantula *et al.,* 1996) with anchored 3' primers (Zietkiewicz *et al.,* 1994) were used. PCR reactions using 45 combinations of anchored di-, tri- and tetranucleotide primers were then undertaken. Six banding patterns generated by PCR were cloned using the cloning kit PGEM T Easy (Promega). Sequences containing microsatellites were recovered by Genome Walking (Siebert *et al.,* 1995). The other method used was FIASCO (Zane *et al.,* 2002) with modifications (M-FIASCO) using the biotinylated probes (TC)15, (CA)15, (TCC)7, and (ATA)7. A detailed protocol of M-FIASCO can be found as Appendix II in this thesis.

Genomic DNA was extracted according to Cortinas *et al.,* (2004). A total of 1µg genomic DNA was pooled from isolates CMW1048 and CMW1026 from South Africa and CMW5236 from Thailand to screen for microsatellites. All *C. zuluensis* isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. Human DNA, was used in parallel with *C. zuluensis* DNA as a positive control.

Modifications of the FIASCO method included preparation of the digestion-ligation using *Mse*I restriction enzyme and a highly concentrated T4 DNA ligase (2000000 U/ ml) (Hamilton *et al.,* 1999). Both enzymes were acquired from New England BioLabs and the same buffer was used. Another modification included the addition of 10 µg of tRNA (Sigma), rather than unrelated PCR product, to the magnetic beads to minimize the non-specific binding of the genomic DNA before mixing with the hybridisation complexes (Zane, pers. com.). Furthermore, a number of A nucleotides, "A tailing", were extended at the 3'end of the PCR fragments immediately before cloning into the TOPO 4- TA Kit (Invitrogen), to increase the cloning efficiency. One µg of cleaned PCR product was mixed with 4 µL dATP (2 mM), 0.2 µL (1 U) *Taq* polymerase (Roche), 2.5 µL of 10x *Taq* polymerase buffer with $MgCl_2$ (500 mM Tris/HCl, 100 mM KCl, 50 mM $(NH4)_2SO_4$, 20 mM $MgCl_2$, pH 8.3) (Roche) and 10.3 µL distilled water. Fragments were incubated at 72 °C for 15 min in an iCycler PCR machine (Bio-Rad).

After cloning, colony-PCRs were carried out by diluting 5 µL of the cell culture suspension in 50 µL of distilled water. Dilutions were incubated for 7 min at 96 °C and 1 µL was used as template in the colony-PCR reactions together with M13 TOPO 4 primers (5'-GTAAAACGACGGCCAG-3'/ 5'-CAGGAAACAGCTATGAC-3') (Invitrogen) as described in Zane *et al.,* (2002). Three µL of PCR products, cleaned with Sephadex G-50 (Sigma), were used in 10 µL total sequencing reactions using Big Dye v3.1 (Applied Biosystems) and the previous TOPO 4 primers using the following thermal profile: 96 °C for 10 s, 56 °C for 30 s, and 60 °C for 4 min for a total of 25 cycles using an iCycler (Bio-Rad) PCR machine. The sequencing extension products were purified using the Ethanol/EDTA/Sodium Acetate precipitation protocol following the manufacturer's protocol. Electrophoresis was carried out on an ABI 3100 auto sequencer (Applied Biosystems).

Eight putative microsatellites were finally recovered after genome walking using RAMS and twenty putative microsatellites were obtained using M-FIASCO. Primers for these microsatellite regions were designed by eye and using Oligo Analyser 3 (Integrated DNA Technologies) available on the internet at http://www.idtdna.com/Home/Home.aspx, to adjust Tm, length and check for the

formation of hairpins, self-dimers and hetero-dimers. To test for polymorphisms, ten isolates were chosen to span a wide range of geographical origins of *C. zuluensis* from South Africa, Thailand and China. PCR using an iCycler (BioRad) were performed in 25 µL reactions containing 100 ng DNA template, 0.2 mM dNTPs (Promega), 0.15 µM of each primer, 0.2 µL Taq Polymerase (Roche), 1x buffer with $MgCl_2$ (50 mM Tris/HCl, 10 mM KCl, 5 mM $(NH4)_2SO_4$, 2 mM $MgCl_2$, pH 8.3) (Roche) and 18µL of distilled water under the following thermal conditions: 96°C 1min, 35 cycles of 94 °C for 30 s, annealing temperature according to Table 1 for 30 s, and extension at 72°C for 1 min. After PCR, products were run on 3 % agarose gels or sequenced to the detect polymorphisms. To sequence the amplicons, the specific designed primers were used using the same PCR sequencing conditions previously described. Two of the putative RAMS loci and seven of the M-FIASCO loci contained polymorphic microsatellites. The forward primers of the polymorphic loci were fluorescently labelled using NED, VIC, FAM or PET dyes for filter set G5 (Applied Biosystems) and tested on DNA from 30 additional isolates (CMW4518, CMW5236, CMW7411, CMW7420, CMW7425, CMW7426, CMW7435, CMW7438, CMW7440, CMW7442, CMW7443, CMW7447, CMW7459, CMW7460, CMW7463, CMW7470, CMW7491, CMW11239, CMW13324, CMW15833, CMW15963, CMW15970, CMW17315, CMW17317, CMW17320, CMW17322, CMW17404, CMW17406, CMW17476, CMW17477). The fragments were electrophoresed on an ABI 3100 auto sequencer (Applied Biosystems). Allele sizes for all the isolates were determined using ABI Genemapper, version 3.0 (Applied Biosystems) using LIZ 500 size standard. The allelic diversity of polymorphic alleles was evaluated according to Nei 1973. Linkage disequilibrium (LD) was calculated using MULTILOCUS 1.2 (Agapow & Burt 2001).

RESULTS AND DISCUSSION

The allelic diversity (Nei 1973) of nine polymorphic alleles ranged from 0.12 to 0.80 with a minimum of two, and a maximum of eight alleles per locus (Table 1). Thirty-seven alleles were observed across the nine loci. No Linkage disequilibrium (LD) was detected between any pair of loci. Cross-species amplification on *Mycosphaerella* spp. (*M. nubilosa*, *M. molleriana*, *M. vespa*, *M. ambiphylla*, *M. cryptica* and *M.*

suttonii) that are phylogenetically related to *C. zuluensis,* produced negative or non-specific amplifications for the nine polymorphic microsatellite loci. The results suggest the fact that these fungi have been reproductively isolated for a significant period of time.

The overall recovery efficiency of putative microsatellite loci, considering the total number of clones sequenced per method was 3.2 % using RAMS (250 clones) and 5.7 % with M-FIASCO (352 clones). In contrast, the human DNA control produced 68 % microsatellite sequences using M-FIASCO (100 clones). The nine polymorphic microsatellite markers developed in this study will now be used to consider the population genetic structure and the reproductive strategy of *C. zuluensis*. They will also be used to determine whether gene flow occurs among populations from different areas of occurrence of the pathogen.

ACKNOWLEDGEMENTS

We thank Dr. Lorenzo Zane for his valuable comments used to improve the original FIASCO protocol. We also acknowledge the National Research Foundation (NRF), members of the Tree Protection Co-operative Programme (TPCP), the THRIP Initiative of the Department of Trade and Industry, South Africa and the Mellon Foundation, for financial support.

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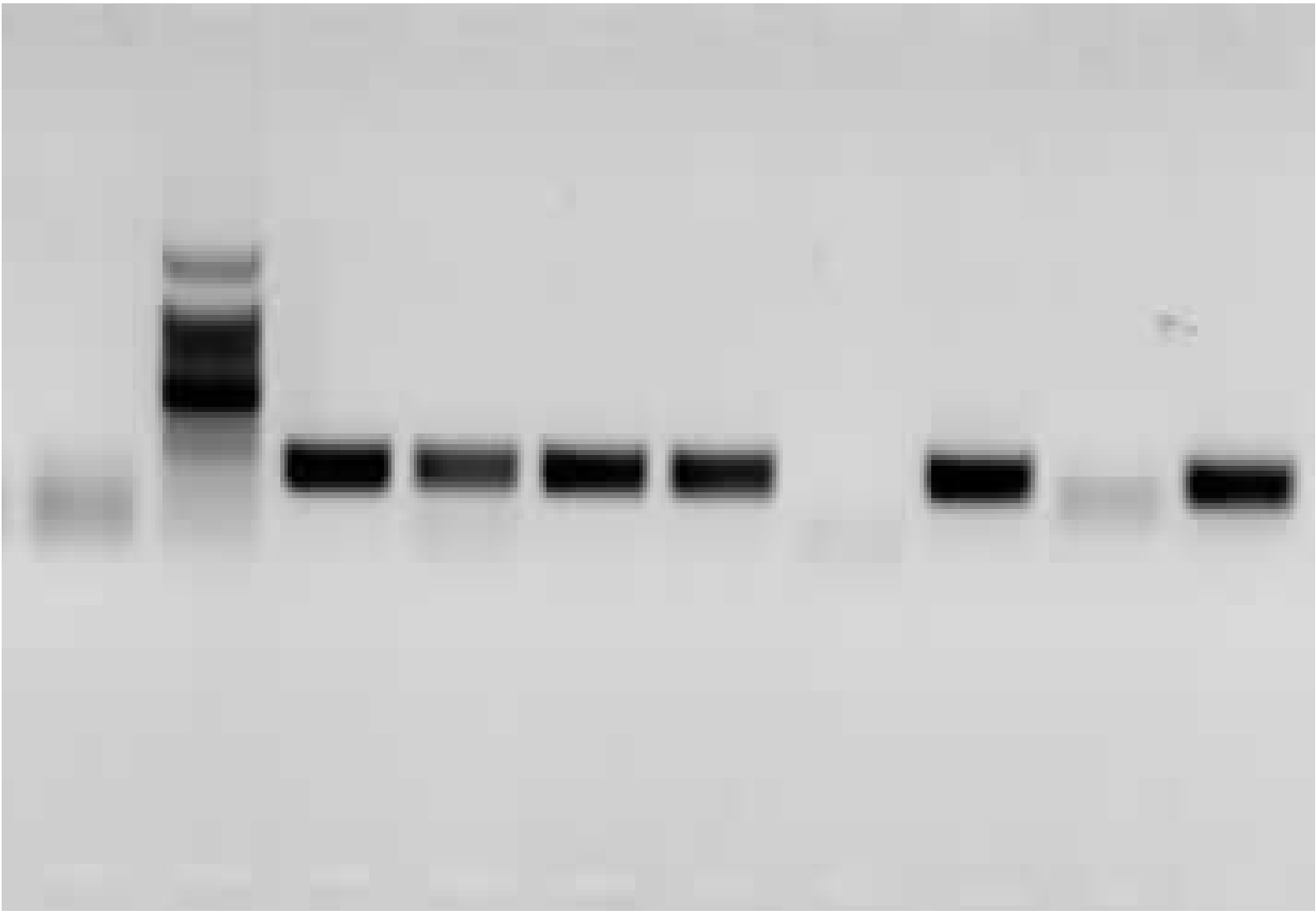
Table 1 Locus and primer names, GenBank Accession numbers, primer sequences, repeat motif, annealing temperature (*T*a), $MgCl_2$ concentration, number and size range of alleles and observed (*H*) allelic diversity (Nei 1973) of the nine polymorphic regions analysed in this study using 30 isolates of *Colletogloeopsis zuluensis*.

Locus name	Primer names	GenBank Accession no.	Primer sequences (5'-3')	Repeat motif	*T*a (^{0}C)	$MgCl_2$ (mM)	No. of alleles	Size range (bp)	Mean *H*	N°. of isolates tested
Czulu 1	Czulu 1F	DQ156110	PET – CTG ATG GCA ATG GGC GTG TGA C	(TG)8	58 °C	3.5	4	153-159	0.35	30
	Czulu 1R		GCC TCT TGC TCT GGC TGT AGG T							
Czulu 2	Czulu 2F	DQ156111	PET – AAG CAT GAA ACG GAC TCT GCG C	(TG)6	61 °C	3.5	4	185-188	0.69	30
	Czulu 2R		GAC GAG GGT GAT GGT CGT TGC							
Czulu 3	Czulu 3F	DQ156112	NED - GGA CAT TGA TTT CAC GCC GAC G	(TGG)9	58 °C	3.5	2	169-172	0.12	30
	Czulu 3F		CTG CAA CGA CAA ATC TCA ACC TG							
Czulu 4	Czulu 4F	DQ156113	FAM - GAC TTT GAC CAG CAT GTC GAC C	(TGG)5	62 °C	3.5	2	149-152	0.23	30
	Czulu 4R		GTG TGG AGG TGG GAA GTG GTG							
Czulu 5	Czulu 5F	DQ156114	FAM - GTT GTG TCC GAT CCT GCG AAG C	(CG)7(AG)21CA(AG)9	62 °C	2.0	7	174-196	0.80	30
	Czulu 5R		CAA GGG CGA AGT CGA GTA TGA GG							
Czulu 6	Czulu 6F	DQ156115	NED – CCA ACC CCA CCA TCA ACC TCA	(TCC)4… 125bp…(CAT)9	61 °C	3.5	5	322-339	0.48	30
	Czulu 6F		TAC CCC CTC CAA AGC TAA CCC							
Czulu7	Czulu 7F	DQ156116	NED – ACA ACC CAC TCC CTA CCC CGG	(ACCCC)6	65 °C	3.5	3	213-225	0.55	30
	Czulu 7R		AAT TGG GCT ATG CTG GTC ACT CG							
Czulu 8	Czulu 8F	DQ156117	VIC – AGC ACG CTG CAC GAG CAA CGG	(TCCC)6… 27bpTC-rich region…	65 °C	2.0	8	185-339	0.76	30
	Czulu 8R		TCG TTT GTG GGG GCC AGC GGC	(GTCTCCCTCTCT)8						
Czulu 9	Czulu 9F	DQ156118	PET - TTA GCC GTC TGG AGT GAA GAG G	(ACC)9 ATCACCACCGTT(ACT)14	58 °C	3.5	2	221-225	0.23	30
	Czulu 9R		GCT TTG TAA GCG CGG TAC GTG							

Chapter 5

Microsatellite markers for the E*ucalyptus* stem canker fungal pathogen *Kirramyces gauchensis*



Chapter 5

Microsatellite markers for the *Eucalyptus* stem canker fungal pathogen *Kirramyces gauchensis*

ABSTRACT

Ten microsatellite markers were developed for the fungus *Kirramyces gauchensis*, which causes an important stem canker disease of *Eucalyptus* trees in plantations. Primers for 21 microsatellite regions were designed from cloned fragments. Fourteen of the primer pairs provided single amplicons and 10 of these were polymorphic for *K gauchensis*. Allelic diversity ranged from 0.24 to 0.76 with a total of 30 alleles. None of the markers was able to amplify in the phylogenetically distinct but morphologically similar species *Kirramyces zuluensis.* The 10 characterized polymorphic microsatellite regions will be studied to determine the population structure of *K gauchensis* in plantations of different countries.

Published as: Cortinas MN, Wingfield BD, Wingfield MJ (2008). Microsatellite markers for the *Eucalyptus* stem canker fungal pathogen *Kirramyces gauchensis* *Molecular Ecology Resources* **8,** 590–592.

INTRODUCTION

Species of *Kirramyces* include important pathogens of *Eucalyptus* leaves, shoots and stems (Andjic *et al.,* 2007). *Kirramyces* (= *Colletogloeopsis*) *gauchensis* is the casual agent of a serious stem canker disease on *Eucalyptus* trees (Cortinas *et al.,* 2006b, c). This fungus is very similar to but phylogenetically distinct from *Kirramyces* (= *Colletogloeopsis*) *zuluensis,* which is also an important *Eucalyptus* stem canker pathogen. *Kirramyces gauchensis* has a wide geographic distribution and has been recorded on *E. grandis, E. tereticornis, E. camaldulensis* and different hybrids in plantations of South American and African countries as well as in Hawaii. The fungus has never been found in the native range of *Eucalyptus* or infecting trees of other genera. At present, the origin of this fungus is unknown. The mycelia of *Kirramyces gauchensis* is haploid as well as the anamorph reproductive structures found in nature, the pycnidias. Like in *K. zuluensis*, sexual or teleomorph reproductive structures have never been reported and is thus *K. gauchensis* is considered an anamorph genus of the teleomorph genus *Mycosphaerella*. However, other closely related species of *Kirramyces* have *Mycosphaerella* sexual states and phylogenetic inference suggests that the same could be true for *K. gauchensis* and *K. zuluensis*.

