Mycoscience (2012) 53:270–283
DOI 10.1007/s10267-011-0164-x

FULL PAPER

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

Matsepo M. Taole · Treena I. Burgess · Marieka Gryzenhout · Brenda D. Wingfield · Michael J. Wingfield

Received: 2 August 2011 / Accepted: 8 November 2011 / Published online: 27 November 2011

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, β-tubulin, and elongation factor-1α genes was sequenced and analyzed for isolates from throughout the range of *T. suttonii* in Australia, and from six countries (China, Indonesia, South Africa, Uruguay, United States, 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. Similarly, 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.

Keywords Eucalyptus diseases · Fungal populations · Fungal taxonomy · Phylogenetics

Introduction

Species of *Eucalyptus* are a primary source of fiber 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 non-native environments has, in part, contributed to the success of introduced *Eucalyptus* plantations (Burgess and Wingfield 2002b; Wingfield et al. 2008). However, the productivity of commercial *Eucalyptus* plantations is increasingly reduced worldwide 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.

M. M. Taole · M. J. Wingfield
Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa

T. I. Burgess
CRC for Forestry, School of Biological Sciences and Biotechnology, Murdoch University, Murdoch 6150, Australia

M. Gryzenhout
Department of Plant Sciences, University of the Free State, Bloemfontein 9301, South Africa

B. D. Wingfield (✉)
Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa
e-mail: Brenda.wingfield@fabi.up.ac.za

2000). They cause a complex of diseases, 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 in *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 families 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*.' Although the origin of *T. destructans* (Wingfield et al. 1996), the most destructive of these pathogens, is unclear (Andjic et al. 2010; 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 caused by these fungi results in the formation of irregular chlorotic or necrotic lesions on both surfaces of the leaves (Andjic et al. 2007a, b, 2010; 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 subgenera *Corymbia*, *Monocalyptus*, *Symphyomyrtus*, and *Indiogenes*. Carnegie et al. (1997) reported *T. suttonii* on three new hosts, namely, *Eucalyptus 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 aging 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 the 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, stage of development of infection, and climatic conditions, a variety of disease symptoms caused by infection with *T. suttonii* can be observed (Pegg et al. 2003; Walker 1962), thus leading to incorrect diagnosis of the pathogen.

A multiple genealogy study based on four gene regions grouped the isolates of *T. suttonii* from Australia, China, Indonesia, and South Africa into three well-supported subclades that could not be related to geographic origin (Andjic et al. 2007b). Several studies have demonstrated

Fig. 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 undersurface of a *Eucalyptus* leaf. **d** Infected leaf of a cutting during the rooting process where the infection by the fungus can reduce root strike

the significance of multilocus sequence typing (MLST) in resolving relationships among fungal strains, characterizing the genetic diversity, and identifying cryptic species (Taylor and Fisher 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 to 1. GSI values at or close to 0 indicate the early stages of lineage sorting and thus the 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 TLD 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 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) (20 g l^{-1} Biolab malt extract, 15 g l^{-1} Biolab agar) and left to hydrate for 5 min. 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 150 μg ml^{-1} streptomycin (Sigma-Aldrich). Cultures were grown at 25°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 in pretoria mycological (PREM, South African National Collection of Fungi).

Multilocus sequence typing

Total DNA was extracted from actively growing cultures by scraping mycelium from the surface of cultures into sterile 1.5-ml Eppendorf tubes and grinding freeze-dried mycelium to a fine powder. This step was followed by DNA extraction using the protocol of Cortinas et al. (2004), modified by adding Proteinase K (1 μg/μl) to the extraction buffer and 5 μl (1 mg/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 ultraviolet light. Subsequently, the concentrations of the extracted DNA were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, DE, USA).

For the purpose of phylogenetic comparisons between isolates, polymerase chain reaction (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-1α) gene region using primers EF1-728F and EF1-986R (Carbone and Kohn 1999). All amplifications were carried out in 25-μl 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.

