PHYLOGENETIC REASSESSMENT AND POPULATION BIOLOGY OF THE *EUCALYPTUS* PATHOGEN *TERATOSPHAERIA SUTTONII* ISOLATED FROM DISEASED *EUCALYPTUS* LEAVES

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Phylogenetic re-assessment and population biology of the *Eucalyptus* pathogen *Teratosphaeria suttonii* isolated from diseased *Eucalyptus* leaves

By

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DECLARATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree Philosophiae Doctor to the University of Pretoria, contains my own independent work and has hitherto not been submitted for any other degree at any other University.

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Matšepo Motšelisi Taole

July 2013

I dedicate this thesis to my husband Mohapinyane and daughters Tsepang, limpho and Bokgabe who spent much of their time without a helping hand of a wife and mother, but together became a strong team that motivated me and gave me hope and strength through difficult times.

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PREFACE

The ability of eucalypt trees to grow in different soil aspects, under variable climatic and environmental conditions and to grow fast consistently producing straight stems has made them preferred plantation trees in many parts of the world. The world's increased demand for pulp wood has been the major contributor to the aggressive extensive development of eucalyptus plantations around the world. The productivity of these trees is however hindered in both native and introduced plantations by pests and pathogens.

Chapter 1 of this thesis is a literature review on *Teratosphaeria suttonii* as the causative agent of Teratosphaeria Leaf Disease on *Eucalyptus*. The chapter highlights the taxonomic placement of *T. suttonii* in the genus *Teratosphaeria* and the name changes of the species as a consequence of molecular taxonomy. The effects of the pathogen on *Eucalyptus* plantations in different parts of the world as well as the incidence and range of *Eucalyptus* species that host the pathogen are presented. In addition the review summarizes much of the information published on the distribution, hosts range and evolutionary relationships of *T. suttonii* with closely related species in the genus *Teratosphaeria.* In conclusion, it highlights the lack of understanding of the degree of diversity of the pathogen from different parts of the world.

In order to characterize and establish the phylogenetic relationships between *T. suttonii* isolates from different countries, chapter 2 of this thesis considered microscopic

examination and DNA sequence comparisons of isolates from its native and introduced localities. The isolates were classified into a single genetically and morphologically diverse group within which representatives of different countries were intermixed in smaller groups. A high level of genetic variation was evident among Australia isolates but despite the diversity there was no overwhelming evidence for sibling species.

In Chapter 3 of this thesis eleven microsatellite markers were developed from three *T. suttonii* isolates obtained from 2 geographic areas. The markers were used in Chapters 4 and 5 to investigate the genetic diversity of *T. suttonii* in both its native and introduced environments and to establish factors underlying such diversity.

The application of the markers in Chapters 4 and 5 provide the first consideration of the diversity of *T. suttonii* in both its native and introduced localities respectively. Both native and introduced pathogen populations exhibit substantially high genotypic diversities. It is evident from these studies that Australia is the point of origin of *T. suttonii* and that anthropogenic activities have resulted in repeated introductions of the pathogen from Australia into many countries.

Teratosphaeria suttonii is of great significance to the eucalyptus plantation industry across the world. Movement of diseased germplasm and introduction of new genotypes favors pathogen adaptability. In order to reduce the distribution of the pathogen into new areas or any other places where it might have negative effects, strict quarantine procedures should be followed.

CHAPTER 1

Teratosphaeria suttonii on *Eucalyptus*: Status and future prospects

Abstract

The expansion of eucalypts plantation areas in their native and introduced environments is hindered by associated pests and pathogens. At the beginning of the establishment of plantations in exotic localities, the eucalypts were successful. Their outstanding performance was attributed to the absence of pests and pathogens naturally associated with these trees in their regions of origin. The ability of these species to resist infection has however been reduced by the genetic uniformity of species planted in plantations, natural and human activities and unfavourable circumstances on site. Fungal foliar pathogens are impacting negatively on the *Eucalyptus* plantation industry worldwide and the species of the genus *Teratosphaeria* with *Kirramyces* anamorphs are important pathogens in this regard. In particular *T. suttonii* has a cosmopolitan distribution and infects a variety of *Eucalyptus* species.

1.0 Introduction

Over 800 eucalypt species are endemic to Australia (Turnbull 2000). Although sparsely distributed in the dry regions of the country, eucalypts dominate the higher rainfall areas. They constitute up to 95% of the continent's forests and outnumber nearly all types of vegetation except rainforest, central and high montane vegetation (Pryor and Johnson 1971). Species in the genera *Eucalyptus*, *Corymbia* and *Angophora* exist in all states of Australia and in almost all habitats, including sea level to 1,800m above sea level and from latitude 7^0N to 40^0S (Turnbull 2000).

The variable climatic and environmental conditions that prevail on the continent of Australia have provided a large variety of habitats to which eucalypts have adapted (Potts and Pederick 2000). Over the years, they have progressively and naturally evolved from rain forest progenitors and have acquired adaptive means, including the ability to grow fast and the potential to withstand and recover from harsh environmental conditions. These mechanisms have enabled them to withstand a wide variety of environmental challenges and out-compete native species in environments outside Australia (Eldridge et al. 1994). Consequently eucalypts exhibit several distinct developmental and genetic properties on which the selection and breeding of a variety of species suited to a wide range of habitats are based (Potts and Pederick 2000).

Eucalypts have been of great significance to native Australians for maintaining the hydrological balance, protecting the soil, providing wood products, medicine and resins and for maintaining the life of animals used as food for people (Turnbull 2000). With the

establishment of state forest services for the management of public forests in Australia at the beginning of the 20th century, commercial exploitation of this natural resource resulted in the production of significant amounts of timber and pulp wood from eucalypt forests. The early 1970's marked an increased pulp wood production which led to the development of an export market for Australian eucalypt woodchips to meet the demands for printing, writing and tissue papers (Turnbull 2000).

When eucalypts were first introduced from Australia to the rest of the world in the 19th century, their value as a source of industrial forest products was not immediately appreciated. However, once domesticated, the ability of some eucalypt species to grow rapidly and produce straight stems was recognized (Turnbull 1999). Owing to their substantially short rotation periods, ability to adapt to different soil aspects and climates, lack of ability to spread as weeds in most environments, ease of management through coppicing, and valuable wood and pulp properties**,** many eucalypts have been successively produced and widely distributed as exotics in plantations (Turnbull 2000, Wingfield et al. 2001). Since the early 20th century, eucalypt forests have served as a major source of wood products (Turnbull 2000).

The world's demand for pulp wood since the early 1980's, has been the driving force behind the rapid expansion of eucalypt plantings in many parts of the world, including Australia (Turnbull 2000). In spite of their natural distribution being limited to Australia, Papua New Guinea, Indonesia and the Philippines (Potts and Pederick 2000, Turnbull

2000), eucalypts are currently widely planted in most tropical and sub-tropical countries of the world (Turnbull 2000). According to Davidson (1995), 15% of the world total area of plantations in more than 70 countries is made up of *Eucalyptus*. Although precise and up to date global estimates of areas planted with eucalypts are difficult to obtain (Eldridge et al. 1994, Turnbull 1999, Turnbull 2000), the total global estimate of 13,000,000 ha for the major eucalypt growing regions and countries of the world for the years 1985, 1993 and 1999 has been reported (Davidson 1995, Eldridge et al. 1994, FAO 1999), 80% of which was reported in Asia and South America. The American statistics are primarily dominated by Brazil, a country reported to have the largest plantation area of eucalypts in the world (Eldridge et al. 1994). India, South Africa, Portugal, Spain, Angola, China, Ethiopia, Argentina, Morocco, Uruguay, Peru and Chile have been documented as some of the countries with the largest eucalypt plantation areas (Eldridge et al. 1994). The FAO (2007) reported 14 million hectares of *Eucalyptus* species and their hybrids in managed plantations.

In both the Northern and Southern Hemisphere, exotic *Eucalyptus* species benefit from being planted in intensively managed plantations in areas with higher rainfall (Eldridge et al. 1994). However, a major economic concern with the rapid expansion of plantation areas is the danger associated with pests and pathogens (Potts and Pederick 2000, Burgess and Wingfield 2002). Natural forest ecosystems display reduced susceptibility to disease epidemics as a result of the co-evolution of the trees and their pathogens and the diverse genetic and age structure of the hosts (Hansen 1999). On the contrary, the genetic uniformity of species planted in plantations together with the different types of natural

and anthropogenic activities (Burgess and Wingfield 2002), and the site circumstances that in general do not favour survival of the species being planted (Potts and Pederick 2000), have reduced the ability of these species to withstand infection and made the trees more susceptible to invasion by non-indigenous pests and pathogens. In the view of Wingfield (1999), the initial success of eucalypts as exotics is likely to be due to the absence of pests and pathogens naturally associated with these trees in their regions of origin.

The failure of the assumed boundaries between pathogens and newly introduced tree species became obvious in South Africa in the 1930s when the negative effects of Teratosphaeria Leaf Disease (TLD) (Hunter et al. 2011) on *E. globulus* lead to the discontinuation of the planting of this species in plantations (Purnell and Lundquist 1986). A similar situation also prevailed on *Eucalyptus* plantations in the equatorial humid tropics (Old et al. 2003a), probably due to humid environments sustaining high levels of leaf and shoot diseases responsible for the depressed growth rates and poor product quality (Old 2002, Old et al. 2003a).

Worldwide, the objectives behind the establishment of commercial plantations are escalated growth, increased canopy leaf area and early canopy closure (Turnbull 1999). Such aims can, however, be invalidated by the inherent destruction due to foliar fungi. Several leaf disease causing fungi are prevalent on eucalypts in native forests and plantations in Australia. Although these fungi hardly cause deleterious effects on native forests, they have turned out to be the main hindrance to cultivating productive

plantations in Australia (Park et al. 2000, Carnegie 2007a). Several foliage pathogens have been introduced into new areas through the germplasm used in the establishment of plantations (Wingfield et al. 2001) and together with local fungi and coevolved pathogens; they have been a serious impediment to the propagation of eucalypt plantations outside Australia (Park et al. 2000).

Shortly after exotic species were introduced many leaf and needle infecting pathogens surfaced in the tropics and Southern Hemisphere (Wingfield et al. 2001). Among the *Eucalyptus* pathogens, species of the genera *Teratosphaeria* and *Aulographina* appeared in the early stages of the establishment of plantations (Chipompha 1987, Crous and Wingfield 1996, 1997, Wingfield et al. 2001). These pathogens were thought to have been introduced from Australia where they occur naturally. The effect of leaf diseases has been most devastating in New Zealand (Dick 1982, 1990), South Africa (Crous et al. 1989a, d), Brazil (Ferreira 1989) and India (Sharma and Mohanan 1981), where the cultivation of eucalypts in plantations has been most extensive. Most species of *Teratosphaeria* infect eucalypt leaves resulting in Teratosphaeria Leaf Disease (TLD) caused by a range of different fungi (Crous and Wingfield 1997). This disease is regarded as one of the important constraints to *Eucalyptus* propagation in various parts of the world (Carnegie 2007a).

Of the various leaf pathogens, species of *Teratosphaeria* with *Kirramyces* anamorphs have been some of the most destructive. Eight species with *Kirramyces* anamorphs are known and these include the type species *T. suttonii*, *T. destructans*, *T. eucalypti*, *T.*

lilianiae, *T. delegatensis*, *T. toledana*, *T. viscidus* and *T. psuedoeucalypti*. These are all involved in causing Teratosphaeria Leaf Diseases (Kirramyces leaf diseases) of *Eucalyptus* species. Five of these species, namely *T. destructans, T. suttonii, T. eucalypti T. viscidus* and *T. pseudoeucalypti* have become known as a threat to the eucalypt plantation industry in the tropical and subtropical areas of Australia (Carnegie 2007a, b, Carnegie et al. 2008).

Teratosphaeria species with *Kirramyces* anamorphs generally infect mature and immature foliage (Andjic et al. 2007a). The characteristic disease symptom on *Eucalyptus* leaves is a sooty appearance due to irregular clusters of conidia exuding from substomatal pycnidia. Pycnidia may be associated with discrete chlorotic or necrotic lesions, or may exist on leaves surfaces with no signs of cell damage. These species are common nursery pathogens that produce very large amounts of inoculum on leaves and survive on fallen leaves (Old et al. 2003a). As a consequence, substantial losses can be incurred should these pathogens exist in young plantations (Wingfield 1990) since trees take many years for their commercial potential to be endorsed.

The appearance and severity of lesions on *Eucalyptus* leaves can be used to recognize the particular species responsible for disease. However accurate interpretation of the causal agent is usually difficult (Andjic et al. 2007a, b, Burgess et al. 2006, Hunter et al. 2011). This is because of the variety of disease symptoms between hosts (Barber 2004, Hunter et al. 2011), stages of development of infection and climatic conditions (Barber 2004, Walker 1962) and resemblance of symptoms associated with infection by the different

species (Andjic et al. 2007b, 2010a, Hunter et al. 2011). For instance, disease symptoms due to infection by the species *T. viscidus* were found to resemble those of *T. destructans* (Andjic et al. 2007c), while the symptoms due to infection by *T. pseudoeucalypti* were similar to those of *T. destructans* and *T. eucalypti* (Andjic et al. 2010b). Differentiation of species can be further complicated by the existence of different species on a single lesion or on one leaf (Burgess et al. 2006) and similar morphological features (Andjic et al. 2010b). Thus, the taxonomy of these fungi relies heavily on DNA sequence comparisons and spore morphology (Andjic et al. 2007a).

The species of *Teratosphaeria* with *Kirramyces* anamorphs infecting *Eucalyptus* leaves are essentially all known from Australia, while some species also occur elsewhere. *T. destructans* is considered a highly virulent pathogen and it was first described from Indonesia (Wingfield et al. 1996). It has subsequently been reported in Thailand, Vietnam, China and northern Australia (Old et al. 2003a, b, Barber 2004, Burgess et al. 2006, Burgess et al. 2007). *T. viscidus* and *T. pseudoeucalypti* have so far been reported only in Australia, where they are most likely endemic (Andjic et al. 2007b, Andjic et al. 2010b). The species *T. delegatensis, T. lilianiae* and *T. toledana* are also known only from Australia (Park and Keane 1984, Walker et al. 1992, Crous et al. 2004). *T. eucalypti* and *T. suttonii* are endemic pathogens to Australia (Carnegie 2007b), *T. eucalypti* has also been reported in New Zealand, while *T. suttonii* has been reported worldwide (Park et al. 2000, Walker et al. 1992).

Several *Eucalyptus* species host the species of *Teratosphaeria* with Kirramyces anamorphs. However specific host species are more susceptible to infection by one pathogen than others, so that while some hosts are shared among different species of the pathogen, other hosts are unique to specific species of the pathogen or the pathogen commonly occurs on the specific hosts. Among the important *Eucalyptus* pathogens, *T. viscidus* and *T. pseudoeucalypti* have been reported on 2 *Eucalyptus* host species (Andjic et al. 2007b, Andjic et al. 2010b), *T. destructans* has been reported on 3 host species (Wingfield et al. 1996, Old et al. 2003b), *T. eucalypti* on 23 different *Eucalyptus* species (Walker et al. 1992, Carnegie 2007a) and *T. suttonii* on 39 different species (Crous et al. 1988, Walker et al. 1992).

Studies based on DNA sequence data of these species have provided evidence on relationships, origin and movement of these pathogens. For instance, a strong phylogenetic relationship was established between *T. destructans* and *T. eucalypti* and *T. viscidus* and *T. pseudoeucalypti* (Andjic et al. 2007c, Andjic et al 2010b). In both studies *T. suttonii* isolates always grouped on their own, distant from the other species. Burgess et al. (2007) confirmed the presence of *T. destructans* in Australia and validated Australia as the place of origin of the pathogen. The genetic movement of *T. destructans* in South-east Asia has been determined (Andjic et al. 2011). The study also confirmed Asia as a foreign locality of the pathogen.

Other important disease complexes include stem and branch canker diseases of *Eucalyptus* and other plantation trees caused by *Botryosphaeria* species (Roux and

Wingfield 1997). Sigakota disease complex of banana caused by three related ascomycetous fungi namely *Mycosphaerella fujiensis, M. musicola* and *M. eumusae* (Crous and Mourichon 2002) and Teratosphaeria stem canker disease (Coniothyrium canker) of *Eucalyptus* species caused by *Teratosphaeria zuluensis* and *T. gauchensis* (Cortinas et al. 2006b).

The canker causing *Botryosphaeria* are endophytes of *Eucalyptus*, the fungi associate with wounds and the disease is expressed when trees are exposed to stressful conditions. Canker diseases due to *Botryosphaeria* species are common in tropical and temperate regions worldwide (Slippers and Wingfield 2007). Virulence of the species is difficult to ascertain since some species tend to be virulent pathogens when plants become stressed, but the most damaging species are those that have wide host and geographic ranges (Slippers et al. 2005a). The greatest negative impacts on *Eucalyptus* have been experienced in non-native environments. Like with TLD conidial characteristics of anamorphs are considered significant to species identification in this genus (Slippers et al. 2004, 2009).

Sigakota disease complex of banana is a threat to banana production worldwide (Crous and Mourichon 2002). Although diseases due to infection by. *M. fujiensis, M. musicola* and *M. eumusae* are referred to as black leaf streak disease, yellow sigakota disease, and eumusae leaf spot respectively, the signs of infection by these fungi are similar; they produce necrotic leaf spots, thus decreasing the photosynthetic efficacy of the plants (Crous and Mourichon 2002, Ploetz 2000). Both *M. fujiensis* and *M. musicola* have

caused great damage in banana plantations, but *M. fujiensis* is more destructive than *M. musicola* (Marin et al. 2003, Romero and Sutton 1997). *M. eumusae* infects varieties that are tolerant to *M. fujiensis* and *M. musicola* (Jones 2002).

The fungi causing Teratosphaeria stem canker disease on *Eucalyptus* are morphologically similar and portray indistinguishable signs of infection. *T. zuluensis* and *T. gauchensis* have resulted in serious losses on clonal *Eucalyptus* plantations in South Africa (Wingfield et al. 1996), and in Africa and South America respectively (Cortinas et al. 2006b). Both fungi occur on *Eucalyptus* species around the world and are closely related to other important leaf and stem infecting pathogens of *Eucalyptus* including *T. suttonii* (Cortinas et al. 2006b).

2.0 *Teratosphaeria suttonii*

2.1 Taxonomy

The development of a sound disease management procedure requires an ability to distinguish different diseases and an understanding of the causal agent (McCartney et al. 2003). Scientific names are used to distinguish organisms and disseminate information about them. In order to reflect newly established phylogenetic relationships, scientific names of fungal species change. The advent of molecular biological techniques has brought substantial genetic information to the fore, which has resulted in significant changes in the classical concepts of systematics and created a growing rigor to the

systematics of pathogenic fungi (Rossman and Hernandez 2008). The systematics of *T. suttonii* is no exception in this regard.

The taxonomic placement of members of *Teratosphaeria* with *Kirramyces* anamorphs has been a subject of debate since the identification of a *Psuedocercospora* species that was causing widespread damage on *Eucalyptus* species in South Africa (Crous et al. 1989b). The following synonyms have been applied to *T. suttonii*;

Readeriella epicoccoides (Cooke & Massee) Crous & U. Braun (2007)
Phaeophleospora epicoccoides (Cooke & Massee) Crous, F. A. Ferreira & Sutton (1997)
Kirramyces epicoccoides (Cooke & Massee) J Walker, B. Sutton & I. Pascoe (1992)
Cercospora epicoccoides Cooke & Massee apud Cooke (1891)
Hendersonia grandispora McAlp. (1903)
Phaeoseptoria eucalypti (Hansf.) J. Walker (1957)
Phaeoseptoria luzonensis T. Kobayashi (1978)

Walker et al. (1992) examined collections of *Cercospora epicoccoides* Cooke & Massee (1891) (Crous et al. 1989b) and made morphological comparisons of *Cercospora epicoccoides*, *Hendersonia grandispora* McAlpine (1903) and *Phaeoseptoria luzonensis* Kobayashi (1978). The study revealed incorrect placement of these species in these genera. These fungi could not be distinguished from the type and several other collections of *Phaeoseptoria eucalypti*. All these species were shown to represent a single fungus; they all had brown, rough-walled, multiseptate conidia that are produced from prominent rough walled, percurrently proliferating conidiogenous cells (Walker et al. 1992). Thus the findings of Swart and Walker (1988) and Kobayashi and Guzman (1988) who could

not distinguish *H. grandispora* and *P. luzonesis* from *Phaeoseptoria eucalypti*, respectively, were confirmed.

The genus *Phaeoseptoria* Spegazzini (1908) was found to be invalid for this group of fungi (Walker et al. 1992), since examination of specimen of the type species *Phaeoseptoria papayae* Speg illustrated the fungus to be pycnidial, with holoblastically produced conidia. The pronounced large, brown, cylindrical, rough walled, percurrently proliferating conidiogenous cells typical of members of the genus *Phaeoseptoria* could not be found. Thus all fungi previously considered as species of *Phaeoseptoria*, including *Phaeoseptoria eucalypti* were removed from the genus (Walker et al. 1992). In the search for an appropriate genus for *P eucalypti*, other related genera including *Scoleasis*, *Sonderhenia* and *Stagonospora* were considered. However these all had fundamental morphological differences from *P. eucalypti*. Therefore, a new generic name, *Kirramyces* J Walker, B. Sutton and I. Pascoe, was recommended for species with pycnidial conidiomata, brown, euseptate, cylindrical to narrowly obclavate rough-walled conidia and brown roughened annellidic conidiogenous cells (Walker et al. 1992). Three taxa, namely the type *K. epicoccoides*, *K. lilianiae* and *K. eucalypti* were placed in the genus. Although the latter species was characterized by pale yellowish-brown, delicately roughened conidia, the former two had characteristic brown rough-walled conidia and conidiogenous cells. The three species were retained in one genus.

The name *Kirramyces* was short lived. Fungi collected from leaf spots on *Eugenia uniflora* matched the definition and interpretation of *Phaeophleospora eugeniae* Rangel

(Crous et al. 1997). The species resembled species of the genus *Kirramyces*. No marked difference was observed between the two genera except colour of conidia and the number of conidial septa. Considering the differences in the pigmentation of conidia of *K. epicoccoides* and *K. lilianiae* from *K. eucalypti*, the observed morphological differences of the two genera were considered inadequate and unreliable to delineate taxa. *Kirramyces* was thus reduced to synonymy with *Phaeophleospora*, which took priority for the genus since it was an older name (Crous et al. 1997).

In conjunction with the traditional circumscription of species in the genus *Teratosphaeria* (then *Mycosphaerella*), (Park and Keane 1982), the sexual state of *Phaeophleospora epicoccoides, Mycosphaerella suttoniae* was described from leaves of a *Eucalyptus sp* from Indonesia (Crous and Wingfield 1997). The study provided a fundamental alliance between members of the two genera. Subsequently the first molecular evidence that *P. eugeniae* (the type species of the genus *Phaeophleospora*) is a member of *Teratosphaeria* was provided by phylogenetic studies based on the ITS region of the ribosomal cistron (Crous et al. 2001). Similar studies based on ITS sequence data (Crous et al. 2004, Andjic et al. 2007a) substantiated the proposed relationship of the genera *Phaeophleospora* and *Kirramyces* (Crous et al. 1997) and confirmed *P. eugeniae* and *P. destructans* to be congeneric.

Phaeophleospora eugeniae was found to be morphologically and phylogenetically distinct from other *Phaeophleospora* species, all occurring on *Eucalyptus* (Andjic et al.

2007b). Consequently, the genus *Kirramyces* was resurrected to accommodate both *Phaeophleospora* and *Colletogloeopsis* species from eucalypts (Andjic et al. 2007b). Previously taxa in the *Teratosphaeria* anamorph genera *Phaeophleospora* and *Colletogloeopsis* were separated based on the pycnidial and acervuloid conidiomata of the former and the later respectively (Crous and Wingfield 1997, Crous et al. 2004). A strongly supported phylogenetic relationship based on LSU rDNA and elongation factor 1-alpha DNA sequence data was established between *Eucalyptus* pathogens assigned to the *Teratosphaeria* anamorph genera *Phaeophleospora* and *Colletogloeopsis* (Hunter et al. 2006, Andjic et al. 2007a, b). Although pycnidial, the newly identified *Eucalyptus* pathogen *Coniothyrium zuluense* could not be accommodated into the anamorph genus *Phaeophleospora* because the type of *Phaeophleospora* clustered distant from the *C. zuluense* clade (Cortinas et al. 2006a). Based on the combined ITS and elongation factor 1-alpha DNA sequence data *P. eugeniae* was separated from other *Phaeophleospora* species (Andjic et al. 2007b). Considering the differences in conidial colour, size and number of septa of *P. eugeniae* that were earlier regarded as minor morphological differences (Crous et al. 1997) to delineate *Phaeophleospora* from *Kirramyces*, the description of the genus *Phaeophleospora* was emended to accommodate *P. eugeniae*. Species producing pycnidial, acervuloid or both types of fruiting bodies and aseptate or eusepate conidia were accommodated into the genus *Kirramyces.*

Crous et al. (2007) moved taxa in the anamorph genus *Kirramyces* to the genus *Readeriella* within the order *Capnodiales*. The rationale behind the transition was a previous confirmation that *Mycosphaerellaceae* resides within the order *Capnodiales*

(Schoch et al. 2006). Furthermore based on the DNA sequence data of the LSU gene, taxa within *Mycosphaerellaceae* were delineated into the families *Capnodiaceae, Trichosphaeriaceae, Davidiellaceae, Schizothyriaceae* and *Mycosphaerellaceae* within the order *Capnodiales* (Crous et al. 2007). Thus the authors confirmed the previous speculation of polyphyly of the genus *Mycosphaerella* based on the diverse anamorph genera related to the genus (Crous and Wingfield 1996) and the established polyphyly based on multi locus sequence typing (Hunter et al. 2006, Schoch et al. 2006). Taxa within the family *Mycosphaerellaceae* formed two phylogenetically and morphologically distinct groups, *Mycosphaerellaceae* and a new family *Teratosphaeriaceae*. The anamorph genera of the *Capnodiales* within the family *Teratosphaeriaceae* consisted of *Cibiessia, Phaeothecoidea, Devriesia, Capnobotryella, Hortaea, Nothostrasseria, Readeriella, Staninwardia, Penediella, Batcheloromyces* and *Catenulostroma* had variable conidiogenous cells, conidia, conidiomata and a range of synanamorphs distributed across the polyphyly. Among them, the anamorph genus name *Readeriella* (1908) was oldest and the genus polyphyletic. Several anamorph taxa within the *Teratosphaeria* clade including species in the genera *Colletogloeopsis*, *Septoria*, *Teratosphaeria* and *Kirramyces* shared the same method of conidiogenesis and displayed the same conidial pigmentation as the type *R. mirabilis*. Thus they were accommodated in *Readeriella,* and *K. epicoccoides* became *Readeriella epicoccoides* (Crous et al. 2007).

The application of a single generic name to anamorphs or teleomorphs in unambiguous monophyletic lineages as outlined by Wingfield et al. (2011) to the families *Mycosphaerellaceae* and *Teratosphaeriaceae* led to the removal of taxa with *Kirramyces*

anamorphs from *Readeriella* to *Teratosphaeria* (Crous et al. 2009b). The procedure followed was in conjunction with the work of Crous et al. (2006), who pursuant to Reynolds and Taylor (1992) grouped fungi in distinct *Botryosphaeriaceae* lineages together regardless of their reproduction state. Although the potential for nucleic acid comparisons to establish relationships in pleomorphic fungi had for a long time been considered (Guadet et al. 1989, Reynolds and Taylor 1991, Berbee and Taylor 1992), it was only then considered significant. The dual nomenclature system of pleomorphic plant pathogenic Ascomycetous fungi according to article 59 of the International Code of Botanical Nomenclature (ICBN) (Taylor 2011), hindered both the classification of this group of fungi and investigations on plant diseases and caused considerable controversy among plant pathologists (Wingfield et al. 2011). Consequently as a means to impede the dual nomenclature system, the "One Fungus = One Name" concept for newly segregated glades of genera, was established. Dual nomenclature has since been considered redundant on the part of fungal taxonomy and has been disregarded (Hawksworth et al. 2011).