Microsatellite markers have been useful in understanding the population biology of many fungal pathogens (e.g. McDonald 1997; Zhan & McDonald 2004; Feau *et al.,* 2005). Initial studies on *K. gauchensis* were frustrated by the fact that microsatellite primers developed for *K. zuluensis* did not amplify any amplicons (Cortinas *et al.,* 2006c). However, this fact and multilocus phylogenetic analyses led to the discovery that isolates initially treated as a single species actually represented distinct taxa (Cortinas *et al.,* 2006c). The objective of this study was, therefore, to isolate and characterize microsatellite loci that can be used to study the population structure of *K. gauchensis,* collected from diseased trees in different countries.

MATERIALS AND METHODS

The microsatellite-containing regions were isolated using a modified form (Cortinas *et al.,* 2006a) of the FIASCO technique of Zane *et al.,* (2002). All isolates used in this

study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. To screen for repetitive sequences, 1 µg of genomic DNA was pooled from the isolates CMW 7474, CMW 7300, CMW 7279 of *K. gauchensis*. Genomic DNA was extracted from cultures using phenol-chloroform following the method described by Cortinas *et al.,* (2006b, c). The biotinylated probes (TC)15, (CA)15 and (GATA)6 were used to enrich the genomic DNA. All PCR's were carried out using an iCycler (Bio-Rad) using the thermal profiles described in Cortinas *et al.,* (2006a).

Of 384 sequenced clones, 21 contained repetitive regions. Primers for these 21 loci were designed visually. OLIGO Analyser 3 (Integrated DNA Technologies) was used to check the melting temperature (Tm), formation of hairpins, self-dimers and hetero-dimers. When the designed primer pairs were tested, 14 primer pairs resulted in single amplicons of the expected size range. One primer from each of the 14 primer pairs was labeled with fluorescent dyes using NED, VIC, FAM or PET dyes for filter set G5 (Applied Biosystems), to allow detection on an ABI 3100 sequencer. PCR amplifications were performed in 25 µL reactions containing 100 ng DNA template, 0.2 mM dNTPs (Promega), 0.15 µM of each primer, 0.2 µL (1U) *Taq* Polymerase (Roche), 1x buffer with $MgCl_2$ (50 mM Tris/HCl, 10 mM KCl, 5 mM $(NH4)_2SO_4$, 2,0 mM $MgCl_2$ or 3,5 mM $MgCl_2$, pH (8.3) (Roche) (Table 1) and 18.0 µL of distilled water. The thermo-cycling conditions were as follows: initial denaturation at 96 °C for 4 min, followed by 10 cycles of denaturation at 94°C for 30 s, annealing temperature according to Table 1 for 30 s, and extension at 72°C for 1 min, followed by 30 cycle repetitions of denaturation at 94°C for 30 s, annealing temperature according to Table 1 for 30 s and extension at 72°C for 1 min (with 5 s increments every 2 repetitions). A final extension was carried out at 72 °C for 45 min. Fragment size analysis was carried out after electrophoresis using the software GENEMAPPER, version 3.0 (Applied Biosystems) and LIZ 500 (-250) size standard (Applied Biosystems).

To assess the level of polymorphism, 21 isolates from Argentina (CMW4915, CMW14336, CMW14337, CMW14338, CMW14339, CMW14343, CMW14345, CMW14347, CMW14348, CMW14349, CMW14351, CMW7345, CMW14510,

CMW14510, CMW14511, CMW14512,CMW14515, CMW14516, CMW7342, CMW1458, CMW15835), and an equal number of isolates from Uruguay (CMW17561, CMW1495, CMW1501, CMW1502, CMW7270, CMW7272, CMW7275, CMW7276, CMW7277, CMW7278, CMW7281, CMW7282, CMW7287, CMW7290, CMW7292, CMW7293, CMW7298, CMW7299, CMW7305, CMW7306, CMW7309) were genotyped. Of the 14 designed primers pairs, 10 loci were polymorphic, two were monomorphic and two yielded complex stutter patterns that were difficult to interpret.

RESULTS AND DISCUSSION

The allelic diversity (Nei 1973) of the 10 polymorphic loci ranged from 0.21 to 0.76 with a minimum of two, and a maximum of six alleles per locus (Table 1). Thirty alleles were found across the 10 loci. Linkage disequilibrium (LD) was calculated using MULTILOCUS 1.2 (Agapow & Burt 2001). Significant LD ($P< 0.05$) was detected for some loci pair comparisons (data not shown), suggesting little evidence for recombination. This indicates that clonal reproduction can be playing an important role in the reproductive structure of this species. Confirmation of this result will be needed with comprehensive population studies. Cross-species amplification between *K. gauchensis* and the closely related *K. zuluensis* (25 isolates) produced negative, incorrect size bands or smeared amplifications, suggesting that the two species no longer share these loci. These primers also failed to amplify amplicons when tested as diagnostic markers on two other related species, *M. nubilosa* and *M. molleriana.*The primers are thus not only useful as population markers but also have the potential to be used as species-specific markers to identify *K. gauchensis* in the development of a DNA-based identification technique.

In this study, 10 microsatellite loci have been characterized and shown to be specific for *K. gauchensis.* These markers can now be applied to populations of the pathogen from different parts of the world, as part of an effort to understand its global diversity and population biology. Such studies will hopefully also enhance efforts to reduce the impact of the pathogen on *Eucalyptus* forestry.

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Table 1 Locus and primer names, GenBank Accession numbers, primer sequences, repeat motif, annealing temperature (*Ta*), $MgCl_2$ concentration, number and size range of alleles and observed (*H*) allelic diversity (Nei 1973) of the ten polymorphic loci analysed in this study using 42 isolates of *Kirramyces gauchensis*.

Locus name	Primer names	GenBank Accession no.	Primer sequences (5'-3')	Repeat motif	*Ta*	$MgCl_2$ (mM)	No. of alleles	Size range (bp)	Mean *H*	No. of isolates tested
K. gauchensis 1	Kgauche 1F	DQ975190	NED - CTC CAT TGC ATC GGG TCT CAT G	(AG)24	59 °C	3.5	6	290-	0.76	42
	Kgauche 1R		GGT GGC AAG TTC GAG CTT CA					327		
K. gauchensis 2	Kgauche 2F	DQ975191	PET - CAA ATC CTC GGC TGC GTC ATG G	(GA)4 TA(GA)4	54 °C	3.5	3	148-	0.50	42
	Kgauche 2R		CAC TGC GCT TTC GTC TCT ACC GA					183		
K.gauchensis 3	Kgauche 3F	DQ975192	NED - AGA TGG CTG TAC GAA GAA TGT CC	(CT)7 AC(TC)17	60 °C	3.5	3	211-	0.42	42
	Kgauche 3F		AAG CCA ATC CAC GCG TCA AGG	TTTCT(GT)12				266		
K.gauchensis 4	Kgauche 4F	DQ975193	VIC - CCG CGA GAG AAA CAA CAT CC	(GA)10	59 °C	3.5	2	251-	0.24	42
	Kgauche 4R		GAT AGG AGG CAC ATA ACC CAA G					260		
K.gauchensis 5	Kgauche 5F	DQ975194	FAM - TTG GCC AGC AGG AAC ATG AGC	(GTGGT)GGT(GTGGT)	62 °C	2.0	2	288-	0.43	42
	Kgauche 5R		CAC TCA TTC ACT TGA CCG CCT C	3 (GGT)2(GTGGT)2				294		
K.gauchensis 6	Kgauche 6F	DQ975195	FAM - CGC CTT ATG CCT TTG ATG GTT GC	(GT)15	56 °C	3.5	4	165-	0.43	42
	Kgauche 6F		GAT TCC TAA ATC GAC CAT CCG C					203		
K.gauchensis7	Kgauche 7F	DQ975196	VIC - ACC AGG GAT GCC GTA TGT GCA G	(TG)9	60 °C	3.5	2	107-	0.46	42
	Kgauche 7R		CAT CAC ACA CCG TCC TCC CAC					109		
K.gauchensis 8	Kgauche 8F	DQ975197	PET - ATC ATC TGC CCT TGG ACG GAC G	(TG)9	59 °C	2.0	3	134-	0.21	42
	Kgauche 8R		CCA TCA CCA CAC GAA ACA TCA AG					150		
K.gauchensis 9	Kgauche 9F	DQ975198	FAM - GAT CAC GCA ATG AGA GTG TCT CC	(ACAG)5	54 °C	3.5	2	89-98	0.52	42

	Kgauche 9R		GGT TTC CGA CTG ATT GGT TCA TC								
K.gauchensis 10	Kgauche 10F	DQ975199	PET – ATA GTA AGA AGA TAA ATA AGG CG	(AAG)53	52°C	3.5	3	134-	0.40	42
	Kgauche 10R		GCG AAG TAG ACT ATA TAA GTA TC					143		

Chapter 6

Genetic diversity in the *Eucalyptus* stem pathogen *Teratosphaeria zuluensis*

Chapter 6

Genetic diversity in the *Eucalyptus* stem pathogen *Teratosphaeria zuluensis*

ABSTRACT

Coniothyrium canker caused by the fungal pathogen *Teratosphaeria (= Coniothyrium) zuluensis* is one of the most important diseases affecting plantation-grown *Eucalyptus* trees. Little is known regarding the pathogen and this study consequently considers the genetic diversity and population structure of *T. zuluensis*. Eleven microsatellites markers, of which six were developed in this study, were used to analyze two temporally-separated populations of *T. zuluensis* from South Africa, one population from Malawi and a population from China. Results showed that the populations of *T. zuluensis* have a moderate to high diversity and that clonal reproduction is predominant. There was also evidence that the genetic diversity of the pathogen in South Africa has increased over time. Comparison of *T. zuluensis* populations from South Africa, Malawi and China suggest that South Africa is most probably not the centre of origin of the pathogen as has previously been suggested.

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INTRODUCTION

Numerous new diseases have emerged in plantations of non-native *Eucalyptus* spp. during the course of the past three decades (Wingfield *et al.,* 2008). This largely coincides with global expansion of *Eucalyptus* plantations in the tropics and sub-tropics (Park *et al.,* 2000; Old *et al.,* 2003; Wingfield 2003). Amongst the most important of these new diseases is Coniothyrium canker (Wingfield *et al.,* 1997; Old *et al.,* 2003), which first appears as small necrotic spots on the young green bark of *Eucalyptus* trees. These can subsequently develop into large girdling stem cankers and in some cases cause tree death (Wingfield *et al.,* 1997; Van Zyl *et al.,* 2002a). The disease has spread rapidly in South Africa, and for a period of about ten years, seriously threatened the rapidly expanding clonal *Eucalyptus* plantations, particularly in the Zululand forestry area.

Coniothyrium canker was first discovered in plantations of *Eucalyptus grandis* in the Zululand forestry area of South Africa in 1991 and the causal agent was described as the new species, *Coniothyrium zuluense* (Wingfield *et al.,* 1997). Consistent with the complex taxonomy of *Coniothryium* that has limited and confusing morphological characteristics, this fungus has undergone various name changes. It was consequently transferred to *Colletogloeopsis* as *Colletogloeopsis zuluensis* (Cortinas *et al.,* 2006) and has more recently treated as *Kirramyces zuluensis* (Andjic *et al.,* 2007) and *Rederiella zuluensis* (Crous *et al.,* 2007). Based on phylogenetic inference, the pathogen was recognised as related to *Mycosphaerella* (Gezahgne *et al.,* 2005; Cortinas *et al.,* 2006) and it is now acknowledged as a member of the Teratosphaeriaceae (Crous *et al.,* 2007) and treated as *T. zuluensis* favouring the sexual (teleomorph) genus *Teratosphaeria* (Crous *et al.,* 2009). Assuming that a decision is made to recognise the value of anamorph characters in the Teratosphaeriaceae and where a single name is used for these, a revision of the taxonomy of this group will most likely favour the name *Colletogloeopsis zuluensis* for the Coniothyrium canker pathogen (unpublished data). However, for the present, the name *Teratosphaeria zuluensis* is most appropriate and it is consequently applied in this manuscript.

Based on DNA comparisons for multiple gene regions, two distinct species, *K. zuluensis* and *K. gauchensis* have been found to cause Coniothyrium canker in

different parts of the world (Cortinas *et al.,* 2006c). *Teratosphaeria zuluensis* occurs in South America, Africa and South-east Asia and has been reported from Thailand (Van Zyl *et al.,* 2002b), Mexico (Roux *et al.,* 2002), Vietnam (Gezahgne *et al.,* 2003; Old *et al.,* 2003), China (Cortinas *et al.,* 2006b) and Malawi (Roux *et al.,* 2005, Cortinas *et al*., 2006c). *Teratosphaeria gauchensis* occurs in South America and Africa and has been reported from Argentina (Gezahgne *et al.,* 2004) and Uruguay, (Cortinas *et al.,* 2006c), Ethiopia and Uganda (Gezahgne *et al.,* 2003, 2005). In contrast to *T. zuluensis*, *T. gauchensis* has never been reported from South-east Asian countries.

Coniothyrium canker caused by *T. zuluense* appeared unexpectedly and spread rapidly in South Africa, initially on a single highly productive *E. grandis* clone. The fact that the disease was first observed in South Africa and that it was unknown elsewhere in the world, led to the suggestion that that the pathogen might be native in the country, possibly having undergone a host shift (Slippers *et al.,* 2005) from native Myrtaceae. An origin on a native South African host and undergoing a subsequent host jump (Slippers *et al.,* 2005) would be similar to that reported for the *Eucalyptus* canker pathogen *Chrysoporthe austroafricana* in Southern Africa (Wingfield 2003; Gryzenhout *et al.,* 2004; Heath *et al.,* 2006; Nakabonge *et al.,* 2006).

The fact that *T. zuluensis* has not yet been observed in the native range of *Eucalyptus* lends support to the host jump hypothesis. However, the close phylogenetic relationship between these canker pathogens and other important leaf pathogens of *Eucalyptus* (Park & Keane, 1982; Carnegie *et al.,* 1998; Hunter *et al.,* 2004, Andjic *et al.,* 2007) which are known to occur in Australia, suggests that *T. zuluensis* is most likely a *Eucalyptus* pathogen that has yet to be discovered in its native range.

Almost nothing is known regarding the biology or genetics of *T. zuluensis*. In nature, asexual pycnidia (Wingfield *et al.,* 1997) are found on lesions on the young green bark and they produce large numbers of asexual mitospores. Sexual reproductive structures have never been observed (Wingfield *et al,* 1997; Cortinas *et al.,* 2006b). This suggests that the fungus is a haploid organism that reproduces

clonally, mainly as a result of mitotic events (Wingfield *et al.,* 1997; Crous 1998; Crous *et al.,* 2004; 2006).

The objective of this study was to consider the genetic structure of a population of *T. zuluensis* and thus to provide some support to tree breeders concerned about the durability of resistance in planting stock. Two temporally -separated populations from South Africa, and smaller available populations of isolates from Malawi and China, were analyzed using eleven microsatellite markers (Cortinas *et al.,* 2006a), of which six were developed in this study. More specifically, the aims were to i) determine whether there has been a change in the genetic variation between isolates sampled during 1997 and 2005 in South Africa, ii) determine whether the South African populations have a high diversity relative to populations from other countries supporting the hypothesis that South Africa might have been a source of *T. zuluensis* to those countries and iii) consider the genetic structure and distribution of variation within populations.

MATERIALS AND METHODS

Sampling and isolation

Isolates of *T. zuluensis* were obtained from cankers on the stems of severely infected *E. grandis* trees, from different localities (Table 1) including those in South Africa, Malawi and China. One population of isolates from South Africa was collected during the initial outbreak of the disease in 1997. Almost all susceptible trees were replaced in South African plantations subsequent to the outbreak of this disease. A second population of isolates was collected approximately nine years later (end of 2005) in remnant plantations of a highly susceptible *E. grandis* clone.

For the South African collections, a hierarchical sampling strategy was used. Infected bark pieces were collected from a single diseased tree at the centre of a plantation selected as the central point for the collection. Samples were taken only from diseased branches showing cankers at approximately 2 m above the ground. This was done as a precaution to avoid possible height differences in the distribution of haplotypes. Additional samples were taken from randomly chosen trees following transects, extending outwards from the central tree. Samples

collected from Malawi and China were from single *E. grandis* trees randomly collected during routine disease sampling.