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

Culture no.[a]	PPRI no.	Herbarium no.	Host[b]	Location[c]	Isolator	GenBank accession no.[d]		
						ITS	EF-1α	β-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.* ×*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.* × *E. c.*	Australia; QLD	G Hardy	JF342919 (4)	JF793425 (1)	JF793335 (1)
CMW31938			*E. g.* × *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.* × *E. c.*	Australia; NSW, Kew	V Andjic	JF342958 (4)	JF793463 (1)	JF793373 (1)
*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.* × *E. u.*	South Africa; Gauteng, Pretoria	MM Taole	JF342924 (4)	JF793430 (1)	JF793340 (2)
CMW29031		PREM60487	*E. g.* × *E. u.*	South Africa; Gauteng, Pretoria	MM Taole	JF342925 (4)	JF793431 (1)	JF793341 (2)
CMW32939		PREM60496	*E. g.* × *E. u.*	South Africa; Gauteng, Pretoria	MM Taole	JF342928 (4)	JF793434 (1)	JF793344 (2)
CMW32940		PREM60494	*E. g.* × *E. u.*	South Africa; Gauteng, Pretoria	MM Taole	JF342927 (4)	JF793433 (1)	JF793343 (2)
*CMW32941		PREM60498	*E. g.* × *E. u.*	South Africa; Gauteng, Pretoria	MM Taole	JF342926 (4)	JF793432 (3)	JF793342 (2)
*CMW32942		PREM60499	*E. g.* × *E. u.*	South Africa; Gauteng, Pretoria	MM Taole	JF342929 (4)	JF793435 (1)	JF793345 (1)
CMW32943		PREM60500	*E. g.* × *E. u.*	South Africa; Gauteng, Pretoria	MM Taole	JF342930 (4)	JF793436 (2)	JF793346 (2)
CMW32781[H]	11219	PREM60492	*E. g.* × *E. u.*	Uruguay; Caldras	MJ Wingfield	JF342933 (4)	JF793439 (2)	JF793349 (2)
*CMW32814		PREM60494	*E. g.* × *E. u.*	Uruguay; La Negra	MJ Wingfield	JF342934 (4)	JF793440 (1)	JF793350 (2)
*CMW32803		PREM60493	*E. g.* × *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)

Table 1 continued

Culture no.[a]	PPRI no.	Herbarium no.	Host[b]	Location[c]	Isolator	GenBank accession no.[d]		
						ITS	EF-1α	β-tub
CMW34087			*E. grandis*	United States of America; Florida	MJ Wingfield	JF342938 (4)	JF793444 (1)	JF793353 (2)
*CMW28688		PREM60484	*E. g.* × *E. u.*	South Africa; Gauteng, Pretoria	MM Taole	JF342923 (5)	JF793429 (1)	JF793339 (2)
*CMW32817[H]	11220	PREM60495	*E. g.* × *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)
CMW31939			*E. g.* × *E. c.*	Australia; QLD	G Hardy	JF342916 (8)	JF793422 (1)	JF793332 (1)
*CMW31933			*E. g.* × *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.* × *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)
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)

Table 1 continued

Culture no.[a]	PPRI no.	Herbarium no.	Host[b]	Location[c]	Isolator	GenBank accession no.[d]		
						ITS	EF-1α	β-tub
*CMW35805		PREM60522	*E. grandis*	Australia; NSW, Morrow	MM Taole & K Taylor	JF342998 (13)	JF793501 (2)	JF793412 (1)
*MUCC428			*E. g.* × *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)

QLD Queensland, *WA* Western Australia, *NSW* New South Wales, *E Eucalyptus*, *ITS* internal transcribed spacer, *EF-1α* elongation factor-1α

[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 = Mycological Culture Collection of the South African Plant Protection Research Institute

[b] *E. g.* × *E. c.* = *E. grandis* × *E. camaldulensis* hybrid; *E. g.* × *E. u.* = *E. grandis* × *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

[H] Isolates that have been deposited in Plant Protection Research Institute (PPRI)

Parsimony analysis was executed using (PAUP) version 4.0b10 (Swofford 2001) and its heuristic search component. The most parsimonious trees were obtained by employing heuristic searches with random stepwise addition in 100 replicates, with 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 1,000 generations trees were saved, resulting in 1,001 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, S11949 (http://www.treebase.org).

Bootstrap consensus trees and trees generated from Bayesian analysis from individual gene trees were uploaded into the online GSI program from http://www.geneologicalsortingindex.org. Each isolate was designated a corresponding ITS haplotype number previously obtained from a heuristic search of the ITS sequence data. Using 1,000 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 or herbarium material. To induce sporulation, isolates were grown on 2% MEA and oatmeal agar (OMA) at 20°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 1,000× magnification, using the Carl Zeiss microscope (Carl Zeiss, Mannheim, West Germany). The extremes of measurements were recorded in parentheses.