The incorporation of several loci to the phylogeny of *Mycosphaerella* facilitated delineation of the genus into several distinct groups (Crous et al. 2007). While taxa in different clades and families proved to have acquired corresponding anamorph morphologies, teleomorph morphological characteristics remained conserved. The situation made it difficult to correlate teleomorphs with corresponding anamorph genera. The interpretations of different groups were further complicated by the difficulty to associate anamorph generic names of polyphyletic taxa to corresponding teleomorph

generic names (Crous et al. 2007). In order to clarify relatedness DNA based comparisons became essential. Information based on DNA sequence data superseded all of the morphological features that were dispersed across the different *Mycosphaerellaceae* and *Teratosphaeriaceae* clades. Irrespective of anamorph or teleomorph, a single generic name was applied to unambiguous monophyletic lineages and taxa previously accommodated into the *Mycosphaerella* species complex including taxa with *Kirramyces* and *Colletogloeopsis* anamorphs were given the genus name *Teratosphaeria* (Crous et al. 2009b). Species with both *Readeriella* and *Kirramyces* anamorphs could be distinguished phylogenetically and morphologically (Crous et al. 2009a, b), thus for *R. epicoccoides* the teleomorph name took precedence and the species became *Teratosphaeria suttonii.* It is for this reason that the name *T. suttonii* is used throughout the current review. This is despite the fact that other names that are now regarded as synonyms of *T. suttonii* are found in the majority of the reports of the biology and distribution of the fungus that follow. Species of *Teratosphaeria* with *Kirramyces* and *Colletogloeopsis* anamorphs and *Batcheloromyces*-like synanamorphs occupy clade 31 of the 31 clades obtained from a phylogeny based on DNA sequence data of the LSU region derived from taxa previously considered as members of the family *Mycosphaerellaceae* (Crous et al. 2009b). Fig 1 depicts *T. suttonii* isolates in a parsimony tree derived from ITS sequence data.

2.2 Occurrence, consequences and host range

The establishment of commercial *Eucalyptus* plantations in non-native areas has often been associated with unintended introductions of their indigenous pests and diseases.

Thus the natural geographic existence of eucalypt pathogens has been changed and modified through human related activities (Perez et al. 2012). *Teratosphaeria suttonii* is one of the most common leaf pathogens of eucalypts that have been reported and investigated from a variety of *Eucalyptus* species in many countries of the world. These countries include Africa, South America, Australia, South-East Asia, Japan, Indonesia, Philippines, New Zealand (Old et al. 2002), Vietnam (Old et al. 2003b) and the subtropics (Barber 2004). The species is prevalent on native eucalypts and in eucalypt plantations in subtropical and tropical Australia (Carnegie 2007a, b). It has been reported as one of the fungal pathogens that is greatly reducing the success of eucalypt plantations in Australia (Carnegie 2007a). Since the first report of this fungus in Sydney (Hansford 1957), *T. suttonii* spread to other parts of New South Wales, where it was reported from several *Eucalyptus* species (Walker 1962). Subsequently the fungus was confirmed as the most prevalent foliage spotting pathogen on a variety of *Eucalyptus* species in different parts of Australia (Table 1). Species widely cultivated in nurseries also suffered severe damage due to infection by this fungus (Walker et al. 1992).

Like many other *Eucalyptus* pathogens native to Australia, *T. suttonii* is speculated to have moved with the eucalypt plant material used for the establishment of plantations (Old et al. 2003b). During the years 1996-1997, *T. suttonii* was recorded from eucalypts in species trials in Indonesia (Old 2002). Significant damage due to the pathogen was also reported in nurseries and plantations in that country (Barber 2004). The fungus was found in nurseries and young *Eucalyptus* plantations in Hawaii (Gardner and Hodges

1988). Padaganur and Hiremath, (1973), Dick (1982) and Simpson et al. (2005) published the first reports of *T. suttonii* in India, New Zealand and China, respectively.

The first report of *T suttonii* in Africa was published by Chipompha (1987) in Malawi, and as in Australia, the fungus was described from *Eucalyptus grandis*. Five other *Eucalyptus* species (Table 2) were found to host the pathogen in that country. Wingfield, (1987) produced the first report of *T. suttonii* on a number of *Eucalyptus* species in many parts of South Africa. A critical survey and examination of diseased eucalypt leaves collected throughout the country (Crous et al. 1988), revealed 22 hosts of the pathogen, from the then four provinces of the country. The largest number of host species, 17, was recorded in Natal, followed by 14 species in Eastern Transvaal. In both South Africa and Malawi *T. suttonii* prevailed in young plantations and nurseries, in particular on *E. saligna* and *E grandis* (Knipscheer et al. 1990, Chipompha 1987). In view of Roux et al. (2005), plantation trees in most parts of Africa are propagated from imported seed with a narrow selection range. Such seeds do not always compliment the new environment. Consequently the trees are susceptible to stress, enhancing the ability of the pathogens to infect them and to evolve and survive. The situation appears to favour the occurrence of *T. suttonii*.

2.3 Diagnostic features

Teratosphaeria suttonii was first diagnosed from living leaves of *Eucalyptus grandis*, (Hansford 1957). The fungus was confirmed as the causative agent of local necrotic lesions on leaves of some *Eucalyptus* species (Heather 1965, Walker 1962). At the first

instance of disease, ostioles of subepidermal or substomatal pycnidia appear as minute black dots on the under surface of leaves (Chipompha 1987, Simpson et al. 2005, Walker 1962). Individual dead spots coalesce as a result of severe infection, forming characteristic angular, irregular purplish-red, brown-black lesions on living leaves (Padaganur and Hiremath 1973, Dick 1982, Chipompha 1987, Gardner and Hodges 1988, Crous et al. 1989b, Old et al. 2003b) (fig 2D). Individual lesions are small, measuring up to 7mm across (Dick 1982, Park et al. 2000). They are usually delimited by veins (fig 2D) and occur in large numbers on both sides of leaves (Gardner and Hodges 1988). Large numbers of conidia are released through the ostioles in the form of cirri that appear hair like on the leaf surfaces (fig 2C) (Chipompha 1987, Crous et al. 1989b), or spread over the leaves surfaces giving them a sooty appearance (fig 2A and B) (Cooke 1891, Chipompha 1987, Crous et al. 1989b, Park et al. 2000). Sometimes conidia occur throughout the leaf surfaces with little development of discrete lesions or cell damage, but infection is visible due to general discoloration giving the leaf a reddish or burgundy appearance (Andjic et al. 2007a, Old et al. 2003b).

Teratosphaeria suttonii has been distinguished from other *Teratosphaeria* species with *Kirramyces* anamorphs by its conidial morphology (fig 3A). Conidia (Table 3) have been reported as both rough walled and smooth walled (Hansford 1957, Walker 1962, Simpson et al. 2005, Gardner and Hodges 1988), with (1-) 3-5 (-7) septa, cylindric fusoid (Cooke 1891, Walker 1962), straight to sparsely curved, gradually tapering to a slender rounded attenuate paler apex and a subtrancate base (Walker 1962, Gardner and Hodges 1988, Crous et al. 1989b, Simpson et al. 2005). Conidia of *T. eucalypti* resemble those of *T.*

suttonii, being straight to slightly curved, but are 0-2 septate and pale brown (Walker et al. 1992). *T. viscidus* and *T. pseudoeucalypti* have hyaline to pale brown, straight to variously curved spores, with 0-3 septa (Andjic et al. 2007a, 2010b), while conidia of *T. destructans* are light brown, variously curved and rarely straight with 1-3 septa (Wingfield et al. 1996). The sexual state of this fungus is characterised by 8 spored bitunicate, fasciculate to broadly ellipsoidal straight to curved 35-45 × 10-12µm asci (fig 3B). Translucent, straight to curved, ovoid (10-) 11-12 (-13) × (2.5-) 3-3.5m ascospores, with broad apex and narrower lower ends. Spores have middle single septa and are not constricted at septa (fig 3B). Spermatia are rod shaped, translucent straight or slightly curved (fig 3B2), and measuring 5-7 x 1µm (Crous and Wingfield 1997).

2.4 Infection and disease development

Different eucalypt subgenera and species exhibit different degrees of susceptibility to infection by *T. suttonii*. While the subgenus *Macranthera* hosts *T. suttonii* in the native forests of Australia, other subgenera such as *Symphyomyrtus*, *Corymbia* and *Monocalyptus* have been reported to host the pathogen in nurseries and greenhouses (Heather 1965) and in plantation forests of Australia (Walker et al. 1992). The species of *Eucalyptus* in the subgenus *Symphyomyrtus* namely *E. grandis*, *E. grandis* clones, *E saligna*, *E. camuldulensis*, *E. globulus*, and *E. tereticornis* are the main hosts of *T. suttonii* (Tables 1 and 2). These species and many more, in the subgenus *Symphyomyrtus* have been shown to be more sensitive to *T. suttonii* (Nichol et al. 1992a). While 80% of the named host species published (Walker et al. 1992) fall into the subgenus *Symphyomyrtus*, the subgenera *Corymbia* and *Monocalyptus* make up 20% of the hosts.

All the *Eucalyptus* species in the subgenus *Monocalyptus* involved in a species trial were found to be resistant to *T. suttonii* (Nichol et al. 1992a).

Variable resistance and or susceptibility to *T. suttonii* have been reported among species in the subgenus *Symphyomyrtus* (Nichol et al. 1992a). Although some members of this subgenus, namely *E. maculata, E. saligna, E. microcorys, E. robusta* and *E. urophylla* were reported as hosts of this pathogen in Australia and other countries, no infection was reported on these species upon inspection in Malawi even though the pathogen was present on other members of the subgenus in that country (Chipompha et al. 1987). Thus, a number of factors play a role in the degree of susceptibility within subgenera and species. In view of Nichol et al. (1992a) the different climatic and edaphic conditions required for growth by each species make it difficult to ascertain differences in susceptibility.

Several biotic and abiotic factors influence the occurrence of disease caused by *T. suttonii* among the different *Eucalyptus* species. Adverse conditions where other pathogens or insects occur and physiological factors associated with the root zone of trees have been postulated to foster a high degree of susceptibility of *Eucalyptus* species to *T. suttonii* (Nichol et al. 1992b). Unsuitable soil conditions, weed competition, frost and drought can be other factors that could contribute to stress (Schoeneweiss 1981). Limited root penetration, increased temperature and canopy formation also could create conditions that enhance disease progress, while high levels of trace elements and fertilizer application could reduce disease incidence (Nichol et al. 1992b). Crous et al. (1989c) established that

both growth and defoliation rates play an important role since the disease was less pronounced on the seedlings of *E. camuldulensis* than of *E. grandis*, where the former species grew more vigorously than the later. Also, *E. nitens* seedlings suffered heavy infection, since the older infected leaves were not readily defoliated and served as a source of inoculum for secondary infection. Chipompha (1987) reported increased disease intensity in closely placed plantings.

The leaf wax and glaucousness of eucalypt leaves plays a significant role in the infection and resistance to pests and pathogens (Heather 1967a). The differences in the degrees of susceptibility of different *Eucalyptus* species to infection by *T. suttonii* are thought to be an attribute of the leaves plate wax layer, which is more granular and thicker in leaves of less susceptible hosts (Crous et al. 1988, 1989c). Increased disease resistance of successively younger *E. bicostata* leaves as well as the high level of infection observed on *Macrantherous* species with green juvenile foliage was explained in terms of increased glaucousness of the leaves (Heather 1967b). Although weathering could reduce the effectiveness of the glaucous barrier to infection, increased disease susceptibility with leaf age could not be implied by the mechanical effect of glaucousness alone since the lower leaves of *E. grandis* a non-glaucous species, had been reported to be 200% more susceptible to *T. suttonii* leaf spot than the upper leaves on the same plant (Heather 1965). The hydrophobic nature of the glaucous leaf coating affects spore deposition (Heather 1967b) and was considered to be more important than chemical inhibition of spore germination in affecting field infection of *E. bicostata* leaves by *T. suttonii***.**

The success of infection by *T. suttonii* is also dependent on the climatic conditions, which should be favourable for spore production, germination and subsequent penetration into the leaves (Knipscheer et al. 1990). Humid conditions have been reported to favour germination on the leaf surfaces. Germinating spores produce germ tubes, which regularly penetrate stomata (Crous et al. 1989c), subsequently the fungus grows intercellularly through the leaf mesophyll and leaf necrosis develops.

2.5 Control of the pathogen

The high demand of fibre for pulp mills across the world has led to rapid establishment of large *Eucalyptus* plantations. In many areas this has resulted in an escalated exchange and importation of seeds and vegetatively generated genotypes from different parts of the world, movement of people and sharing of forest products, equipment and staff among companies (Wingfield et al. 2008). This is in addition to possible host shift events between pathogens of other trees and eucalypts (Slippers et al. 2005b). These factors are making it difficult to predict the most likely pests and pathogens to be introduced into new environments and to control the introduction of associated pests and pathogens (Wingfield et al. 2008). In spite of efforts to apply integrated pest and pathogen management techniques (Turnbull 1999), the implied disease problems are steadily increasing in recently established non-native eucalypt plantations (Wingfield et al. 2008). Selection and breeding for resistant or tolerant germplasm has been proposed as the only meaningful, management approach for plantations (Old et al. 2003b, Crous et al. 1989c, Barber 2004).

Teratosphaeria suttonii affects seedlings, clonal hedges and young and mature eucalypt trees (Walker 1962, Heather 1965, Chipompha 1987, Gardner and Hodges 1988, Crous et al. 1988). Thus, control of this pathogen has been successful where intense nursery and plantation hygiene procedures such as the early detaching of diseased leaves, isolation and eradication of diseased seedlings and contaminated plant material from below seedlings (Jamaluddin et al. 1985, Crous et al. 1989c) and cutting down and burning of infected branches (Chipompha 1987) were practiced. Avoiding factors that favour disease development such as excessive water logging, frequent irrigation during winter and dense growth of stock (Jamaluddin et al. 1985, Harsh et al. 1987) have also helped contain the pathogen in many situations. The disease has also been successfully controlled by spraying with fungicides such as Mancozeb (Harsh et al. 1987) and Zineb (Chipompha 1987).

Other general recommendations for control of plantation forest pathogens include the detection and diagnosis before the planting stock is moved to plantations (Barber 2004), plant isolation from others until it is known that they are free from disease, and getting rid of alternative hosts on arable land adjacent to stands (Chipompha 1987). Quarantine restrictions on plant movement have also been recommended (Wingfield et al. 2001, Barber 2004, Brown and Ferreira 2000, Chipompha 1987).

3.0 Conclusion

Species of *Eucalyptus* are extensively planted in commercial forests worldwide. The wide distribution of these trees is attributed to their ability to survive in a broad range of

climates and soil conditions, their comparatively short rotation period and commendable fibre and pulp characteristics. A wide range of pests and pathogens have detrimental effects on eucalypts in their natural geographic range. When eucalypt plantations were first established in non native locations, their development improved, in comparison to that accomplished in their place of origin. The absence of any of their natural enemies supported the vigorous growth. But the condition only lasted for a short period. The trees were soon infected by new pests and pathogens native to the countries where they were propagated and introduced pests and pathogens associated with imported seeds. Natural and human disturbances have been reported to reduce the ability of these trees to withstand infection

Teratosphaeria disease of *Eucalyptus* is caused by a variety of fungal pathogens in the genus *Teratosphaeria*. The disease is causing substantial damage to *Eucalyptus* trees grown in plantations in Australia and in exotic plantations. Among these pathogens, *T suttonii* has a cosmopolitan distribution. In this review 36 *Eucalyptus* species and 7 clones have been reported to host this pathogen in 8 countries including Australia, South Africa, Malawi, China, Zambia, Hawaii, Vietnam and India. The majority of the species are members of the subgenus *Symphyomyrtus*, of which 73% were reported in Australia. *E. grandis* is the common host of *T. suttonii*, being reported in 6 countries, followed by *E. camuldulensis* reported in 4 countries.

Teratosphaeria suttonii exhibits both the sexual and asexual modes of reproduction. It infects *Eucalyptus* species in all stages of development resulting in continuous

defoliation, reduced growth and survival capacity and ultimately tree death. At present *T. suttonii* is considered one of the pathogens with a significant effect on the productivity of eucalypts.

It has been difficult to ascertain differences in the susceptibility of the hosts of this pathogen since different *Eucalyptus* species require different climatic and edaphic conditions for growth. Also, the occurrence of this disease among the different *Eucalyptus* species is influenced by several biotic and abiotic factors. Although several control strategies including the use of fungicides, nursery and plantation hygiene have been recommended and successful in the control of this pathogen, the disease continues to cause devastating effects in many parts of the world.

The correct identity and biology of pests and pathogens is necessary for the implementation of sound management strategies. Like many other fungi in the genus *Teratosphaeria* the taxonomy of *T. suttonii* has over the years been subject to debate.
First identified as *Phaeoseptoria eucalypti*, the nomenclature of this pathogen has changed over time. As new molecular and morphological data was obtained on the fungi and related genera, new relationships were discovered and old relationships found to be incorrect. Like other species and anamorphs of *Teratosphaeria*, *T. suttonii* was found to have evolved from several ancestral types, the situation which complicates the taxonomy of species. The application of a single genus name to both anamorphs and teleomorphs in unambiguous monophyletic lineages of the families *Mycosphaerellaceae* and *Teratosphaeriaceae* resulted in the present name *T. suttonii*.

Teratosphaeria suttonii is certainly moving around the world, and has devastating effects on *Eucalyptus* plantations. Although the origin, distribution, host range, and evolutionary relationships with closely related species in the genus *Teratosphaeria* is known, changes in the composition of this species over time and diversity of pathogen populations from different parts of the world have not been substantiated. When they are dispersed, pathogen populations do not conform to well defined modes of dissemination. Population genetics studies are however contributing significantly to the interpretation of their distribution.

The primary aims of investigations developed in this thesis will be to contribute to the regulation of the intensity and range of Teratosphaeria Leaf Disease due to *T. suttonii*, by confirming the classification, identification and diagnosis of isolates of *T. suttonii* collected from different parts of the world. Furthermore microsatellite markers will be used to conduct population genetic studies to establish the evolutionary potential of pathogen populations and genetic variation within and among population. Sources of introduction and patterns of spread of this pathogen will also be established. Molecular phylogenetics techniques will be used to substantiate macro evolutionary changes that have occurred within the species. Information acquired from these studies will help determine aspects associated with the genetics of the pathogen and can be used to conduct subsequent regulatory procedures for this pathogen.

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Table 1 Distribution and classification of species of *Eucalyptus* recorded as hosts of *T. suttonii* in different parts of Australia.

Host	Subgenus Classification	Location	Author
E. grandis	S	N.S.W.	Hansford (1957); Walker (1962); Walker et al. (1992); Carnegie et al. (2007a)
		Q.	Walker et al. (1992); Pegg et al. (2003)
E. camaldulensis	S	Q.	Walker et al. (1992); Pegg et al. (2003)
		V.	Walker et al. (1992)
		N.S.W	Carnegie (2007b)
E. marcathuri	S	N.S.W	Walker (1962); Walker et al. (1992)
E. maculata	C	N.S.W	Walker (1962); Walker et al. (1992)
E. saligna	S	N.S.W	Walker (1962); Walker et al. (1992)
		Q.	Walker et al. (1992)
E. citriodora	C	Q.	Walker et al. (1992); Pegg et al. (2003)
E. globulus	S	N.S.W	Walker et al. (1992)
		Q.	Walker et al. (1992); Pegg et al. (2003)
E. tereticornis	S	Q.	Walker et al. (1992); Pegg et al. (2003)
E. camuldulensis x grandis		Q.	Pegg et al. (2003);
		N.S.W	Carnegie (2007b)
E. sideroxylon	S	N.S.W	Walker (1962); Walker et al (1992)
		Q.	Walker et al (1992)
E. urophylla	S	*Q.	Walker et al. (1992)
E. microcorys	S	*Q.	Walker et al. (1992)
E. crebra	S	Q.	Walker et al. (1992)
E. dealbata	S	Q.	Walker et al. (1992)
E. drepanophylla	S	Q.	Walker et al. (1992)
E. exserta	S	Q.	Walker et al. (1992)
E. longifolia	S	N.S.W	Walker et al. (1992)
E. major	S	Q.	Walker et al. (1992)
E. punctata	S	Q.	Walker et al. (1992)
E. ampilifolia	S	N.S.W	Walker et al. (1992)
E. globulus ssp. Bicostata	S	N.S.W	Walker et al. (1992)
E. platypus	S	N.S.W	Walker et al. (1992)
E. radiate ssp. Robertsonii	M	V.	Walker et al. (1992)
E. resinifera	S	*V.	Walker et al. (1992)

Table 1 continued

Host	Subgenus Classification	Location	Author
E. robusta	S	*V.	Walker et al. (1992)
E. viminalis	S	V.	Walker et al. (1992)
E. delegatensis	M	T.	Walker et al. (1992)
E. cloeziana	I	Q.	Pegg et al. (2003)
E. argophloia		N.S.W (N)	Carnegie (2007a)
E. scias		N.S.W (N)	Carnegie (2007a)
E. longirostrata		N.S.W (N)	Carnegie (2007a)
E. grandis x urophylla		N.S.W	Carnegie (2007b)
E. grandis x tereticornis		N.S.W	Carnegie (2007b)

Subgeneric classification according to Pryor and Johnson (1971): C=Corymbia; M=Monocalyptus; S=Symphyomyrtus; I=Indiogenes. Distribution: N.S.W. = New South Wales; Q. = Queensland; T. = Tasmania; V = Victoria

Table 2 Distribution and classification of species of *Eucalyptus* recorded as hosts of *T. suttonii* in Africa and other localities of the world.

Host	Subgenus classification	Country	Author
E. grandis	*S*	South Africa	Crous *et al.* (1988)
		Malawi	Chipompha (1987)
		China	Simpson (2005)
		Zambia	Chungu *et al.* (2010)
		Hawaii	Gardner and Hodges (1988)
E. camaldulensis	S	South Africa	Crous *et al.* (1988)
		Vietnam	Old *et al*. (1999a)
		Malawi	Chipompha (1987)
E. globulus	S	South Africa	Crous *et al.* (1988)
		India	Padaganur and Hiremath (1973)
E. saligna	S	South Africa	Crous *et al.* (1988)
		Hawaii	Gardner and Hodges (1988)
E. tereticornis	S	South Africa	Crous *et al.* (1988)
		Malawi	Chipompha (1987)
E. grandis x urophylla		South Africa	Crous *et al.* (1988)
		China	Simpson (2005)
E. bicostata	S	South Africa	Crous *et al.* (1988)
E. cladocalyx	S	South Africa	Crous *et al.* (1988)
E. dunni	S	South Africa	Crous *et al.* (1988)
E. grandis x Camaldulensis		South Africa	Crous *et al.* (1988)
E. grandis x cladocalyx		South Africa	Crous *et al.* (1988)
E. grandis x nitens		South Africa	Crous *et al.* (1988)
E. grandis x tereticornis		South Africa	Crous *et al.* (1988)
E. macarthuri	S	South Africa	Crous *et al.* (1988)
E. macarthuri x grandis		South Africa	Crous *et al.* (1988)

Table 2 continued

Host	Subgenus classification	Country	Author
E. maidenii	S	South Africa	Crous *et al.* (1988)
E. nitens	S	South Africa	Crous *et al.* (1988)
E. nova-anglica	S	South Africa	Crous *et al.* (1988)
E. resinifera	S	South Africa	Crous *et al.* (1988)
E. urophylla	S	South Africa	Crous *et al.* (1988)
E. pellita	S	Malawi	Crous *et al.* (1988)
E. punctata	S	Malawi	Crous *et al.* (1988)
E. cloeziana	I	Zambia	Chungu *et al*. (2010)

Subgeneric classification according to Pryor and Johnson (1971): C=Corymbia; M=Monocalyptus; S=Symphyomyrtus; I=Indiogenes

Table 3 Reported size, number of septa and colour of *T. suttonii* conidia from eucalypts.

	Length (µm)	**Width (µm)**	**Number of septa**	**Colour**
Cooke, (1891)	50	5	3-5	-
Hansford, (1957)	-	-	(1) 3-5 (7)	Brown-pale olive
Walker, (1962)	(21) 36-57 (61)	3-5.5 (7)	(1) 3-5 (7)	Brown
Chipompha, (1987)	-	-	5 celled	Black
Gardner and Hodges (1988)	40-60	4-6	2- several	Yellowish-brown to Brown
Crous *et al*. (1989)	40-(47)-55	4 (5)-6	3-(4)-7	-
Simpson *et al*. (2005)	33-58	3.2-5.2	2-5	Brown

Figure 1 One of the most parsimonious trees constructed from ITS DNA sequence data derived from taxa considered as members of *Teratosphaeria*. *T. suttonii* isolates are highlighted.

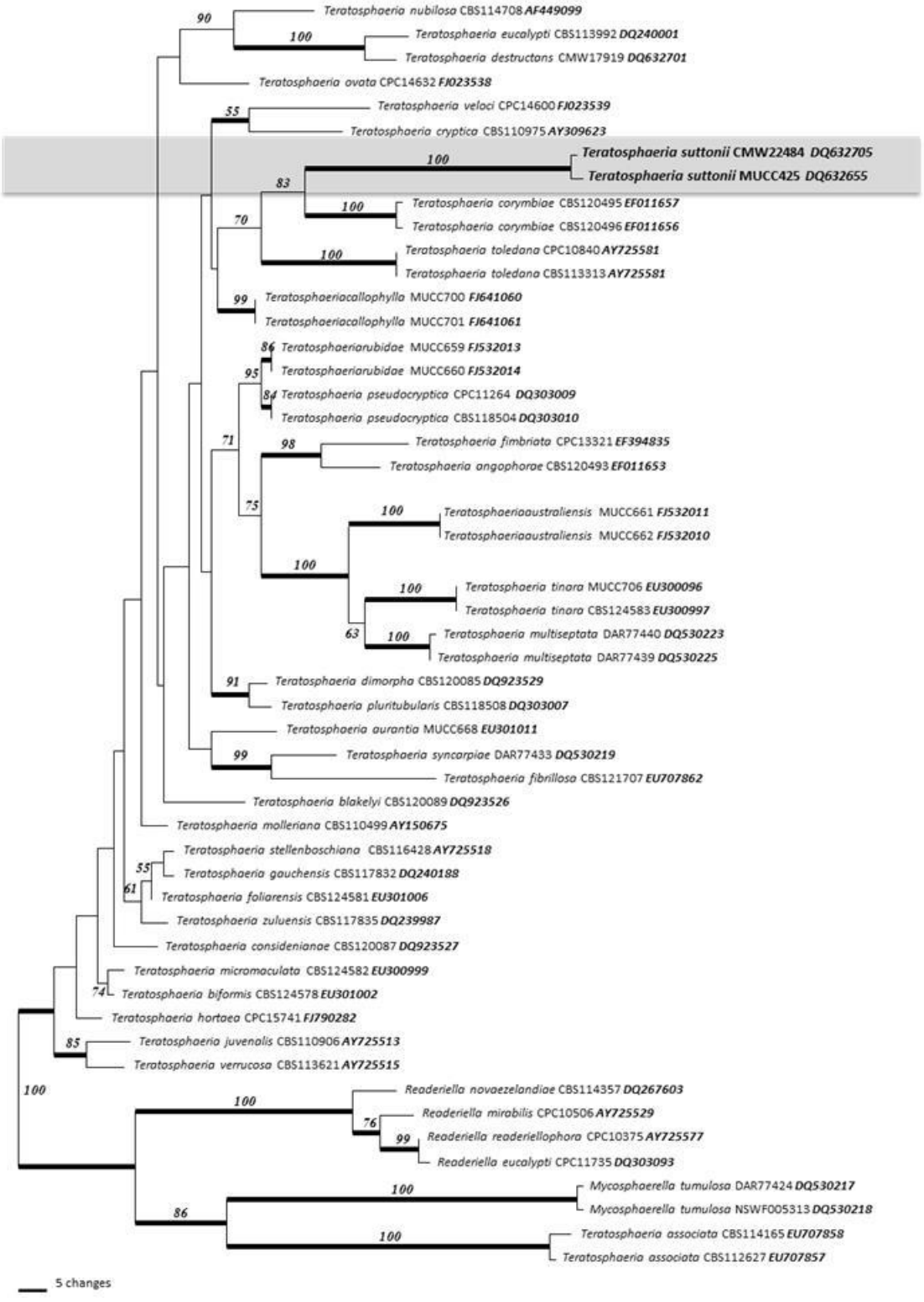


Figure 2 Symptoms on *Eucalyptus* leaves due to infection by *T. suttonii*. (A) and (B) sooty appearance of leaves due to accumulation of spore masses, (C) hair-like cirri of conidia on the leaf surface and (D) conidia associated with irregular purplish-red blotch

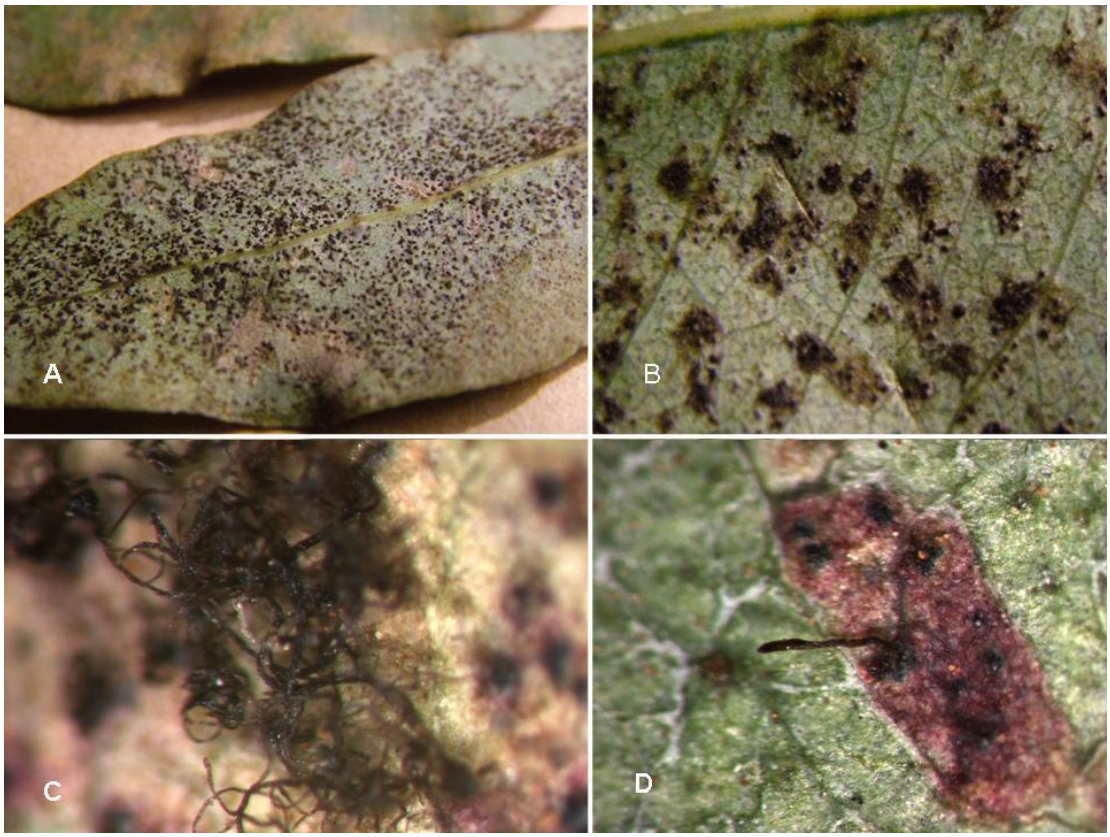
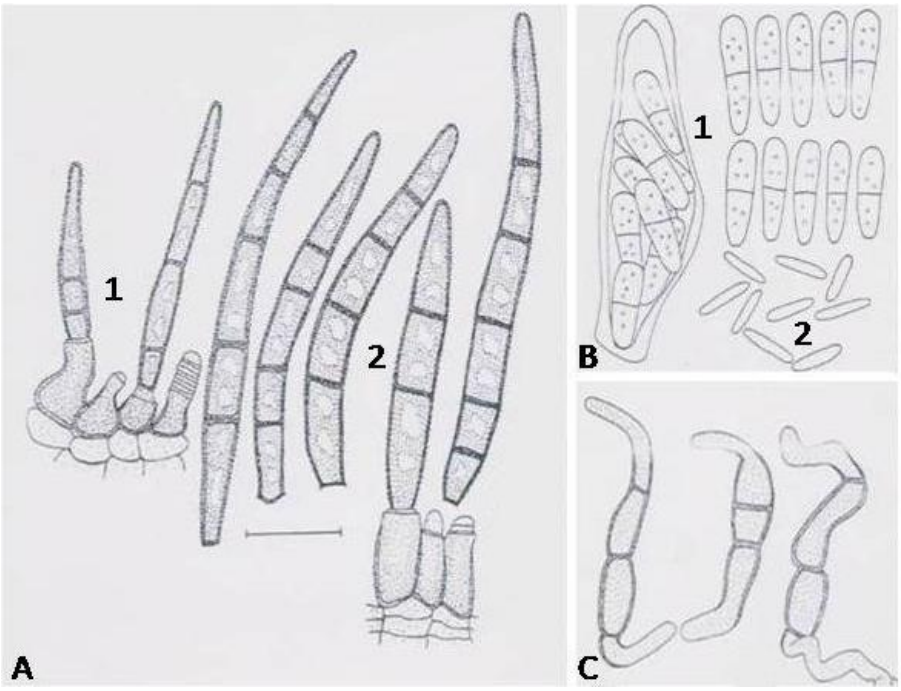


Figure 3 *Teratosphaeria suttonii* conidiogenous cells and conidia (A1) in vitro and (A2) in vivo; (B1) asci and ascospores; (B2) spermatia; (C) germinating ascospores.