Single conidial isolates were generated from the bark samples as described previously (Van Zyl *et al.,* 1997; Cortinas *et al.,* 2006b; 2006c). Cultures obtained from the samples were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction

Isolates were grown on 2% malt extract agar (MEA) plates, for 30 days at 25°C (5 to 6 cm diameter). Mycelium from these actively growing single-conidial cultures was scraped from surface of the agar in the Petri dishes. The fungal material was freeze-dried, immersed in liquid nitrogen until frozen and ground to a fine powder. DNA was extracted using a phenol-chloroform method described by Cortinas *et al.,* (2006b).

Polymorphic microsatellite loci

Eleven polymorphic loci for all samples were amplified using five pairs of fluorescently labelled primers designed previously (Cortinas *et al.,* 2006a) and an additional six primer pairs developed as part of this study (Table 2). The additional primers were developed and characterized using the same methods as described by Cortinas *et al.,* (2006a). Amplicons obtained by PCR were size separated on an ABI 3100 Automated DNA Sequencer (Applied Biosystems, Foster City, USA) together with the internal size standard GENSCAN LIZ 500 (-250) (Applied Biosystems). Fragment size analysis was carried out using the software GENEMAPPER, version 3.0 (Applied Biosystems). Different alleles at each locus were identified based on the size of each amplicon and each allele was given an alphabetical designation. Multilocus haplotypes were generated by using the letters assigned to each isolate across the eleven loci. Isolates with the same haplotype were considered to be clones.

In order to check whether increasing the number of loci would modify the values of genotypic diversity, a plot of Mean Genotypic Diversity against the number of loci was performed using MULTILOCUS 1.3 (Agapow & Burt 2001). The

program samples randomly from 1 to m-1 loci (m= number of loci) from the dataset and calculates the number of different genotypes and the genotypic diversity.

Population genetic analysis

Genetic diversity, richness and evenness

Gene diversity was calculated in POPGENE using the algorithm (*H*) of Nei (1973) (Yeh *et al.,* 1999). Genotypic diversity (*G*) was calculated using Stoddart & Taylor (1988) and different sample sizes were compensated for by calculating the maximum percentage of genotypic diversity as G/N x 100. A *t*- test was used to determine whether the genotypic diversities of the populations were significantly different from each other (Chen *et al.,* 1994).

GENCLONE 2.0 (Arnaud-Haond & Belknir 2007; Rozenfeld *et al.,* 2007) was used to describe the clonal diversity of the populations in terms of richness, evenness and heterogeneity. This program was specially developed to deal with clonal organisms and uses a 'round-robin' method to calculate the allelic frequencies in order to avoid the overestimation of the low frequencies alleles. The Shannon -Weiner index for calculating richness and the corresponding evenness index (V') were used and Pareto distributions (richness and evenness integrated) were constructed to calculate heterogeneity.

The Shannon index is 0 for populations with only a single haplotype and increases in populations with many different haplotypes. For evenness (V), values between 0 and 1 are expected. The clonal evenness is used to describe the equal distribution of sampling units (haplotypes). The log-log transformation of the Pareto distribution gives an integrated representation of both richness and evenness (heterogeneity). The parameter □ calculated by regression (r^2) (the -1 x regression slope) from the Pareto distribution, increases exponentially with increasing evenness.

Population differentiation and assignment tests

Differences in allele frequencies between populations of *T. zuluensis* were calculated from clone corrected datasets using POPGENE. The significance of

differences in allelic frequencies between populations across the eleven loci was tested using Chi square tests (Workman & Niswander 1970).

The differentiation among populations was measured as theta (θ) (Weir 1996), which is a modification of F_{ST} (Wright 1978). Theta (θ) values were calculated using MULTILOCUS, version 1.3 (Agapow & Burt 2001) using the equation $\theta = Q\text{-}q / 1\text{-}q$, where Q is the probability that two alleles from the same population are the same and q is the probability that two alleles from differing populations are the same. For multiple loci, Q and q are summed across the evaluated loci. The significance of θ was evaluated by comparing the observed value to that of 1000 randomizations in which individuals were randomized across populations.

STRUCTURE version 2.2 (Pritchard *et al.,* 2000; Falush *et al.,* 2003) was used to carry out the assignment of individuals into 'K' number of clusters/populations based on their allelic frequencies for the South African, China and Malawi populations. The analyses were carried out in two steps. An initial analysis was carried out to determine the optimal 'K' using an admixture ancestry model and an independent allele frequency model. A hundred thousand runs were carried out (burn-in set at 10 000 runs) with 10 iterations. The analysis was repeated for the most likely K obtained using 1 000 000 runs (burn-in was set at 100 000). In both cases, the likelihood values were plotted against the delta likelihood values to determine the K with lower standard deviation and higher likelihood (Evanno *et al.,* 2005).

Recombination analyses

The random association of alleles was tested by calculating linkage disequilibrium (LD) for all pairs of loci and as a multilocus measure using the Index of Association (I_A), both implemented in MULTILOCUS version 1.3 (Maynard Smith *et al.,* 1993; Agapow & Burt 2001). The LD for all pairs of loci and I_A values were determined for all populations using clone corrected data matrices. The significance of the LD for all pairs of loci and the I_A observed values were determined by comparing the observed values with that of a distribution of a randomly mating population using 1000 randomizations of the allelic frequencies.

Analyses of clonal structure in the temporally-separated South African populations

Pairwise genetic differences among individuals were studied using GENCLONE 2.0 (Arnaud-Haond & Belknir 2007; Rozenfeld *et al.,* 2007) between the two temporally separated South African populations (SA 1997 and SA 2005) to determine clonal lineages that might constitute clusters of slightly different multilocus haplotypes, possibly derived from an original individual by mutation. The program makes use of microsatellite motif length differences to calculate a genetic distance index using a stepwise model of mutation and builds a histogram showing the distribution of pairwise genetic distances. The genetic distance index matrix generated for the two South African populations (SA 1997 and SA 2005) was imported into MEGA 4 (Tamura *et al.,* 2007) to perform cluster analyses using the unweighted pair group method with arithmetic mean (UPGMA). Furthermore, P_{gen}, the likelihood that two individuals with the same multilocus genotypes are the same clone and P_{sex}, the likelihood that individuals sharing the same multilocus genotype were derived from a distinct sexual reproductive event, were calculated using GENECLONE 2.0.

An examination was made as to whether pairs of individuals in the SA1997 and SA2005 populations that were separated by a defined spatial-interval, were more similar, or dissimilar to that expected from pairs of individuals that were randomly associated using spatial autocorrelation analysis as implemented in GENCLONE 2.0. A grid was superimposed on the localities sampled within South Africa (Table 7, Fig 2). Geographical x and y coordinates were assigned to the South African localities for both the SA1997 and SA2005 populations according to the position on the grid (Table 7). The Ritland (1996) co-ancestry coefficients (Fij) (the average genetic distance between pairs of individuals) were calculated using GENCLONE 2.0. Six distances classes were arbitrarily chosen and the grouping of the isolates determined.

RESULTS

Isolates

A total 248 isolates of *T. zuluensis* were obtained from the isolations from trees in South Africa. Of these, 75 were from the 1997 and 110 were from the 2005 South

African collections. From the single plantation in Malawi, 41 isolates were collected and 22 isolates were obtained from a single plantation in China (Table 1).

Polymorphic microsatellite loci

From the collection of 248 isolates of *T. zuluensis,* the 11 species-specific polymorphic microsatellite markers amplified a total of 68 different alleles (Table 3). In the two South African populations, 41 and 50 different alleles were observed for the 1997 and 2005 populations respectively. Forty five alleles were found for the Malawian isolates and 42 alleles were detected in the Chinese collection of isolates. The number of alleles per individual locus ranged from three to 14. Private alleles were observed in all populations. In total, 18 private alleles were identified, of which four were detected in the SA1997 population and nine in the SA population collected in 2005. Three private alleles were found in the Malawian population and two were observed in the Chinese collection of isolates. The majority of private alleles showed frequencies ranging from 3.5% to 10%. No monomorphic loci were detected in the South African populations although the locus Czulu3 (Table 3) was monomorphic in the Chinese and Malawian populations.

Genetic analysis of populations from South Africa, Malawi and China

Genetic diversity, richness and evenness

The plot of mean genotypic diversity against the number of loci constructed using MULTILOCUS 1.3 showed that a plateau of genotypic diversity was reached using the set of 11 microsatellite markers developed for *T. zuluensis* (data not shown). This provided statistical support for the assumption that the total diversity of the populations had been adequately sampled.

The levels of gene diversity in the *T. zuluensis* populations were moderate. Values were H= 0.51 for Malawi, H= 0.53 for China, H= 0.44 for SA1997 and H= 0.51 for SA 2005 (Table 3). One hundred and eighty-eight genotypes were identified across all the *T. zuluensis* isolates. The levels of clonality within populations ranged from 0% in China to 43% in SA1997.

The maximum genotypic diversity ranged from a minimum of $\hat{G}$ = 24% for the SA1997 population to a maximum value of $\hat{G}$ =100% for the Chinese population.

The populations from Malawi ($\hat{G}$ = 84%), and SA2005 ($\hat{G}$ = 43%) showed intermediate values (Table 3). No significant difference (P < 0.05) in genotypic diversities was found between the populations from South Africa and Malawi. Only one genotype was shared between the populations studied and this was for the South African population sampled in 1997 and 2005.

The relative richness, evenness and heterogeneity (richness and evenness integrated) gave Shannon–Weiner index values ranging from 3.09 for China and 4.35 for SA2005. The corresponding evenness index, V', ranged from 0.90 for SA1997 to 0.99 for China (Table 3). These values indicated moderate to high heterogeneity for all the populations in the study with the SA2005 population having the highest level of heterogeneity, and groups of clones within populations, had a similar size. The evenness index was also high for all the populations. The highest level of evenness was observed in the Chinese and Malawian populations.

The Pareto distributions determined for SA1997 and SA2005 showed good regression fits. The slopes of the regression lines were different for both populations (r^2=9.99, p<0.0001 in SA1997; r^2=0.97, p<0.0001 in SA2005). They suggested high diversity and low heterogeneity (low dominance of haplotypes relative to other haplotypes within populations). Nevertheless, the slope obtained for the SA1997 population was shallower (1,658) than that determined for the SA2005 (2,779) population, indicating lower heterogeneity among the haplotypes obtained in 1997 than those in 2005. It was not possible to calculate the Pareto distribution and the associated parameters for the China and Malawi populations. This was due to the haplotypes in both populations having approximately the same number of replicates (maximum evenness) which would not produce sufficient pairwise point comparisons between haplotypes to calculate the parameter β by regression (Arnaud-Haond *et al.,* 2007; Rozenfeld *et al.,* 2007).

Population differentiation and assignment tests

Significant differences were found between loci for the clone-corrected populations in the majority of the pairwise comparisons, including the two temporally-separated South Africans populations (Table 4). These results suggest that the RSA populations belong to different gene pools.

For the theta (θ) calculations, only the Malawian population showed significant Chi-square values ($P < 0.05$) when compared with other populations (Table 5). The differentiation between the SA populations ($\theta = 0.10$) was the smallest. The largest differentiation was observed between Malawi and the SA2005 ($\theta = 0.18$) populations.

The assignment tests indicated that the number of groups obtained with the highest likelihood and lowest standard deviation was K= 5.The majority of isolates from SA2005 were assigned to G1 (Fig 1). Groups G2 and G3 also consisted of mainly South African isolates while G4 and G5 were assigned the majority of isolates from China and Malawi respectively.

Recombination analyses

Pairwise comparisons between loci detected linkage disequilibrium (LD) in the populations of *T. zuluensis* (Table 6). The values were moderate with a maximum in the SA2005 population where almost half of the loci were in LD (values ranging from 8/49 to 21/49). The multilocus Index of Association (I_A) results were comparable to the LD results obtained by the pairwise analyses (Table 6). Significant departures from gametic equilibrium were detected for all populations (0.41 to 0.75) except China (0.17). The observed values of I_A for all the populations except the China population was significantly different to the value expected from a randomised distribution of allelic frequencies, suggesting that recombination has occurred only in the China population. This is also the only population that showed 100% genotypic diversity.

Analyses of clonal structure in the temporally-separated South African populations

Differences were found in the distribution of haplotypes between the temporally-separated South African populations SA1997 and SA2005. Using GENCLONE, 43 different haplotypes were identified in the SA1997 population, 12 of which were repeated in the population (replicates of the same haplotype). These identical haplotypes formed clusters containing two to 12 replicates each (Fig 3). In contrast, 86 different haplotypes were identified in SA2005 and 13 haplotypes formed clusters with between two to four replicates. The P_{gen} calculated for both

populations suggested that the majority of haplotypes were most likely a result of clonal reproduction (all $P_{gen} < 0.002$). In addition, within the different haplotype clusters, the probability that the haplotype replicates originated from different sexual events (P_{sex}) was very low ($P_{sex} < 0.03$) in the majority of cases.

The distribution of clones and haplotypes in the populations was further evaluated by plotting histograms to show the frequency distribution of genetic distances among haplotypes. A bimodal distribution pattern of frequencies was obtained for SA1997 and SA2005 indicating there are two main groups of clones within these populations (Fig 3). The global shape of the histograms was also informative as it was possible to visualize a decreased homogeneity of SA2005 population relative to SA1997 population. The bimodal pattern observed was further analysed using UPGMA analysis (Figs 4, 5) to examine whether there was an association between the groups and localities. The trees generated resulted in two main clusters for both the SA1997 and SA2005 populations. The trees showed no association between localities and clusters.

The overall results of tests for correlation between genetic and geographic distance of the SA1997 and SA2005 populations were significant ($p < 0.05$). Using the complete data set, the values were 1 for SA1997 and SA2005 and using the clone corrected data, 0.99 for SA1997 and 1 for SA2005. In both cases, the results suggested genetic structuring by means of gene flow restrictions at the scale at which the isolates were sampled.

DISCUSSION

In this study, eleven microsatellite markers were used to consider the population biology and structure of the *Eucalyptus* stem canker pathogen *T. zuluensis* in South Africa. Despite an observable reduction of pathogen population size on *Eucalyptus* across plantations in South Africa, there was an increase in genetic diversity during the period between 1997 and 2005. Two small populations collected from Malawi and China for comparative purposes were more diverse compared with two temporally-separated populations from South Africa. This result does not support the hypothesis (Wingfield *et al.,* 1997) that South Africa represents the original source of *T. zuluensis.*

Because the majority of susceptible trees in South African plantations were replaced with trees resistant to *T. zuluensis,* it was expected that the genetic diversity of the pathogen would be substantially lowered in the population of isolates collected nine years after the onset of the disease. Further, that the population diversity of *T. zuluensis* collected in 2005 would either reflect the one collected in 1997 or show a reduction of genetic diversity due to increased random genetic drift (Wright 1931; Young *et al.,* 1996). Results of this study showed no evidence of such a decrease in genetic diversity. Populations of *T. zuluensis* collected in 1997 and 2005 showed significant levels of differences in genetic diversity including allelic richness and evenness (homogeneity) and a shift of allelic frequencies. Recent studies have shown that the capacity of populations to recover genetic diversity after a reduction in population size is not easily predicted (Young *et al.,* 1996; Lowe *et al.,* 2005). The outcome depends on a combination of factors that are frequently unknown such as the original population size and other parameters related to the life history and reproductive structure of the populations (Young *et al.,* 1996; Edwards *et al.,* 2005; Lowe *et al.,* 2005; Reusch 2006). For instance, the reduction of population size from an original, highly diverse population can produce enhanced opportunities for a different group of haplotypes (including better adapted haplotypes) to replace those that were there in the first place (McNelly & Roose 1984; Watkinson & Powell 1993; Hughes & Stachowicz 2004; Kohn 2005).

The populations of *T. zuluensis* showed a broad global range of genotypic diversity (between 24% and 100%) but the South African populations had the lowest levels of genotypic diversity (SA1997, 24%; SA2005, 43%). In comparison, high genotypic diversities (84% to 100%) were detected in the Malawian and Chinese populations, despite the fact that the sample size for these populations was relatively small. Native populations typically have higher diversity than introduced populations (McDonald 1997; Stukenbrock *et al.,* 2007; Hunter *et al.,* 2008). Thus, our results fail to support the view that *T. zuluense* originated in South Africa (Wingfield *et al.,* 1997). This speculative view emerged due to the fact that the pathogen first appeared in South Africa and that it had never been found in the native range of *Eucalyptus* spp. (Wingfield 2003).