Results

Multilocus sequence typing

The three gene regions ITS, EF-1α, 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 asterisk in Table 1) and including the outgroups.

The aligned ITS sequence data consisted of 617 characters, 127 base pairs of which were from 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, resulting 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 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; and 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 isolates included the combinations Australia, South Africa, Uruguay, and United States (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, whereas 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-1α 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 three polymorphic loci, which resulted in four 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, United States, 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 two 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 two polymorphic loci, which resulted in 2 BT haplotypes (Table 1; Fig. 2c), thus reducing the 14 ITS haplotypes to 2 haplotypes. The smaller BT subclade had equally high bootstrap values in parsimony and Bayesian analyses; the other subclade was not supported by posterior probability values. The smaller BT subclade consisted of the ITS haplotypes 2, 4, 5, 9, and 11. The group included isolates from Australia, South Africa, Uruguay, and United States. The larger subclade 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.

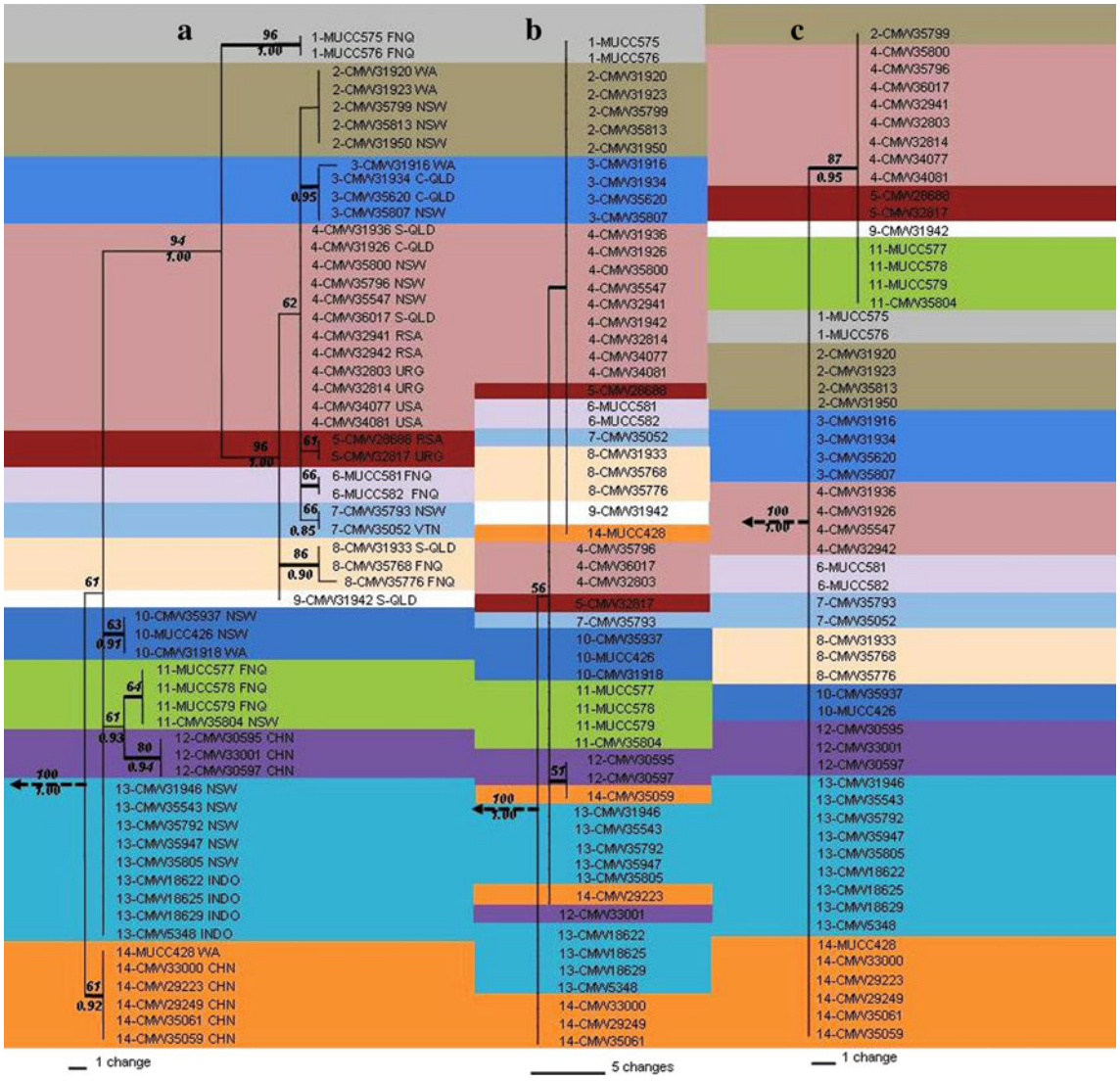