CHAPTER 2

DNA sequence incongruence and inconsistent morphology obscure species boundaries in the *Teratosphaeria suttonii* species complex.

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Abstract

Teratosphaeria suttonii (= *Kirramyces epicoccoides*) is a leaf pathogen that can cause premature defoliation, reduced growth and vigor and subsequent tree death of many *Eucalyptus* species. Although the fungus primarily infects mature leaves in the lower canopy, infections can spread to younger leaves during continued epidemics or when trees are stressed. *Teratosphaeria suttonii* has a wide distribution in Australia and has been introduced to many other parts of the world, most probably with germplasm used to establish plantations. The aim of this study was to establish the phylogenetic relationships between *T. suttonii* isolates from different countries and to consider whether cryptic species exist in a species complex. DNA from parts of the nuclear ribosomal internal transcribed spacer (ITS), β-tubulin and elongation factor-1α genes was sequenced and analysed for isolates from throughout the range of *T. suttonii* in Australia, and from six countries (China, Indonesia, South Africa, Uruguay, USA and Vietnam) where the pathogen is introduced. Morphometrics of conidia produced both *in vivo* and *in vitro* were also considered. Analysis of the sequence data resulted in incongruent genealogies. Furthermore, groups of isolates in the genealogies could not be linked to area of origin. Likewise differences in conidial morphology could not be linked to any of the phylogenetic groups. There was no evidence of distinct species boundaries and isolates from Australia were closely related to those from other parts of the world. The results of this study support the treatment of *T. suttonii* as a morphologically and genetically

diverse species in its natural range in Australia. The diversity is reflected in introduced populations.

Introduction

Species of *Eucalyptus* are a primary source of fibre for the international paper and pulp industry (Turnbull 2000). The trees are propagated extensively as exotics in different parts of the world. The absence of pests and pathogens affecting trees in their natural environments has, in part, contributed to the success of introduced *Eucalyptus* plantations (Burgess and Wingfield 2002a, Wingfield et al. 2008). However, the productivity of commercial *Eucalyptus* plantations is increasingly reduced world-wide by diseases caused by fungi and insects (Wingfield et al. 2008). Of these, foliar fungi have been implicated in reduced growth, reduced canopy leaf area and delayed canopy closure in commercial plantations (Carnegie 2007b).

Teratosphaeria species are the most significant foliar pathogens of eucalypts (Carnegie et al. 1997, Park et al. 2000). They cause a complex of disease known as Teratosphaeria Leaf Disease (TLD) which has been reported to affect the eucalypt plantation industry in the subtropical and tropical areas of Australia (Andjic et al. 2010, Carnegie 2007a, b, Hunter et al. 2011). Species with *Kirramyces* anamorphs involved in this complex include *T. suttonii T. destructans T. eucalypti T. viscidus* and *T. pseudoeucalypti*. Previously placed in the genus *Kirramyces* and later *Readeriella*, these species were transferred to the genus *Teratosphaeria* following the application of one genus name for anamorphs or teleomorphs in unambiguous monophyletic lineages in the family's *Mycosphaerellaceae* and *Teratosphaeraceae* (Crous et al. 2009). The older name of the

teleomorph state of the fungus *T. suttonii* took precedence over the epithet *'epicoccoides'*. While the origin of *T. destructans* (Wingfield et al. 1996), the most destructive of these pathogens is unclear (Andjic et al. 2010a, Burgess et al. 2007), *T suttonii, T. eucalypti, T. viscidus* and *T. pseudoeucalypti* are endemic to Australia (Hansford 1957). These species have morphologically similar spores, display similar symptoms on infected *Eucalyptus* leaves and share common hosts. Unequivocal identification thus depends on DNA sequence comparisons (Andjic et al. 2007a, b, Burgess et al. 2006). Infection due to these fungi results in the formation of irregular chlorotic or necrotic lesions on both surfaces of the leaves (Andjic et al. 2007a, b, 2010a, Burgess et al. 2007). These lesions are usually associated with substomatal pycnidia that exude conidia. Spore masses appear as irregular brown-black clusters or smears mostly on the lower leaf surfaces.

Among the species in this group, *T. suttonii* has a cosmopolitan distribution. It has been reported in native *Eucalyptus* forests and plantations in Australia (Carnegie 2007a, b, Walker et al. 1992) and other countries where these trees are grown as non-natives (Chipompha 1987, Chungu et al. 2010, Crous et al. 1988, Gardner and Hodges 1988, Padaganur and Hiremath 1973, Simpson et al. 2005) from a variety of *Eucalyptus* species (Crous et al. 1988, Sankaran et al. 1995) in the sub-genera *Corymbia, Monocalyptus*, *Symphyomyrtus* and *Indiogenes*. Carnegie et al. (2007b) reported *T. suttonii* on three new hosts, namely *E. argophloia*, *E. scias* and *E. longirostrata.*

Teratosphaeria suttonii infects seedlings, clonal hedges and mature trees (Crous et al. 1988, Sankaran et al. 1995, Walker et al. 1992). It first manifests itself on ageing leaves in the lower canopy or on leaves of stressed trees (Carnegie 2007a, Crous et al. 1988, Nichol et al. 1992). The disease can spread to younger leaves higher in the canopies of trees during continued epidemics (Park et al. 2000). It has led to extensive defoliation of plantations in Australia and Indonesia (Old et al. 2003) and caused death of young plants in Malawi and South Africa (Chipompha 1987, Crous et al. 1989). It has recently caused substantial and comprehensive damage in young *Eucalyptus* plantations in New South Wales, Australia (Carnegie 2007b).

Leaf disease caused by *T. suttonii* is characterized by small, angular, irregular, purplish-red blotches on living leaves (Fig.1b, c) (Andjic et al. 2007b). Individual lesions are delimited by veins and occur in large numbers on both sides of leaves (Hodges and Gardner 1984). Pycnidia exude spores in grey-brown to black cirri that form hair-like extrusions on the leaf surfaces or conidia can spread over the leaf surfaces giving them a sooty appearance (Fig. 1c, d) (Walker et al. 1992). However, depending on the host, stages of development of infection and climatic conditions a variety of disease symptoms due to infection with *T. suttonii* can be observed (Pegg et al. 2003, Walker 1962), thus leading to incorrect diagnosis of the pathogen.

A multiple geneology study based of four gene regions grouped the isolates of *T. suttonii* from Australia, China, Indonesia, and South Africa into three well supported sub-clades that could not be related to geographical origin (Andjic et al. 2007b). Several studies have demonstrated the significance of Multi-Locus Sequence Typing (MLST) in resolving relationships among fungal strains, characterizing the genetic diversity and identifying cryptic species (Taylor and Fischer 2003). However, lack of congruence among gene-trees and species-trees often present problems when interpreting species relationships. The Genealogical Sorting Index (GSI) has been introduced to detect the progression from polyphyly to monophyly in diverging populations and approximate genealogical incongruence among taxa from which gene copies were sampled (Cummings et al. 2008). The GSI has recently been used to delineate a fungal species within the *Neofusicoccum parvum-N. ribis* species complex (Sakalidis et al. 2011). The level of monophyly of a group as represented by the GSI value can range from 0-1. GSI values at or close to 0 indicate the early stages of lineage sorting and thus absence of exclusive ancestry. The GSI value of 1 marks the end of the divergence process. For a given tree topology the statistical support for the observed monophyly is denoted by the corresponding p-value. The overall GSI value for gene trees from multiple unlinked loci is provided by the $GSI_{T,}$ which indicates the degree of distinctiveness of the genome of a group, relative to that of another group (Cummings et al. 2008), thus GSI is applicable for both single and multiple gene phylogenies.

The aims of this study were to use MLST to confirm the identity of the causal agent of Teratosphaeria Leaf Disease on eucalypts from various locations and hosts by comparing isolates collected from leaves of a variety of *Eucalyptus* species with known *T. suttonii* isolates and closely related species. A further aim was to establish the phylogenetic relationship of *T. suttonii* isolates from the different parts of the world and because differences have been observed in the morphology of conidia of this fungus, to consider whether collections do not encompass cryptic species.

Materials and Methods

Origin and sampling of fungal isolates

A collection of isolates used in this study were obtained from diseased *Eucalyptus* leaves sampled from Australia, China, Indonesia, South Africa, United States of America, Uruguay and Vietnam. Using a dissection microscope, conidia exuding from single pycnidia were collected at the tip of a sterile needle. Spores were placed on malt extract agar (MEA) (20g.L^{-1} Biolab malt extract, 15g.L^{-1} Biolab agar) and left to hydrate for 5min. Under a dissecting microscope, a sterile needle was used to draw conidia across the agar surface, after which single spores were picked from the agar and transferred onto new MEA plates with 150µgml^{-1} streptomycin (Sigma-Aldrich). Cultures were grown at 25^{o}C for 2 weeks, after which they were transferred to new MEA plates. Additional isolates from Australia, China and Indonesia were obtained from the Murdoch University Culture Collection (MUCC), Perth, Western Australia and from the collection (CMW) of

the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. All cultures are maintained in the CMW collection. Leaf specimens were deposited with (PREM), the South African National Mycological Herbarium.

Multi-Locus Sequence Typing

Total DNA was extracted from actively growing cultures by scraping mycelium from the surface of cultures into sterile 1.5ml Eppendorf tubes, and grinding freeze dried mycelium to a fine powder. This was followed by DNA extraction using the protocol of Cortinas et al. (2004), modified by adding Proteinase K (1µg/µl) to the extraction buffer and 5µl (1mg/ml) RNAse to the final product (incubated overnight at room temperature) to digest RNA. The presence of DNA was confirmed by electrophoresis on 2% (w/v) agarose gels (Roche Diagnostics) stained with ethidium bromide and visualized under Ultra Violet light. Subsequently, the concentrations of the extracted DNA were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies Rockland USA).

For the purpose of phylogenetic comparisons between isolates, PCR was used for the partial amplification of three gene regions. These included the internal transcribed spacer regions ITS1, ITS2 and 5.8S of the rDNA operon using the primers ITS-1 and ITS-4 (White et al. 1990), part of the β-tubulin genes using primers BT2a and BT2b (Glass and Donaldson 1995) and the transcription elongation factor-1α (EF-1 α) gene region using

primers EF1-728F and EF1-986R (Carbone and Kohn 1999). All amplifications were carried out in 25µl reaction mixtures, with cycling conditions as described previously (Cortinas et al. 2006) using the GeneAmp PCR system 2700 thermal cycler (Applied Biosystems). PCR products were purified and sequenced as described previously (Andjic et al. 2007b).

The resulting sequences of both strands were edited individually, and assembled into contigs using Vector NTI Advance ™ 11. An initial identification of the isolates was done by performing a similarity search of the ITS rDNA sequences (standard nucleotide BLAST) against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). Additional sequences of related species were retrieved from GenBank (Table 1). Sequences were aligned using Mafft version 6. Where required, adjustments were made manually by inserting gaps.

Parsimony analysis was executed using (PAUP) version 4.0 b10 (Swofford 2001), and its heuristic search component. The most parsimonious trees were obtained by employing heuristic searches with random stepwise addition in 100 replicates, the tree bisection-reconnection branch swapping option (TBR) on and the steepest-descent option off. The analysis excluded all parsimony-uninformative and ambiguous characters, and gaps were considered as a fifth character. Approximate levels of homoplasy and phylogenetic signal, tree length (TL), retention index (RI) and the consistency index (CI) were

determined. Trees were unlimited, tree branches of zero length were collapsed and the multiple equally parsimonious trees generated were saved. The bootstrap proportions (Felsenstein 1985) were used to estimate confidence levels of the phylogenies. Related species, including *T. destructans* (CMW17919), *T. eucalypti* (CMW17917), *T. cryptica* (CMW3279), *T. viscidus* (MUCC452), *T. zuluensis* (CBS117262), *T. nubilosa* (CMW11560) and *T. molleriana* (CMW4940), were treated as outgroup taxa. All sequences generated in this study were deposited in GenBank (Table 1).

The aligned data sets used in the parsimony analysis were subjected to Bayesian analysis. The process involved determining the best nucleotide substitution model using MrModeltest version 25 (Nylander 2004), Evolutionary Biology Centre, Uppsala University. Subsequently the specifications of the general time-reversible (GTR) substitution model and a proportion of invariable site (I) were used to integrate variable rates across sites as part of performing phylogenetic analyses with MrBayes version 3.1 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). Beginning at random tree topology, the Markov Chain Monte Carlo (MCMC) analysis of four chains went up 1,000,000 generations. Every 1000 generations trees were saved, resulting in 1001 saved trees. Burn-in was set at 50,000 generations (after the likelihood values converged to stationary), 950 trees were left, from which the consensus tree and posterior probabilities were calculated. The consensus tree was reconstructed in PAUP 4.0b10, and maximum posterior probabilities were allocated to branches following the construction of

a 50% majority rule consensus tree from sampled trees. Sequence alignments have been deposited in TreeBASE, submission S11949 (www.treebase.org).

Bootstrap consensus trees and trees generated from Bayesian analysis from individual gene trees were uploaded into the online Genealogical Sorting Index (GSI) program from www.geneologicalsortingindex.org. Each isolate was designated a corresponding ITS haplotype number previously obtained from a heuristic search of the ITS sequence data. Using 1000 permutations, the GSI was calculated following the methods of Cummings et al. (2008).

Morphological characterization

For the purpose of comparing *in vivo* and *in vitro* spore characteristics (i.e. spores obtained from specimen and cultures respectively), a sterile needle was used to obtain conidia from cultures and/or herbarium material. In order to induce sporulation, isolates were grown on 2% MEA and oatmeal agar (OMA) at 20^{o}C in the dark for 30 days. Conidia from leaf material and squash mounts of fruiting structures were mounted in lactic acid and water (1:1 volume), and observed under a compound microscope. Characteristics determined for each isolate or specimen included conidial size, shape, number of septa and pigmentation. Fifty measurements of spore length and width were recorded for each isolate or specimen at 1000X magnification, using the Carl Zeiss

microscope (Carl Zeiss Ltd., Mannheim, West Germany). The extremes of measurements were recorded in parenthesis.

Results

Multilocus sequence typing

The three gene regions ITS, EF-1 α and β-tubulin were sequenced for a total of 100, 90, and 88 isolates respectively including the ex-type sequences obtained from GenBank (CMW5348) (Table 1). A few isolates to be sequenced were selected at random from each of the populations from each country sampled. Phylogenetic analysis of the generated DNA sequence data was initially performed on data for the three gene regions separately. For each gene region, isolates with identical sequence data were given the same haplotype number (Table 1). The final analysis performed on the three gene regions separately was performed on a reduced number of isolates, which included representatives of all polymorphisms observed (indicated by an asterix in Table 1) and including the out groups.

The aligned ITS sequence data consisted of 617 characters, 127 base pairs of which were due to a large indel, in some isolates from Australia (47) and all isolates from South Africa, United States of America, Uruguay and Vietnam. Two Australian isolates had a shorter indel of 77 base pairs. The indel was coded and treated as a single evolutionary

event thus leaving 106 parsimony informative characters that were used in the analysis. The data contained significant phylogenetic signal (P<0.01; g1=-1.01) to allow for meaningful analysis. Heuristic searches of unweighted characters in PAUP resulted in six most parsimonious trees of 167 steps (CI=0.82, RI=0.96), of similar topology, of which one is presented in Fig. 2A.

There were 25 polymorphic loci for the *T. suttonii* isolates and this resulted in 14 ITS haplotypes (Table 1, Fig. 2). These were distributed into two main groups, the first containing haplotypes 1–9 and the second containing haplotypes 10–14. Haplotype 4 comprised of the highest number of isolates (30) followed by haplotypes 13 and 8. Haplotypes 1, 2, 3, 6, 8, 10 and 11 were found only in Australian isolates; haplotypes 1, 6 and 8 were restricted to Queensland isolates, haplotypes 2, 3, 10 and 11 were found in isolates from different parts of Australia. Haplotypes 4, 5, 7, 13 and 14 included isolates from Australia and other countries. These included the combinations Australia, South Africa, Uruguay and USA (haplotype 4), South Africa and Uruguay (haplotype 5), New South Wales and Vietnam (haplotype 7), New South Wales and Indonesia (haplotype 13) and Western Australia, Vietnam and China (haplotype 14). Haplotype 12 occurred only in China isolates and haplotype 9 was a single isolate from Queensland, Australia. ITS haplotypes 1, 8 and 12 resulted in equally high bootstrap values in parsimony and Bayesian analysis (Fig. 2A). ITS haplotypes 5, 6 and 11 had moderate parsimony and no posterior probability values, while ITS haplotypes 7, 10 and 14 had moderate parsimony and high posterior probability values. ITS haplotype 3 had no bootstrap support and a

high posterior probability and ITS haplotypes 2, 4, 9 and 13, were not supported. Isolates representing ITS haplotypes, marked with a superscript H in table 1, were deposited with the mycological culture collection of the South African Plant Protection Research Institute.

The aligned EF-1 α data set consisted of 290 characters, of which 112 were parsimony informative and included in the analysis. The data contained significant phylogenetic signal ($P<0.01$; g1 = -2.97). Heuristic searches of unweighted characters in PAUP resulted in a single most parsimonious tree of 261 steps (CI = 0.801, RI = 0.881) as represented in Fig. 2B. Among isolates of *T. suttonii* there were 3 polymorphic loci which resulted in 4 EF haplotypes (Table 1, Fig. 2B). The smallest supported group of isolates in the EF tree consisted of three isolates from China, corresponding to isolates from ITS haplotypes 12 and 14. The other three EF haplotypes were made up of ITS haplotypes 1, 2, 3, 4, 5, 6, 7, 8, 9 and 14 including isolates from Australia, South Africa, Uruguay, USA, and Vietnam; ITS haplotypes 4, 5, 7, 10 and 11 representing isolates from Australia and Uruguay; and ITS haplotypes 12, 13 and 14, representing isolates from Australia, China and Indonesia.

The β-tubulin dataset consisted of 381 characters, of which 2 were parsimony informative and were used in the analysis. The data contained significant phylogenetic signal ($P<0.01$; g1 = -2.25). Heuristic searches of unweighted characters in PAUP resulted in 2

trees of 140 steps (CI= 0.79, RI= 0.91). One of the trees was saved for representation (Fig. 2C). Among isolates of *T suttonii*, there were 2 polymorphic loci which resulted in 2 BT haplotypes (Table 1, Fig. 2C), thus reducing the 14 ITS haplotypes to 2 haplotypes. The smaller BT sub-clade had equally high bootstrap values in parsimony and Bayesian analyses; the other sub-clade was not supported by posterior probability values. The smaller BT sub-clade consisted of the ITS haplotypes 2, 4, 5, 9 and 11. The group included isolates from Australia, South Africa, Uruguay and USA. The larger sub-clade included isolates from Australia, China, Indonesia, South Africa and Vietnam, corresponding to ITS haplotypes 1, 2, 3, 4, 6, 7, 8, 10, 12, 13 and 14.

For the GSI analysis (Table 3), 31 isolates in 9 of the 14 ITS haplotypes, namely haplotypes 1, 5, 6, 7, 8, 10, 11, 12 and 14, had a genealogical divergence of 1.000. Haplotypes 2, 3 and 4 had low genealogical divergence values 0.118, 0.087 and 0.369 respectively, while haplotype 13 scored a moderate GSI value of 0.688. Taxa in all haplotypes except 2 and 3 showed significant estimates of measures of exclusive ancestry. Statistics could not be produced for the ITS haplotype 9 since it consists of a single haplotype. Significant measures of exclusive ancestry were estimated for taxa of the ITS haplotypes 1, 11 and 13 in BT, but overall low to moderate genealogical divergence was found for all isolates at the BT and EF loci scoring GSI values ranging from 0.002–0.824 and 0.004– 0.075 respectively. Failure to separate isolates on the BT and EF trees (Fig. 2B, C; Table 3) supported the low levels of genealogical divergence at these loci.

Morphological characterization

Variable conidial shapes were observed amongst the isolates examined (Figs. 3, 4). Spores were straight, slightly curved and curved. Spores from all isolates and herbarium material were generally brown, with a few that were faint brown at the narrow ends. A collection of spore measurements was obtained from representative isolates and herbarium material (Table 2). The lengths and widths of spores *in vivo* ranged from 28.49 µm to 77.49 µm and 2.06 µm to 7.62 µm respectively. *In vitro* spore length and width ranged from 16.07 µm to 66.57 µm and 2.30 µm to 6.98 µm respectively. The shortest spores were recorded *in vitro* from isolate CMW31916, while the longest spores were recorded *in vivo* from specimen PREM60495. The narrowest and widest spores were recorded *in vivo* from leaf material (PREM60540 and PREM60536). Where both *in vivo* and *in vitro*, measurements could be recorded lengths of conidia from herbarium material was generally greater than for the *in vitro* measurements. Conidial widths did not differ significantly for material from culture or from leaf tissue. The average length of spores for isolates CMW28689, CMW29223, and CMW32939 and from corresponding leaf material was similar. The number of septa recorded in the conidia ranged from 1–10. Where spores were observed both *in vitro* and *in vivo*, often fewer septa were recorded for those produced *in vitro*. In a few instances, the number of septa was the same both *in vivo* and *in vitro* collections. Single septate spores were obtained only *in vitro*.

Discussion

A multi-gene phylogeny was constructed for a large collection of *T. suttonii* isolates including those from across the natural range of this species, as well as from many countries where it has been introduced. Initial examination of ITS data alone revealed several distinct and strongly supported terminal clades suggesting that *T. suttonii* represents a species complex. This was consistent with early observations of considerable variability in the morphology of the conidia. However, much lower variability was observed in BT and EF sequence data. Additionally, the trees obtained for the different gene regions were not congruent and apart from the ITS region, there was no GSI support for monophyly for the other gene regions. The lack of clarity in the molecular support was also confounded by the wide range of conidial morphology in the fungus, with no relationships emerging consistent with the morphological and the molecular divisions. We therefore, conclude that *T. suttonii* represents a single, highly variable species. Such considerable genetic instability has also been observed in *Fusarium avenaceum* and *Fusarium heterosporum* isolates from the same host and geographic origin (Benyon et al. 2000). The high level of genetic variability was considered to be a factor of genetic instability. Similar to *T. suttonii* in this study, *Catenulostroma wingfieldii* has been reported to display variable spore characteristics (Crous et al. 2008).

Isolates from Australia were scattered throughout the phylogenetic trees and they included 12 of the 14 ITS haplotypes identified for *T. suttonii* in this study. This result was not surprising given the broad natural distribution of the fungus in eastern Australia

(Park et al. 2000; Walker et al. 1992). The two haplotypes not represented in Australia either represent unsampled haplotypes from Australia or they might be explained by a post-introduction mutation as Pérez et al. (2010) have shown for the related *T. nubilosa*. Isolates obtained from other countries where *T suttonii* is believed to be an introduced pathogen were considerably less variable, and represented by only one or two ITS haplotypes. According to the results, the haplotypes introduced into Asia are different to those introduced into South Africa, USA and Uruguay and they thus represent independent introductions, probably directly from Australia. It was, however, not possible to determine whether isolates found in Florida and Uruguay originated from Australia or South Africa since both the South Africa and Australia isolates share similar haplotypes with isolates from the former two countries. *Eucalyptus* planting stock, particularly in the form of seed has been widely traded around the world and it is believed that this has been one of the important sources of pathogens moving into new areas (Andjic et al. 2011; Wingfield et al. 2008). For example, *T. nubilosa*, an important *Eucalyptus* pathogen and relative of *T. suttonii* moved from Australia into South Africa and the latter country provided a beachhead for movement of the pathogen into Africa and Europe (Hunter et al. 2008). Thus, the origin of populations of forest pathogens such as *T suttonii* in countries can often be linked to global trade in forest products and not necessarily to introductions from countries where these pathogens are native as has for example been established for *T. nubilosa* and *D. pinea* (Hunter et al. 2008).

Interestingly, *T. suttonii* is not endemic to Western Australia (Jackson et al. 2008). In contrast, it was probably introduced in the early 2000's when the rapidly expanding eucalypt plantation industry was sourcing seedlings from nurseries all down the eastern seaboard of Australia. This was also reflected in the haplotypes of the pathogen observed in Western Australia in the current study where they were representative of isolates commonly found over a wide geographic range in Australia.

A high level of variability in conidial size, number of septa and shape was observed between isolates irrespective of their molecular aggregation. Conidial characteristics could not be related to origin, host or whether they were produced *in vivo* or *in vitro* under controlled conditions. Variation in spore morphology has previously been reported in *T. suttonii* (Knipscheer et al. 1990), although these authors did not mention all the different forms of variation observed in the current study. Based on the species description, *T. suttonii* is characterized by straight to slightly curved spores, that are brown, rough-walled with usually 3–5 septa, sinuate, narrowing to the rounded, slightly paler apex, sometimes slightly constricted at septa and measuring (21–)36–57(–61) × 3–5.5(–7) µm (Crous et al. 1989; Hansford 1957; Padaganur 1973; Walker 1962). In general, conidia from different isolates observed in this study fit the original and subsequent descriptions of *T. suttonii*. This study has used nucleotide sequences for three genes and phenotypic characteristics to identify fungal isolates from leaves with typical Teratosphaeria Leaf Disease symptoms thought to be associated with *T. suttonii* and to infer their evolutionary history. The morphological and molecular data at hand, the

indistinguishable symptoms of infection and similar biological niches provide convincing evidence for a single but diverse species rather than a species complex. Repeated recombination, interrupted deviation over time and incomplete geographical containment may have resulted in introgression between previously defined species (Taylor et al. 1999). Further studies using deep sequencing for the identification of SNP's (Perez 2010) may still elucidate species boundaries within this complex.