On a global scale, the allele frequency theta (θ) and assignment tests indicated significant differentiation across the *T. zuluensis* populations. Multiple clusters were formed according to the assignment tests showing that the majority of individuals from the populations in China and Malawi are different to the individuals reflecting the two South African populations. The large numbers of private alleles in the populations, together with the genetic diversity results, negate the possibility that South Africa represents a centre of origin for *T. zuluensis.* What is, however, clear is that there is no significant gene flow between the populations that were examined in this study. This suggests that *T. zuluensis* in South Africa, Malawi and China have originated independently of each other but from an unknown source.

The observed differentiation between the two temporally separated populations from South Africa (SA1997 and 2005) was unexpected. The genetic distances and cluster analyses within these populations revealed a level of population structure. Two major groups of intermingled haplotypes from different localities were recovered as bimodal distributions in both populations. The spatial correlation analysis provided additional evidence of structure at the "with-in" population level indicating there were restrictions to gene exchange at the sampled scale. The best explanation for these observations is that the two populations arose as the result of loss of haplotypes and subsequent introduction of new haplotypes. The restricted gene exchange also provides evidence that dispersal occurs mainly by conidia as is the case with other closely related fungi (Feau *et al.,* 2005; Milgate *et al.,* 2005; Hunter *et al.,* 2008) that show predominantly clonal population structure.

Linkage disequilibrium analyses showed significant departure from random mating for all populations studied with the exception of the population from China. The fact that sexual structures have never been observed for *T. zuluensis* in South Africa or elsewhere does not preclude the existence of cryptic sexual recombination. Results of this study, however, suggest that sexual recombination is not the predominant form of reproduction in the *T. zuluensis* populations in South Africa and Malawi. The fact that evidence for recombination was observed in the Chinese population, which is also the most genetically diverse, is enigmatic as this fungus has only recently been observed in that country and on *Eucalyptus* which is

not native to this region. While, *T. zuluensis* might therefore have its origin in South-east Asia, the fact that is not known in Australia does not imply that it is not present also there. This would be consistent with the fact that there are growing numbers of examples of *Eucalyptus* pathogens being reported for the first time in plantations outside Australasia and thus before they are detected in that country (Wingfield *et al.,* 1996; Burgess *et al.,* 2007) and this could also be the case for *T. zuluensis*.

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Table 1 *Teratosphaeria zuluensis* isolates used in this population study.

Country	Host	Collection date	Collector	No. Isolates
South Africa 1997 (SA1997)	*E. grandis*	1997	L Van Zyl	75
South Africa 2005 (SA2005)	*E. grandis*	2005	MJ Wingfield/ MN Cortinas	110
Malawi	*E. grandis*	2004	J Roux	41
China	*E. urophylla*	2004	T Burgess	22
Total				248

Table 2 Locus and primer names, primer sequences with florescent labels, repeat motif, annealing temperature (T_a), $MgCl_2$ concentration and size ranges of the alleles for six additional species-specific *Teratosphaeria zuluensis* microsatellites loci developed in this study and used in the population analyses of *T. zuluensis* isolates.

Locus name	Primer names	Primer sequences (5'- 3')	Repeat motif	T_a (°C)	$MgCl_2$ (mM)	Size range (bp)
Kzulu5	F18F/ F18RC1	FAM- GTT GTG TCC GAT CCT GCG AAG C GGATCTCCTCAATCACTTACTGC	$(CG)_7(AG)_{21}CA(AG)_9$	56	3.5	192-265
Kzulu10	F19W2/ F19FL2	PET- CCG CTG TGG CAT CCA AAT TCC GGC GCT CTG TCA CTG CTA AGG C	$(TATCAACACC)_8$	59	3.5	321-426
Kzulu11	F25F1/ F25RC2	PET- CGC TAT TTG CTG CTT TTG GAA CC AGG GGC TGT ATG TAG ATG CCG	$(AG)_7$	59	3.5	101-124
Kzulu12	F27F/ F27RC1	PET- GGA TCA GAA ATG CGA GGA CGA GG CTACCACGACTTTCCTCACTACG	(TG)rich	63	3.5	275-304
Kzulu13	F33F1/ F33RC1	VIC- AGT GAG ACA TAG GCA CGG GTA GG GGT ACG CTT GAA CAC ACA CA	$(TG)_{12}$	58	3.5	123-154
Kzulu14	Ms42RC1/ Ms42F1	VIC- GCT CGA CCA CGC CTG ACT TAA GG ACG ATG GCG GCA GTG AAG GAG	$(TG)_{12}$	59	3.5	254-282

Table 3 Allelic frequencies and other diversity indices of clone-corrected populations of *Teratosphaeria zuluensis* from Malawi, China and South Africa at 11 microsatellite loci.

Loci	Alleles	Malawi	China	SA-1997	SA-2005	Total
Czulu1	A	0.737	0.181	0.2434	0.091	
	B	0.184	0.455	0.659	0.523	
	C			0.024	0.261	
	D		0.364		0.114	
	E	0.079		0.073		
	F				0.011	
Czulu2	A		0.183	0.122	0.012	
	B	0.263	0.366	0.390	0.391	
	C	0.737	0.455	0.488	0.598	
Czulu3	A				0.250	
	B			0.097	0.031	
	C	1.000	1.000	0.902	0.716	
Kzulu5	A	0.027		0.024	0.114	
	B	0.108	0.050	0.024		
	C	0.216	0.100			
	D			0.024	0.273	
	E				0.011	
	F	0.027	0.050			
	G	0.270	0.200	0.195	0.273	
	H	0.027				
	I	0.027				
	J	0.027		0.024	0.068	
	K	0.243	0.550	0.707	0.114	
	L	0.027	0.050		0.023	
	M				0.091	
	N				0.034	
Kzulu10	A		0.095	0.04 9	0.205	
	B	0.447	0.191	0.220	0.398	
	C	0.237	0.286	0.7073	0.2273	
	D		0.238	0.024	0.0909	
	E	0.316	0.095			
	F		0.095		0.011	
	G				0.068	
Kzulu11	A				0.034	
	B	0.158	0.091	0.024	0.056	
	C	0.737	0.818	0.927	0.738	
	D				0.045	
	E	0.105	0.091	0.049	0.091	
	F				0.034	
Kzulu12	A	0.398	0.227	0.854	0.773	
	B	0.526	0.727		0.068	
	C	0.026	0.045	0.049	0.1591\	
	D	0.053		0.049		
	E			0.049		
Kzulu13	A	0.158	0.524	0.200	0.049	
	B	0.053	0.191	0.318	0.602	
	C	0.553	0.191	0.366	0.349	
	D	0.237	0.095	0.098		
Czulu6	A	0.394	0.455	0.634	0.840	

	B	0.026	0.364		0.159	
	C	0.316	0.182			
	D	0.263				
	E			0.293		
	F			0.024		
	G			0.049		
Czulu7	A	0.316	0.190	0.146	0.023	
	B	0.553	0.810	0.781	0.716	
	C	0.053		0.073	0.216	
	D	0.079			0.046	
Kzulu14	A	0.053	0.227	0.195	0.0342	
	B	0.026	0.227			
	C	0.026	0.091	0.342	0.568	
	D	0.026	0.091		0.011	
	E		0.046			
	F		0.046			
	G	0.579	0.189	0.366	0.364	
	H	0.263	0.091	0.097	0.011	
	I	0.026			0.011	
N		41	22	75	110	248
Nc		37 (7.5%)	22 (0%)	43 (22,9%)	86 (11.1%)	188
Na		45	42	41	50	
Number of private alleles		3	2	4	9	18
H		0.51	0.53	0.44	0.51	
Number of polymorphic loci		10	10	11	11	
G		34.48	22	18,18	47,61	
Ĝ		84%	100%	24%	43%	
S		3.68	3.09	3.4	4.35	
V’		0.98	0.99	0.90	0.97	
β				1.658	2.779	

N = Number of isolates (non clone-corrected)

Nc=Number of haplotypes in the clone-corrected populations

Na = Observed number of alleles

H = Nei’s Gene Diversity according (1973)

G = Genotypic Diversity (Stoddart & Taylor, 1988)

Ĝ = G/N% = percentage maximum diversity

S = Shannon–Weiner index

V’ = Evenness index derived from Shannon-Weiner

β= β parameter of Pareto distribution

Table 4 Pairwise Chi-square comparisons of allelic frequencies between *Teratosphaeria zuluensis* populations from Malawi, China, South Africa 1997 and South Africa 2005. The total number of loci whose frequency differ significantly from each other (as indicated by *), in the pairwise comparison, is shown in the last column.

Pairwaise populations		Czulu 1	Czulu2	Czulu3	Kzulu5	Kzulu10	Kzulu11	Kzulu12	Kzulu13	Czulu6	Czulu7	Kzulu14	Number of significantly different loci
Malawi and China	chi2	27.19*	9.13*	0.000	7.79	21.69*	0.61	3.42	14.45*	17.41*	4.93	22.76*	6 out of 11
	df	3	2	0	9	5	2	3	3	3	3	8	
Malawi and SA1997	chi2	21.20*	7.61*	3.90*	25.41*	27.91*	5.64	30.26*	11.49*	40.89*	7.37	21.67*	9 out of 11
	df	3	2	1	10	4	2	4	3	6	3	6	
Malawi and SA2005	chi2	67.61*	2.46	13.46*	5.67*	42.20*	7.481	41.31*	44.39*	62.35*	26.35*	44.69*	9 out of 11
	df	5	2	2	13	6	5	3	3	3	3	6	
China and SA1997	chi2	18.31*	0.41	2.29	10.44	19.25*	1.92	40.81*	6.19	31.22*	1.71	21.98*	5 out of 11
	df	4	2	1	8	5	2	4	3	5	2	7	
China and SA2005	chi2	14.21*	11.77*	8.08*	46.14*	20.60*	2.96	47.78*	41.00*	23.12*	14.35*	55.70*	10 out of 11
	df	4	2	2	11	6	5	2	3	2	3	8	
SA1997 and SA2005	chi2	25.6*	7.85*	13.58*	54.45*	28.98*	7.17	14.35*	20.19*	40.26*	12.25*	17.69*	10 out of 11
	df	5	2	2	9	5	5	4	3	4	3	5	

Table 5 Population differentiation values, represented as Theta (*θ),* for the *Teratosphaeria zuluensis* populations.

T. zuluensis	China	SA1997	SA2005
Malawi	0.11*	0.17*	0.18*
Thailand	0.10	0.20	0.18
China		0.13	0.16
SA1997			0.10

*significant Chi-square values (P < 0.05)

Table 6 Two-locus linkage disequilibrium analysis (LD) expressed as the number of loci with significant differences over the total pairwise loci comparisons, observed value of Index of Association (I_A) and range of I_A values obtained after 1000 randomizations. In the last column recombination is indicated as a 'yes' based on the observation that the observed I_A value falls within the randomized dataset values.

	LD between pairs of loci	Obs. I_A	Range of obtained I_A values after 1000 randomizations	Obs. I_A within randomized the data range. (i.e. evidence for recombination)
T. zuluensis				
China	8/49	0.17	-0.003- 0.24	yes
Malawi	15/49	0.75*	-0.002- 0.13	no
SA1997	8/49	0.70*	-0.02- 0.28	no
SA2005	21/49	0.41*	-00008- 0.17	no
All	14/49	0.37*	-0.0033- 0.15	no

*significant $p<0.05$

Table 7 Localities sampled from in South Africa in 1997 and 2005 including x and y coordinates and number of isolates obtained from each location.

Locality	Locality abbreviation	X Coord.	Y Coord.	Number of isolates
1997				
Aboyoni	A	4,5	6,0	4
Honey Farm	H	5,0	6,0	9
Palm Ridge	P	4,5	8,5	11
Shire	S	3,0	3,5	4
Teranera	Te	4,5	4,5	5
Teza	T	6,0	6,5	17
Trust	Tr	4,5	7,5	6
Fair Breeze	FB	1,5	2,0	1
Kwambonambi	K1	5,0	6,0	7
2005				
Kwambonambi	K2	4,5	6,0	8
Venters	V	4,5	6,0	42
Mtubatuba	M	4,75	7,5	44
Mtunzini	Mt	1,5	2,0	14
Moba Dam	MD	4.5	5,5	3

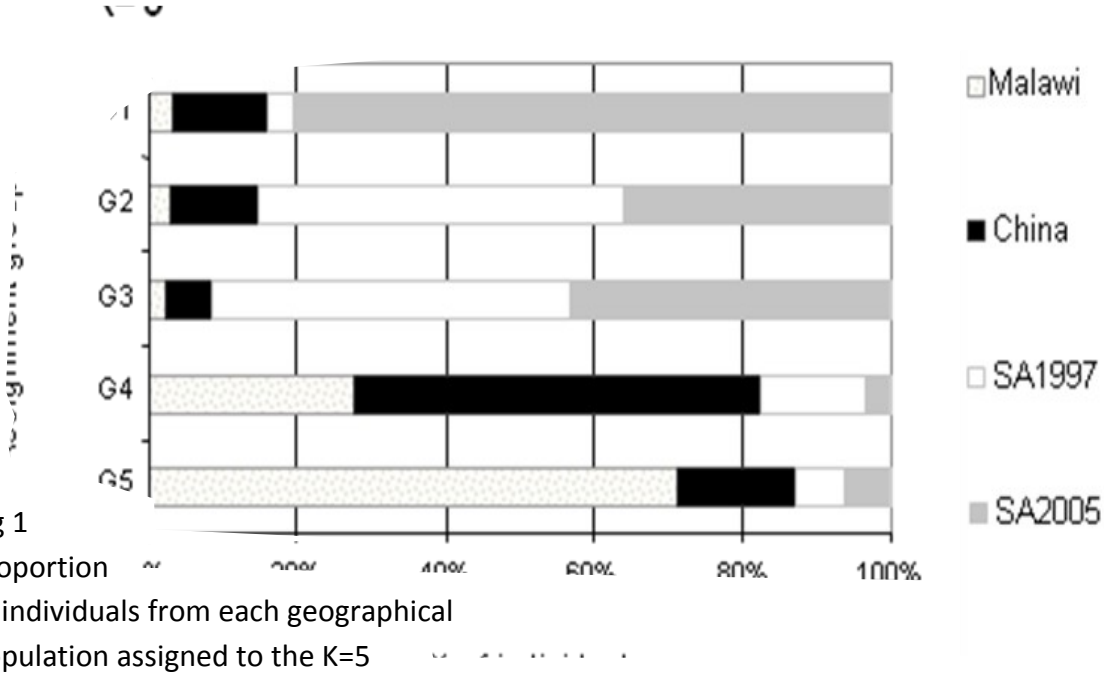


Fig 1 Proportion of individuals from each geographical population assigned to the K=5 groups (G1 to G5). In three out of the 5 groups (G1to G3), the majority of SA1997 individuals group with SA2005 individuals. The majority of the Chinese and Malawi individuals group in distinctive groups (G4, G5).

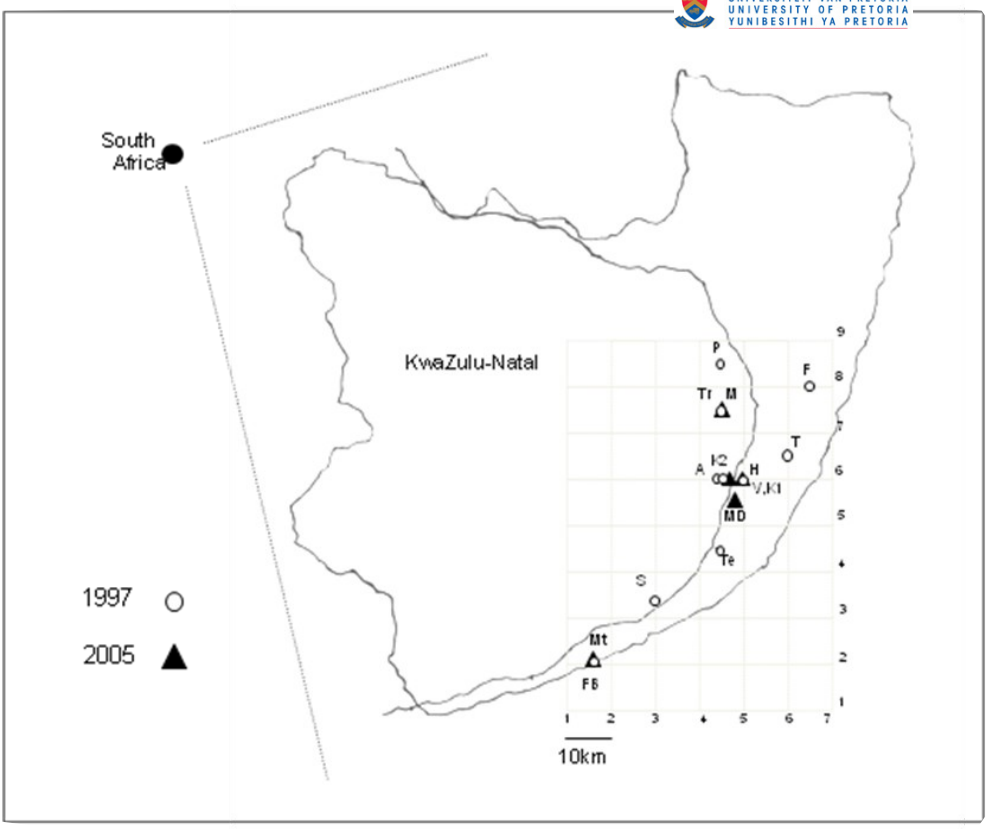


Fig 2 Location of sites sampled in 1997 and 2005 in KwaZulu Natal, South Africa (see Table 7).