Fig. 2 Phylogenetic relationships among *Teratosphaeria suttonii* isolates included in the study. The trees present a summary of the parsimony and Bayesian analyses of DNA sequence data obtained from the internal transcribed spacer (ITS) (**a**), elongation factor (EF)-1α (**b**), and β-tubulin (**c**) 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, showing how they have been reduced and intermixed in the EF and BT phylogenies

For the GSI analysis (Table 2), 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 of 0.118, 0.087, and 0.369, respectively, whereas 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 because 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 to 0.824 and from 0.004 to 0.075, respectively. Failure to separate isolates on the BT and EF trees (Fig. 2b, c; Table 2) supported the low levels of genealogical divergence at these loci.

Morphological characterization

Variable conidial shapes were observed among 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

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

herbarium material (Table 3). The lengths and widths of spores in vivo ranged from 28.49 to 77.49 µm and from 2.06 to 7.62 µm, respectively. In vitro spore length and width ranged from 16.07 to 66.57 µm and from 2.30 to 6.98 µm, respectively. The shortest spores were recorded in vitro from isolate CMW31916; 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, length 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 to 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 multigene 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 finding was consistent with early observations of considerabe 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

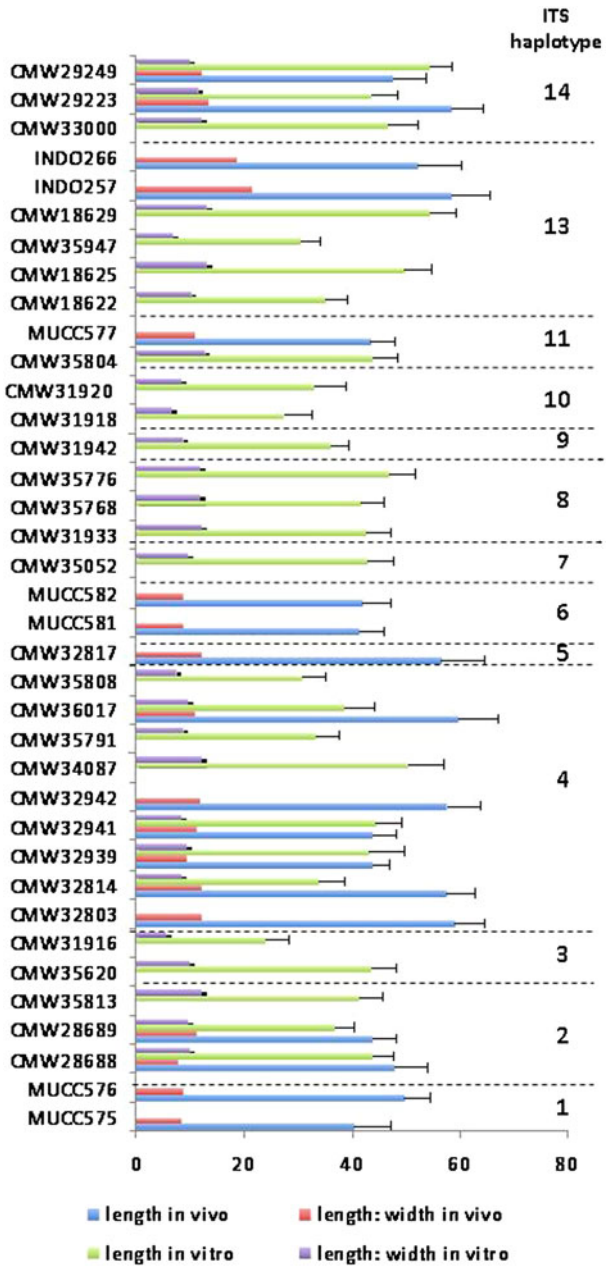

Fig. 3 Average lengths and widths of spores obtained from cultures and leaves. *Error bars* represent the standard deviation