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Table 1 Isolates of *Teratosphaeria suttonii* considered in the phylogenetic study.

[A]Culture no.	PPRI no.	Herbarium no.	[B]Host	[C]Location	Isolator	[D]GenBank Accession No.		
						ITS	EF1-α	ß-tub
*MUCC575		PREM60538	*E. camaldulensis*	Australia; QLD, Davies Creek Plantation	TI Burgess	JF342909(1)	JF793414 (1)	JF793325 (1)
*MUCC576		PREM60539	*E. camaldulensis*	Australia; QLD, Davies Creek Plantation	TI Burgess	JF342910 (1)	JF793415 (1)	JF793326 (1)
*CMW31920			*E. grandis*	Australia, WA,	S Jackson	JF342911 (2)	JF793417 (1)	JF793327 (1)
*CMW31921			*E. grandis*	Australia, WA,	S Jackson	JF342913 (2)	JF793419 (1)	JF793329 (1)
CMW31924			*E. grandis*	Australia, WA,	S Jackson	JF342914 (2)	JF793420 (1)	JF793330 (1)
*CMW31923			*E. grandis*	Australia, WA,	S Jackson	JF342915 (2)	JF793421 (1)	JF793331 (1)
*CMW35799		PREM60518	*E. longifolia*	Australia; NSW, Garas Trial Plantation	AJ Carnegie	JF342944 (2)	JF793450 (1)	JF793359 (2)
*CMW35813[H]	11217	PREM60526	*Eucalyptus* sp.	Australia; NSW, Tunglebung	MM Taole & K Taylor	JF342954 (2)	JF793459 (1)	JF793369 (1)
*CMW31950			*Eucalyptus* sp	Australia; NSW	TI Burgess	JF342965 (2)	JF793416 (1)	JF793380 (1)
*CMW31916			*E. grandis*	Australia, WA,	S Jackson	JF342912 (3)	JF793418 (1)	JF793328 (1)
*CMW31934			*E. g x E. c*	Australia; QLD	G Hardy	JF342917 (3)	JF793423 (1)	JF793333 (1)
*CMW35620		PREM60506	*E. grandis*	Australia; Central QLD, Koumala	MM Taole & K Taylor	JF342949 (3)	JF793454 (1)	JF793364 (1)
CMW35650[H]	11218	PREM60507	*E. grandis*	Australia; Central QLD, Koumala	MM Taole & K Taylor	JF342950 (3)	JF793455 (1)	JF793365 (1)
CMW35685		PREM60508	*E. grandis*	Australia; Central QLD, Koumala	MM Taole & K Taylor	JF342951 (3)	JF793456 (1)	JF793366 (1)
*CMW35807		PREM60523	*Eucalyptus* sp	Australia; NSW, Tunglebung	MM Taole & K Taylor	JF342952 (3)	JF793457 (1)	JF793367 (1)
*CMW31936			*E. g x E. c*	Australia; QLD	G Hardy	JF342919 (4)	JF793425 (1)	JF793335 (1)
CMW31938			*E. g x E. c*	Australia; QLD	G Hardy	JF342920 (4)	JF793426 (1)	JF793336 (1)
*CMW31926			*Eucalyptus* sp	Australia; QLD, Mackay	TI Burgess	JF342922 (4)	JF793428 (1)	JF793338 (1)
*CMW35800		PREM60519	*E. longifolia*	Australia; NSW, Garas Trial Plantation	AJ Carnegie	JF342945 (4)	JF793451 (1)	JF793360 (2)
*CMW35796		PREM60516	*E. saligna*	Australia; NSW, Myrtle Greek	AJ Carnegie	JF342946 (4)	JF793452 (2)	JF793361 (2)
CMW35798		PREM60517	*E. saligna*	Australia; NSW, Myrtle Greek	AJ Carnegie	JF342947 (4)	JF793452(2)	JF793362 (1)
CMW35808		PREM60524	*Eucalyptus* sp	Australia; NSW, Tunglebung	MM Taole & K Taylor	JF342953 (4)	JF793458 (1)	JF793368 (1)
CMW35801		PREM60520	*E. grandis*	Australia; NSW, Morrow	MM Taole & K Taylor	JF342955 (4)	JF793460 (2)	JF793370 (2)
CMW35791		PREM60512	*E. grandis*	Australia; NSW, Burns	MM Taole & K Taylor	JF342956 (4)	JF793461 (1)	JF793371 (2)
CMW35536		PREM60504	*E. g x E. c*	Australia; NSW, Kew	V Andjic	JF342958 (4)	JF793463 (1)	JF793373 (1)

Table 1 continued

[A]Culture no.	PPRI no.	Herbarium no.	[B]Host	[C]Location	Isolator	[D]GenBank Accession No.		
						ITS	EF1-α	ß-tub
*CMW35547		PREM60505	*E. dunnii*	Australia; NSW, Emu Creek	V Andjic	JF342959 (4)	JF793464 (1)	JF793374 (1)
*CMW36017		PREM60536	*E. grandis*	Australia; Southern QLD, Imbill SF	MM Taole & K Taylor	JF342962 (4)	JF793467 (2)	JF793377 (2)
CMW36016		PREM60536	*E. grandis*	Australia; Southern QLD, Imbill SF	MM Taole & K Taylor	JF342961 (4)	JF793466 (1)	JF793376 (2)
CMW36011		PREM60535	*E. grandis*	Australia; Southern QLD, Imbill SF	MM Taole & K Taylor	JF342963 (4)	JF793468 (2)	JF793378 (1)
CMW35997		PREM60534	*E. grandis*	Australia; Southern QLD, Imbill SF	MM Taole & K Taylor	JF342964 (4)	JF793469 (1)	JF793379 (1)
CMW36020		PREM60537	*E. grandis*	Australia; Southern QLD, Imbill SF	MM Taole & K Taylor	JF342960 (4)	JF793465 (2)	JF793375 (1)
CMW28708		PREM60486	*E. g x E. u*	South Africa; Gauteng, Pretoria	MM Taole	JF342924 (4)	JF793430 (1)	JF793340 (2)
CMW29031		PREM60487	*E. g x E. u*	South Africa; Gauteng, Pretoria	MM Taole	JF342925 (4)	JF793431 (1)	JF793341 (2)
CMW32939		PREM60496	*E. g x E. u*	South Africa; Gauteng, Pretoria	MM Taole	JF342928 (4)	JF793434 (1)	JF793344 (2)
CMW32940		PREM60494	*E. g x E. u*	South Africa; Gauteng, Pretoria	MM Taole	JF342927 (4)	JF793433 (1)	JF793343 (2)
*CMW32941		PREM60498	*E. g x E. u*	South Africa; Gauteng, Pretoria	MM Taole	JF342926 (4)	JF793432 (3)	JF793342 (2)
*CMW32942		PREM60499	*E. g x E. u*	South Africa; Gauteng, Pretoria	MM Taole	JF342929 (4)	JF793435 (1)	JF793345 (1)
CMW32943		PREM60500	*E. g x E. u*	South Africa; Gauteng, Pretoria	MM Taole	JF342930 (4)	JF793436 (2)	JF793346 (2)
CMW32781 [H]	11219	PREM60492	*E. g x E. u*	Uruguay; Caldras	MJ Wingfield	JF342933 (4)	JF793439 (2)	JF793349 (2)
*CMW32814		PREM60494	*E. g x E. u*	Uruguay; La Negra	MJ Wingfield	JF342934 (4)	JF793440 (1)	JF793350 (2)
*CMW32803		PREM60493	*E. g x E. u*	Uruguay; La Negra	MJ Wingfield	JF342935 (4)	JF793441 (2)	JF793351 (2)
CMW34074			*E. grandis*	United States of America; Florida	MJ Wingfield	JF342937 (4)	JF793443 (1)	-
*CMW34077			*E. grandis*	United States of America; Florida	MJ Wingfield	JF342939 (4)	JF793445 (1)	JF793354 (2)
*CMW34081			*E. grandis*	United States of America; Florida	MJ Wingfield	JF342940 (4)	JF793446 (1)	JF793355 (2)
CMW34087			*E. grandis*	United States of America; Florida	MJ Wingfield	JF342938 (4)	JF793444 (1)	JF793353 (2)
*CMW28688		PREM60484	*E. g x E. u*	South Africa; Gauteng, Pretoria	MM Taole	JF342923 (5)	JF793429 (1)	JF793339 (2)
*CMW32817 [H]	11220	PREM60495	*E. g x E. u*	Uruguay; La Negra	MJ Wingfield	JF342936 (5)	JF793442 (1)	JF793352 (2)
*MUCC581		PREM60491	*Eucalyptus* sp	Australia; North QLD, Cairns	TI Burgess	JF342931 (6)	JF793437 (1)	JF793347 (1)
*MUCC582		PREM60491	*Eucalyptus* sp	Australia; North QLD, Cairns	TI Burgess	JF342932 (6)	JF793438 (1)	JF793348 (1)
**CMW35793		PREM60514	*E. saligna*	Australia; NSW, McMullen Road	AJ Carnegie	JF342948 (7)	JF793453 (2)	JF793363 (1)
**CMW35052 [H]	11221	PREM60501	*E. grandis*	Vietnam	TI Burgess	JF342957 (7)	JF793462 (1)	JF793372 (1)

Table 1 continued

[A]Culture no.	PPRI no.	Herbarium no.	[B]Host	[C]Location	Isolator	[D]GenBank Accession No.		
						ITS	EF1-α	ß-tub
CMW31939			*E. g x E. c*	Australia; QLD	G Hardy	JF342916 (8)	JF793422 (1)	JF793332 (1)
*CMW31933			*E. g x E. c*	Australia; QLD	G Hardy	JF342921 (8)	JF793427 (1)	JF793337 (1)
*CMW35768 [H]	11222	PREM60509	*E. grandis*	Australia; QLD, Davies Creek Plantation	MM Taole & K Taylor	JF342941 (8)	JF793447 (1)	JF793356 (1)
*CMW35776		PREM60511	*E. grandis*	Australia; QLD, Davies Creek Plantation	MM Taole & K Taylor	JF342943 (8)	JF793449 (1)	JF793358 (2)
CMW35771		PREM60510	*E. grandis*	Australia; QLD, Davies Creek Plantation	MM Taole & K Taylor	JF342942 (8)	JF793448 (1)	JF793357 (1)
*CMW31942 [H]	11223		*E. g x E. c*	Australia; QLD	G Hardy	JF342918 (9)	JF793424 (1)	JF793334 (2)
*CMW31918			*E. grandis*	Australia, WA,	S Jackson	JF342966 (10)	JF793470 (2)	-
*CMW35937 [H]	11224	PREM60529	*E. grandis*	Australia; NSW, Wedding Bells SF	MM Taole & K Taylor	JF342995 (10)	JF7934498 (2)	JF793409 (1)
*MUCC426			*E. globulus*	Western Australia	S Jackson	**DQ632704 (10)**	**DQ632715 (2)**	**DQ632620 (1)**
*MUCC577			*E. camuldulensis*	Australia; QLD, Davies Creek Plantation	TI Burgess	JF342974 (11)	JF793477 (2)	JF793388 (2)
*MUCC578			*E. camuldulensis*	Australia; QLD, Davies Creek Plantation	TI Burgess	JF342975 (11)	JF793478 (2)	JF793389 (2)
*MUCC579			*E. camuldulensis*	Australia; QLD, Davies Creek Plantation	TI Burgess	JF342976 (11)	JF793479 (2)	JF793390 (2)
*CMW35804 [H]	11225	PREM60521	*E. grandis*	Australia; NSW, Morrow	MM Taole & K Taylor	JF342997 (11)	JF793500 (2)	JF793411 (2)
*CMW30595 [H]	11226		*Eucalyptus* sp.	China	TI Burgess	JF342977 (12)	JF793480 (4)	JF793391 (1)
*CMW33001			*Eucalyptus* sp.	China	TI Burgess	JF342978 (12)	JF793481 (2)	JF793392 (1)
*CMW30597			*Eucalyptus* sp.	China	TI Burgess	JF342979 (12)	JF793482 (4)	JF793393 (1)
*CMW31946			*Eucalyptus* sp	Australia; NSW	TI Burgess	JF342967 (13)	JF793471 (2)	JF793382 (1)
CMW31947			*Eucalyptus* sp	Australia; NSW	TI Burgess	JF342968 (13)	JF793472 (2)	JF793383 (2)
*MUCC431			*E. grandis*	Australia; NSW	TI Burgess	**DQ530227 (13)**	-	-
*CMW35543		PREM60564	*E. grandis*	Australia; NSW; Kimbell Plantation	V Andjic	JF342987 (13)	JF793490 (2)	JF793401 (1)
*CMW35792		PREM60513	*E. saligna*	Australia; NSW, McMullen Road	AJ Carnegie	JF342988 (13)	JF793491 (2)	JF793402 (1)

Table 1 continued

[A]Culture no.	PPRI no.	Herbarium no.	[B]Host	[C]Location	Isolator	[D]GenBank Accession No.		
						ITS	EF1-α	ß-tub
CMW35794		PREM60515	*E. saligna*	Australia; NSW, McMullen Road	AJ Carnegie	JF342989 (13)	JF793492 (2)	JF793403 (1)
CMW35934		PREM60531	*E. grandis*	Australia; NSW, Wedding Bells SF	AJ Carnegie	JF342992 (13)	JF793495 (2)	JF793406 (1)
CMW35941 [H]	11227	PREM60532	*E. grandis*	Australia; NSW, Wedding Bells SF	AJ Carnegie	JF342990 (13)	JF793493 (2)	JF793404 (1)
CMW35943		PREM60527	*E. grandis*	Australia; NSW, Wedding Bells SF	AJ Carnegie	JF342991 (13)	JF793494 (2)	JF793405 (1)
*CMW18622			*Eucalyptus* sp	Indonesia	MJ Wingfield	JF342970 (13)	JF793473 (2)	JF793384 (1)
*CMW18625			*Eucalyptus* sp	Indonesia	MJ Wingfield	JF342971 (13)	JF793474 (2)	JF793385 (1)
*CMW18629			*Eucalyptus* sp	Indonesia	MJ Wingfield	JF342972 (13)	JF793475 (2)	JF793386 (1)
CMW18641			*Eucalyptus* sp	Indonesia	MJ Wingfield	JF342973 (13)	JF793476 (2)	JF793387 (1)
*CMW5348			*Eucalyptus* sp	Indonesia	MJ Wingfield	**AF309621(13)**	**DQ240170 (2)**	**DQ240117 (1)**
*CMW35947		PREM60533	*E. grandis*	Australia; NSW, Wedding Bells SF	AJ Carnegie	JF342993 (13)	JF793496 (2)	JF793407 (1)
CMW35935		PREM60528	*E. grandis*	Australia; NSW, Wedding Bells SF	MM Taole & K Taylor	JF342994 (13)	JF793497 (2)	JF793408 (2)
CMW35940		PREM60530	*E. grandis*	Australia; NSW, Wedding Bells SF	MM Taole & K Taylor	JF342996 (13)	JF793499 (2)	JF793410 (2)
*CMW35805		PREM60522	*E. grandis*	Australia; NSW, Morrow	MM Taole & K Taylor	JF342998 (13)	JF793501 (2)	JF793412 (1)
* MUCC428			*E. g x E. c*	Australia; QLD	TI Burgess	**DQ632707 (14)**	**DQ632717(1)**	**DQ632618 (1)**
CMW30584 [H]	11228		*Eucalyptus* sp	China; Kaiping	MJ Wingfield	JF342980 (14)	JF793483 (2)	JF793394 (1)
*CMW33000			*Eucalyptus* sp	China; Kaiping	MJ Wingfield	JF342981 (14)	JF793484 (2)	JF793395 (1)
CMW30585			*Eucalyptus* sp	China; Kaiping	MJ Wingfield	JF342982 (14)	JF793485 (2)	JF793396 (1)
*CMW29223		PREM60488	*Eucalyptus* sp	China; Fujian Province	MJ Wingfield	JF342983 (14)	JF793486 (2)	JF793397 (1)
*CMW29249		PREM60489	*Eucalyptus* sp	China; Fujian Province	MJ Wingfield	JF342984 (14)	JF793487 (2)	JF793398 (1)
*CMW35061		PREM60503	*E. urophylla*	Vietnam	TI Burgess	JF342985 (14)	JF793488 (2)	JF793399 (1)
*CMW35059		PREM60502	*E. urophylla*	Vietnam	TI Burgess	JF342986 (14)	JF793489 (4)	JF793400 (1)

[A] Designation of isolates and culture collections: CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; MUCC = Murdoch University culture collection, Australia; PPRI = Mycolological Culture Collection of the South African Plant Protection Research Institute
[B] Eg x Ec = *E. grandis* x *E. camaldulensis* hybrid; Eg x Eu = *E. grandis x E. urophylla* hybrid
[C] SF = State Forest
[D] Haplotypes for the isolates indicated in brackets next to GenBank numbers; Bold gene numbers represent sequence data from other studies
QLD = Queensland; WA = Western Australia; NSW = New South Wales
E = *Eucalyptus, ITS internal transcribed spacer, EF-1α elongation factor 1α*

Table 2 Characteristic features of conidia obtained from leaves and cultures.

Culture number	Herbarium number	Conidial length in vivo (µm)	Conidial length in vitro (µm)	Conidial width in vivo (µm)	Conidial width in vitro (µm)	No. of septa	Pigmentation
DC1.1 (1)	PREM60538	28.49 (33.83) – (47.21) 52.49	n/a	3.06 (3.40) – (6.40) 7.13	n/a	3-6	brown
DC1.2 (1)	PREM60539	43.00 (44.83) – (54.59) 64.44	n/a	4.38 (1.85) – (13.39) 7.62	n/a	5-10	brown
CMW31920 (2)		n/a	21.12 (26.86) – (39.02) 45.31	n/a	3.13 (3.40) – (4.52) 5.09	1-4	brown
CMW35813 (2)	PREM60526	n/a	32.03 (36.63) – (45.91) 52.19	n/a	2.52 (2.99) – (3.87) 4.47	2-4	brown
CMW31916 (3)		n/a	16.07 (19.75) - (28.25) 37.44	n/a	3.60 (3.94) - (4.86) 5.53	1-3	brown
CMW35620 (3)	PREM60506	n/a	32.55 (38.87) - (48.11) 57.17	n/a	3.03 (3.88) – (4.92) 5.43	3-7	brown
CMW35808 (4)	PREM60524	n/a	23.02 (26.67) – (35.15) 40.23	n/a	3.28 (3.69) – (4.73) 5.66	1-2	brown
CMW36017 (4)	PREM60536	41.59 (52.18) - (67.14) 74.68	25.5 (33.1) - (44.2) 47.51	3.87 (4.77) - (6.19) 7.02	2.86 (3.6) - (4.52) 5.29	2-5/1-3	brown
CMW34087 (4)		n/a	32.06 (44.07) -(57.03) 63.68	n/a	2.84 (3.59) - (4.81) 5.73	2-4	brown
CMW35791 (4)	PREM60512	n/a	24.55 (29.03) – (37.73) 39.72	n/a	2.52 (3.35) – (4.39) 5.46	1-3	brown
CMW32941 (4)	PREM60498	36.11 (39.38) - (48.38) 54.15	32.61 (39.98) – (49.18) 55.50	3.06 (3.37) - (4.53) 6.62	4.08 (4.58) - (6.02) 6.98	2-5	brown
CMW32942 (4)	PREM60499	44.04 (51.22) – (63.74) 67.69	n/a	3.9 (4.12) – (5.68) 6.78	n/a	3-4	brown
CMW32939 (4)	PREM60496	37.89 (40.57) – (46.99) 49.39	30.98 (36.78) – (49.77) 66.57	3.64 (4.04) – (5.40) 5.85	3.38 (3.94) - (5.4) 6.29	4-5/3-4	brown
CMW32803 (4)	PREM60493	49.49 (53.31) - (64.67) 69.67	n/a	4.07 (4.38) – (5.4) 6.03	n/a	3-6	brown
CMW32814 (4)	PREM60494	38.89 (52.08) – (62.9) 67.75	23.52 (28.86) - (38.71) 44.64	3.76 (4.3) – (5.22) 5.66	2.60 (3.49) - (4.57) 5.49	2-5/1-3	brown
CMW28688 (5)	PREM60484	30.56 (41.85) – (54.13) 63.84	36.23 (40.33) – (47.73) 52.10	3.57 (5.63) – (6.59) 6.11	3.52 (3.86) – (4.86) 5.6	2-5	brown
CMW32817 (5)	PREM60495	40.26 (48.48) – (64.74) 77.49	n/a	3.29 (4.09) – (5.19) 5.81	n/a	3-6	brown
CMW31930 (6)	PREM60491	36.11 (39.38) – (48.38) 54.15	28.01 (32.99) – (40.39) 44.89	3.06 (3.37) – (4.53) 6.62	2.90 (3.34) - (4.26) 5.10	2-4	brown
TIN2.2 (6)	PREM60491	30.01 (37.06) - (47.26) 52.22	n/a	3.38 (4.18) – (5.42) 6.33	n/a	3-6	brown
CMW35052 (7)	PREM60501	n/a	33.20 (37.98) – (47.74) 53.84	n/a	3.01 (3.85) – (5.07) 5.72	1-4	brown
CMW35768 (8)	PREM60509	n/a	31.31 (37.25) - (46.05) 52.43	n/a	2.63 (3.07) - (3.91) 4.47	3-5	brown
CMW35776 (8)	PREM60511	n/a	36.30 (42.24) -(51.56) 56.5	n/a	2.730 (3.44) -(4.40) 5.00	2-4	brown
CMW31933 (8)		n/a	34.85 (37.94) – (47.38) 54.22	n/a	2.79 (3.19) - (3.89) 4.39	2-4	brown
CMW31942 (9)		n/a	29.05 (32.80) – (39.58) 44.80	n/a	3.20 (3.65) – (4.75) 5.75	2-4	brown
CMW31918 (10)		n/a	17.69 (22.32) – (32.54) 39.43	n/a	3.39 (3.49) – (4.91) 6.31	1-3	brown
DC10.2 (11)		35.57 (39.10) – (48.08) 54.15	n/a	3.06 (3.37) – (4.53) 6.62	n/a	3-6	brown

Table 2 continued

Culture number	Herbarium number	Conidial length in vivo (µm)	Conidial length in vitro (µm)	Conidial width in vivo (µm)	Conidial width in vitro (µm)	No. of septa	Pigmentation
CMW35804 (11)	PREM60521	n/a	35.52 (39.25) - (48.67) 54.77	n/a	2.55 (2.90) - (3.94) 4.57	3-4	brown
CMW35947 (13)	PREM60533	n/a	22.79 (26.73) - (34.11) 37.42	n/a	3.35 (3.79) - (5.01) 6.06	1-3	brown
CMW18622 (13)		n/a	28.48 (31.18) - (39.18) 42.23	n/a	2.70 (3.03) - (3.77) 4.19	2-4	brown
CMW18625 (13)		n/a	42.02 (44.95) – (54.93) 61.51		2.79 (3.38) – (4.34) 5.08	3-4	brown
CMW18629 (13)		n/a	43.57 (49.43) – (59.53) 65.83		3.02 (3.56) – (4.82) 5.42	3-4	brown
CMW28689 (13)	PREM60485	36.11 (39.38) _ 48.38 (54.15	28.01 (32.99) - (40.39) 44.89	3.06 (3.39) - (4.53) 6.62	2.90 (3.34) - (4.26) 5.10	2-4	Brown
HERB2577 (13)	PREM60540	37.82 (51.23) – (65.77) 71.86	n/a	2.06 (2.37) – (3.05) 3.48	n/a	2-6	brown
HERB2676 (13)	PREM60541	32.01 (44.14) – (60.44) 71.49	n/a	2.15 (2.50) – (3.12) 3.36	n/a	2-3	brown
CMW29249 (14)	PREM60489	38.15 (41.76) – (53.72) 52.98	32.27 (50.48) – (58.46) 50.60	2.65 (3.53) – (4.43)5.25	3.08 (4.87) – (5.91) 5.39	3-6/3-5	brown
CMW29223 (14)	PREM60488	45.35 (52.79) – (64.39) 68.48	33.90 (38.71) – (48.51) 53.52	3.14 (3.74) – (5.06) 6.20	2.88 (3.37) – (4.19) 4.62	4-6/2-4	brown

The numbers in bracket next to the isolate numbers are the ITS haplotype numbers.
n/a = not applicable (conidial measurements not obtained)

Table 3 Genealogical Sorting Index (GSI) and probability values for the consensus bootstrap trees from parsimony analysis of sequence data from the three gene regions

ITS haplotype	N	ITS	EF	BT
1	2	1.000 P=0.002	0.005 P=0.575	0.824 P=0.001
2	5	0.118 P=0.032	0.024 P=0.185	0.007 P=0.519
3	4	0.087 P=0.084	0.017 P=0.268	0.027 P=0.150
4	12	0.369 P<0.001	0.075 P=0.012	0.029 P=0.201
5	2	1.000 P=0.004	0.006 P=0.576	0.051 P=0.069
6	2	1.000 P=0.004	0.006 P=0.569	0.002 P=0.934
7	2	1.000 P=0.002	0.006 P=0.575	0.008 P=0.459
8	3	1.000 P<0.001	0.012 P=0.367	0.018 P=0.257
9	1			
10	3	1.000 P<0.001	0.012 P=0.394	0.005 P=0.850
11	4	1.000 P<0.001	0.017 P=0.267	0.600 P=0.004
12	3	1.000 P<0.001	0.004 P=0.751	0.018 P=0.260
13	5	0.688 P<0.001	0.008 P=0.592	0.080 P=0.012
14	10	1.000 P<0.001	0.026 P=0.195	0.047 P=0.054

Figure 1. Symptoms associated with infections by *Teratosphaeria suttonii*. (a). Trees severely defoliated. (b). Branch showing infections typically on the older leaves. (c). Sporulation of the fungus from stomata on the under surface of a *Eucalyptus* leaf. (d). Infected leaf of a cutting during the rooting process where the infection by the fungus can reduce root strike.

Figure 2. Phylogenetic relationships among *Teratosphaeria suttonii* isolates included in the study. The trees present a summary of the Parsimony and Bayesian analyses of DNA sequence data obtained from the ITS (A), EF-1 α (B) and β-tubulin (C) gene regions. Estimates of nodal support have been deduced from Parsimony jackknifing (above nodes) and Bayesian inference analysis (below nodes). The different colors represent the 14 ITS haplotypes, how they have been reduced and intermixed in the EF and BT phylogenies.