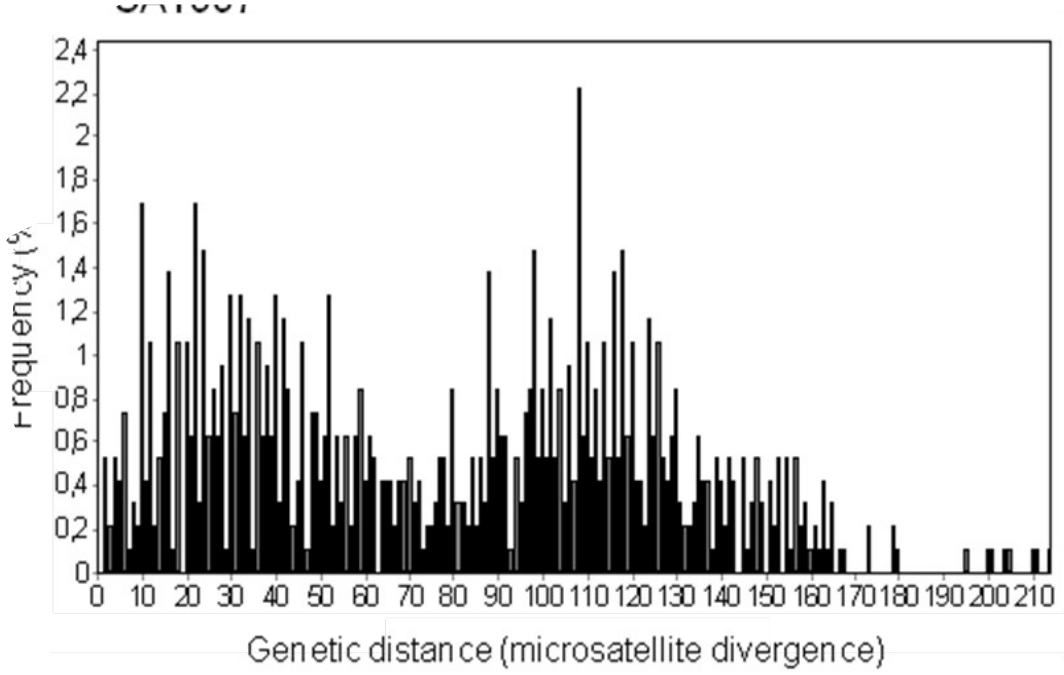
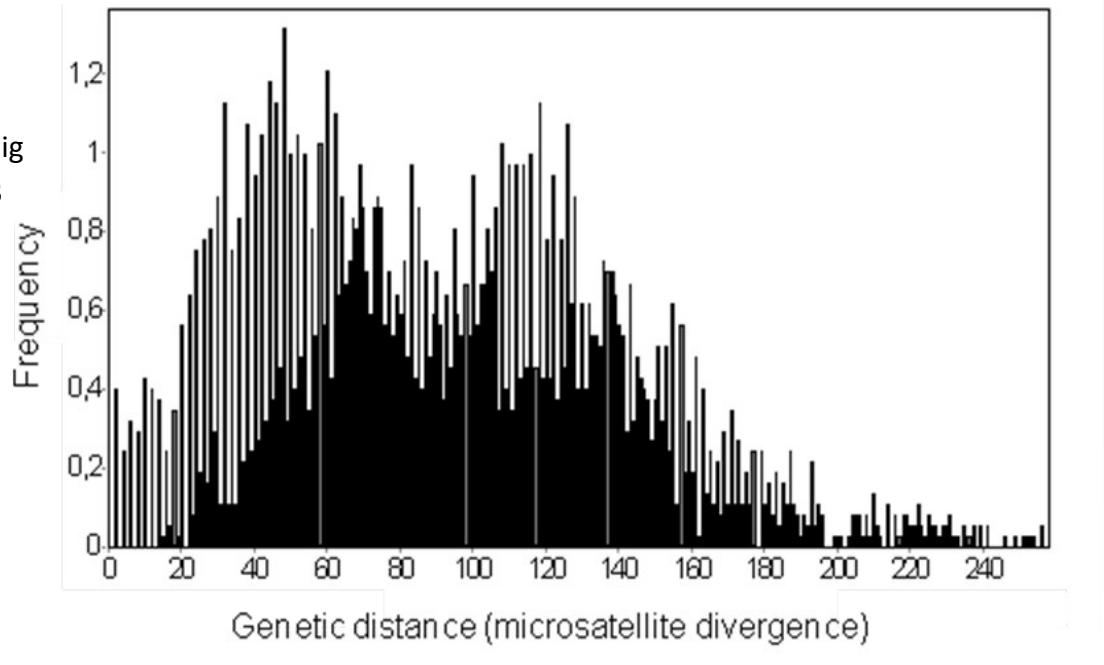


Fig 3



Frequency distribution of microsatellite divergence amongst pairs of isolates A. for the SA1997 population and B. for the SA2005 population.

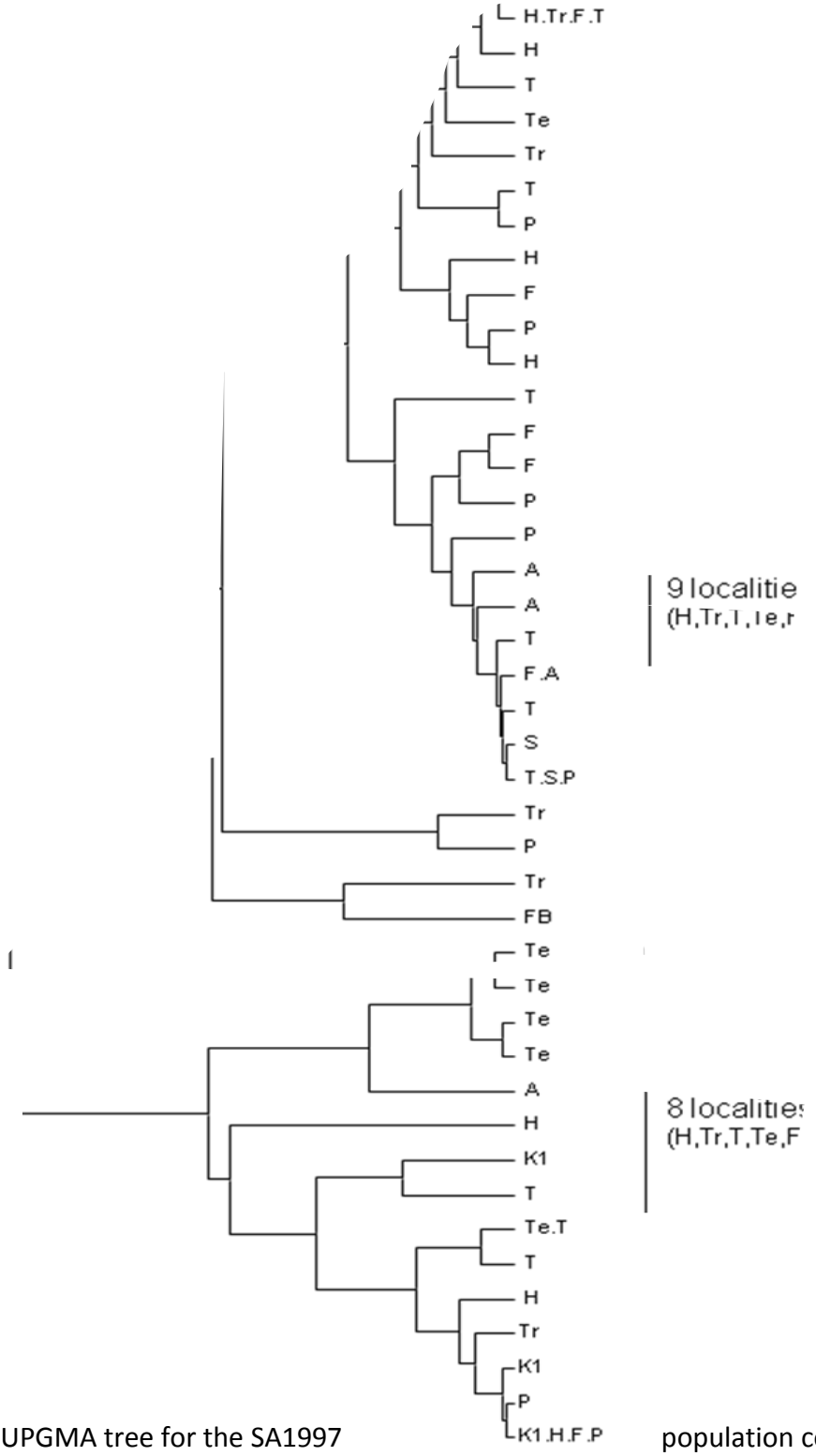


Fig 4 UPGMA tree for the SA1997 population constructed in MEGA 4 using the distance matrix calculated in GENCLONE 2.0. The branches include samples recognized as part of the clone in GENCLONE 2.0. The letters indicate the original sampling location of the clones included in the branch as indicated

in Table 3. Two main clusters of clones emerge in the tree. Each cluster includes clones from the majority of the sampled locations.

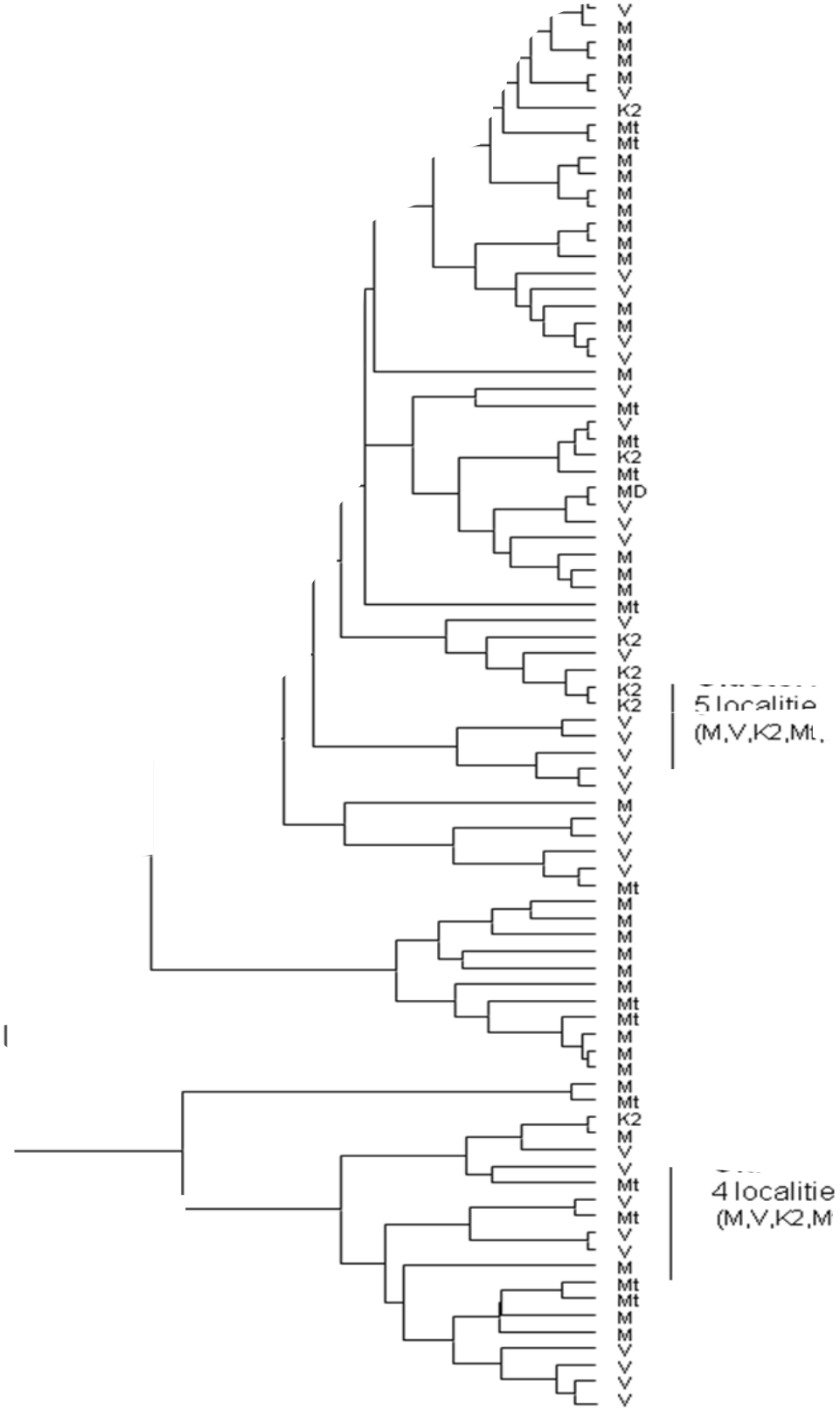


Fig 5 UPGMA tree for the SA2005 population constructed in MEGA 4 using the distance matrix calculated in GENECLONE 2.0. The branches include samples recognized as part of the clone in GENCLONE 2.0. The letters indicate the original sampling location of the clones included in the branch as indicated in Table 3. Two main clusters of clones emerge in the tree. Each cluster includes clones from the majority of the sampled locations.

Chapter 7

Unexpected genetic diversity revealed in the *Eucalyptus* canker pathogen *Teratosphaeria gauchensis*

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ABSTRACT

Teratosphaeria gauchensis causes a serious canker disease on *Eucalyptus* spp. in plantations in South America and Africa. The pathogen is closely related to, but distinct from *T. zuluensis* that causes a similar stem canker disease on *Eucalyptus.* The objective of this study was to use 10 previously developed polymorphic microsatellite markers to study the population diversity of *T. gauchensis*, based on collections of the fungus made in Argentina and Uruguay. The alleles were size -analyzed to determine population genetic parameters of the *T. gauchensis* populations. The results showed that isolates from the two collection sites represent the same population. Overall, the genetic diversity amongst isolates was higher than expected and inconsistent with the notion that the pathogen represents a recent introduction into South America.

INTRODUCTION

Teratosphaeria gauchensis (M.N. Cortinas, Crous & M.J. Wingf.) M.J Wingf. & Crous Andjic & M.J. Wingf. and the related *Teratosphaeria zuluensis* (M.J. Wingf., Crous & T.A. Cout.) M.J. Wingf. & Crous cause a disease known as Coniothyrium canker on *Eucalyptus* spp. *Teratosphaeria zuluensis* was the first of these fungi to be described after it was discovered causing serious damage to the stems of clonally propagated *Eucalyptus grandis* in the Kwa-Zulu Natal province of South Africa (Wingfield *et al.,* 1997). The disease spread rapidly in the 1990's and became one of the most serious impediments to in *Eucalyptus* plantation forestry in that country (Old *et al*., 2003).

Due to the serious economic impact of Coniothyrium canker on plantations in South Africa, there were various studies undertaken to better understand the relevance and biology of *T. zuluensis* (Van Zyl, 1999, Van Zyl *et al.,* 2002). Some years later, a very similar disease was discovered on *E grandis* clones in Argentina and Uruguay and surprisingly, the causal agent was found to be different to *T. zuluense* (Cortinas *et al*., 2006b). The causal agent of the disease was a fungus that was provided with the name *Teratosphaeria gauchensis*. *Teratosphaeria gauchensis* and *T. zuluensis* are morphologically almost indistinguishable and they give rise to the same symptoms after infection. Thus, the only reliable means to distinguish between the two fungi is via DNA sequence comparisons. Both fungi were initially described as mitotic species and residing in the teleomorph genus *Mycosphaerella* based on phylogenetic inference (Cortinas *et al.,* 2006b; Andjic *et al.,* 2007) but recent taxonomic re-evaluation has relegated them to anamorphs of *Teratosphaeria* in the Teratosphaeriaceae (Crous *et al.,* 2007; Crous *et al.,* 2009).