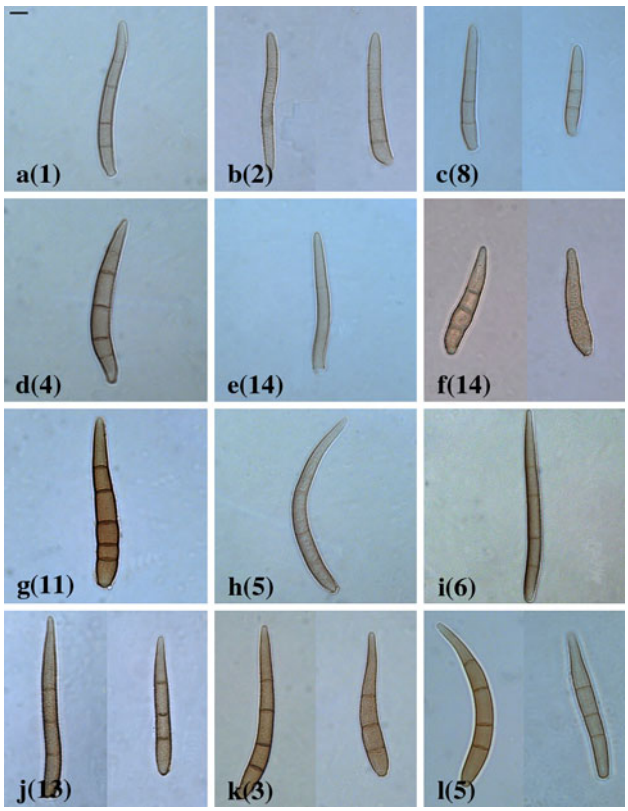

Fig. 4 Conidia obtained from isolates of *Teratosphaeria suttonii*. **a** CMW35813, **b** CMW35768, **c** CMW35791, **d** CMW29223, **e** CMW18629, **f** CMW31916, and leaf specimen **g** PREM60538, **h** PREM60488, **i** DC10, **j** PREM60484, **k** PREM60491, **l** PREM60495. Where two spore images 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

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 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 postintroduction mutation, as Perez (2010) has 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, represented by only one or two ITS haplotypes. According to the results, the haplotypes introduced into Asia are different from those introduced into South Africa, United States, 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 because 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

Table 3 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
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

Table 3 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
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 brackets next to the isolate numbers are the ITS haplotype numbers

N/A not applicable (conidial measurements not obtained)

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* (Burgess and Wingfield 2002a; 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 2000s when the rapidly expanding eucalypt plantation industry was sourcing seedlings from nurseries all down the eastern seaboard of Australia. This idea 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 and Hiremath 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 TLD 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 geographic containment may have resulted in introgression between previously defined species (Taylor et al. 1999). Further studies using deep sequencing for the identification of single nucleotide polymorphisms (SNPs) (Perez 2010) may still elucidate species boundaries within this complex.

Acknowledgments We acknowledge the National Research Foundation (NRF), members of the Tree Protection Co-operative Program

(TPCP), the Centre of Excellence in Tree Health Biotechnology (CTHB), University of Pretoria, South Africa, National Manpower Development Secretariat (NMDS), Lesotho and Murdoch University for financial support. The CRC for Forestry Ltd. is thanked for supporting the visit to Australia of M. Taole. Invaluable assistance for field collections in Australia was provided by Katherine Taylor.

References

Andjic V, Barber PA, Carnegie AJ, Pegg GS, Hardy GESJ, Wingfield MJ, Burgess TI (2007a) *Kirramyces viscidus* sp. nov., a new eucalypt pathogen from tropical Australia closely related to the serious leaf pathogen, *Kirramyces destructans*. Australas Plant Pathol 36:478–487

Andjic V, Hardy GESJ, Cortinas MN, Wingfield MJ, Burgess TI (2007b) Multiple gene genealogies reveal important relationships between species of *Phaeophleospora* infecting *Eucalyptus* leaves. FEMS Microbiol Lett 268:22–33

Andjic V, Pegg GS, Carnegie AJ, Callister A, Hardy GESJ, Burgess TI (2010) *Teratosphaeria pseudoeucalypti*, new cryptic species responsible for leaf blight of *Eucalyptus* in subtropical and tropical Australia. Plant Pathol 59:900–912

Andjic V, Dell B, Barber PA, Hardy G, Wingfield M, Burgess TI (2011) Plants for planting; indirect evidence for the movement of a serious forest pathogen, *Teratosphaeria destructans*, in Asia. Eur J Plant Pathol 131(1):49–58