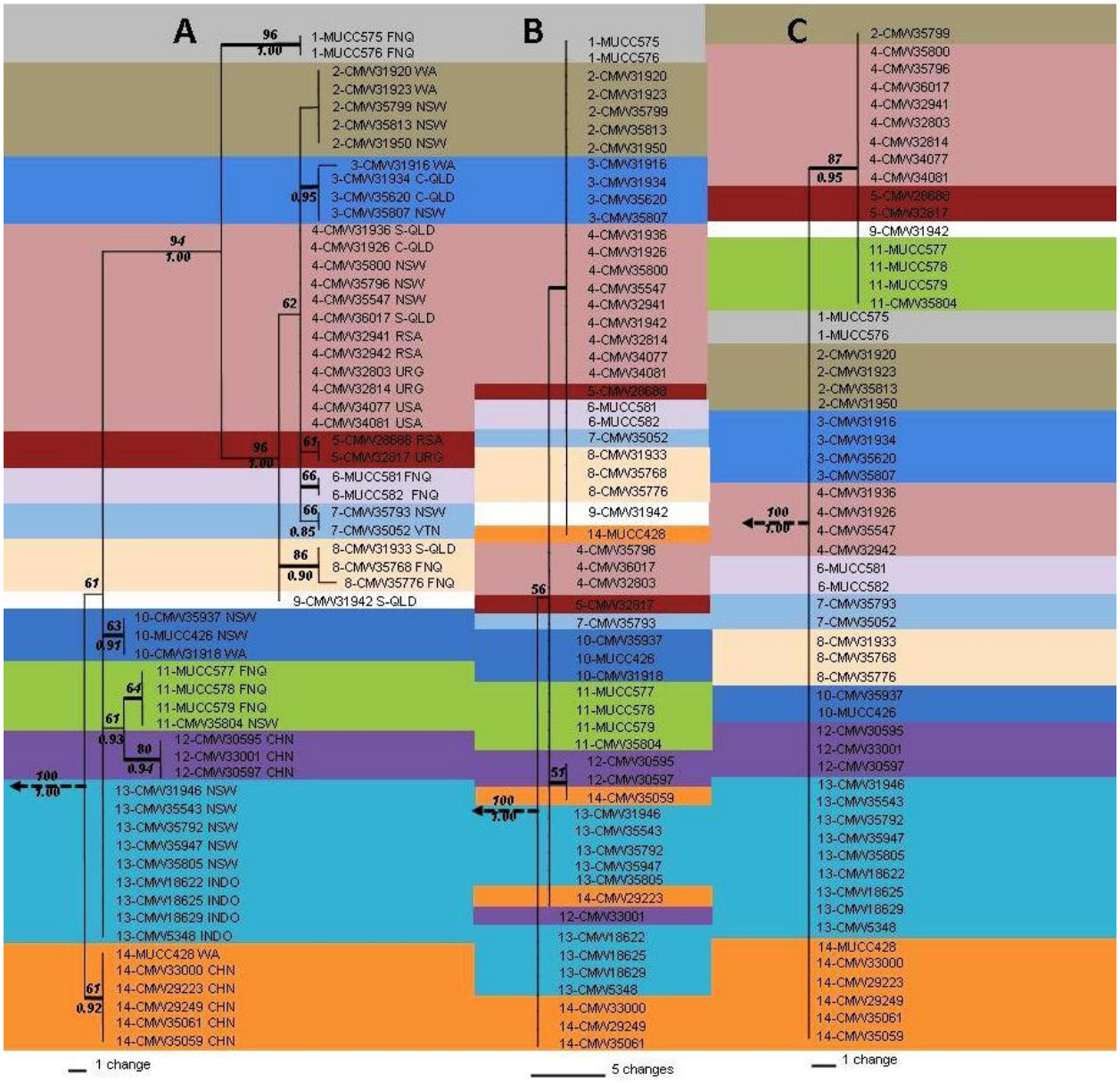


Figure 3. Average lengths and widths of spores obtained from cultures and leaves. Error bars represent the standard deviation

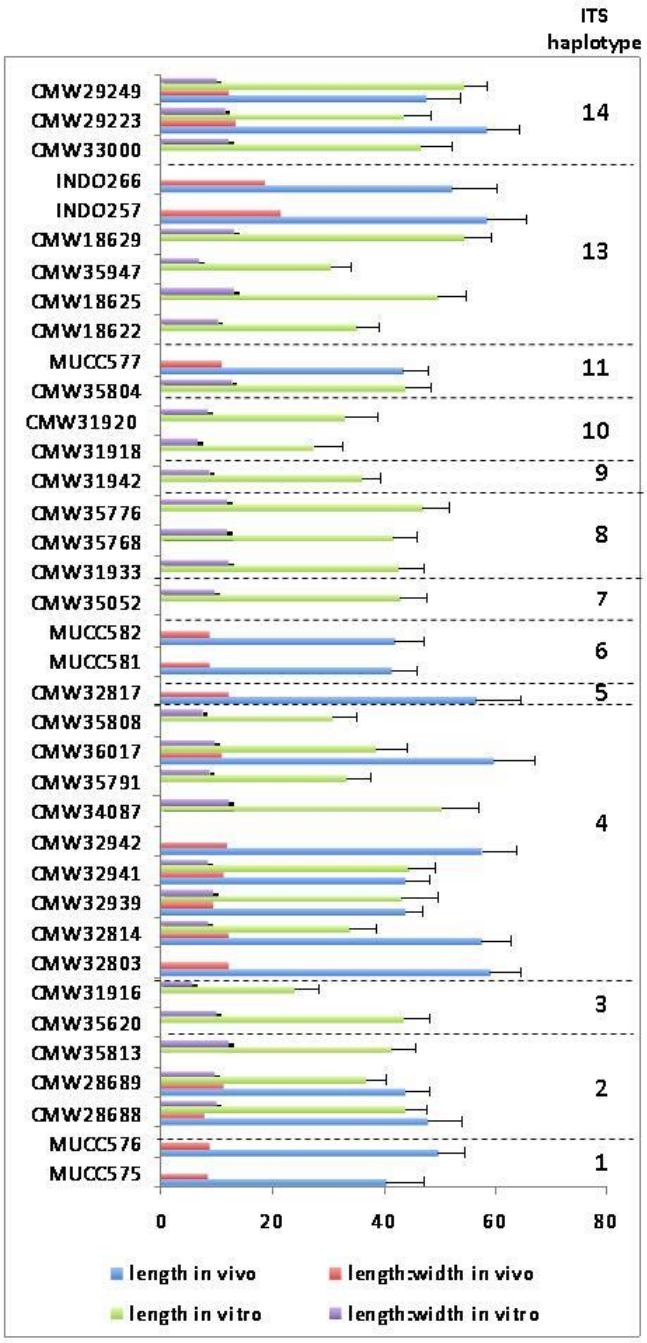
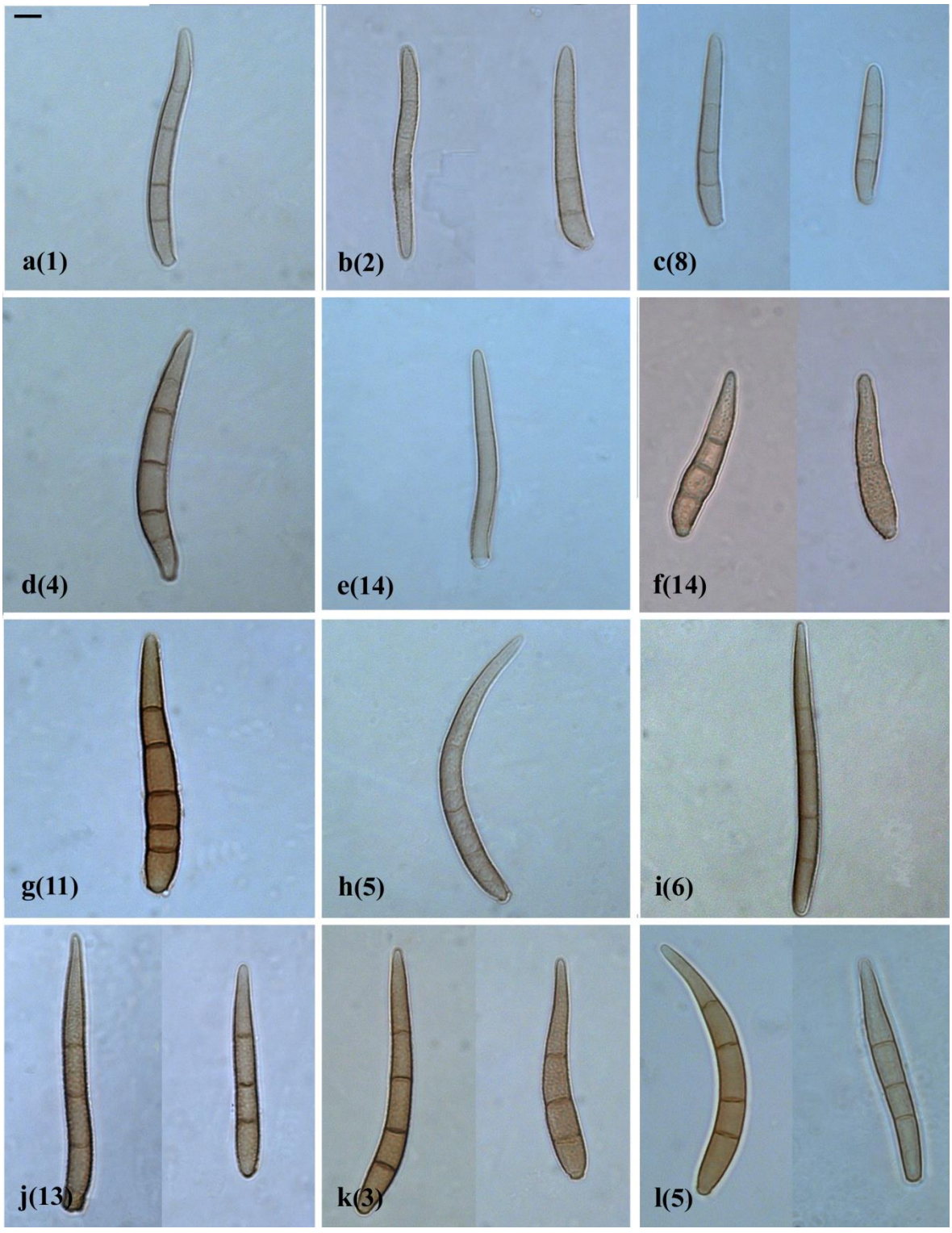


Figure 4. Conidia obtained from isolates of *Teratosphaeria suttonii*. (a) CMW35813, (b) CMW35768, (c) CMW35791, (d) CMW29223, (e) CMW18629, (f) CMW31916 and leaf specimen (g) PREM60538, (h) PREM60488, (i) DC10, (j) PREM60484, (k) PREM60491, (l) PREM60495. Where two spore images are given, as for b, c, f, j, k and l, the image on the right represents spores from culture and the one on the left from an infected leaf. The numbers in brackets represent those for ITS haplotypes.



CHAPTER 3

Polymorphic microsatellite markers developed for the *Eucalyptus* leaf pathogen *Teratosphaeria suttonii*.

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Abstract

Eleven microsatellite markers were obtained and characterized for *Teratosphaeria suttonii*, a fungus that causes Teratosphaeria Leaf Disease (TLD) of *Eucalyptus* trees. Forty primer pairs from potential microsatellite regions were designed from ISSR-PCR generated fragments. Eleven of these bound to target regions and were polymorphic for *T. suttonii*. A total of 52 alleles with allelic diversity ranging from 0.38 to 0.83 were recognized in 62 isolates of *T. suttonii*. These microsatellite markers can now be applied to study the population biology of *T. suttonii* which is believed to be native in Australia but spread to many countries where *Eucalyptus* spp. sustain major forestry industries.

Introduction, materials, methods, results and discussion

Despite substantial efforts to control diseases using chemical fungicides and genetic resistance, dramatic economic losses due to pathogens have prevailed in agriculture and forestry (McDonald and McDermott 1993). Over successive generations, the genetic composition of pathogen populations changes allowing adaption and survival in new environments. Intensive forestry based on plantations of *Eucalyptus* species has expanded dramatically in recent years and there has been a growing tendency to propagate clones of selected rapidly growing species and hybrids (FAO, 2010). The practice has, however, exposed these plantations to elevated risk from endemic and introduced pests and pathogens (Wingfield et al. 1989, Wingfield et al. 2008). Indeed there is growing evidence of the adaptation of pathogens to be able to infect trees, previously known to be resistant to them (Andjic et al. 2010, Cortinas et al. 2010). With the increasing awareness of the negative environmental effects and high cost of chemical control, forestry industries are relying on genetic resistance. In this regard, it is increasingly important to understand the structure of pathogen populations and to link this to the likely durability of resistance in the host trees (McDonald and McDermott 1993)**.**

A complex of shoot and foliage diseases of *Eucalyptus* caused by species of *Teratosphaeria* (previously *Kirramyces/Phaeophleospora*), affects the productivity of eucalypt plantation industries in subtropical and tropical areas of Australia (Andjic et al. 2010, Carnegie 2007a, b) and many other parts of the world. *Teratosphaeria suttonii* is one of the most abundant and obvious pathogens in this regard. The aim of this study was to develop and characterize polymorphic microsatellite markers to consider the population structure of *T. suttonii* within and between populations from diseased trees in different countries of the world.

Total genomic DNA was extracted from *T. suttonii* isolates CMW18634, CMW18636 (from Indonesia) and CMW32944 (from South Africa) following the protocols used by Cortinas et al. (2004). Random amplified microsatellites (RAMS) polymerase chain reaction (ISSR-PCR) (Zietkiewicz et al. 1994) and pyrosequencing (Santana et al. 2009) were used to generate and sequence microsatellite rich loci from DNA.

Sequence reads recovered from pyrosequencing (Roche 454 GS-FLX) were assembled into contigs. Microsatellites were identified in the resulting sequences and primer pairs for the amplification of microsatellite sequences designed (Santana et al. 2009). Primer properties including melting temp (Tm), formation of hairpins, self dimmers and hetero-dimmers were verified on the CLC Bio Work bench 4.1.1 (CLC Bio A/S, Aarhus, Denmark).

Thirty percent of all the contigs generated from the three isolates contained microsatellites (Table 1). However the number of sequences from which primers could be successfully designed was considerably less, since many contigs were unsuitable as they contained more than one microsatellite locus or the microsatellite region was within 20 base pairs of the 5´ and 3´ ends of the sequences.

Forty oligonucleotide primers flanking repeat sequences were designed from sequence data. PCR was performed using an iCycler (Bio-Rad) set at standard thermal conditions using 25µl reactions containing 50ng DNA, 1x PCR buffer (Roche), 0.2mM dNTPs (Promega), 0.2pm of each primer, 0.02U*Taq* polymerase (Roche) and millipore water. Appropriate $MgCl_2$ concentrations and annealing temperatures were used for each primer pair. PCR amplification

was verified under UV light with 2% agarose gels. Of these synthesized primers, optimum working conditions could be established for 23 primer pairs.

PCR products from the 23 primer pairs were purified using Sephadex G-50 (Sigma Aldrich) in Centri-sep Spin Columns (Princeton Separations) and used as template DNA for cycle sequencing reactions using the ABI PRISM BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems). The primers used to generate the PCR fragments were used to prime the sequencing reactions. Where ambiguous nucleotides were obtained in a sequence, PCR fragments were cloned into the pGEM-T easy cloning vector (Promega, Madison, WI, USA). This was followed by a colony PCR of the recombinant colonies with standard PCR conditions and vector specific primers at annealing temperature 50^{o}C. The resultant amplicons were sequenced.

Of the 23 primer pairs, 11 were able to bind to target loci and were polymorphic (Table 2). Forward primers of the 11 polymorphic loci were labeled with NED, VIC, FAM and PET fluorescent dyes (Applied Biosystems) and PCR was performed as above on 62 *T. suttonii* isolates from Australia (46), China (7), South Africa (7) and Indonesia (2). PCR products were separated by electrophoresis on an ABI PRISM 3100 auto sequencer (Applied Biosystems). Allele sizes at each locus were identified for each isolate using ABI GENEMAPPER, version 3.0 (Applied Biosystems) and LIZ-600 size standard. Data was analyzed using genescan and genemapper software (Applied Biosystems).

PCR with the 11 SSR primers resulted in single bands from which a total of 52 alleles were obtained from the 62 isolates. Various allele sizes and diversities were scored at each locus (Table 3). The highest gene diversity was observed for locus TE11 (0.83) and the lowest gene diversity was recorded at two loci TE8 and TE9 (0.38).

DNA sequence data revealed polymorphism in the repeat regions and in flanking regions, in the form of base pair substitutions, insertions, deletions; repeat length differences and indels. For example, seven polymorphic sites were obtained in the sequences flanking the GTT repeat at locus TE2 in seven China isolates and length variation in the number of repeats at the microsatellite region. Such polymorphism could not be observed directly from the gels. The implication is that the observed genetic diversity (Table 3) is not entirely due to variation in the repeat region.

The microsatellite primers developed in this study will be used to determine the level of genetic variation within and between populations of *T. suttonii* collected in many parts of the world. They will also be used to determine evolutionary factors affecting the population genetic structure of this pathogen in different parts of the world. These data will further inform efforts by the *Eucalyptus* plantation industry to reduce the impact of disease caused by *T. suttonii*.

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Table 1 Sequence data obtained from different isolates by pyrosequencing. Microsatellite types obtained and number of primers designed from each isolate.

Isolate	Sequence reads	Contigs assembled	Contigs size range (bp)	Contigs with SSRs	Di-nucleotides	Tri-nucleotides	Tetra-nucleotides	Contigs with more than one motif	Primers designed
CMW18636	36971	539	40-1084	126 (23%)	126	29	-	43	**10**
CMW32944	3776	214	112-1091	80 (37%)	75	14	1	31	**10**
CMW18634	3099	174	159-1968	72 (41%)	71	12	7	36	**20**
Total		**927**		**278 (30%)**	**272**	**53**	**8**	**112**	**40**

Table 2 Primer names, primer sequences and their corresponding GenBank Accession numbers, SSR structure in amplicons, PCR annealing temperatures (Ta), and $MgCl_2$ concentrations established for 11 polymorphic loci investigated in 62 isolates of *T. suttonii* from Queensland Australia, China, South Africa and Indonesia.

Primer Names	Locus Name	Primer sequence (5´-3´)	GenBank Accession no.	Repeat motif	Ta (°C)	$MgCl_2$ (mM)	Cloned fragment (bp)
MT1F MT1R	TE1	TCCCTCCTGGTGATACATCC TTAGTTGGTCGGTTGGTTGG	JN133903	$(TGAC)_{16}$	48	2.5	600
MT2F MT2R	TE2	TCCATCCACAACTGCTCAAC ACCACCACCACCACAACAG	JN133904	$(GTT)_{11}CCT(GTT)_3CCT$ $(GTT)_4$	56	5	200
MT3F MT3R	TE3	CAGGCAACATAAACACACTCG CACACACACACCGCATTC	JN133905	$(T)_7$	50	5	200
MT4F MT4R	TE4	CCCACATCCATCTCCATACC GTTCGGCACATCCCTTACC	JN133906	$(CATC)_7$	55	5	160
MT5F MT5R	TE5	GGTCGTCAGTTCTACACACACAC CGACGACGACGACTACTTTG	JN133907	$(AC)_{16}$	49	2.5	160
MT6F MT6R	TE6	ACCACCATCGAGTCGAAATC CACACACACGCACCATACAC	JN133908	$(GA)_3CA(GA)_5$	49	2.5	150
MT7F MT7R	TE7	ATGAAGTGGCGTGGTTCG TGAATGGCAACGGAAAGAAG	JN133909	$(GGT)_7$	55	2.5	200
MT8F MT8R	TE8	CCAAGATCTCGGAACATGAAG GCCTTGGTCCACATAGTTGC	JN133910	$(CCT)_5G$ CCT	40	3.0	200
MT9F MT9R	TE9	CGAGGACGCAAACATCAAC TGGTGAGAAGGCAGATTTCG	JN133911	$(GAT)_7$	41	3.0	250
MT10F MT10R	TE10	CAGACAGACAGAAAGTCATAAGAGG CTACCTTGGCCAACACCTTC	JN133912	$(CAGT)_9$	45	2.5	180
MT11F MT11R	TE11	ACGACGACAGCATCTTCTCC GGGTGGTGGATTTGAGTGG	JN133913	$(CAA)_2$ CAG $(CAA)_{17}$	51	3.5	210

Table 3 Microsatellite allele sizes, number of alleles and observed allelic diversity (*H*) established for 11 polymorphic loci investigated in 62 isolates of *T. suttonii*

Locus	**SSR alleles sizes (bp)**	**Total number of alleles**	**Allelic diversity (H)**
TE1	457, 465, 470, 472, 490, 495, 499	7	0.79
TE2	209, 210, 212, 213, 217, 220, 223, 224, 224, 226	10	0.67
TE3	204, 205, 206,, 207	4	0.47
TE4	151, 154, 171, 175	4	0.54
TE5	140, 143, 145, 147	4	0.64
TE6	97, 99, 100	3	0.52
TE7	196, 199, 202	3	0.47
TE8	196, 197	2	0.38
TE9	242, 252	2	0.38
TE10	172, 180, 200, 208	4	0.64
TE11	174, 175, 177, 178, 180, 148, 186, 187, 199	9	0.83
Mean H			0.58

Allelic diversity H; (Nei 1973)

CHAPTER 4

High diversity and geographic isolation characterize *Teratosphaeria suttonii* populations in Australia.

Abstract

Polymorphic microsatellite markers were used to establish the genetic structure of a collection of isolates of *T. suttonii*, one of the causal agents of Teratosphaeria Leaf Disease (TLD) of *Eucalyptus* species in Australia. The pathogen is naturally distributed in tropical and subtropical regions of eastern Australia. The population genetic structure of 201 isolates from Queensland, New South Wales and Western Australia was analyzed by assigning them in seven groups corresponding to geographic regions. Moderate to high levels of genetic diversity (H=0.45-0.55) and genotypic diversity (G*=39-92%) were observed in the populations. In general, populations were clonal; however, there was evidence for recombination in one population from southern Queensland. Isolates from Western Australia were monomorphic at most loci and exhibited very low diversity confirming the hypothesis that *T. suttonii* was recently introduced to the region. Population differentiation tests based on F_{st} showed that the four populations were genetically distinct. Results reflect limited gene flow among the populations, which is attributed to the movement of infected germplasm probably with *Eucalyptus* seedlings used to establish plantations.

Introduction

Consistent with the high demands for timber and pressure to minimize utilization of native forests, the plantation area in Australia has increased since 2001 (Gavran 2012). The establishment of commercial *Eucalyptus* plantations has involved movement of these trees from their natural localities to different previously unused areas (Potts et al. 2004). The trees were moved along with their related natural pathogens, acquired from the chemically and taxonomically related native forests (Strauss 2001, Wingfield et al. 2008, Wingfield et al. 2010). Thus human activities influenced and magnified the geographic composition of *Eucalyptus* pathogens in Australia (Perez et al. 2012). Both natural and commercial *Eucalyptus* stands in Australia have been reported to harbor *Teratosphaeria* species, particularly at the early stages of leaf development (Old et al. 2003, Hunter et al. 2011). Due to infection by these fungi, photosynthetic ability is reduced; leading to premature leaf fall and retarded growth, as a consequence industrial forestry companies can incur substantial economic losses (Chipompha 1987, Carnegie 2007a, Milgate et al. 2005).

Teratosphaeria species with *Kirramyces* anamorphs have resulted in significant damage of *Eucalyptus* plantations in the tropical and subtropical areas of Australia (Carnegie 2007b, Andjic et al. 2010). Their negative impacts on the yield of plantations in Australia and other parts of the world have also been studied (Carnegie et al. 1997, Barber et al. 2003, Maxwell et al. 2003, Hunter et al. 2011). Among the species of *Teratosphaeria*, *T. suttonii* is the most ubiquitous, having been reported on native eucalypts of the subgenus *Macranthera* and in plantations in subtropical and tropical Australia (Carnegie 2007a, b),

from a variety of *Eucalyptus* species and clones in the subgenera *Corymbia*, *Monocalyptus, Symphyomyrtus, Indiogenes* (Walker et al. 1992, Carnegie 2007b). *Teratosphaeria suttonii* infects the leaves of seedlings, plants in clonal hedges and young as well as mature eucalypt trees (Chipompha 1987, Gardner and Hodges 1988, Crous et al. 1988).

Teratosphaeria suttonii was first reported in Sydney, New South Wales from *E. grandis* and described as *Phaeoseptoria eucalypti* (Hansford 1957). The geographic range of the pathogen subsequently extended to other parts of eastern Australia, where it was reported from several *Eucalyptus* species (Walker 1962). It was later noted as the most predominant foliage-infecting fungus in Australia (Walker et al. 1992) and has been reported as one of the fungal pathogens that substantially reduces the success of eucalypt plantations in Australia (Carnegie 2007a) and other parts of the world (Chipompha 1987, Knipscheer et al. 1990). The fungus has undergone several name changes (Crous et al. 2009) and variation in ITS sequence data has led several authors to propose that *T. suttonii* represents a species complex. But the application of a combination of multiple gene phylogeny and morphological assessment for collections from various parts of the world led to the recent conclusion that *T. suttonii* is a single but morphologically variable species (Taole et al. 2011).

As a means of surviving in a constantly changing natural environment, pathogen populations undergo gradual adaptation. The characterization of plant pathogen populations in terms of their genetic structure provides insight into the evolutionary

processes that have influenced the population in the past and allows a perspective of the inherent evolutionary ability of pathogen populations (McDonald 1997). Knowledge of population's genetic diversity, mode of reproduction, the presence or absence of interbreeding with members of the same species as well as methods of exchange of genes and genotypes with separated populations can be used to predict their evolutionary potential and to determine the risks associated with their evolution (McDonald and Linde 2002). Investigations into the natural occurrence and arrangement patterns of pathogens can also reveal pathogen dispersal patterns in different countries and around the world (McDonald and Linde 2002). Clearly, the development and implementation of rational control strategies requires an understanding of the population biology of plant pathogenic fungi and the evolutionary factors underlying their genetic structure (McDonald and McDermott 1993).

Unambiguous and informative molecular markers are used in the population genetic analysis of plant pathogens (McDonald and McDermott 1993, Taylor et al. 1999, McDonald and Linde 2002). The hyper variability and co-dominant nature of microsatellites as well as the reproducibility of these markers has resulted in their widespread use (e. g. Barnes et al. 2005, Hunter et al. 2008, Perez et al. 2010). Following this view, a set of microsatellite markers was developed for *T. suttonii* (Taole et al. 2012/ Chapter 3). The aim of this study was to establish the population structure and genetic diversity of *T. suttonii* within its natural range in Australia using these microsatellite markers.

Materials and Methods

Fungal isolates

A total of 201 isolates of *T. suttonii* collected in Australia from different parts of Queensland (QLD, five locations), New South Wales (NSW, six locations) and Western Australia (WA, one location) (Table 1) were used in this study. Of these, more than 20 isolates were collected from four locations, namely Davis Creek in northern Queensland (DC), Koumala in central Queensland (KMA), Imbil in southern Queensland (IMB) and Wedding Bells (WB) in northern NSW and these were treated as populations. Thus there were a total of 156 isolates in the four populations and an additional 45 isolates from QLD, WA and NSW (Table 1, Fig. 1), which were not part of the populations.

Isolates were acquired from diseased *Eucalyptus* leaves with typical *T. suttonii* symptoms as previously described (Taole et al. 2011). All isolates used in this study have been deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) culture collection housed at University of Pretoria.

DNA extraction, SSR-PCR and determination of allele sizes

DNA was extracted from cultures grown on 2% malt extract agar ($20gL^{-1}$ malt extract, $20gL^{-1}$ agar; Biolab) at $20^{o}C$, using the CTAB protocol as described by Andjic et al. (2007). Eleven pairs of primers specifically designed for the amplification of 11 polymorphic microsatellite loci of *T. suttonii* isolates (Taole et al. 2012), were used to amplify microsatellite loci in 25µl PCR reactions. The forward primers of each pair were fluorescently labeled. PCR products were stained with GelRedTM nucleic acid stain and

amplifications verified on 2% agarose gels exposed to UV illumination. For primer pairs that failed to amplify expected PCR products, a range of $MgCl_2$ and annealing temperatures were applied. Complete failure of primers to produce expected products led to the loci concerned being excluded in the data analysis. Depending on the size and concentration of amplified DNA fragments and the fluorescent label of the primers, PCR products were multiplexed and diluted for Genescan analysis. Samples were separated using an ABI PRISM^{TM} 3100 Automated DNA sequencer (Applied Biosystems, Forster City USA). Estimates of allele sizes for the DNA fragments were determined by comparison with the internal size standard GENSCAN LIZ 600 (Applied Biosystems, Foster City USA) and with the software GENMAPPER version 3.0 (Applied Biosystems).

Gene and Genotypic diversity

Alleles at each locus were provided with an alphabetical letter and the letters representing the different alleles together, constituted multi locus genotypes for each isolate. Similar genotypes were regarded as clones. Statistical analyses were carried out on clone-corrected populations that included isolates from DC, KMA, IMB and WB populations. Other isolates from QLD, WA and different parts of NSW were few and thus not included in the statistical analyses; the isolates were, however, included in the population structure analysis.

For each clone corrected population the frequency of alleles at each locus was calculated. Gene diversity (H) was determined using the program POPGENE version 1.31 (Yeh et al.

1999) and the equation H=1-$\sum x_k 2$, where x_k represents the frequency of the k^{th} allele (Nei 1973). Chi-square (X^2) tests (Workman and Niswander 1970) were used to establish the significance of differences in allele frequencies for each locus among the clone corrected populations. Gene diversities at all loci were regarded considerably distinct when the calculated X^2 values were higher than the value on the contingency Chi^2 table, at $P<0.05$ at corresponding degrees of freedom. An estimate of genotypic diversity (G) was calculated using the equation $G = 1/\sum pi^2$, where Pi is the observed frequency of the i^{th} phenotype (Stoddart and Taylor 1987). A comparison of the population's genotypic diversities was deduced from the maximum percentage genotype diversity (G*) calculated for each population using the formula G* = G/N x 100, where N = number of isolates (McDonald et al. 1994).

Linkage disequilibrium

An estimate of the Index of Association I_A was used as a measure of the amount of gametic disequilibrium among the 11 microsatellite loci for each clone corrected population. The tests were executed by running 1000 simulated randomizations in the program MULTILOCUS version 1.3 (Agapow and Burt 2000). Association between alleles at different loci was established by comparing the observed value with an output of a 1000 randomizations.

Population differentiation

Population differentiation as a consequence of allele or gene frequencies theta (θ) was quantified among pairs of clone corrected populations in the program MULTILOCUS

version 1.3 (Agapow and Burt 2000), using Wright's F_{st} index for haploids. The statistical significance of θ was determined from a comparison of the observed value with a value obtained when individuals were randomized 1000 times among populations.