Teratosphaeria gauchensis causes cankers on young branches and on tree trunks although it has also been isolated from leaf spots on *E. maidenii* and *E. tereticornis* in Uruguay (Pérez *et al.,* 2009a). The typical stem and trunk lesions caused by this fungus are necrotic and have a characteristic dark oval shape (Cortinas *et al.,* 2006b). The extent of the lesions varies depending on the susceptibility of the infected trees. Severe infections arise from small cankers that merge to cover large areas of the trunks. Both the soft tissue and wood become malformed resulting in retarded growth and girdling can be observed at the tree

tops. Kino pockets are formed as part of the defence response of the trees. Kino that exudes from the cankers can cause the stems to become a black colour. In some cases, diseased trees also produce epicormic shoots alongside the cankers that can cause the terminal parts of the branches and stems to die (Wingfield *et al.,* 1997; Cortinas *et al*., 2006b).

Very little is known regarding the biology of *T. gauchensis*. It is presumed that the fungus exists in a haploid state (Wingfield *et al.,* 1997; Crous, 1998; Crous *et al.,* 2004; 2006). In nature, only asexual pycnidia are found on the bark lesions. These structues give rise to mitospores (conidia) that are presumably responsible for short distance dispersal, as is the case for closely related fungi (Feau *et al.,* 2005; Milgate *et al.,* 2005; Hunter *et al.,* 2008). Sexual structures have never been observed in nature nor have they been produced in culture.

The origin of *T. gauchensis* is not known. Its distribution is limited to Uganda and Ethiopia (Gezahgne 2003; Gezahgne *et al.,* 2005), Argentina and Uruguay (Gezahgne *et al.,* 2004; Cortinas *et al.,* 2006b) and Hawaii (Cortinas *et al.,* 2004). It is has also never been found on any host other than *Eucalyptus* species, which is an exotic in all these countries. The current distribution of *T. gauchensis* does not overlap with the distribution of the sibling species *T. zuluensis* (Cortinas *et al.,* 2006b). The fact that *Eucalyptus* species are not native to any of the countries where *T. gauchensis* is found, and its close phylogenetic relationship to other *Terathosphaeria* species on *Eucalyptus,* suggests that it is a *Eucalyptus*-specific pathogen, which has yet to be discovered in its native range. If that is the case, then one would expect to find fungal populations with low genetic diversity in areas where it has been introduced, which is true for the related *M. nubilosa* (Pérez *et al.,* 2009).

The aim of this study was to investigate the population diversity and structure of *T. gauchensis* found on non-native *Eucalyptus* in plantations of Argentina and Uruguay where the associated disease has been particularly serious. To achieve this goal, ten polymorphic microsatellite markers, recently developed for this species (Cortinas *et al.,* 2008), were used to calculate estimates of haplotype richness and evenness, haplotypic diversity and genetic differentiation for isolates collected in Argentina and Uruguay.

MATERIALS AND METHODS

Sampling and isolations

Necrotic lesions on the bark of infected *Eucalyptus* clones were sampled from plantations in the neighbouring provinces of Entre Ríos, Corrientes and Misiones in Argentina and from two areas (Rivera and Paysandú), in the Northern part of Uruguay. The sampling area covered a range of approximately 450 km in a North-South direction and 300 Km in an East- West direction (Table 1). Samples were collected as part of a disease evaluation project in Uruguay and Argentina between 1999 and 2005. Samples were taken from one lesion per tree on the stems of randomly chosen trees approximately 2 m above the ground.

One hundred and thirty one single conidial isolations were made from lesions as described previously (Cortinas *et al*., 2006a). These single conidial cultures were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, where they are maintained in long-term storage facilities.

DNA extraction and microsatellite loci

Single conidial isolates were grown on 2% malt extract agar (MEA) in Petri dishes for 30 days at 25°C. The fungal mycelium was scraped from the colonies, freeze dried, immersed in liquid nitrogen until frozen and ground to a fine powder. DNA extraction followed, from a total of one hundred and thirty-one isolates of *T. gauchensis,* using the phenol-chloroform method as described by Cortinas *et al.,* (2006a).

Ten pairs of fluorescently labelled primer sets for 10 polymorphic microsatellite loci of *T. gauchensis* (Cortinas *et al.,* 2008) were used in this study. The microsatellite loci were amplified by PCR and the amplified products were size-separated on an ABI 3100 Automated DNA Sequencer (Applied Biosystems, Foster City, USA) using GENSCAN LIZ 500 (-250) (Applied Biosystems) as internal size standard. Thirty-eight isolates were analysed from Argentina. These included 10 isolates from the Entre Rios province, 17 from the Corrientes province, eight from the Misiones of Argentina and three from undefined source within these provinces.

Ninety-three isolates were obtained/ analysed from Uruguay including 33 from the Paysandú department and 60 from the Rivera department (Table 1). GENEMAPPER, version 3.0 (Applied Biosystems) software package was used to carry out the fragment size analysis. Based on size differences of the amplicons produced for each locus, different alleles were identified. For further analyses, each allele was designated by their size in nucleotides or by a letter of the alphabet.

Genetic diversity

Gene diversity (*H*) was estimated using the algorithm of Nei (1973) as implemented in POPGENE (Yeh *et al*., 1999). Genotypic diversity (*G*) was calculated (Stoddart and Taylor 1988). To compensate for differences in sample size, the maximum percentage of genotypic diversity (G/N x 100) was used. The significance of differences in haplotypic diversity between populations was determined using a *t*-test (Chen *et al.,* 1994).

Richness and evenness

The clonal diversity of the populations in terms of richness and evenness was studied using GENCLONE 2.0 (Arnaud-Haond & Belknir, 2007; Arnaud-Haond *et al.,* 2007). This program was specially developed to deal with clonal organisms. The program has a 'round-robin' algorithm implemented to avoid the overestimation of the rare allele frequencies. As implemented in GENECLONE 2.0, richness and evenness of the populations were studied using the Shannon -Weiner index (richness), the complementary index (V') (evenness) and Pareto distributions to examine richness and evenness as a whole.

Population differentiation and assignment tests

The program POPGENE was used to determine differences in allele frequencies between populations of *T. gauchensis.* Clone-corrected datasets were analysed to avoid over representation of genotypes produced by asexual reproduction or by sampling at different spatial scales. Differences in allelic frequencies between

populations across the ten loci were tested using Chi square tests (Workman & Niswander, 1970).

The program MULTILOCUS version 1.3 (Agapow & Burt, 2001) was used to estimate the amount of differentiation among populations. The program estimates theta (θ) (Weir, 1996); a modification of the original F_{ST} of Wright (1978). An evaluation of the level of significance of θ was carried out by comparing the observed value to the values obtained by a thousand randomizations of the individuals across populations.

Assignment of individuals into a number of clusters/populations (K) was carried out for the Uruguayan and Argentinean populations using STRUCTURE version 2.2 (Falush *et al.,* 2003). Individuals are assigned to one (K=1), two or more populations where their allelic frequencies were indicative of admixture. To determine the "optimal K", one hundred thousand runs were performed with 10 iterations using an admixture ancestry model and an independent allele frequency model. The burn-in was set at 10 000 runs. Assignments of individuals to the optimal "K" populations was carried out using 1 000 000 runs with a burn-in of 100 000.

Recombination analyses

MULTILOCUS version 1.3 (Agapow & Burt, 2001) was used to test for random association of alleles by calculating linkage disequilibrium (LD) for all pairs of loci and the Index of Association (I_A), using clone corrected data matrices. To determine the significance of the LD and I_A observed values, a distribution of values from a randomly mating population was simulated by performing 1000 randomizations of the allelic frequencies. The LD and I_A observed values were then compared with those obtained for the simulated distribution.

RESULTS

Allele and genetic diversity

Forty- three different alleles were recovered for the 131 isolates of the *T. gauchensis* collected and analysed. Individually, 31 different alleles were recovered from the Argentinean samples and 35 from the Uruguayan population (Table 2). The

number of alleles at individual loci, for both populations, ranged from two to eight. Private alleles were observed in both populations; five from Argentina and nine from Uruguay. The majority of these alleles were present with frequencies higher than 3%. No monomorphic loci were observed.

The gene diversity (H) calculated for *T. gauchensis* was 0.43 in Argentina and 0.42 in Uruguay (Table 2). Ninety-one different genotypes were identified across the two *K. gauchensis* populations (Table 2). One genotype was found to be shared between the Argentinean and Uruguayan populations. The number of repeated genotypes was 26.3% for the Argentinean population and 33.3% for the Uruguayan population. The maximum genotypic (haplotype) diversity was similar for Uruguay ($\hat{G}$ = 50%) and Argentina ($\hat{G}$ = 54%) (Table 2). The *t* test (P < 0.05) showed no significant differences between the genotypic diversities of the Argentinean and Uruguayan populations.

Richness and evenness

The heterogeneity within the populations (relative richness and evenness) values obtained were S= 3.29 and V'= 0.965 for Argentina and S= 3.96 and V'= 0.967 for Uruguay, very similar for both populations. Both had regression values = 1 and similar slopes (β= 2.64 for Argentina and β = 2.43 for Uruguay). Together, these results showed moderate to high haplotype heterogeneity and a high level of evenness (groups of clones of similar membership size). The majority of repeated haplotypes in Argentina and Uruguay formed groups of two individuals.

Population differentiation and assignment tests

The allelic frequencies across populations were compared by calculating the differences in allelic frequencies per locus and between pairwise populations (Table 3). The analysis of the loci showed that the frequencies of the alleles between the populations of Argentina and Uruguay were only significantly different at one (Locus 6) of 10 loci. The theta value of 0.011 (P< 0.05) indicated no differentiation among populations.

No admixture patterns were detected using STRUCTURE as clusters were not detected. The assignment diagrams showed that the majority of individuals

assigned to all different K groups in similar proportions in the tested range between K=1 to K=10.

Recombination analyses

In *T. gauchensis,* low LD was found using two-locus pairwise analyses: zero out of 45 comparisons in the Argentinean population and four of 45 comparisons in the Uruguayan population showed linkage disequilibrium (Table 4). The results obtained from the multilocus Index of Association (I_A) analyses were comparable to the LD results calculated using the pairwise method (Table 4). The observed values of I_A in *T. gauchensis* fell within the randomized distribution of allelic frequencies suggesting that recombination could be occurring in both *T. gauchensis* populations.

DISCUSSION

Teratosphaeria gauchensis is a pathogen of growing importance to a rapidly expanding *Eucalyptus* plantation industry in South America. This study provides the first consideration of its genetic diversity and thus, long term durability of resistance in intensively propagated planting stock. As such, populations of *T. gauchensis* from Argentina and Uruguay showed a genetic structure that is very different to one expected for a recently introduced pathogen. These populations contained moderate levels of genetic variation, homogeneous distribution of haplotypes, no differentiation between populations and indications that recombination is occurring.

The moderate to high levels of genetic diversity found in the *T. gauchensis* populations from South America were unexpected as the disease was only discovered in Argentina and Uruguay in the last two decades. Thus, a low genetic diversity and a small number of predominant haplotypes (clones) were expected in the populations of *T. gauchensis*. This would be similar to a number of other closely related *Eucalyptus* pathogens recently reported in Uruguay (Balmelli *et al.,* 2004; Pérez *et al.,* 2009). For example, the *Eucalyptus* leaf blotch pathogen *T. nubilosa* was found to be clonal, which suggests a recent, localized introduction in the area (Pérez *et al.,* 2009).

The levels of genetic diversity of *T. gauchensis* found in this study were comparable with the genetic diversities of other phylogenetically related *Mycosphaerella* and *Teratosphaeria* species from their native ranges. These species include *M. musicola* (Hayden *et al.,* 2003b; 2005; Zandjanakou-Tachin *et al.,* 2009), *M. fijiensis* (Carlier 2004; Hayden *et al*., 2003a) and *T. nubilosa* (Hunter *et al.,* 2008; 2009). Interestingly, with the exception of *T. gauchensis*, all these species have well characterized sexual states that would promote their genetic diversity.

Results of this study showed evidence of recombination in the studied *T. gauchensis* population from Argentina. This result was unexpected as sexual structures have never been found in the field for this fungus. Nonetheless, there is precedence for finding evidence of recombination in apparently asexual fungi (Taylor *et al.,* 1999; Zhou *et al.,* 2007). From this study we can conclude that *T. gauchensis* in all likelihood has a mixed mode of reproduction and has asexual and sexual reproductive structures similar to the most closely related *Mycosphaerella* spp. (Cortinas *et al.,* 2010; Crous *et al.,* 2004; 2006; Hunter *et al.,* 2008; Pérez *et al.,* 2010). A more exhaustive survey should be conducted in the future to find the teleomorph in the field.

Population genetic analyses showed that the two collections of isolates from Argentina and Uruguay can be considered as part of the same genetic pool, rather than two separate and unrelated populations. Thus, the differentiation tests showed weak to no differentiation between the two *T. gauchensis* populations. These results were further supported by the assignment tests whereby the individuals from Argentina and Uruguay, regardless of the number of clusters tested, were separated in equal proportions among clusters, indicating a lack of population structure for the isolates (Pritchard *et al.,* 2000).

Analyses of *T. gauchensis* isolates from Argentina and Uruguay are not compatible with the hypothesis that this is a recently introduced pathogen. One possible explanation for this result is that the fungus originated in Australasia where *Eucalyptus* is native, as in the case of *T. nubilosa* (Hunter *et al.,* 2008; 2009). This would be consistent with recent well documented examples of new *Eucalyptus* pathogens first being described from plantations outside the native range of *Eucalyptus* and later being discovered in Australia (Wingfield *et al.,* 1996; Burgess *et*

al., 2007). An alternative interpretation is that the pathogen has undergone a host shift from native ad Myrtaceae in Argentina and Uruguay. There are a growing number of *Eucalyptus* pathogens that have undergone host jumps (Slippers *et al.*, 2005) from native Myrtaceae and Melastomataceae (Myrtales) in countries where *Eucalyptus* spp. have been planted as exotics (Wingfield 2003; Wingfield *et al.,* 2008; Glen *et al*., 2007) Many of these examples are from South America including Uruguay (Pérez 2008). The most recent examples are *Quambalaria eucalypti* (Pérez *et al.,* 2008), *Neofusicoccum eucalyptorum* (Pérez *et al.,* 2009b), *Puccinia psidii* (Pérez *et al.,*2010a, in press) and members of Botryosphaeriaceae (Pérez *et al.,*2010b). It would not be unusual for *T. gauchensis* to have behaved in a similar fashion.

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Table 1 List of T. *gauchensis* isolates included in this population study.

Country	Province /Department	Host	Collection Period	Collector	Number of isolates
Argentina	Total 3provinces	*E. grandis*	2001/2003/2004	MJ Wingfield/ MN Cortinas	38
	Entre Ríos				10
	Corrientes				17
	Misiones				8
	Undefined within the 3 provinces				3
Uruguay	Total 2 departments	*E. grandis*	1999/2001/2005	MJ Wingfield/ MN Cortinas	93
	Paysandú				33
	Rivera				60

Table 2 Allelic frequencies and other diversity indices of the clone-corrected populations from Argentina and Uruguay at 10 microstellite loci.