Benyon FHL, Burgess L, Sharp P (2000) Molecular genetic investigations and reclassification of *Fusarium* species in sections *Fusarium* and *Roseum*. Mycol Res 104:1164–1174

Burgess TI, Wingfield MJ (2002a) Impact of fungal pathogens in natural forest ecosystems: a focus on *Eucalyptus*. In: Sivasithamparam K, Dixon KW (eds) Microorganisms in plant conservation and biodiversity. Kluwer, Dordrecht, pp 285–306

Burgess TI, Wingfield MJ (2002b) Quarantine is important in restricting the spread of exotic seed-borne tree pathogens in the southern hemisphere. Int For Rev 4(1):56–65

Burgess TI, Andjic V, Hardy GESJ, Dell B, Xu D (2006) First report of *Phaeophleospora destructans* in China. J Trop For Sci 18:144–146

Burgess TI, Andjic V, Wingfield MJ, Hardy GESJ (2007) The eucalypt leaf blight pathogen *Kirramyces destructans* discovered in Australia. Australas Plant Dis Notes 2:141–144

Carbone I, Kohn LM (1999) A method for designing primer sets for speciation studies in filamentous Ascomycetes. Mycologia 91:553–556

Carnegie AJ (2007a) Forest health condition in New South Wales, Australia, 1996–2005. I. Fungi recorded from eucalypt plantations during forest health surveys. Australas Plant Pathol 36:213–224

Carnegie AJ (2007b) Forest health condition in New South Wales, Australia, 1996–2005. II. Fungal damage recorded in eucalypt plantations during forest health surveys and their management. Australas Plant Pathol 36:225–239

Carnegie AJ, Keane PJ, Podger FD (1997) The impact of three species of *Mycosphaerella* newly recorded on *Eucalyptus* in Western Australia. Australas Plant Pathol 26:71–77

Chipompha NSW (1987) *Phaeoseptoria eucalypti*: a new pathogen of *Eucalyptus* in Malawi. S Afr For J 142:10–12

Chungu D, Muimba-Kankolongo A, Wingfield MJ, Roux J (2010) Identification of fungal pathogens occurring in eucalypt and pine plantations in Zambia by comparing DNA sequences. Forestry 83:507–515

Cortinas M, Koch N, Thain J, Wingfield BD, Wingfield MJ (2004) First record of the *Eucalyptus* stem canker pathogen, *Coniothyrium zuluense* from Hawaii. Australas Plant Pathol 33:309–312

Cortinas MN, Crous PW, Wingfield BD, Wingfield MJ (2006) Multigene phylogenies and phenotypic characters distinguish two species within the *Colletogloeopsis zuluensis* complex associated with *Eucalyptus* stem cankers. Stud Mycol 55:133–146

Crous PW, Knox-Davies PS, Wingfield MJ (1989) Infection studies in *Phaeoseptoria eucalypti* and *Coniothyrium ovatum* on *Eucalyptus* spp. S Afr For J 149:30–35

Crous PW, Knox-Davies PS, Wingfield MJ (1988) *Phaeoseptoria eucalypti* and *Coniothyrium ovatum* on *Eucalyptus* species in South Africa. Phytophylactica 20:337–340

Crous P, Summerell B, Mostert L, Groenewald J (2008) Host specificity and speciation of *Mycosphaerella* and *Teratosphaeria* species associated with leaf spots of *Proteaceae*. Persoonia 20:59–86

Crous PW, Summerell B, Carnegie A, Wingfield MI, Hunter GC, Burgess TI, Andjic V, Barber P, Groenewald J (2009) Unravelling *Mycosphaerella*: do you believe in genera? Persoonia 23:99–118

Cummings MP, Neel MC, Shaw KL, Otto S (2008) A genealogical approach to quantifying lineage divergence. Evolution 62:2411–2422

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Gardner DE, Hodges CS (1988) Hawaiian forest fungi. IX. *Botryosphaeria pipturi* sp. nov. and miscellaneous records. Mycologia 80:460–465

Glass NL, Donaldson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol 31:1323–1330

Hansford GC (1957) Australian fungi. IV. New records and revisions. Proc Linn Soc N S W 82:209–229

Hodges CS, Gardner DE (1984) Hawaiian forest fungi. IV. Rusts on endemic *Acacia* species. Mycologia 76:332–349