Population's genetic distance and assignment

Investigations of genetic distance and structure of populations were carried out on haplotypes of clone corrected populations and isolates from QLD, NSW and WA that were not part of the populations, using (PAUP) version 4.0 b10 (Swofford 2003), and the model based Bayesian clustering procedure in STRUCTURE version 2.2 (Pritchard et al. 2000) respectively. The optimum number of populations (K) was determined from STUCTURE and on the basis of their genotype data individual isolates were allocated to respective populations. An analysis of Molecular Variance (AMOVA) was conducted in GenAlEx version 6.2 (Peakall and Smouse 2006) to establish the difference in the amount of variation among and within the populations and among regions. The complete data set was subjected to 1000 permutations in order to establish the significance.

Results

Polymorphic microsatellite loci

All isolates in IMB and WB populations produced null alleles at loci TE1 and TE3 and as a result the two loci were excluded from further analysis thus leaving 9 loci for analysis of populations. Furthermore, some isolates from QLD and WA, which were not part of the four defined populations, also produced null alleles at locus TE9. For this reason, the

STRUCTURE and distance analyses of all the isolates from Australia included only 8 loci.

A total of 88 alleles were obtained from amplification of 9 loci; 72 of these were found among the 156 isolates corresponding to four populations of *T. suttonii* from KMA, DC, IMB and WB (Table 2). The number of alleles per population ranged from 27 to 43, while alleles per locus ranged from 2 to 16. Ten percent of the alleles were present in all populations. Alleles with low frequencies (<0.02) were found in 6 of the 9 loci examined. TE10 was the most polymorphic locus with 16 alleles, followed by TE1 with 13 alleles. Locus TE9 was monomorphic in the KMA, DC and WB populations, while locus TE6 was monomorphic in the WB population. Eleven isolates from WA were monomorphic at 7 of the 9 loci tested.

Among the 9 SSR loci investigated**,** unique alleles were obtained in 7 loci. Thirty three unique alleles were present across all the populations. The greatest number of unique alleles (11) was scored in the WB population, followed by populations IMB, DC and KMA with 10, 7 and 6 unique alleles respectively.

Gene and genotypic diversity

One hundred and twenty six genotypes were found across the four Australian *T suttonii* populations. The observed genotypic diversities (G*) for the populations were considerably different (Table 2), ranging from 39% to 92%. There were no shared genotypes among the four populations. The highest gene diversity (H) within a

population was 0.55; while the mean total gene diversity across all populations was 0.67. Thus there was higher diversity between populations than within populations. Chi-square tests confirmed a significant difference in allele frequencies at all loci for the entire clone corrected populations (Table 3). Only, three genotypes were obtained among the 11 WA isolates with a G* of 22.5%.

Linkage disequilibrium

The I_A and r_d values for populations from KMA, DC, and WB were significantly greater than for the randomized data set ($P<0.001$), implying clonality of strains (Table 4). The observed I_A and r_d values for the IMB population, however, fell within the randomized distribution range of allelic frequencies, implying recombination in this population (Table 4).

Population distinction, genetic distance and structure

Population differentiation (θ) values ranged from 0.241-0.352 at $P<0.001$ (Table 5). The values indicated significant difference among the four population pairs. The proportions of genetic variation obtained from analysis of molecular variance (AMOVA) were 3% among regions, 29% among populations and 68% within populations. Based on the highest likelihood (ln and delta k) and lowest standard deviation, the largest estimated number of groups (K) among all isolates was 7 (Fig. 2-3; Table 6). In general, NSW and QLD haplotypes were allocated to 5 and 6 groups respectively, while WA haplotypes were allocated to 1 group. IMB haplotypes were distributed among 3 groups, DC and KMA haplotypes were assigned to 2 groups and WB haplotypes confined to 1 group. A

majority of the haplotypes from IMB (67%), DC (91%), WB (100%), and KMA (84%) were assigned to groups 1, 4, 5 and 6 respectively. Groups 2 and 3 consisted of a majority of the QLD and NSW haplotypes that were not assigned to populations and a minority of the KMA, IMB and DC haplotypes. Group 7 consisted of MV and MJ haplotypes (Table 6).

The distance tree analysis (Fig. 4) also separated the haplotypes of *T. suttonii* into 7 groups. Consistent with results of STRUCTURE analysis, 5 of the groups were comprised mainly of haplotypes belonging to geographic locations from which the isolates had been collected, while the other 2 groups comprised of haplotypes from different parts of QLD and NSW (Fig. 4). Overall, most haplotypes from predefined populations fell into a single group; however in some cases there were a few haplotypes which fell into other groups.

Discussion

Results of the study showed that multiple unique genotypes of the *Eucalyptus* leaf pathogen *T. suttonii* exist in different locations of Australia. High genetic diversity, significant population differentiation and low gene flow characterized the populations. At three of the four populations allelic linkage suggested clonal reproduction.

The occurrence of both different haplotypes and different dominant haplotypes of *T. suttonii* in different geographic locations is most likely a consequence of asexual propagation. These factors together with the observed high levels of genetic diversity were expected since Australia is considered to be the centre of origin of *T. suttonii*. The results indicate that the pathogen consists of a high number of unique individuals. The moderate to high gene and genotypic diversity in hypothesized centers of origin have been reported for other phylogenetically closely related species such as *Mycosphaerella musicola* (Hayden et al. 2003), *Mycosphaerella fijiensis*, (Hayden and Carlier 2003) and *Teratosphaeria nubilosa* (Hunter et al. 2008, Perez 2012) in South-East Asia, Papua New Guinea, Australia respectively.

Although the majority of populations of *T. suttonii* appeared to be clonal, it was clear that recombination is occurring in the population from southern Queensland and this accounted for the high level of diversity there. This is very interesting as the only time that a sexual state for the fungus has ever been found was from collections made in Queensland (Crous et al. 1998). There is no clear reason why sexual reproduction would

only be found in this area but the data suggest that other populations in Australia might have originated here.

It is known that *T. suttonii* is not endemic to WA, having been introduced during the rapid plantation expansion phase in the early 2000's when nursery material was sourced from eastern Australia (Jackson et al. 2008). *T. suttonii* has most likely been recently introduced to WA from QLD. This is reflected in the low gene diversity of WA isolates and the close association of WA and QLD haplotypes in the STRUCTURE and distance analyses. Lower diversity has also been observed for known introduced populations of other *Teratosphaeria* species (Andjic et al. 2011, Hunter et al. 2008, Perez et al. 2012).

The haplotypes of isolates from MV did not cluster together with other central Queensland haplotypes. The implication is that the set of alleles in the MV isolates have most probably been acquired from native *Eucalyptus* trees in that region (Slippers et al. 2005); or possibly the isolates are representatives of a well established population that has accumulated unique alleles over a long period of time (Taylor et al. 1999).

The assignment and distance analyses in this study substantiated a significant amount of geographic structure on the basis of the distribution and frequency of different alleles, indicating independent evolution of populations that could have been sustained by restricted gene flow between the populations. This would be similar to what has been found for the pine infecting fungus *Diplodia scrobiculata* populations in North America (Burgess et al. 2004). Based on structure and distance analysis, some admixture was,

however, observed among the QLD and NSW isolates, probably indicating that the pathogen in different localities was initially introduced from the same source perhaps as a consequence of a common source of seedlings for plantation development (Andjic et al. 2011) as has also been observed for *Diplodia pinea* (Bihon et al. 2011).

Considering the high proportion of common alleles between the populations and low numbers of unique alleles that occurred in each population, it is highly likely that the differentiation observed between the populations is a consequence of mutations (Zhan and McDonald 2004). Loci that could not be amplified in some of the populations were excluded from the analysis of data in order to avoid the possibility of bias of results due to null alleles. As a consequence data has been lost, since a total of 73 alleles of which 12 were unique were not included in the data analysis. Thus, the observed population diversity is actually underestimated.

Teratosphaeria suttonii is a significant pathogen of *Eucalyptus* plantations in Australia and different countries of the world. This study is the first to investigate the genetic diversity and thus evolutionary potential of this pathogen in comprehensively managed industrial plantations. The results of this study have provided an insight into the biology of *T. suttonii* in Australia. A high level of genetic diversity has been shown to exist within the investigated populations. Restricted movement of planting stock in Australia should be maintained in order to avoid the introduction of new genotypes of this pathogen into new areas.

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Table 1. *T. suttonii* isolates used in this population study, their localities and collection dates.

Populations	Other isolates	origin	No. of isolates	Collector	Collection date
1 (DC)		Davies Creek, North - QLD	27	MM Taole & K Taylor	April 2010
2 (KMA)		Koumala, Central - QLD	46	MM Taole & K Taylor	April 2010
3 (IMB)		Imbil, South – QLD	47	MM Taole & K Taylor	April 2010
4 (WB)		Wedding Bells, NSW	36	AJ Carnegie	February 2010
	MV	Miriamvale, QLD	8	V Andjic	2009
	MJ	Manjimup, WA	11	T Burgess	October 2007
	EC	Emu Creek, NSW	3	AJ Carnegie	February 2010
	KW	Kew, NSW	6	V Andjic	2009
	KM	Kimbel, NSW	4	V Andjic	2009
	NVS	Neaves, NSW	2	V Andjic	2009
	TGB	Tunglebung, NSW	3	MM Taole & K Taylor	April 2010
	MW	Morrow, NSW	3	MM Taole & K Taylor	April 2010
	MC	Myrtle Creek, NSW	3	AJ Carnegie	February 2010
	GS	Garas, NSW	2	MM Taole & K Taylor	April 2010

Table 2. Allele sizes and frequencies in populations at 9 loci for *T. suttonii* isolates collected from Koumala, Davies Creek, Imbil and Wedding Bells.

Locus	Allele	KMA	DC	IMB	WB
TE2	209				0.0741
	210				0.0370
	212				0.0741
	217	0.0312			0.1852
	220	0.3125	0.2727	0.0667	0.1111
	223	0.1250			
	224	0.3750	0.1818	0.2000	0.2963
	225		0.2727	0.0667	0.2222
	226	0.1562	0.1818		
	227		0.0455	0.4444	
	230			0.2667	
	233			0.2889	
	NULL		0.0455	0.0667	
TE4	138				0.1852
	142		0.0909	0.2889	0.2222
	146			0.2667	0.5926
	151			0.1111	
	154	0.7812		0.2889	
	158			0.2667	
	163		0.9091		
	175	0.2188			
TE5	136		0.4545	0.8444	0.5185
	138				0.3704
	140		0.5000	0.1333	0.0741
	143	0.2500		0.0222	
	145	0.7188			
	NULL	0.0312	0.0455		0.0370
TE6	97	0.2812		0.0444	
	99	0.7188	0.6818	0.9333	
	100		0.2727	0.0222	1.0000
	NULL		0.0455		
TE7	150			0.0222	
	189		0.7727		
	193		0.0455	0.0889	
	196	0.3438		0.5111	0.0741
	199	0.6562	0.0909	0.3333	0.4444
	202				0.4444
	205				0.0370
	NULL		0.0909	0.0444	
TE8	196	0.5000	0.5455	0.6000	0.5926
	197	0.5000	0.4545	0.4000	0.4074
TE9	242			0.1556	
	252	1.0000	1.0000	0.6889	1.0000
	NULL			0.1556	

Locus	Allele	KMA	DC	IMB	WB
TE10	163				0.4074
	164		0.0909	0.1111	0.5185
	172				0.0370
	175		0.7273		
	176		0.0455		
	180	0.3438			
	188			0.0444	
	192		0.0455		
	194		0.0455		
	196			0.4222	
	197		0.0455	0.0667	
	198			0.0222	
	200	0.1250			
	204	0.0312			
	208	0.3750		0.2444	
	NULL	0.1250		0.0889	0.0370
TE11	175	0.3125	0.2727	0.1778	
	176		0.0909		
	178	0.0625	0.3182	0.0667	0.1852
	180			0.5333	0.0741
	187	0.3750			0.0741
	193		0.0455	0.0222	0.0741
	196			0.0444	0.1481
	197		0.1364	0.0444	0.1481
	199	0.1875		0.0889	
	202		0.0909		0.0370
	205				0.1852
	NULL	0.0626	0.0455	0.0222	0.0741
Ni		46	27	47	36
Ng		32	22	45	27
Na		27	34	43	35
Nua		6	7	9	11
Npl		8	8	9	7
G		18.42	17.78	43.31	20.25
G*		39.66	65.85	92.15	56.25

Ni Total number of isolates (non clone corrected)

Ng Number of genotypes

Na Number of alleles

Nua Number of Unique alleles

Npl Number of polymorphic loci

G Genotypic Diversity (Stoddart and Taylor 1988)

G= G/N% Percentage maximum diversity of genotypes

Table 3. Gene diversity and Chi^2 tests for differences in allele frequencies for nine SSR loci

		Gene diversity (H)				
Locus	Koumala	Davies Creek	Imbil	Wedding Bells	X^2	df
TE2	0.72	0.78	0.79	0.80	330.61***	36
TE4	0.34	0.17	0.75	0.57	294.75***	24
TE5	0.42	0.54	0.27	0.59	369.24***	15
TE6	0.40	0.46	0.13	0.00	452.90***	9
TE7	0.45	0.38	0.62	0.59	396.36***	21
TE8	0.50	0.49	0.48	0.48	18.31***	3
TE9	0.00.	0.00	0.48	0.00	21.64***	6
TE10	0.71	0.45	0.73	0.56	421.69***	45
TE11	0.72	0.79	0.67	0.86	172.04***	33
Mean	0.47	0.45	0.55	0.50		

H Gene Diversity (Nei 1973)

Significant differences in allele frequencies (P<0.005) based on Chi^2 tests.

Degrees of freedom = (no. populations -1) X (no. alleles -1)

Table 4. Multilocus disequilibrium estimate I_A for 1000 randomized data sets.

Population	**I_A**	**I_A range**	**$\bar{r}D$**	**$\bar{r}D$ range**	**P value**
Koumala (C-QLD)	0.227*	-0.407 – 0.229	0.032	-0.026 - 0.033	<0.001
Davies Creek (N-QLD)	0.844*	-0.310 - 0.429	0.123	-0.045 – 0.062	<0.001
Imbil (S-QLD)	0.130	-0.198 – 0.354	0.016	-0.025 – 0.045	<0.054
Wedding Bells (NSW)	0.314*	-0.221 – 0.321	0.054	-0.038 – 0.055	<0.001

*Significant P<0.001

Table 5. Pairwise comparison of population differentiation (θ) among 4 populations from Australia

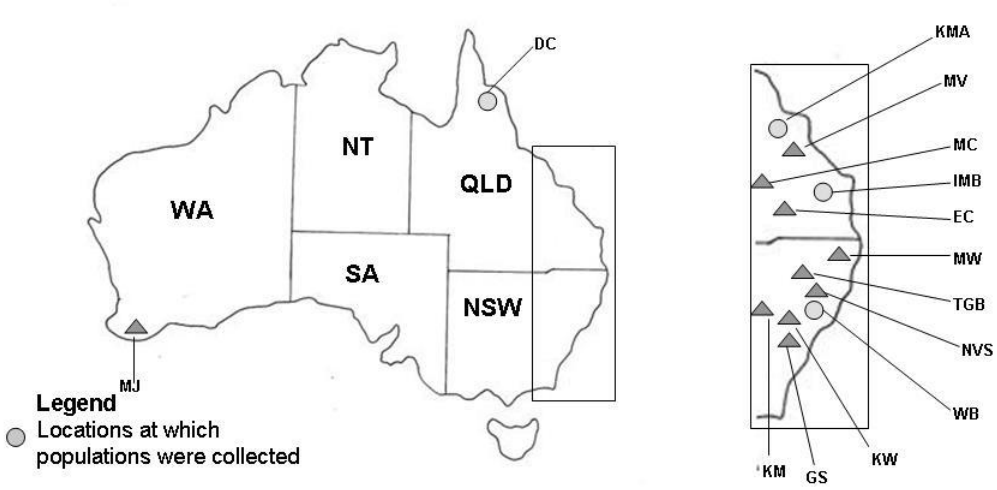
	Wedding Bells	Imbil	Davies Creek
Koumala	0.352*	0.241*	0.347*
Wedding Bells	-	0.277*	0.326*
Imbil	-	-	0.276*

*Significant P<0.001

Table 6. Haplotypes of *T. suttonii* assigned to 7 groups obtained from analysis in STRUCTURE.

				K=7						
Location		**No. isolates**	**No. genotypes**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
DC	FNQ	46	22				**20**	**2**		
KMA	C-QLD	36	32		**1**	**4**			**27**	
IMB	S-QLD	47	45	**31**	**3**	**11**				
WB	NSW	36	27					**27**		
MV	C-QLD	8	8							**8**
MJ	WA	11	3							**3**
EC	S-QLD	3	-		**3**					
KW	NSW	5	-		**5**					
KM	NSW	4	-		**2**	**1**		**1**		
NVS	NSW	2	-		**2**					
TGB	NSW	3	-					**1**	**1**	**1**
MRW	NSW	3	-	**1**		**1**		**1**		
MC	C-QLD	3	-			**3**				
GS	NSW	2	-	1		1				

Figure 1. Map showing the different locations in Australia where *T. suttonii* isolates were collected.



DC=Davies Creek, KMA=Koumala, MV= Miriam Vale, MC= Myrtle Creek, IMB= Imbil, EC=Emu Creek, MW= Morrow, TGB= Tunglebung, NVS= Neaves, WB= Wedding Bells, KW= Kew, GS=Garas, KM= Kimbell, MJ= Manjimup

Figure 2. Optimum number of populations (K=7) established from LnK and DeltaK values obtained from STRUCTURE analysis.

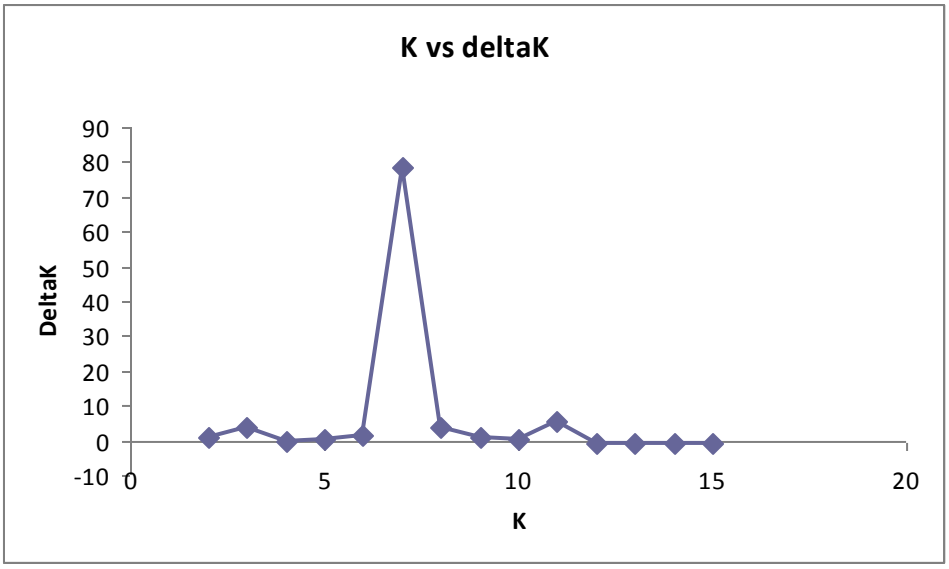
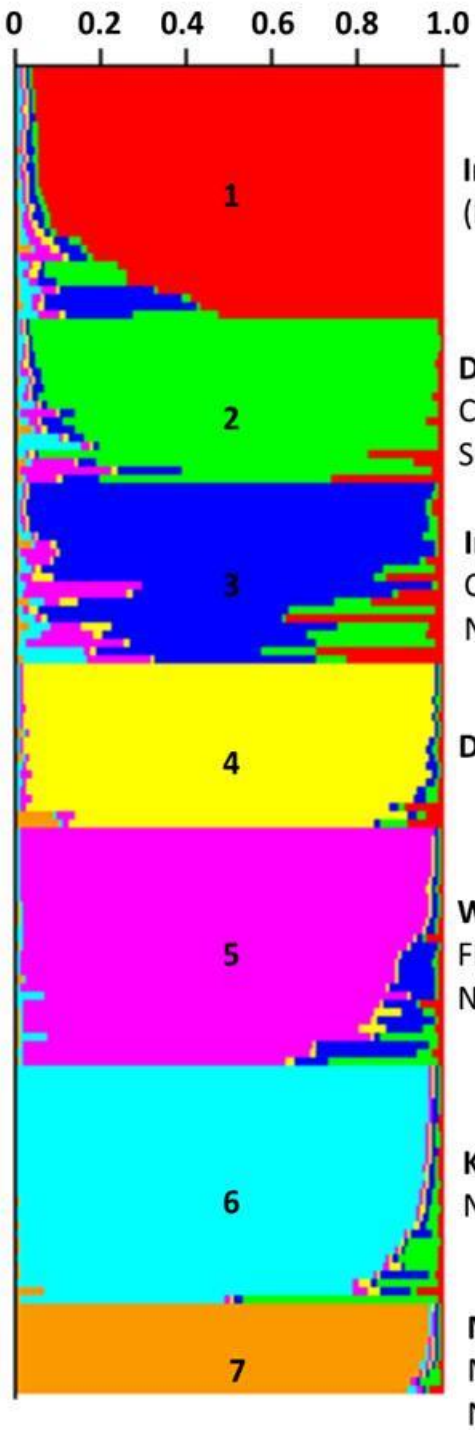


Figure 3. An assignment diagram showing the allocation of individual isolates based on allele frequencies as determined by STRUCTURE 2.2. The different coloured sections represent 7 underlying genetic populations identified from the genotyped individuals. The multicoloured vertical bars show characteristic genotypes affiliated with several populations. The height of each colour within an individual provides a measure of the amount of affiliation of an individual to different populations.



Imbil (S-QLD)
(NSW)

Different locations (NSW)
C-QLD
S-QLD

Imbil (S-QLD)
C-QLD
NSW

Davies Creek (FNQ)

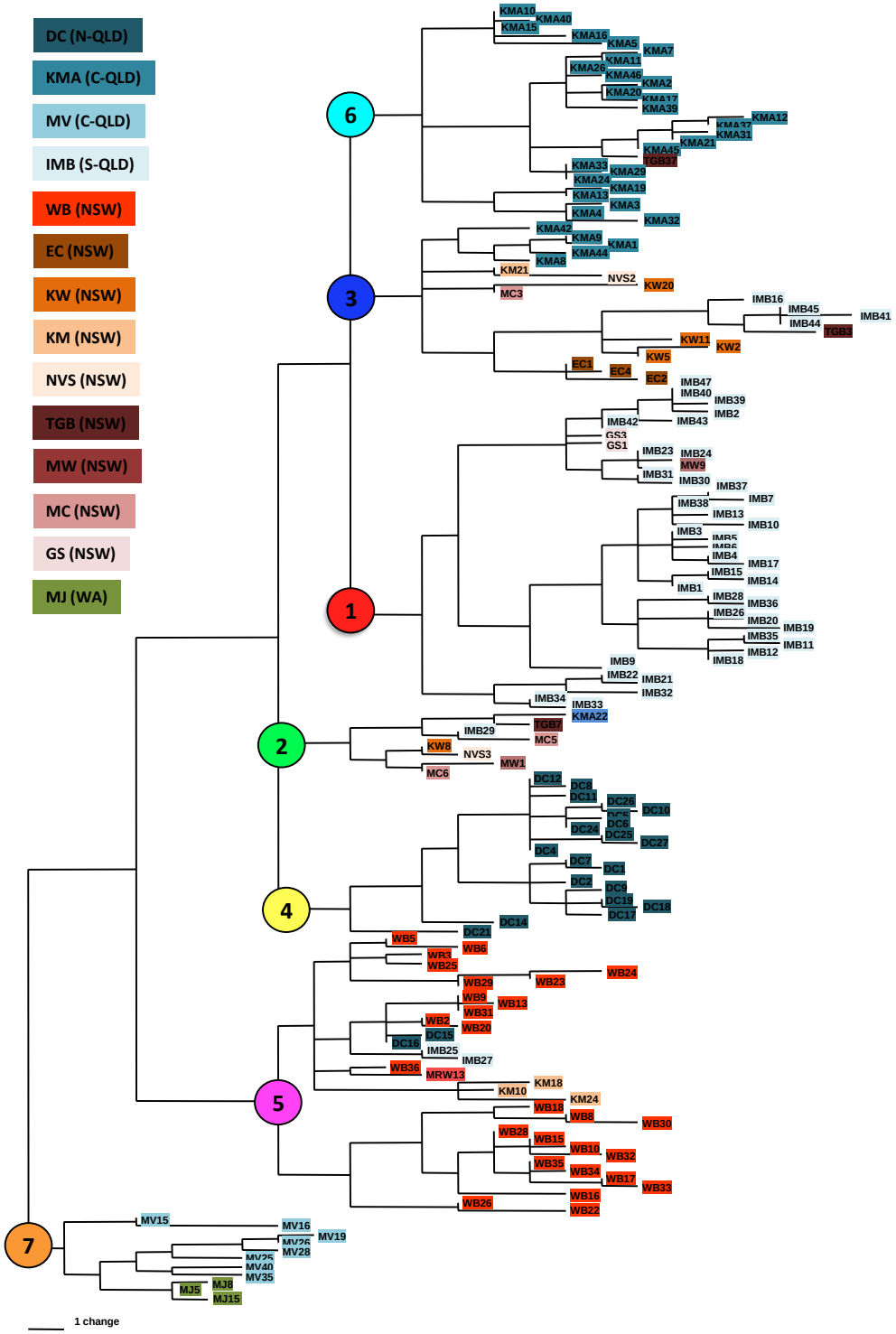
Wedding Bells (NSW)
FNQ
NSW

Koumala (C-QLD)
NSW

Miriamvale (C-QLD)
Manjimup (WA)
NSW

Numbers 1-6 correspond to group numbers on neighbor joining tree
Predominant members of the group in bold

Figure 4. Neighbor-joining tree diagram of *T. suttonii*, illustrating 7 haplotype groups. The numbers given to each of the groups correspond with the numbers awarded the groups obtained from STRUCTURE analysis.



CHAPTER 5

Movement of the *Eucalyptus* pathogen *Teratosphaeria suttonii* from native Australian forests to plantations world-wide

Abstract

Teratosphaeria suttonii is a commonly occurring leaf pathogen of *Eucalyptus* species, especially those residing in the sub-genus *Symphyomyrtus.* Although it has generally been regarded as a minor pathogen commonly infecting older leaves, repeated infections can result in severe defoliation and tree deaths. Eleven polymorphic microsatellite markers were used to assess the genetic structure of seven introduced populations of *T. suttonii* from *Eucalyptus* plantations in China, Indonesia, South Africa, the United States of America, Uruguay and Vietnam. This was compared with the genetic structure of populations of isolates from native Australian forests and plantations. In general the genetic diversity of the pathogen populations was high. Some introduced populations appeared to be clonal with low diversity supporting recent introduction of a limited number of haplotypes. The random association of alleles in other populations could be intrepreted as evidence for sexual recombination. However, even in native populations sexual reproduction is rare and the observed high allelic diversity is probably evidence of multiple introductions from different locations followed by genetic drift. The high levels of diversity of these introduced populations provide a high evolutionary potential increasing the risk of novel genotypes being moved to new environments and to adapt to local conditions.

Introduction

The ascomycete fungus, *Teratosphaeria suttonii* (=*Phaeoseptoria epicoccoides*, *Phaeophloespora epicoccoides* and *Kirramyces epicoccoides*) is a well-known pathogen of *Eucalyptus* foliage (Hunter et al. 2011). The asexual form of the fungus is most frequently encountered but the sexual state has been observed infrequently (Barber 2004). The asexual state of the fungus is one of the most prevalent leaf pathogens of *Eucalyptus* spp. grown as non-natives in plantations in the tropics and southern hemisphere (Walker et al. 1992, Burgess et al. 2006, Andjic et al. 2010). *T. suttonii* is known to be endemic to eastern Australia, the place where it was fist isolated (chapter 4, Hansford 1957, Walker 1962) and has been introduced to Western Australia (chapter 4).

Generally *T suttonii* is considered to be a relatively unimportant pathogen that infects old and stressed leaves (Knipscheer et al. 1990). But there are also reports of significant damage due to this pathogen in the sub-tropics, both in *Eucalyptus* plantations in Australia (Andjic et al. 2011, Carnegie 2007a, b) and plantations of non-native *Eucalyptus* spp. in other parts of the world including South Africa (Crous et al. 1998, Nichol et al. 1992a, b), South America (Perez et al. 2009), China (Burgess et al. 2006), Japan, Indonesia, Philippines, New Zealand (Old et al. 2003), Vietnam (Pegg et al. 2003) and Zambia (Chungu et al. 2010).