Loci	Alleles	Argentina	Uruguay
K. gauchensis 1	A	0.036	0.064
	B	0.179	0.302
	C	0.536	0.508
	D	0.179	0.079
	E	0.036	0.016
	F		0.016
	G		0.016
	H	0.036	
K. gauchensis 2	A	0.643	0.429
	B	0.215	0.427
	C	0.143	0.127
	D		0.016
K. gauchensis 3	A	0.500	0.508
	B	0.036	
	C	0.429	0.429
	D	0.036	0.032
	E		0.032
K. gauchensis 4	A	0.964	0.968
	B	0.036	
	C		0.016
	D		0.016
K. gauchensis 5	A	0.679	0.740
	B	0.286	0.222
	C	0.036	0.032
K. gauchensis 6	A	0.607	0.571
	B	0.143	0.397
	C		0.032
	D	0.250	
K. gauchensis 7	A	0.679	0.6825
	B	0.286	0.2857
	C	0.036	0.0317
K. gauchensis 8	A	0.071	
	B	0.036	
	C	0.893	0.984
	D		0.016
K. gauchensis 9	A	0.464	0.333
	B	0.536	0.667
K. gauchensis 10	A	0.679	0.571
	B	0.321	0.381
	C		0.032
	D		0.016
N		38	93

Nc	28	63
Na	31	35
Number of private alleles	5	9
H	0.43	0.42
Number of different genotypes (haplotypes)	28	63
G	20.41	46.29
$\hat{G}$	54%	50%
S	3.29	3.96
V'	0.963	0.967
β	2.64	2.43

N= Number of isolates (non clone-corrected)
Nc= Number of haplotypes in the clone-corrected populations
Na= Observed number of alleles
H = Gene Diversity according to Nei (1973)
G = Genotypic Diversity (Stoddart and Taylor, 1988)
$\hat{G}$ = G/N% = percent maximum diversity
S= Shannon–Weiner index
V'= Evenness index derived from Shannon-Weiner (V')
β= β parameter of pareto distribution

Table 3 Pairwise Chi-square comparisons of allelic frequencies between *T. gauchensis* populations of Argentina and Uruguay.

Locus/clone corrected populations		*T. gauch.* 1	*T. gauch.* 2	*T. gauch. 3*	*T. gauch.* 4	*T. gauch.* 5	*T. gauch.* 6	*T. gauch.* 7	*T. gauch.* 8	*T. gauch.* 9	*T. gauch.* 10
Argentina and Uruguay	Chi^2	6.63	4.73	3.15	3.13	0.45	20.60*	0.009	7.36	1.42	1.89
	df	7	3	4	3	2	3	2	3	1	3

*significant Chi-square values (P < 0.05)

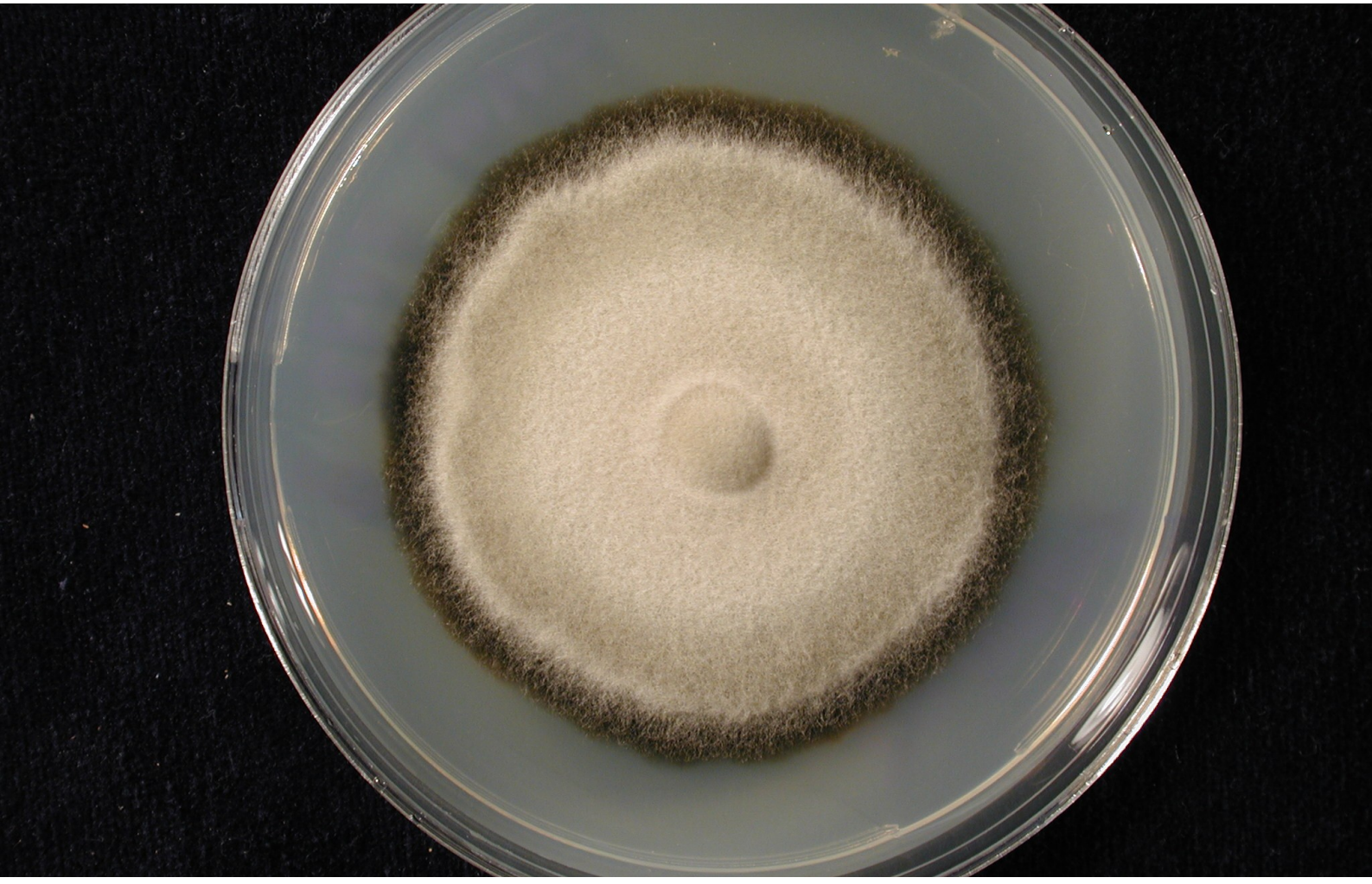
Table 4 Two-locus linkage disequilibrium analysis (LD) expressed as the number of loci with significant differences over the total pairwise loci comparisons, observed Index of Association (I_A) value and range of I_A values after 1000 randomisations. In the last column recombination is indicated as a 'yes' based on the observation that the observed I_A value falls within the randomized dataset values.

	LD between pairs of loci	Obs. I_A	Range of obtained I_A values after 1000 randomizations	Obs. I_A within the randomized data range. (i.e. evidence for recombination)
Argentina	0/45	0.22	-0.0005- 0.33	Yes
Uruguay	4/45	0.08*	-0.0066- 0.13	Yes
All	4/45	0.13	-0.00015- 0.15	Yes

*significant p<0.05

Appendix I

First record of the *Eucalyptus* stem canker pathogen, *Coniothyrium zuluense* from Hawaii



APPENDIX I

First record of the *Eucalyptus* stem canker pathogen, *Coniothyrium zuluense* from Hawaii

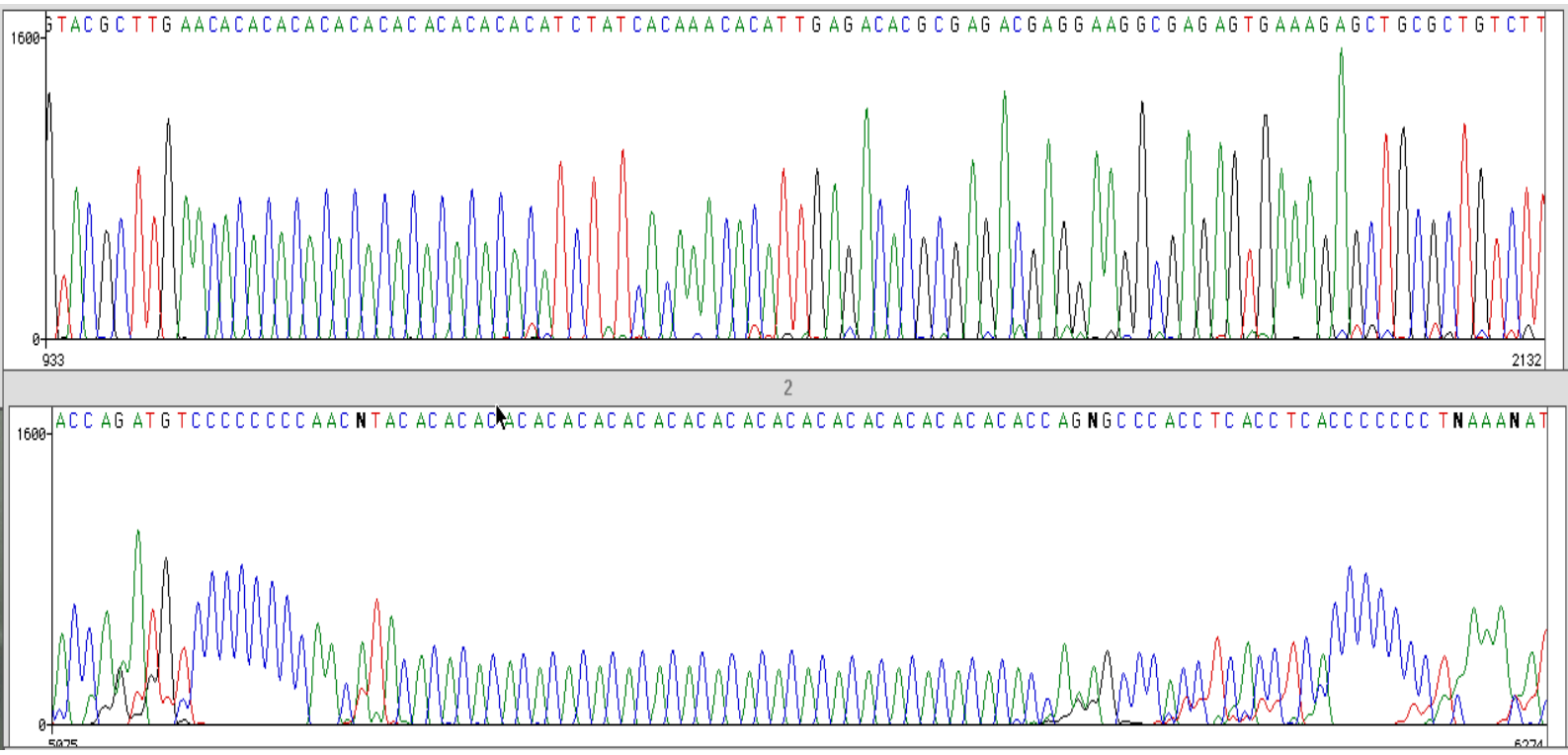
ABSTRACT

A new stem canker disease on *Eucalyptus grandis* in Hawaii is recorded. Symptoms are similar to those of *Coniothyrium* canker on *Eucalyptus* in South Africa. A fungus resembling *Coniothyrium zuluense* was found on lesions and analysis of ITS sequences confirmed this identification. *Coniothyrium* canker is a serious disease of *Eucalyptus* in South Africa and strategies to reduce its impact in Hawaii may be required.

Published as: Cortinas MN, Koch N, Thane J, Wingfield BD, Wingfield MJ (2004d). First record of the *Eucalyptus* stem canker pathogen, *Coniothyrium zuluense* from Hawaii *Australasian Plant Pathology* **33**, 309–312.

Appendix II

M - FIASCO protocol @ FABI



APPENDIX II

M - FIASCO at FABI

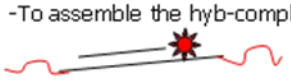
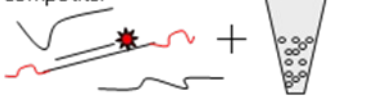
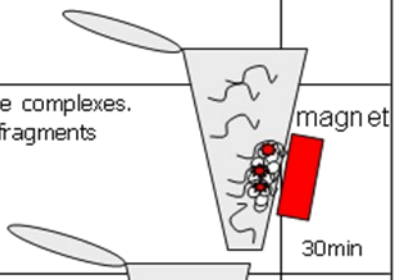
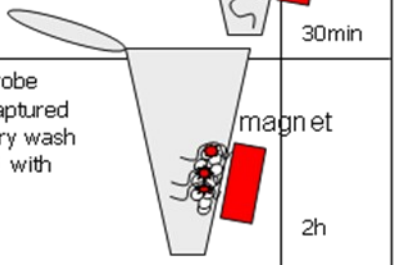
Capture of microsatellite sequences by enrichment procedures.

Version 1.4, May 2007.

This protocol was compiled as part of the PhD project of María Noel Cortinas. It was developed from a combination of pre-existent protocols included in the references section of the protocol.

The protocol was described in: Cortinas MN, Barnes I, Wingfield BD, Wingfield MJ (2006a). Polymorphic microsatellite markers for the *Eucalyptus* fungal pathogen *Colletogloeopsis zuluensis. Molecular Ecology Notes* **6**, 780–783.

Flow chart of microsatellites capture procedure in 5 steps

	Steps	Why do you do this? What is the result?	Timetable min, hs, days	
1.	**DNA Preparations**			1 or 2 days
	1.1 Pre-selection of RE	-To ensure your digestion was succesfull (smear)	3h	
	1.2 Digestion-ligation of adaptors	-To be able to amplify the fragments of DNA	3h	
	1.3 PCRs of **D**igested **D**NA with **A**daptors	-To increase amount of fragments that can be probed -To check ligation of adaptors -To repair nicks -To verify if size selection is needed	3h 1h (gel) 3h 1h (gel)	
2.	**Hybridizing DDA with µsat probes**			1 or 2 days
	2.1 Incubation of DNA together with biotinylated probes	-To assemble the hyb-complexes (DAA/DNA-probe) 	1h	
3.	**Capture of microsatellites (enrichment)**			
	3.1 Incubation with streptavidin-coated magnetic beads and competitor 	-Formation of µsats capture complexes. Non complementary DNA fragments remain in solution 	30min	
	3.2 Washes - 3 non stringent - 3 stringent	-Only complexes ssDNA-probe containing µsats remain captured with the beads during every wash (separation is acomplished with the aid of the magnet) 	2h	
	3.3 Elution	-To dismantle the complexes formed by ssDNA, probes, and beads complexes	15min	
	3.4 PCR post-capture. -Maybe you have to try more than once	-Back to double stranded DNA -To Increase amount of DNA -To verify quality of enrichment. You are looking for an homogeneous representation of fragments = smears	3h 1h (gel)	
	3.5 Freeze enrichments: optional	-If not prepared for cloning or you want to make more enrichments before cloning.		
4.	**Cloning: e.g. TOPO4 Kit**			2 days
5.	**Screening**			2 days
	5.1 Colony PCR		3h, 1h (gel)	
	5.2 Sequencing PCR		3h, 1h (purif)	
			O.N.	6 to 8

M-FIASCO @ FABI

Fundamentally Based on Hamilton *et al.* 1999 and Zane *et al.* 2002

http://fabinet.up.ac.za/personnel/showperson.php?id=marianoel

	Volume (µl)		Temp. (oC)	For how long? (Cycles or h)	
1. DNA preparations					
Adaptor Preparation					
Fiasco1 A (10 µM)	100,0 µl		96	2min	
Fiasco2 B (10 µM)	100,0 µl		94	1min	
Total	200,0 µl		Bench	until RT	
1.1 y 1.2 Genomic DNA Digestion					
Digestion Test					
Enzyme:	**MSE I**				
DNA (aprox. 1 µg)	10,0 µl				
Enzyme buffer (NEB 2)	2,0 µl		37	O.Night	
BSA 100X	0,2 µl				
H20	7,0 µl				
Enzyme	1,0 µl				
Total	20,0 µl				
Gel 0.8% agarose	Run gel				
if test is OK, procede with the definitive digestion-ligation reaction					
DNA (aprox. 1 µg)	80,0 µl				
10x Enzyme buffer (NEB 2)	10,0 µl	Enzymes from New England,			
BSA 100X	1,0 µl	buffer NEB2 compatible with both			
ddH20	6,0 µl				
Enzyme	2,0 µl				
Ligase (High conc. 2000 U/ µl)	1,0 µl				
ATP (1mM final)	10,0 µl				
Adaptor (10 µM)	10,0 µl				
Total	100,0 µl				
Incubation			37	O.Night	
Inactivation			65	20min	
Gel 0.8% agarose	Run gel				
Cleaning of Lig-reactions with Sigma Purification columns for high MW DNA "GenElute" (NA 1020)					
Make PCR dilutions in ddH2O	1:5	or 1:10			
1.3 PCR post- ligation					
DNA	5,0 µl		94	2min	
Buffer 10X with 15mM MgCl2	2,5 µl		94	30s	17, 20
MgCl2	2,0 µl		53	1min	25, 30
Primer Fiasco MseI- N (4 bases)	3,0 µl		72	1min	cycles
dNTPs (10 µM)	4,0 µl		72	7min	
H2O	8,1 µl		4	infinite	
Taq (Normal or Expand Roche)	0,4 µl				
Total	25,0 µl				