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755

Hunter GC, Van Der Merwe NA, Burgess TI, Carnegie AJ, Wingfield BD, Crous PW, Wingfield MJ (2008) Global movement and population biology of *Mycosphaerella nubilosa* infecting leaves of cold-tolerant *Eucalyptus globulus* and *E. nitens*. Plant Pathol 57:235–242

Hunter GC, Crous PW, Carnegie AJ, Burgess TI, Wingfield MJ (2011) *Mycosphaerella* and *Teratosphaeria* diseases of *Eucalyptus*: easily confused and with serious consequences. Fungal Divers. doi:10.1007/s13225-011-0131-z

Jackson SL, Maxwell A, Burgess TI, Dell B, Hardy GES (2008) Incidence and new records of *Mycosphaerella* species within a *Eucalyptus globulus* plantation in Western Australia. For Ecol Manag 255:3931–3937

Knipscheer NS, Wingfield MJ, Swart WJ (1990) Phaeoseptoria leaf spot of *Eucalyptus* in South Africa. S Afr For J 154:56–59

Nichol NS, Wingfield MJ, Swart WJ (1992) Differences in susceptibility of *Eucalyptus* species to *Phaeoseptoria eucalypti*. Eur J For Pathol 22:418–423

Nylander JAA (2004) MrModeltest v25. Program distributed by the author. Evolutionary Biology Centre, Uppsala University 2

Old KM, Wingfield MJ, Yuan ZQ (2003) A manual of diseases of eucalypts in South-East Asia. Center for International Forestry Research

Padaganur GM, Hiremath PC (1973) *Phaeoseptoria eucalypti* Hansf. A new record in India. Mysore J Agric Sci 7:336–338

Park RF, Keane PJ, Wingfield MJ, Crous PW (2000) Fungal diseases of eucalypt foliage. In: Keane PJ, Kile GA, Podger FD, Brown BN (eds) Diseases and pathogens of eucalypts. CSIRO, Melbourne, pp 153–259

Pegg GS, Brown BN, Ivory M (2003) Eucalypt diseases in hardwood plantations in Queensland. Hardwood Queensland Report no. 16. Forestry research. Agency for Food and Fibre Sciences, DPI

Perez AG (2010) Global genetic diversity of the *Eucalyptus* leaf pathogen *Teratosphaeria nubilosa* species complex in native forests and commercial plantations. PhD dissertation, University of Pretoria South Africa

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572

Sakalidis ML, GEStJ Hardy, Burgess TI (2011) Use of the Genealogical Sorting Index (GSI) to delineate species boundaries in the *Neofusicoccum parvum–N. ribis* species complex. Mol Phylogenet Evol 60:333–344

Sankaran K, Sutton BC, Minter D (1995) A checklist of fungi recorded on *Eucalyptus*. Mycol Pap 170:1–376

Simpson JA, Xiao Y, Bi H-Q (2005) Phaeophleospora leaf disease of *Eucalyptus* in China. Aust Mycol 24:13–14

Swofford DL (2001) PAUP*: phylogenetic analysis using parsimony, version 4.0 b6. Sinauer, Sunderland

Taylor JW, Fisher MC (2003) Fungal multilocus sequence typing—it's not just for bacteria. Curr Opin Microbiol 6:351–356

Taylor JW, Jacobson DJ, Fisher MC (1999) The evolution of asexual fungi: reproduction, speciation and classification. Annu Rev Phytopathol 37:197–246

Turnbull JW (2000) Economic and social importance of eucalypts. In: Keane PJ, Kile GA, Podger RD, Brown BN (eds) Diseases and pathogens of eucalypts. CSIRO, Melbourne, pp 1–7

Walker J (1962) Notes on plant parasitic fungi. I. Proc Linn Soc N S W 87:162–176

Walker J, Sutton BC, Pascoe IG (1992) *Phaeoseptoria eucalypti* and similar fungi on *Eucalyptus*, with description of *Kirramyces* gen. nov. (Coelomycetes). Mycol Res 96:911–924

White T, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Michael I, Thomas WG, John JS (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 315–322

Wingfield MJ, Crous PW, Boden D (1996) *Kirramyces destructans* sp. nov., a serious leaf pathogen of *Eucalyptus* in Indonesia. S Afr J Bot 62:325–327

Wingfield MJ, Slippers B, Hurley B, Coutinho T, Wingfield BD, Roux J (2008) Eucalypt pests and diseases: growing threats to plantation productivity. South For 70:139–144