Species of *Eucalyptus* residing in the sub-genus *Symphyomyrtus* are most susceptible to infection by *T. suttonii* (Walker 1962, Walker et al. 1992, Pegg et al. 2003, Carnegie et al. 2007a, b, Nichol et al. 1992a). Several biotic and abiotic factors influence the disease

occurrence in *Eucalyptus* species. In general stress favours infection by the pathogen (Nichol et al. 1992b, Carnegie et al 2007b) and this is presumably why it occurs predominantly on old rather than juvenile leaves. However, when inoculum levels are high and conditions humid, *T suttonii* can infect juvenile foliage causing repeated tip dieback. Infection due to this fungus reduces the surface area of leaves available for photosynthesis, resulting in defoliation, reduced growth and in some cases death of trees (Knipscheer et al. 1990).

As is true for many other *Eucalyptus* pathogens native to Australia, *T. suttonii* is suspected to have spread from that country to other areas of the world where *Eucalyptus* spp. have been used to establish plantations (Old et al. 2003, Wingfield et al. 2008). However, this has never been tested experimentally. Within its natural environment in eastern Australia the diversity of *T suttonii* is high and the gene flow between populations is low resulting in a high level of structure (chaper 4). Additionally, in most populations in Australia alleles were linked indicating clonal reproduction. However, nothing is known regarding the population structure of the pathogen in introduced situations. This is despite the fact that it would be valuable to have knowledge of the population diversity of the pathogen and its potential to adapt to new host material, especially where large plantation programmes are being established (McDonald and McDermott 1993). The aims of this study were, therefore to determine the genetic diversity of *T. suttonii* for seven populations of isolates collected where the fungus has apparently been introduced. Furthermore, we compared these data with those for native populations of the pathogen determined in a previous study (Chapter 4).

Materials and methods

Sampling and Isolations

One hundred and eighty six single conidial cultures (Table 1) of *T. suttonii* were obtained from lesions on *Eucalyptus* leaves collected from plantations in Uruguay (URY-L and URY-C), the United States of America (USA), China (CHN), Indonesia (IDN), Vietnam (VNM), and a nursery in South Africa (ZAF). Isolation of the fungus from conidia was achieved as previously described by Taole et al. (2011). A further 201 isolates from various areas in Australia including Far North Queensland (FNQ), central Queensland (C-QLD), southern Queensland (S-QLD), New South Wales (NSW) and Western Australia (WA), previously considered in a population biology study (Chapter 4) were included in the Structure and distance analysis for comparative purposes.

DNA extraction and amplification of microsatellite loci

DNA was extracted from all cultures as previously described (Taole et al. 2011). For each isolate, 11 SSR loci were amplified in SSR-PCRs using 11 fluorescently labelled primer pairs specifically designed for *T. suttonii* ; (http://tomato.biol.trinity.edu/). The resulting fluorescently labelled PCR products were separated on an ABI Prism DNA sequencer (Applied Biosystems). Estimates of allele sizes were determined by a comparison of the mobility of the PCR products with that of the LIZ 600 internal size standard (Applied Biosystems), using the GeneScan analysis software.

Analysis of Gene and genotypic diversity

The frequency of alleles at individual loci and Gene diversity (H) were computed using the program POPGENE (Yeh et al. 1999); Estimates of Genotypic diversity (G) (Stoddart and Taylor 1988) and maximum percentage genotypic diversity (G*) for individual populations were determined. Chi square (X^2) tests for the dissimilarity of allele frequencies for each locus for all clone corrected populations were calculated.

Population differentiation and Linkage disequilibrium

Population divergence (θ) due to allele frequency differences was analysed between pairs of clone corrected populations using Wrights F_{st} in the program MULTILOCUS version 1.3 (Agapow and Burt 2000). The null hypothesis that an apparent differentiation exists between populations was validated when determined "θ" values were considerably different from the 1,000 times randomized data sets at $P<0.05$. Analysis of Index of Association (I_A) was performed on clone corrected populations in the program MULTILOCUS in order to evaluate sexual recombination within individual populations. Random mating was further quantified by analysing rbarD (r^-D) in the same program. The null hypothesis that random association between alleles at different loci exists in a population was supported when the observed I_A values fell within the range of 1,000 times simulated random mating data and insignificant difference was observed in the probability (P) values at $P<0.05$. Haplotypes of the two Uruguay populations were combined for the purpose of determining recombination.

Population's genetic structure and distance analysis

Assignment of isolates was performed in STRUCTURE (Pritchard et al. 2000) on the entire collection of clone corrected haplotypes used in this study and those previously analysed *T. suttonii* isolates from Australia (Chapter 4). Isolates were characterised by a collection of allele frequencies at each locus and then assigned to respective populations. Genetic distance of the isolates was determined in (PAUP) version 4.0 b10 (Swofford 2003). Furthermore analysis of Molecular Variance (AMOVA) was executed in GenAlEx version 6.2 (Peakall and Smouse 2006) in order to establish the characteristic degrees of variation within and among populations.

Results

Alleles and genetic diversity

All loci other than TE10 were at least monomorphic in one population and the largest number of monomorphic loci (8) was observed for the population from URY-L. Locus TE11 was most polymorphic including 12 alleles. A total of 71 alleles were obtained across 11 microsatellite loci from the 186 isolates of *T. suttonii* from *Eucalyptus* leaves on trees growing in non-native plantations (Table 2). One allele at each of the loci TE3, TE8 and TE9 was shared by all populations and 4, 3, 10, 15 and 19 alleles were common among 6, 5, 4, 3, and 2 populations respectively. The populations URY-L and VNM had a single unique allele, URY-C and USA populations had two unique alleles and CHN and ZA populations had three unique alleles. There were 4 unique alleles in the IDN population (Table 2). The IND population had the largest number of alleles (43) and the URY-L population had the smallest (14) total number of alleles.

One hundred and twelve haplotypes were identified among the seven *T. suttonii* populations. The populations from ZAF, CHN, IDN, VNM, USA, URY-L, URY-C had 25, 22, 21, 17, 12, 4 and 11 genotypes respectively (Table 2). The maximum percentage genotypic diversity for the IDN population was (G*=91%) and this was in comparison to the high levels of genotypic diversity (G*=83%, 56%, 52%, and 42%) observed for the VNM, USA, CHN and ZAF populations respectively and the low values (G*=16% and 4%) for the URY-C and URY-L populations. Gene Diversity (H) was also high for IND, moderate for most other populations and low for URY-C. The mean total (H) for all the

T. suttonii populations combined was 0.68, higher than the values obtained for the individual populations (Table 2).

Results of the X^2 tests for the 11 polymorphic microsatellite loci showed significant differences in allele frequencies ($P<0.05$) at all loci for the clone corrected populations (Table 3).

Population differentiation, assignment and genetic distance

Based on the calculated theta values no significant genetic variability exists between the 2 Uruguay populations and the populations URY-L and ZAF and URY-L and IDN. However, a significant genetic difference was present between all other populations at $P<0.001$ (Table 4).

STRUCTURE analysis assigned the haplotypes into 6 groups (Figs.1 and 2 and Table 5). Of these, group 1 included haplotypes from Australia (FNQ and N-QLD, NSW, WA) and USA. Group 2 included isolates from Australia (NSW), URY-C, USA, ZAF, CHN, VNM and IDN. Group 3 included only Australia haplotypes from S-QLD and NSW. Group 4 contained haplotypes from Australia (FNQ) and IDN while group 5 included haplotypes from Australia, (C-QLD and NSW), USA, URY-C, ZAF and VNM. Group 6 included Australian haplotypes (C-QLD and NSW) as well as some haplotypes from CHN, VNM, URY-L, URY-C, USA and ZAF. Thus USA haplotypes were represented in 4 of the 6 groups, ZAF and VNM haplotypes were represented in 3 of the 6 groups and CHN and IDN haplotypes represented in 2 of the 6 groups. Australia haplotypes were represented

in all the 6 groups (Table 5). Group 2 was the most diverse, consisting of haplotypes from 7 different countries. There was a high level of admixture among the haplotypes (Fig. 2). The distance analysis (Fig. 3) segregated the haplotypes of *T. suttonii* into 2 large groups A consisting of representatives of structure groups 2, 4, 5 and B containing structure groups1, 3, 6 respectively. Thus group A consisted of all the WA haplotypes, the bulk of C-QLD, N-QLD, CHN, IDN, VNM halotypes and a minority of the ZAF and URY haplotypes while group B consisted of the bulk of NSW, S-QLD, USA, URY, ZAF, haplotypes and a few IDN and CHN haplotypes. The division of the isolates corresponded well to that of structure analysis with the exception that MV (C-QLD) and MJ (WA) haplotypes placed in group 1 in structure analysis were now clustered within group 2 in distance analysis. Generally as in structure analysis Australia haplotypes were present in all six groups and groups 1, 3, 4 and 5 consisted of a majority of the haplotypes from predefined Australia populations, while groups 2 and 6 consisted of mainly haplotypes from the introduced populations. Analysis of Molecular Variance showed 4% genetic variation among populations and 96% genetic variation within populations.

Mode of Reproduction

For the Linkage disequilibrium analysis the observed I_A and $r\bar{}D$ values for the combination of all genotypes in all populations and for the populations USA, IDN and CHN were greater than the output of 1,000 times simulated random mating, indicating a high level of clonality of these populations (Table 6). On the contrary no significant difference was established between the observed I_A and $r\bar{}D$ values and the simulated random mating data sets for the populations ZAF and VNM and the URY populations

combined (Table 6)**.** The null hypothesis that there is free recombination of alleles in these populations could not be rejected.

Discussion

The populations of isolates of *T. suttonii* collected from *Eucalyptus* leaves in six countries where these trees are grown as non-natives in plantations showed a range of genetic diversity. However, the overall trend was that diversity in populations from all countries examined except Uruguay was high. Genetic diversity of the Indonesian population was higher than for any of the populations previously studied from Australia (Chapter 4). However, while the diversity was high there were relatively few unique alleles in any of the populations, compared to what has been found for *T. suttonii* in native populations (Chapter 4). A significant amount of differentiation was established between a majority of the populations. Evidence for both sexual and asexual methods of reproduction was established in the populations.

The populations of isolates of *T. suttonii* from trees in introduced situation would have been expected to have low levels of genetic diversity and small numbers of rare alleles, typical of introduced pathogens (McDonald and McDermott 1993). The relatively high levels of genetic diversity observed for some introduced populations could be a consequence of multiple introductions of the pathogen from numerous sources within Australia or from other exotic plantations (Burgess et al. 2001). The high diversity observed in the populations from South Africa and Vietnam could also be a consequence of recombination as is reflected in the index of association values obtained for these

populations (Fig. 4). In well established fungal populations, in the absence of sexual reproduction, apparent diversity can also be increased by the stepwise accumulation of mutations at microsatellite loci, resulting in new haplotypes that are only slightly different to the original haplotype (Perez et al. 2010, 2012, Fig. 4). However there is no evidence for this process in the studied populations since only a small number of unique alleles were established in individual populations. In contrast, the low diversity observed in a population from Uruguay indicates that it represents a newly established founder population (Goodwin et al. 1994) as has been explained for low diversity observed in introduced *T. nubilosa* populations (Perez et al. 2012).

Four of the seven introduced populations show evidence of recombination, which was observed in only one of the native populations. It is therefore possible that the high allelic diversity observed in introduced populations is due to multiple introductions from different locations and genetic drift (Fig. 4)**.** Considering the different ways by which alleles and genotypes can be added to or acquired into non native plant pathogen populations (Fig. 4), the URY populations and the populations IDN, USA and CHN have probably obtained alleles and genotypes through patterns A and C respectively. Patterns G, H and I have probably been involved in the observed genetic composition of populations ZAF and VNM.

Assignment of introduced individuals in STRUCTURE reflected admixture for the introduced isolates. Consistent with this outcome, analysis of molecular variance showed low genetic variability among introduced populations and huge variation within

populations. This is in stark contrast with the native populations from Australia which had high genetic variability and low gene flow between populations (chapter 4). The implication is that there has been a substantial exchange of seeds and vegetative material among countries, in particular among plantation forestry companies that own land in several countries (Wingfield et al. 2008; Andjic et al. 2011). Similar findings of lack of geographical structure as a result of human mediated movement of infected germplasm between populations or common origin for different populations have also been reported among other introduced populations including *T. gauchensis* (Cortinas et al. 2011), *T. destructans* (Andjic et al. 2011), *T. nubilosa* (Hunter et al. 2008, Perez et al. 2010), *Chrysoporthe cubensis* (Nakabonge et al. 2007), *Ophiostoma ips* (Zhou et al. 2007).

A substantial amount of differentiation was observed among most of the populations and among the introduced and native populations. The results appear inconsistent with the observed admixture among populations. However, this can be explained by the fact that introduced populations consist of individuals from different origins. Assortment of alleles as a result of recombination, selection, genetic drift and bottlenecks among these populations can result in the loss of some alleles and or increased frequency of other alleles leading to apparent differentiation among populations. However, the original alleles still remain even if their frequency is low and the pattern of introduction can be inferred. Similar contrary diversity and assignment tests results have been reported for *Ophiostoma ipis* populations from different continents (Zhou et al. 2007). Insignificant differentiation obtained between the populations in Uruguay and between South Africa

and Indonesia populations could reflect high gene flow between populations or be an indication that the populations have recently been part of the same gene pool.

The allocation of *T. suttonii* haplotypes in STRUCTURE revealed a strong link of the Australia populations to geographic location as well as a link between introduced populations and populations in Australia. All the 6 groups obtained from STRUCTURE analysis consisted of Australia haplotypes together with haplotypes from different introduced populations. In line with these results the distance analysis grouped the haplotypes into 6 groups in which the Australia haplotypes were represented. The assumption is that each of the non native populations is a result of numerous introductions which originated in Australia (but may have come via an intermediary country) where the sources of these introductions still remain. Consequently the non-native populations are allocated into several structure groups. The implication is that *T suttonii* has been introduced to Indonesia, China, Vietnam, South Africa, Uruguay, and USA from the sub-tropics of eastern Australia (Fig. 5). A high proportion of haplotypes from China (95%), Indonesia (94%) and Vietnam (59%) were clustered with haplotypes from Queensland.

Structure group 2 has almost virtually no representatives from Australia. The source of the introduced populations in this group is Australia, but common alleles are missing between introduced populations in this group and Australian isolates. This most likely reflects incomplete sampling from Australia. Alternatively, these populations could have been isolated from Australian populations for a long time and as a consequence of genetic

drift and selection, different allele frequencies but not different alleles are observed in the introduced populations.

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Table 1 Isolates of *Teratosphaeria suttonii* investigated in this study

Origin	Code	Host	No. of Isolates	Date of Collection
Uruguay; La Negra	URY-L	*E. grandis x E. urophylla*	38	February 2009
Uruguay; Caldras	URY-C	*E. grandis x E. urophylla*	23	February 2009
United States of America; Florida	USA	*E. grandis*	18	
China; Fujian Province	CHN	*Eucalyptus sp.*	32	
Indonesia; Aek Nauli, Sumatra	IDN	*Eucalyptus sp.*	22	
Vietnam, Dai Lai, Vinh Phuc province	VNM	*E. urophylla*	17	
South Africa; Pretoria	ZAF	*E. grandis x E. urophylla*	36	August 2007
Total			**186**	

Table 2 Allele sizes and frequencies and other indicators of diversity of the 7 populations of *T. suttonii* obtained from the amplification of the 11 microsatellite loci.

Locus	Allele	La Negra	Caldras	Florida	Pretoria	Vietnam	China	Indonesia
TE1	457	1.0000	0.9091	0.3333	0.8400	-	0.0909	0.0952
	464	-	-	0.0833	-	-	-	-
	465	-	-	-	-	1.0000	0.5000	0.0476
	472	-	-	0.2500	0.1600	-	-	-
	497*	-	0.0909	-	-	-	-	0.6190
	498*						0.1818	
	NULL*	-	-	0.3333	-	-	0.2273	0.2381
TE2	212	-	-	-	-	0.5882	-	-
	213*	-	-	-	-	-	0.0909	-
	215*	-	-	-	-	-	-	0.3333
	216*	-	-	-	-	-	0.2273	0.1905
	217	-	-	0.6667	-	-	0.4545	0.1429
	218*	-	-	-	-	-	0.0455	0.1905
	223	-	0.3636	0.3333	-	0.4118	-	0.0476
	224	1.000	0.6364		0.9600		0.0909	
	226	-	-	-	0.0400	-	-	-
	NULL	-	-	-	-	-	0.0909	-
TE3	189*	-	-	-	0.0400	-	-	0.0476
	204	-	0.0909	-	0.2800	-	-	0.3333
	205	-	-	-	0.0400	0.2353	0.9091	0.1905
	206	0.5000	0.7273	1.0000	0.4800	0.7647	0.0455	0.1429
	207	0.5000	0.1818	-	-	-	0.0455	-
	NULL	-	-	-	0.1600	-	-	0.1429
TE4	142	1.0000	0.9091	0.7500	0.6400	-	0.1818	0.0476
	151	-	-	-	-	0.8235	0.2273	0.2381
	154	-	-	-	-	-	0.5909	0.3810
	175	-	0.0909	0.2500	0.3600	0.1765	-	-
	NULL*	-	-	-	-	-	-	0.3333
TE5	136	1.0000	0.7273	0.5000	0.7600	-	0.0455	0.6667
	138	-	-	-	-	-	0.0455	0.1429
	140	-	-	-	-	0.7647	0.7727	-
	143	-	0.1818	-	0.1200	-	-	-
	145	-	0.0909	0.5000	0.0800	0.2353	-	0.1429
	NULL	-	-	-	0.0400	-	0.1364	0.0476
TE6	82*	-	-	0.0833	-	0.4118	-	-
	83*	-	-	0.9167	-	0.2353	-	-
	99	1.0000	0.9091	-	0.9600	-	-	0.1905
	100	-	0.0909	-	0.0400	-	1.0000	0.7619
	NULL	-	-	-	-	0.3529	-	0.0476
TE7	196	0.7500	0.8182	-	0.8800	0.7647	-	0.1905
	197*	0.2500	0.1818	-	0.0400	0.0588	0.0909	0.0952
	199	-	-	1.0000	-	0.1765	0.0455	-
	202	-	-	-	-	-	0.0636	0.4286
	NULL	-	-	-	0.0800	-	-	0.2857
TE8	196	-	-	-	0.4800	-	-	-
	197	1.0000	1.0000	1.0000	0.4400	1.0000	0.9545	1.000
	NULL*	-	-	-	0.0800	-	0.0455	-
TE9	242	-	-	-	-	0.9412	0.8182	0.8571
	251*	-	0.8182	-	-	-	-	-

Locus	Allele	La Negra	Caldras	Florida	Pretoria	Vietnam	China	Indonesia
	252	1.0000	0.0909	1.0000	1.0000	0.0588	0.0455	0.0476
	NULL	-	0.0909	-	-	-	0.1364	0.0952
TE10	164	0.7500	0.8182	0.6667	0.7600	-	0.0455	-
	172	-	-	-	-	0.9412	0.6818	0.1429
	176	0.2500	-	-	-	-	-	-
	180	-	0.0909	0.3333	0.2400	-	0.0455	-
	184*	-	-	-	-	-	-	0.0952
	194	-	-	-	-	-	-	0.0476
	204	-	0.0909	-	-	0.5880	0.1818	0.5238
	NULL	-	-	-	-	-	0.0455	0.1905
TE11	175	-	0.7273	-	0.7200	0.5294	-	-
	176	1.0000	-	-	-	-	0.1364	
	177*	-	-	-	0.0400	-	-	-
	178	-	0.0909	0.1667	0.0400	0.1176	-	-
	184*	-	0.0909	-	-	0.2941	0.5455	0.2486
	194*	-	0.0909	-	-	-	-	-
	196	-	-	-	0.0400	-	0.0455	-
	197	-	-	0.2500	0.0800	-	-	0.1905
	199	-	-	-	-	-	-	0.2858
	202	-	-	0.5000	-	-	-	-
	205	-	-	0.8333	0.0800	-	-	0.0476
	NULL	-	-	-	-	0.0588	0.2727	0.0476
Ni		**38**	**23**	**18**	**36**	**19**	**32**	**22**
Ng		**4**	**11**	**12**	**25**	**17**	**22**	**21**
Na		**14**	**27**	**22**	**32**	**24**	**38**	**43**
Nua		**1**	**2**	**2**	**3**	**1**	**2**	**4**
Npl		**3**	**10**	**7**	**10**	**9**	**10**	**10**
G		**1.61**	**3.86**	**10.125**	**15.069**	**15.695**	**16.51**	**80.645**
G*		**4%**	**16%**	**56%**	**42%**	**83%**	**52%**	**91%**
H		**0.11**	**0.2900**	**0.2980**	**0.3238**	**0.3058**	**0.3847**	**0.5364**

Ni Total number of isolates (non clone corrected)
Ng Number of genotypes
Na Number of alleles
Nua Number of Unique alleles
Npl Number of polymorphic loci
G Genotypic Diversity (Stoddart and Taylor 1988)
G*= G/N% Percentage maximum diversity of genotypes
H Gene diversity (Nei 1973)
Highlighted alleles are unique within specific populations
* Alleles unique to introduced populations

Table 3 Gene diversity and Chi^2 tests for differences in allele frequencies for the 11 microsatellite loci across the clone corrected *T. suttonii* populations.

			Gene diversity (H)						
Locus	**La Negra**	**Caldras**	**Florida**	**Vietnam**	**Pretoria**	**China**	**Indonesia**	***X*^{2}**	**df**
TE1	0.00	0.17	0.71	0.00	0.27	0.65	0.55	309.39	30
TE2	0.00	0.46	0.44	0.48	0.07	0.71	0.78	428.62	45
TE3	0.50	0.43	0.00	0.36	0.66	0.17	0.69	103.43	25
TE4	0.00	0.17	0.38	0.29	0.46	0.57	0.68	381.01	20
TE5	0.00	0.43	0.50	0.36	0.40	0.38	0.51	195.78	25
TE6	0.00	0.17	0.15	0.65	0.08	0.00	0.38	347.17	20
TE7	0.38	0.30	0.00	0.38	0.22	0.24	0.69	138.76	20
TE8	0.00	0.00	0.00	0.00	0.57	0.09	0.00	113.84	10
TE9	0.00	0.31	0.00	0.11	0.00	0.31	0.25	307.63	15
TE10	0.38	0.31	0.44	0.11	0.36	0.50	0.66	231.87	35
TE11	0.00	0.45	0.65	0.62	0.46	0.61	0.69	511.57	55
Mean	**0.11**	**0.29**	**0.30**	**0.31**	**0.32**	**0.38**	**0.54**		

H Gene Diversity (Nei 1973)

Significant differences in allele frequencies (P<0.005) at all loci based on Chi^2 tests.

df Degrees of freedom = (no. populations -1) X (no. alleles -1)

Table 4 Pairwise comparison of population differentiation (θ) among the 7 *T. suttonii* populations from Uruguay, USA, Vietnam, South Africa, China and Indonesia

	Uruguay-Caldras	Florida	Vietnam	Pretoria	China	Indonesia
Uruguay-La Negra	0.284	0.481***	0.628***	0.229	0.577***	0.405
Uruguay-Caldras	-	0.498***	0.567***	0.237***	0.574***	0.413***
Florida	-	-	0.576***	0.489***	0.567***	0.441***
Vietnam	-	-	-	0.594***	0.424***	0.402***
Pretoria	-	-	-	-	0.601***	0.469***
China	-	-	-	-	-	0.253***
Indonesia	-	-	-	-	-	-

*Significant P<0.001

Table 5 Number of *T. suttonii* haplotypes allocated to the 6 groups derived from STRUCTURE analysis. N/A denotes isolates not assigned to any of the 2groups

Origin of isolates	No. of haplotypes	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	N/A
DC (N-QLD)	22	**2**	-	-	**19**	-	-	
KMA (C-QLD)	32	-	-	-	-	**32**	-	
IMB (S-QLD)	43	-	-	**36**	-	-	**7**	
WB (NSW)	27	**27**	-	-	-	-	-	
MV (C-QLD)	8	**8**	-	-	-	-	-	
MJ (WA)	3	**3**	-	-	-	-	-	
EC (NSW)	3	-	-	**3**	-	-	-	
MC (NSW)	3	-	**1**	-	-	**1**	**1**	
KW (NSW)	5	-	-	**4**	-	-	-	
KB (NSW)	4	**2**	-	**1**	-	**1**	-	
NVS (NSW)	2	**1**	-	**1**	-	-	-	
TGB (NSW)	3	-	-	-	-	**3**	-	
MRW (NSW)	3	**1**	-	**1**	-	-	**1**	
GS (NSW)	2	-	-	-	-	-	**2**	
La Negra	4	-	-	-	-	-	**4**	
Caldras	11	-	**1**	-	-	**1**	**9**	
Florida	12	**4**	**3**	-	-	**3**	**1**	**1**
Pretoria	25	-	**9**	-	-	**2**	**14**	
China	22	-	**21**	-	-	-	**1**	
Vietnam	17	-	**10**	-	-	**1**	**5**	**1**
Indonesia	17	-	**16**	-	**1**	-	-	

DC Davies Creek, KMA Koumala, IMB Imbil, WB Wedding Bells, MV Mariamvale, MJ Manjimup, Emu Creek, MC Myrtle Creek, KW Kew, KB Kimbell, NVS Neaves, TGB Tunglebung, MRW Morrow, GS Garas,

Table 6 Multilocus disequilibrium estimate I_A for 1000 randomized data sets.

Population	**Observed I_A**	**Range obtained I_A values**	**P value**
Uruguay	0.8168	-0.4673-1.3855	P=0.008
Florida	1.6123	-0.3962-0.8491	P<0.001
Vietnam	0.3356	-0.3981-0.8034	P=0.049
Pretoria	0.0418	-0.4043-0.7265	P=0.375
China	0.6369	-0.4306-0.6369	P=0.001
Indonesia	0.6881	-0.3033-0.0459	P=0.049
All	1.8573	1.4883-1.8573	P<0.001

*Significant P<0.001

Figure 1 Optimum number of populations (K=6) determined from LnK and DeltaK values obtained from STRUCTURE analysis.