	2.	**Hybridizing your genomic DNA**					
	2.1	**Probing reactions (adjusted to 100 µl)**			in a termocycler		
					you can try different hyb. profiles		
		in eppe 0,5 ml add:			96	10min	
					62	1h	
		DNA	10,0 µl				
		Biotinylated Probes (10 µM)	6,0 µl		96	10min	
		Hybridization solution	82,0 µl		40	1h	
		H2O	0,0 µl				
		Total	100,0 µl		96	10min	
					RT	1h	
		before capturing, cleaning through Sephadex is also possible at this step					
	3.	**Capture of microsatellites (enrichment)**					
	3.1	**Incubation with the beads**					
		Use 1mg of beads per each hybridization mix you want to enrich					
		1 mg of beads (DYNAL, 1mg =100 µl)					
		Wash together all the beads you will use 3 to 5 times					
		Wash adding buffer TEN100	100,0 µl	for each 1mg of beads			
		Magnetize, remove supernatant		3 to 5 times			
		After the last wash resuspend in					
		same buffer	40,0 or 50,0 µl	for each 1mg of beads			
		Add to the resuspended beads:					
		tRNA (Sigma, R- 5636)	5-10 µl	(10 µg)			
		Mix well!!					
		and add:					
		hyb mixes	100,0 µl				
		TEN 100	300,0 µl				
		Incubate @			RT with agit.	30-60 min	
					(150-200rpm)		
				or	33	3h	
	3.2	**Enriching Washes (with gentle agitation)**					4-6h
		1 non Stringent TEN1000	400 µl			5min	
		2 non Stringent TEN1000	400 µl			5min	
		3 non Stringent TEN1000	400 µl			5min	
		4 Stringent Solution	400 µl			5min	
		5 Stringent Solution	400 µl			5min	
		6 Stringent Solution	400 µl		42	5min	
	3.3	**Elution**					
		Add 150 µl TLE or ddH2O			95	10min	
		After magnetizing collect in a clean tube					
		Precipitation					
		Add 1 vol isopropanol	150,0 µl				
		NaOAc 3M	7,5 µl				
		O.N -20oC				O.N	
		Centrifuge				15-30 min	
		Wash with EtOH 70%					
		Centrifuge				5-10 min	

		Resuspend in H2O	30,0 µl				
		Store at -20oC					
	3.4	**PCR post-capture**					
					94	2min	
		DNA	2,0 µl		94	30s	
		Buffer 10X	2,5 µl		53	1min	30 cycles
		MgCl2	2,0 µl		72	1min	
		Primer Fiasco N (4 bases)	3,0 µl		72	7min	
		dNTPs (10 µM)	4,0 µl		4	infinite	
		ddH2O	11,1 µl				
		Taq (normal or Expand, Roche)	0,4 µl				
		Total	25,0 µl				
		Agarose Gel		0,8 to 1%			
		Cleaning of PCR products					
		Sephadex G-50					
		Taq 3' tailing					
		DNA (Clean PCR product)	8,0 µl				
		2mM dATP	4,0 µl				
		Buffer 10X with 15mM MgCl2	2,5 µl		72	30min	
		Normal Taq polymerase	0,2 µl (1,0U)				
		ddH2O	10,3 µl				
		Total	25 µl				
	4.	**Cloning**					
		Ligations	**PGEM**		**TOPO**		
		DNA	2,5 µl	DNA	2,5 µl		
		Ligase Buffer	5,0 µl	Salt	1,0 µl		
		Vector	0,5 µl	Vector	1,0 µl		
		ddH20	0,5 µl	ddH2O	1,5 µl		
		Ligase	1,5 µl				
		Total	10,0 µl		6,0 µl		
					incubate for 30min		
		Follow the instrcutions of manufacturers for transforming and growing cells					
	5.	**Screening**					
	5.1	**Colony preparation**					
		Pick 20 colonies and grow in tubes in 2ml media			37	grow O.N	
		with antibiotics (LB or terrific Broth)					
		alternative:					
		Grow in 96 microtitre plates with 150- 200 µl LB + antibiotic			37	grow O.N	
		in each well					
		(add Glycerol for long term storage after colony PCR)					
		Dilute O.N cultures with ddH2O					
		Cell suspention	5,0 µl				
		H2O	45,0 µl	It depends on concentrations of cells			
		Total	50,0 µl	obtained in the O.N growth			
		Alternative: you can try growing the bacteria for only 3 h and make the Colony PCR					
		using the cell suspentions directly without dilutions					

		Denaturation in termocycler			96	7- 10min	
		(to open cells and liberate the DNA)			On ice until PCR		
	5.2	**Do colony PCR**					
		DNA	1,0 µl		96	5min	
		Buffer 10X	2,5 µl		94	30s	
		dNTPs (10 µM)	2,5 µl		53	1min	30 cycles
		MgCl2 (25mM)	2,0 µl		72	1min	
		Primer M13 TopoF (10 µM)	1,0 µl		72	7min	
		Primer M13 TopoR (10 µM)	1,0 µl		4	infinite	
		Taq (normal or Expand, Roche)	0,12 µl				
		H2O	16,38 µl				
		Total	25,0 µl				
		Cleaning of the PCR products before sequencing					
		Sephadex G-50 or Exo-Sap treatment					
	5.3	**Sequencing**					
		DNA	3,0 µl				
		Big Dye v3.1	2,0 µl		96	10s	
		Buffer 5X	2,0 µl		50	5s	25 cycles
		Primer (10 µM)	1,0 µl		60	4min	
		ddH2O	2,0 µl		4	infinite	
		Total	10,0 µl				
		References					
		Hamilton *et al.* protocol 1999					
		Hamilton MB, Pincus EL, Di Fiore A, Fleischer C (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *BioTechniques* **27**, 500-507.					
		Zane *et al.* 2002 protocol					
		Zane L, Baegelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology* **11,** 1-16.					
		Apendix					
		Topo (M13) primers					
		5' GTA AAA CGA CGG CCA G	16bp				
		5' CAG GAA ACA GCT ATG AC	17bp				
		Sephadex G-50 Recipe to clean PCR and Sequencing products					
		Disolve 2g in 30ml ddH2O					
		boil in microwave for 30 seconds					
		Use @ RT. Mix well before use					
		Store @ 4oC					
		Procedure:					
		Fill CentriSep plastic columns with					
		Sephadex G-50	650,0 µl				
		Centrifuge* with a collector tube				2 min*	
		and discard ddH2O					
		Add PCR or Seq products to the	10,0 µl - 60,0 µl				
		centre of the packed column					
		Centrifuge* and collect purif. DNA				2 min*	
		in a new clean tube		* 0.7, 0.8 g = 2800 rpm in eppendorf 5415D			
		Additional for Sequencing…					
		Dry in a vacuum centrifuge				aprox. 15 min	

		Exo-SAP					
		Prepare a solution of 1:1 Exonuclease I					
		and Shrimp Alkaline Phosphatase					
		mixing the enzymes in ddH2O. Store @ -20oC					
		Use 0.5-1 U of each enzyme for every 20 ul of PCR reaction product					
		incubate			37	15min	
					80	15min	
		PCR product ready to use					
		Cleaning of sequencing reactions					
		Sephadex G-50 or					
		96 well Ethanol precipitation (Ethanol/EDTA/Sodium Acetate precipitation protocol from ABI					
		(Applied Biosystems, Protocol booklet 4337035 Rev. A, CA, USA)					
		Solutions as in Zane *et al.* 2002					
		TEN 100	(10mM Tris-HCl, 1mM EDTA, 100mM NaCl, pH 7.5)				
		TEN 1000	(10mM Tris-HCl, 1mM EDTA, 1M NaCl, pH 7.5)				
		Stringent solution	(SSC 0.2X, 0.1% SDS				
		Hybridization solution	(SSC 4.2X, SDS 0.7%)				

SUMMARY

Coniothyrium canker is a fungal disease of *Eucalyptus* spp. grown in plantations. It was first discovered in South Africa in 1989 on *Eucalyptus grandis* trees in plantations of Kwa-Zulu Natal. The pathogen was only described in 1997 when it became economically important to the forestry industry. Since this first report in South Africa, the disease has been reported from other African, South-east Asian and South American countries and the island of Hawaii. The fungus has the capacity to infect a wide range of new clones, hybrids and *Eucalyptus* species. Isolates obtained from single conidia are pleomorphic and lack definitive morphological characteristics. DNA sequence comparisons are therefore, essential for identification. In this study taxonomic questions regarding the causal agents of Coniothyrium canker are addressed using morphological and multilocus phylogenetic sequence analyses. Furthermore, this work includes the first studies on the population genetics on the causal agents of Coniothyrium canker. Polymorphic miscrostellites DNA regions were isolated and pairs of fluorescent primers were designed to amplify the microsatellites alleles using PCR technology. The analyses of the alleles showed that isolates from Coniothyrium canker represent two major independent lineages. During the course of this study, the taxonomic status of the Coniothyrium canker pathogens changed in several occasions including placement in genera such as *Coniothyrium*, *Colletogloeopsis*, *Kirramyces* and *Teratosphaeria*. Morphological and DNA phylogenetic studies identified differences to justify the separation of two major lineages that are now treated as *Teratosphaeria zuluensis* and *Teratosphaeria gauchensis*. The allelic analyses of the microsatellites regions confirmed the separation of lineages as there was no cross amplification between the species. Moderate levels of variation were found for both species but important differences were found regarding the composition and distribution of the genetic variation. Sexual recombination appeared not to be important in *T. zuluensis* but important in the population biology of *T. gauchensis.* Both species most probably did not originate in the areas where they were found and studied. Overall, this study has provided the methodological and theoretical foundation that will promote future work aimed at

understanding Coniothyrium canker and reducing damage due to this important disease.

CONCLUSIONS

Current scientific contributions of this study and future research directions

The taxonomic contribution of this work was to provide evidence that Coniothyrium canker on *Eucalyptus* is caused by two cryptic species, *Teratospaheria zuluensis* and *T. gauchensis* and not by one as previously thought. A re-evaluation of morphological characteristics revealed only minuscule differences in the conidiogeneous cells and conidial size that can be used to discriminate between these species. Temperature growth studies and DNA sequence analyses, however, allowed a clear separation between these two taxa.

Phylogenetic results showed that both *T. zuluensis* and *T. gauchensis* can be accommodated within the genus *Teratospaheria.* Beyond the pure taxonomic interest, the clarification of the taxonomic status of the cause of Coniothyrium canker was important to help interpret further results within an accurate historical and biological context. Interestingly, the closest known phylogenetic relatives of these two species are also pathogens of *Eucalyptus.* Evidence emerged to support the fact that some of these relatives are native Australian species associated with *Eucalyptus* trees in their centre of origin.

The development of microsatellites markers provided tools to gain additional evidence to support the separation of both these *Teratosphaeria* species. The flanking primers developed to amplify the microsatellite regions of *T. zuluensis* could not be used to amplify microsatellites regions on *T. gauchensis.* Likewise primers developed for *T. gauchensis,* when applied to *T. zuluensis,* often did not result in any amplification. This reinforced the conclusion that there is enough genetic divergence between these species to consider them as two different taxa.

The phylogenetic analysis using the ATP6 DNA region detected a common mitochondrial ancestor between both species. This probably reflects the speciation events leading to the final separation of these species. It would be interesting to further explore the sequence information contained in the flanking sequences of

the microsatellite regions and also to find appropriate nuclear information to investigate the historical connections between these two taxa.

Population studies with both *T. zuluensis* and *T. gauchensis* using the DNA microsatellites regions identified in this study produced results that were different than expected. Globally, the *T. zuluensis* populations were shown to contain moderate levels of genetic variation. Sexual recombination seems not to be frequent and there is thus no genetic support for the notion that *T. zuluensis* from South Africa is the source of the *T. zuluensis* populations in the other countries where it was reported. The *Teratospaheria gauchensis* populations in South America were initially thought to be recently introduced into these regions. Results from the population analysis, however, revealed these populations to be well established with high genetic variation. In addition, there was evidence of recombination. Consequently, it is most likely that the pathogen is native to South America.

The population genetic data for *T. zuluensis* from different countries showed that the different populations were in different epidemiological phases. Whereas there are indications that the populations are shrinking in South Africa, it is possible that populations are expanding in China. *Teratospaheria zuluensis* in Asia showed high variation and recombination which is compatible with a scenario of a species experiencing a population expansion phase. Additional collections and appropriate genetic analyses will be necessary to refine and test these new scenarios in the future.

Future research on *T. zuluensis* and *T. gauchensis* will require special attention to the sampling strategies. It will be crucial to chose the right scale and conduct adequate samplings in accordance with the questions that need to be answered. In this way it will be possible to increase the level of confidence of the analyses that are done.

The DNA regions studied and microsatellite markers developed in this study proved to be sensitive enough to detect good levels of singularities within populations and to be useful in the investigation of some of the population dynamics of these populations. In this regard there are two aspects that I feel would be worthy of further study. The first is the bimodal distribution in the South African

T. zuluensis populations. This is also reflected in the phylogenetic data. It would be very interesting to uncover the reasons for the persistence of this internal structure. For example, the bimodal distribution could indicate some sort of ecological adaptation, distribution of mating types or even pathogenicity differences.

The second aspect that is particularly worthy of further study is the fact that both species have been reported in the African continent: *T. zuluensis* in the south and *T. gauchensis* in the north. It would be interesting to investigate whether these two species have an overlapping geographical range and if so, to study the populations in those areas. Finding these species coinciding in one region would not be entirely unexpected as other related *Teratosphaeria* species have been found co-infecting *Eucalyptus* plantations. If these two species co-occur within the same niche, it would be important to investigate to what extend these two species are sharing the same resource. This is important information that would have vital consequences in making global quarantine decisions and also would contribute in the field of ecology to address questions in the context of the niche theory.

The question of the origin, sources and dispersal for the populations of the *T. zuluensis* and *T. gauchensis* changed substantially as a result of this study. Based on the phylogenetic results, the most logical explanation would be that these species originated in the native range of *Eucalyptus* as has been shown for other pathogens of the same phylogenetic group. It is, however, not always trivial to find pathogens in their native range. In addition, *Eucalyptus* trees have been present in South America and Southern Africa for more than one hundred years, originally introduced as ornamentals or used as wind breaks. This situation could have favoured the development of large saprophytic populations of fungi from which some could jump hosts and infect the more recently established *Eucalyptus* plantations. With time, an additional problem is that the historical signal is most probably tainted in all these countries by the sporadic introduction of genetic material by humans in the form of informal exchanges of seeds, infected plant material or transmission of material via clothes and shoes. The importance of transportation of new inoculum in this ways is difficult to measure and is generally agreed that it is underestimated. Therefore, it is possible that the starting

populations in the countries receive multiple introductions from time to time from a different population sources.

Future studies should aim at narrowing down the number of alternative hypothesis relating to the origin, establishment and dispersal *of T. zuluensis* and *T. gauchensis* populations. This could be achieved, in part, by finding answers for some of the basic biological questions regarding these fungi and by achieving a closer cooperation with the forestry sector to collect relevant information on frequency and routes of exchanges of plant material. From the biological point of view, it would be very important to locate the teleomorphs of these species. This would provide the opportunity to re-evaluate how important clonality is for these species and to get better insights as to how variation is created and maintained in these populations. Another important question will be to determine to what extent these organisms can survive as saprobes or, as demonstrated recently in Uruguay, whether in some circumstances they only cause mild symptoms making these pathogens more difficult to detect. Understanding these basic biological questions is particularly important in terms of quarantine.

By the time this study was started, only five articles were published on Coniothyrium canker disease. During the period of this study this number was doubled. These studies showed that the disease is caused by two symptomatically indistinguishable species, *T. zuluensis* and *T. gauchensis*. Although morphological and phylogenetically closely related, they showed that the establishment of populations worldwide was very different and that both species have very different population structures. The polymorphic microsatellite markers that are now available should make it easier to perform additional studies aimed at acquiring a profound knowledge on the population genetic of these species. As these two species are closely related, I envision new contributions going beyond the individual species level to perform comparative studies. Thus, studies of *T. zuluensis* and *T. gauchensis* offer excellent opportunity to those who want to contribute to the field of emergent pathogen diseases, query about the movement of mitotic fungi around the world and answer questions on the speciation process of fungal species.