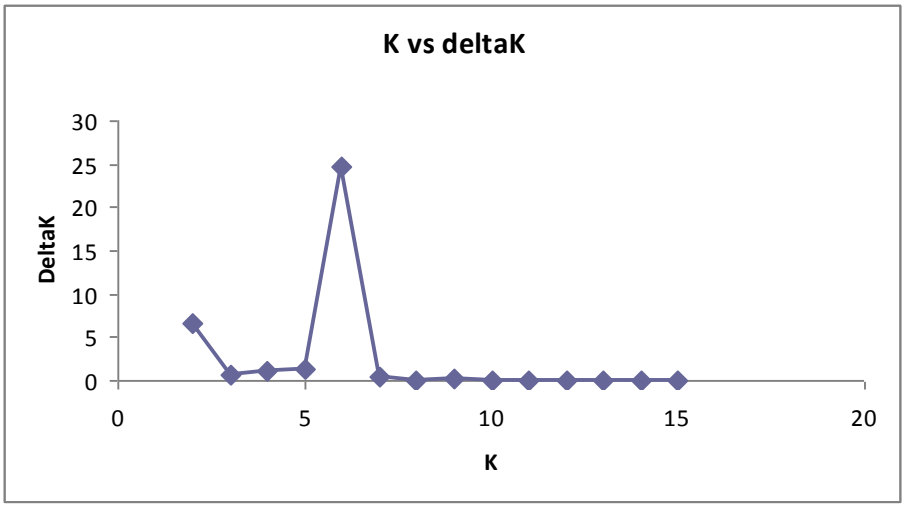
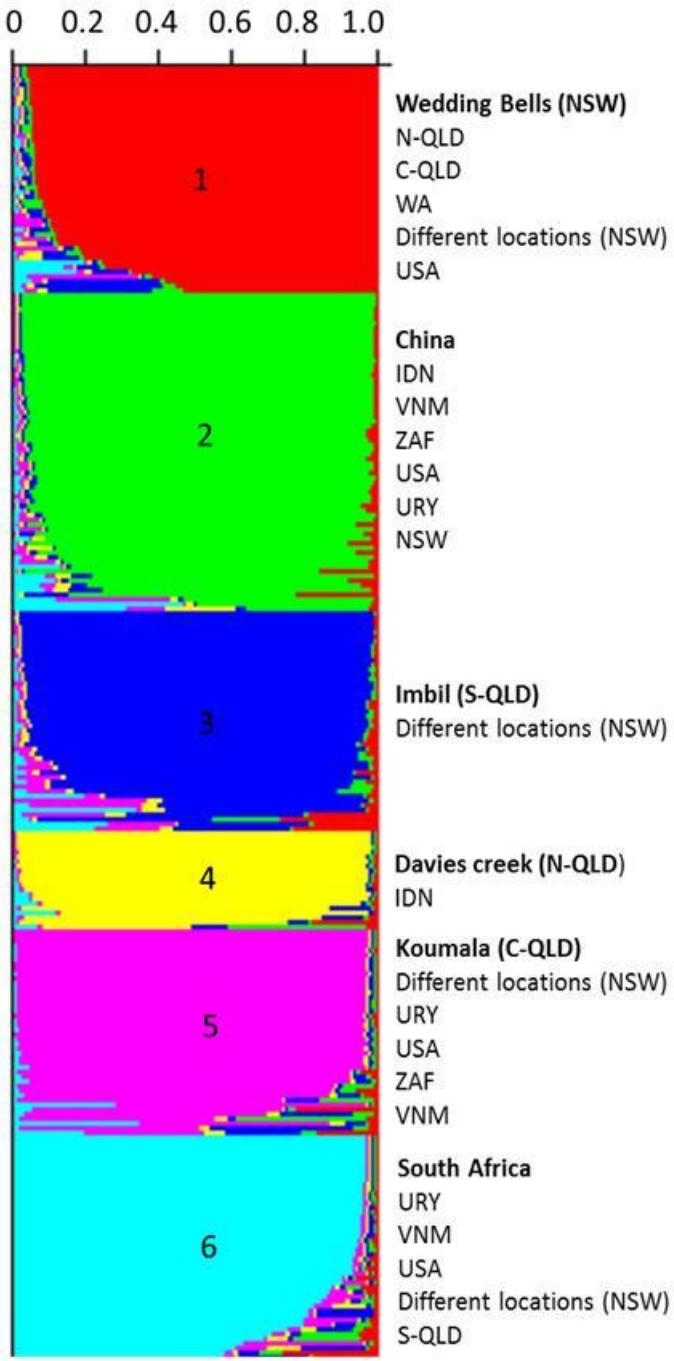
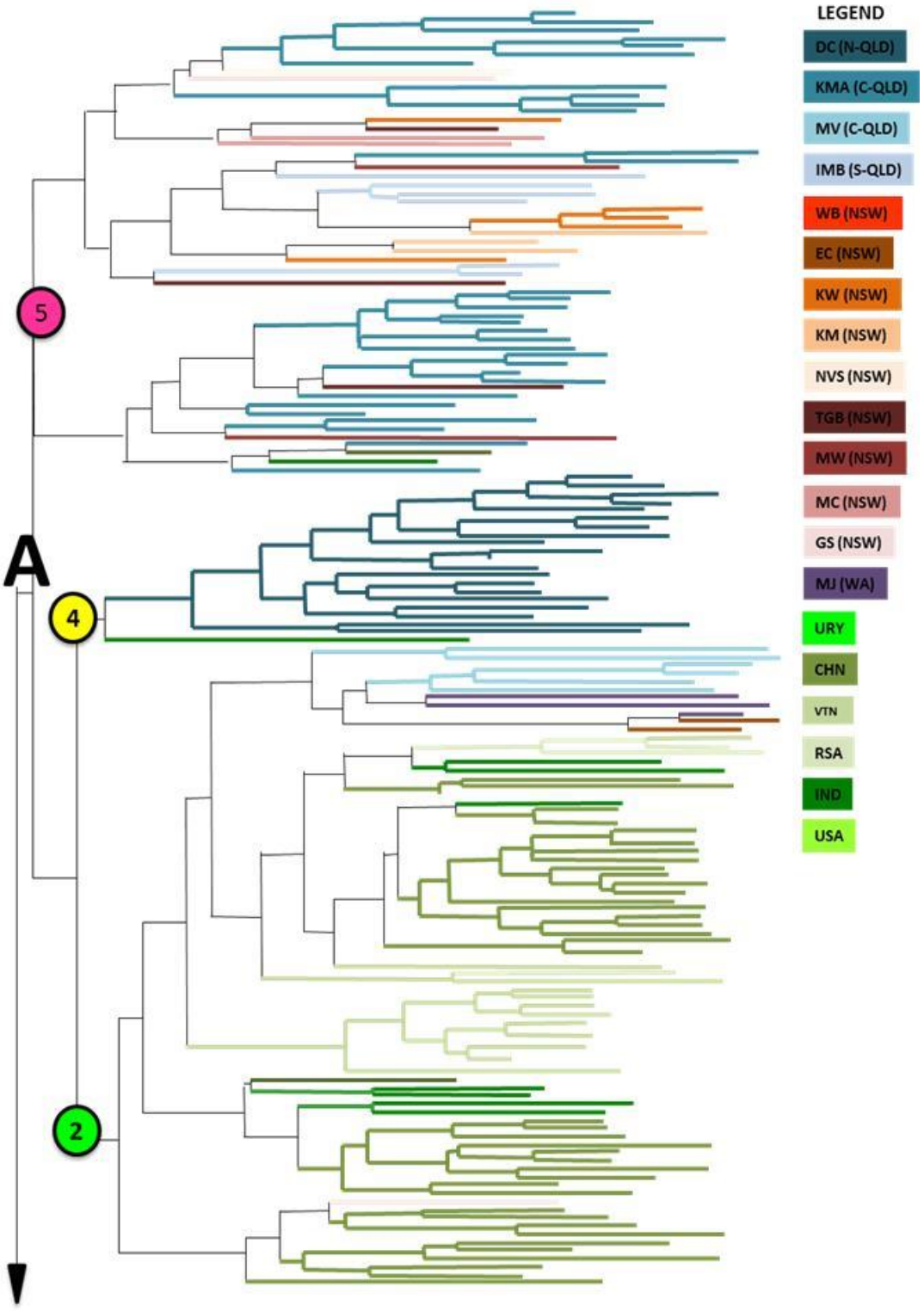


Figure 2 An assignment diagram showing the assignment of individual isolates established from allele frequencies as determined by STRUCTURE 2.2. The coloured sections rpresent 7 underlying genetic populations identified from the genotyped individuals. Individual genotypes affiliated with several populations are represented by the multicoloured vertical bars. The height of each colour within an individual provides a measure of the amount of affiliation of an individual to different populations.



Numbers 1-6 correspond to group numbers on neighbor joining tree
Predominant members of the group in bold

Figure 3 Neighbour joining tree of *T. suttonii* demonstrating relationships among haplotypes from native and non native populations. The Australian populations were genetically distinct from one another.



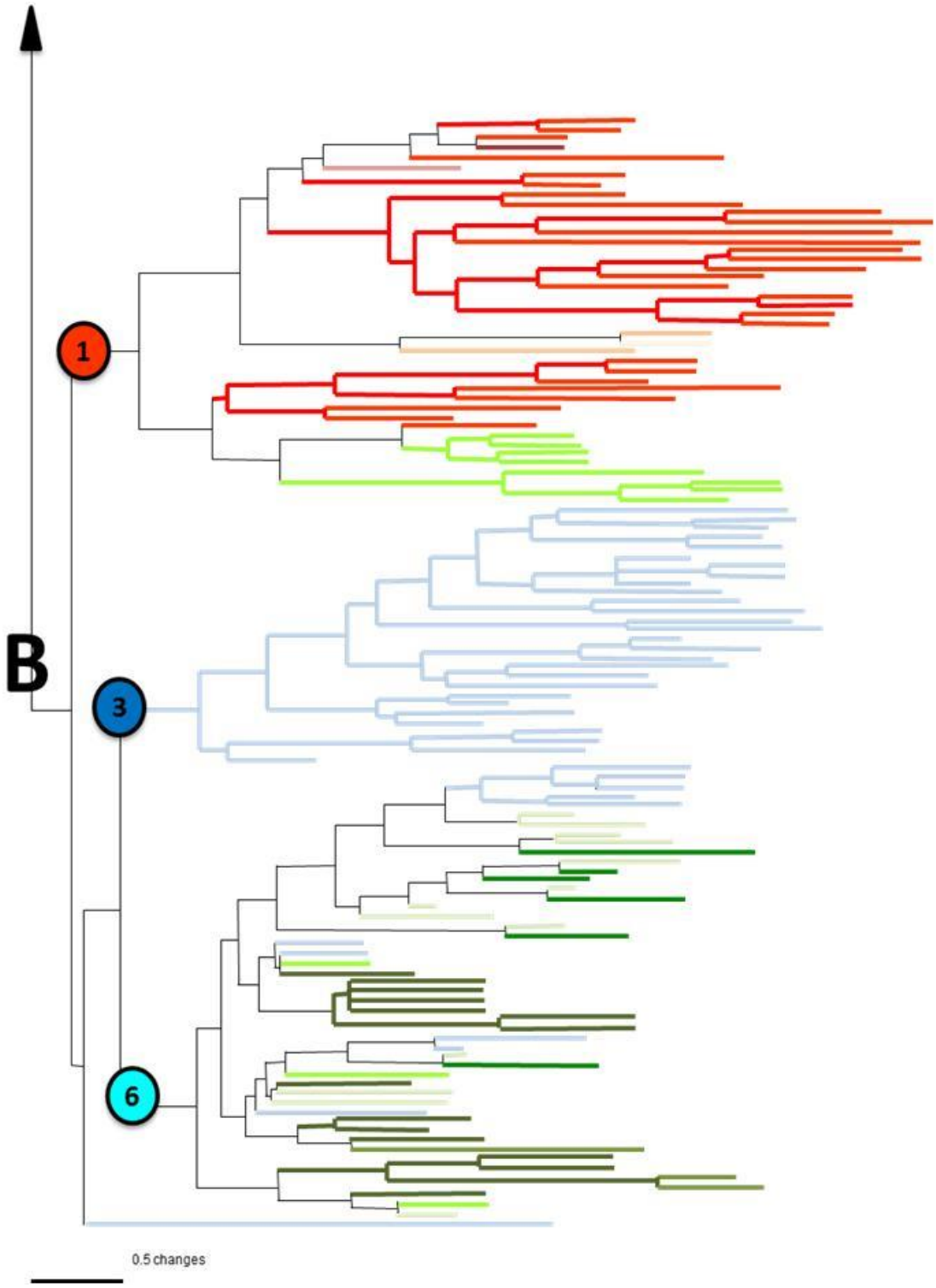


Figure 4 Some of the assumed possible models through which alleles and genotypes can be introduced into or acquired by introduced pathogen populations over time. **A.** Small founding population characterized by few genotypes. Clonal reproduction over time generates a population with low allelic and genotypic diversity. **B.** Large founding population in which several genotypes were added simultaneously and reproduction is clonal over time. A population with high allelic diversity and moderate genotypic diversity results. **C.** Repeated introductions represented by many genotypes, in a clonally reproducing population result in a population with high allelic diversity and moderate genotypic diversity. **D**. A small founding population characterized by few genotypes, clonal reproduction and mutation, produce high allelic diversity and moderate genotypic diversity in the resulting population. **E.** Large founding population showing several genotypes, selection favours some genotypes resulting in a population with low allelic and genotypic diversities. **F**. Small founding population showing few genotypes, outcrossing over time generates low allelic diversity and high genotypic diversity in the population. **G**. Small founding population characterized by few genotypes, outcrossing and mutation through time result in a population with high allelic and genotypic diversities. **H**. Large founding population with several genotypes. Clonal reproduction and mutation over time generate high allelic and genotypic diversities in the population. **I**. Large founding population with several genotypes, Clonal reproduction, mutation and selection result in a population with high allelic and genotypic diversities.

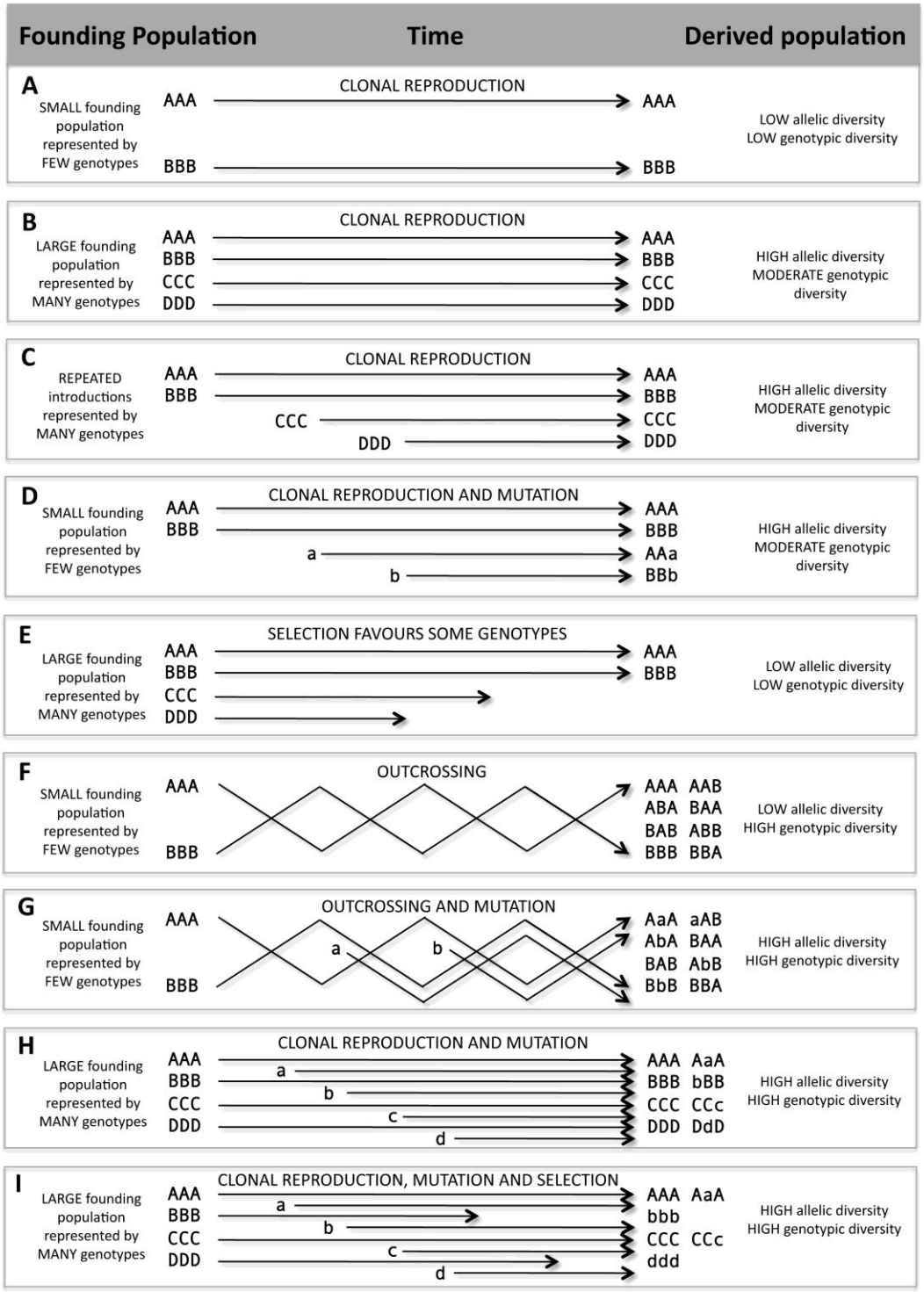
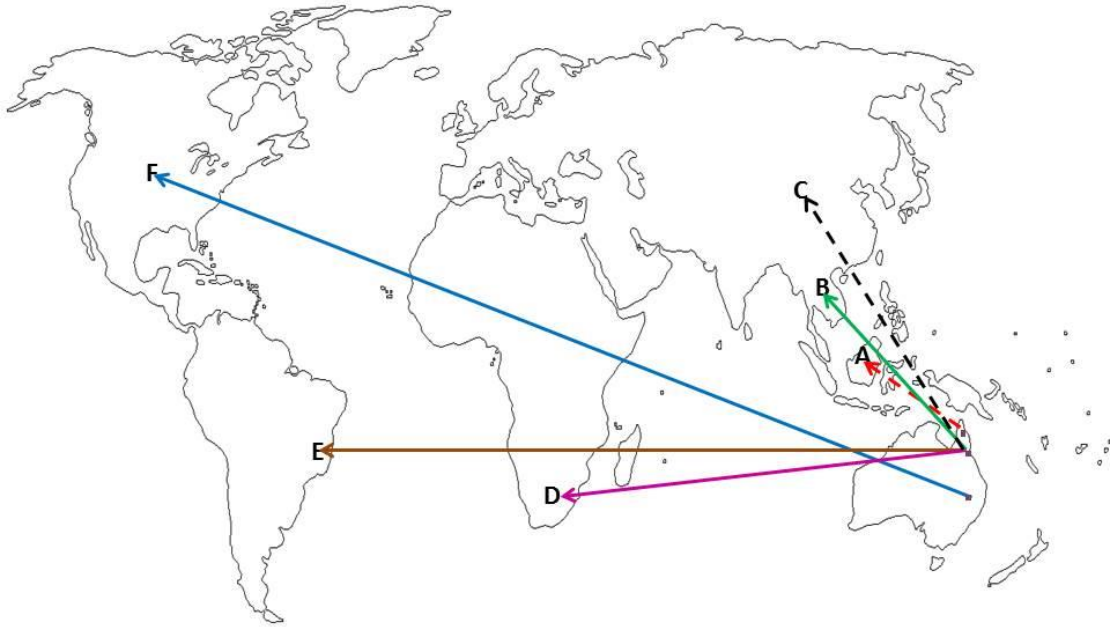


Figure 5 An outline of the world map showing the possible dispersal routes of *T.suttonii* from Australia to A. Indonesia, B. Vietnam, C. China, D. South Africa, E. Uruguay and F. USA. Solid arrows represent the mist likely sources of isolates in Australia, the dotted lines represent questionable likely souces of isolates in Australia.



SUMMARY

Teratosphaeria leaf disease due to *T. suttonii* is a ubiquitous disease of a variety of important *Eucalyptus* species in both their native and introduced environments in many parts of the world.

Research in this thesis focused on the taxonomy, phylogenetics and population genetics of *T. suttonii* from different parts of the world. Multigene genealogies confirmed the isolates of *T. suttonii* from different geographic origins as one genetically and morphologically diverse species and provided evidence that isolates from different non native origins were closely associated with Australia isolates. In order to evaluate the population dynamics of *T. suttonii*, eleven polymorphic microsatellite markers were designed and used to carry out population genetic studies of local and introduced populations of the fungus. Population genetics revealed high levels of genetic diversity of both native and non native populations of *T. suttonii*. There were no shared genotypes among native populations, non native populations and between the native and non native populations. Isolates of *T. suttonii* from the native range were allocated to groups according to their geographic origin. But isolates from introduced localities were divided among groups, in close association with isolates from different parts of Australia, implying independent introductions of the pathogen from different localities in Australia. Lack of association between alleles supports recombination in one Australia population and four introduced populations. However, it is believed that the profile in introduced populations was a result of repeated introductions and drift and not necessarily recombination. Essentially the results indicate that extensive movement of individual fungal isolates between

different geographic locations has led to high diversity of the pathogen and interrupted the processes related to local differentiation and isolation.

CONCLUSION

Teratosphaeria leaf disease is an important disease of natural forests and commercial eucalypt plantations in their native Australia and across the world. The disease is caused by Ascomycetous fungi in the family *Teratosphaeriaceae*. Among the species, *Teratosphaeria suttonii* has the widest distribution having been introduced to other countries most likely with diseased plant material used in the establishment of plantations.

Control of plant diseases is essential for the production of good quality plantation products. Plant pathogenic fungi have caused loss of forest products. The situation has compelled plant pathologists to engage in the development strategies towards inhibiting, reducing or regulating diseases. The utilization of chemical fungicides and the selective breeding to manipulate the genetic ability of the host plant to withstand infection have been widely used to combat plant diseases. But with the development of rapidly growing clonal plantations, conditions favorable to the rapid evolution and adaptation of pathogen populations have been created.

In order to implement an effective Teratosphaeria leaf disease management strategy accurate identification and understanding of the biology of *T. suttonii* is required. This thesis is concerned with the taxonomy, phylogenetics and population genetics of *T. suttonii* from different parts of the world. Initial observations suggested that *T. suttonii* may be a species complex.

Thus the aims of this thesis were to:

1. Study the phylogenetic relationship between a diverse set of isolates and coupled with morphological measurements determine if this is a complex of species or a single species
2. Study the population genetics of *T. suttonii* to understand the structure within natural and introduced populations and understand the movement of this pathogen around the world.

The current scholarly information on *T. suttonii* was reviewed and reported in chapter 1. Chapter 2 of this thesis focused on the comparisons of gene sequence data obtained from three gene regions and spore morphology to characterize and determine the taxonomic classification of isolates of *T. suttonii* collected from Australia and six other countries. ITS DNA sequence data elucidated the isolates of *T. suttonii* into a number of distinguishable strongly supported clades, supporting the hypothesis that *T. suttonii* was a species complex. But BT and EF DNA sequence data were less variable. In general genealogies were not congruent and a close relationship was established among isolates from different parts of the world and Australia isolates, so that groups of isolates could not be related to place of origin. Furthermore, extensive conidial morphology was observed, but could also not be related to phylogenetic groups. The results indicated that *T. suttonii* is a morphologically and genetically diverse species but not a species complex. A number of assumptions were made at this point from the results.

1. The incongruence in the genealogies of different genomic regions of DNA was assumed to be a result of alleles at different loci being shuffled by genetic exchange and or recombination to create new genotypes that can result in organisms exhibiting different traits.
2. Since isolates from Australia were observed to be scattered throughout the phylogenetic trees and in general highly supported subclades containing isolates from different countries were obtained, an assumption was made that the isolates have crossed their evolutionary boundaries, have been united and evolved as a single unit as was evidenced by
3. The close proximity of Australian isolates with isolates from other countries was considered confirmation that the pathogen has been introduced from Australia to the rest of the world and an indication of the high genetic diversity of this pathogen in Australia.

Microsatellites have been qualified as useful markers to investigate the population dynamics of fungal pathogens. In order to establish an understanding into the genetics and movement of *T. suttonii* across countries, 11 polymorphic microsatellite markers were developed in chapter 3. The procedure involved designing primer pairs from potential microsatellite regions obtained from Pyrosequencing of ISSR-PCR generated fragments, from three *T. suttonii* isolates from two countries. The markers were used to screen populations of *T. suttonii* from different localities in Australia in chapter 4 and from different localities where the pathogen has been introduced in chapter 5.

The application of microsatellite markers to *T. suttonii* isolates from Australia (Queensland and New South Wales), provided evidence that *T. suttonii* populations from Australia are characterized by high genetic diversity. Based on population differentiation and assignment tests and distance analysis, the populations were found to be genetically distinct. The observed independent evolution of the pathogen in different localities was considered to be a result of limited gene flow. Results of the study further provided evidence of recombination in one of the four investigated populations and that *T. suttonii* has been newly introduced into Western Australia from central Queensland.

A population genetics study of seven introduced *T. suttonii* populations indicated a general high genetic diversity. Evidence of recombination was provided in four of the seven investigated populations. Considering limited recombination previously observed in the place of origin of the pathogen, Australia, the later situation was explained in terms of numerous introductions of the pathogen from different sources. Furthermore genetic drift was considered to be a possible factor for the observed recombination. Based on both STRUCURE and distance analysis Australia haplotypes were grouped according to geographic locations, while members from different introduced populations were placed in groups together with Australian counterparts. Such admixture within the introduced populations was interpreted as an indication that the pathogen has originated from different areas in Australia and that there has been multiple introductions of the pathogen from Australia and other exotic localities into different non-native areas.

Both phylogenetics and population genetics studies have supported the notion that Australia represents the centre of origin of *T. suttonii* and that the pathogen has been introduced from Australia to different parts of the world. The studies have demonstrated high diversity of *T. suttonii* in Australia and in countries where the pathogen has been introduced. While Australia populations are strongly linked to their geographic locations indicating limited movement of infected germplasm across localities, introduced populations lack geographic subdivision. The situation is largely a consequence of anthropogenic activities related to the establishment of plantations (plants for planting). Haplotypes from different origins formed distinct groups which could be related to the Australian groups. Thus the pattern of introduction from Australia could be established.

Results of the population genetic studies support a high evolutionary potential of *T. suttonii*. For the introduced populations, this feature does not support adaptation and hence complicates attempts to control the pathogen. Continued introductions of *T. suttonii* into non native populations have complicated the determination of the distribution pattern of this fungus.

This study has revealed the relationships of isolates of *T. suttonii* from different countries as well as the diversity and evolutionary potential of this pathogen in different countries. Data obtained from this thesis is valuable since it shows that the common method of disease control (development of resistant genotypes) will not be effective against *T. suttonii*. Other genetic factors such as increased thickness of palisade layer and decreased air paces within cells in leaves, which have been found to convey resistance to *T. nubilosa* and *T. cryptica* in *E. nitens*, will probably help

reduce the impact of species of *Teratosphaeria* on *Eucalyptus*. Reducing host stress will only help establish good hygiene in nurseries and healthy plantations, but not help combat the disease.

The question which still remains is, does recombination really exist in *T. suttonii*? It would be appropriate to assess the isolates of *T. suttonii* for the presence of mating type loci and establish how they compare with homologues of genes that confer mating in related sexually reproducing fungi in order to answer this question.

ANNEXURE

Microsatellite loci polymorphisms between different *T. suttonii* isolates

Figure 1 Polymorphism at locus TE1.

Figure 2 Polymorphism at locus TE2.

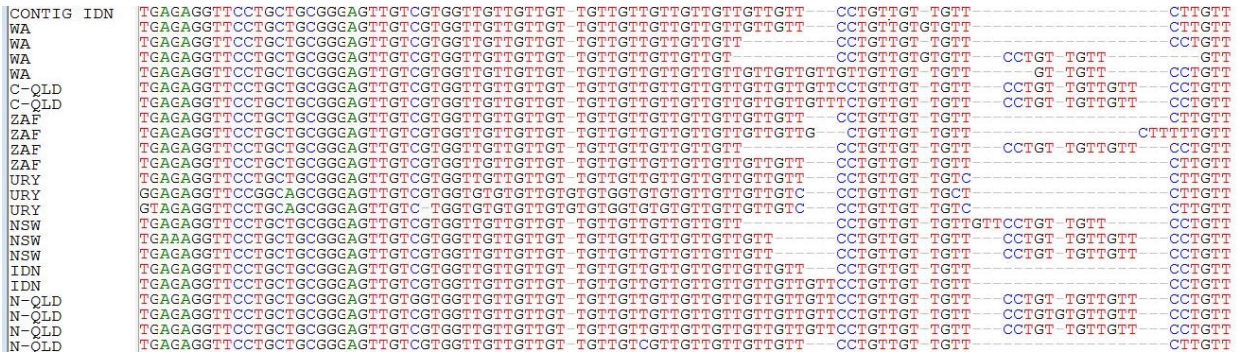


Figure 3 Polymorphism at locus TE3

Figure 4 Polymorphism at locus TE4

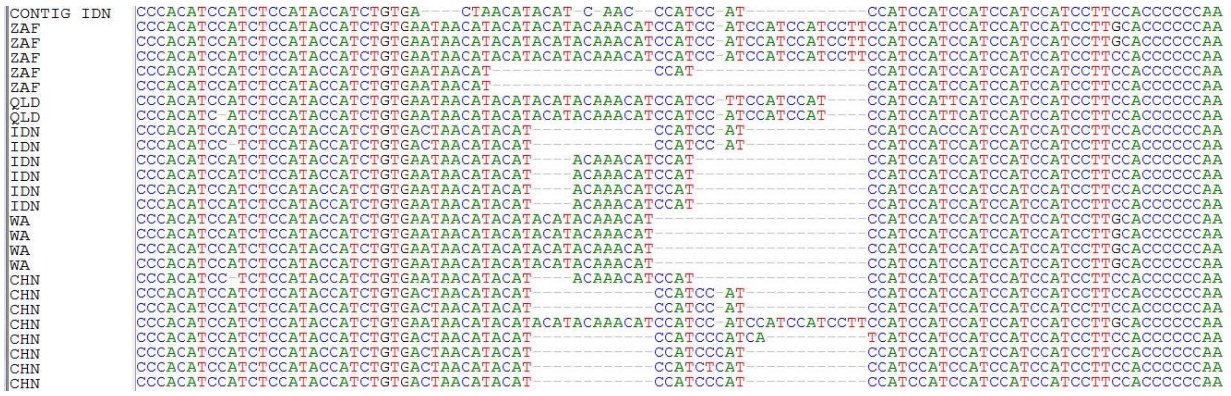


Figure 5 Polymorphism at locus TE5

Figure 6 Polymorphism at locus TE6

Figure 7 Polymorphism at locus TE7

Figure 8 Polymorphism at locus TE8

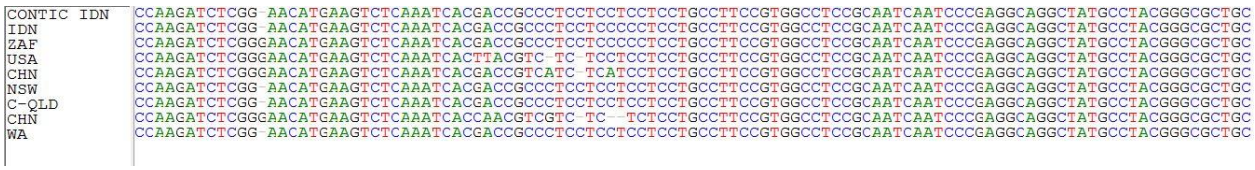


Figure 9 Polymorphism at locus TE9

Figure 10 Polymorphism at locus TE10

Figure 11 Polymorphism at locus TE11