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Teratosphaeria pseudoeucalypti, new cryptic species responsible for leaf blight of *Eucalyptus* in subtropical and tropical Australia

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Sub-tropical and tropical plantations of *Eucalyptus grandis* hybrids in eastern Australia have been severely affected by anamorphs of *Teratosphaeria* (formerly *Kirramyces*) causing a serious leaf blight disease. Initially the causal organism in Queensland, Australia, was identified as *Teratosphaeria eucalypti*, a known leaf parasite of endemic *Eucalyptus* spp. However, some inconsistencies in symptoms, damage and host range suggested that the pathogen in Queensland may be a new species. Isolates of *T. eucalypti* from throughout its known endemic range, including Queensland and New Zealand, where it is an exotic pathogen, were compared using multiple gene phylogenies. Phylogenetic studies revealed that the species responsible for leaf blight in Queensland represents a new taxon, described here as *Teratosphaeria pseudoeucalypti*. While the DNA sequence of *T. pseudoeucalypti* was more similar to *T. eucalypti*, the symptoms and cultural characteristics resembled that of *T. destructans*. The impact of this disease in central Queensland has increased annually and is the major threat to the eucalypt plantation industry in the region.

Keywords: clone evaluation, DNA sequence, *Eucalyptus* spp., haplotypes, kirramyces leaf blight, phylogeographic analysis

Introduction

Kirramyces leaf diseases, caused by anamorphs of species of *Teratosphaeria* (formerly *Kirramyces*) (Crous *et al.*, 2009a,b), have emerged as significant diseases impacting on the eucalypt plantation industry in subtropical and tropical areas of Australia (Carnegie, 2007a,b; Carnegie *et al.*, 2008). Three symptom types have been identified within this disease complex: charcoal leaf disease (caused by *T. suttonii*), halo leaf spot (caused by *T. eucalypti*) and kirramyces leaf blight (caused by *T. viscida* and other *Teratosphaeria* spp, only found in Queensland). Carnegie (2007b) included *T. suttonii* and *T. eucalypti* under a single disease complex, kirramyces leaf disease (KLD), describing it as the 'most devastating disease in *E. grandis* and *E. grandis* × *E. camaldulensis* plantations' in northern New South Wales (N-NSW), Australia.

During forest health surveys between 1996 and 2005 in NSW, *T. eucalypti* was observed causing significant and repeated damage to plantations of *E. nitens* and hybrids of *E. nitens* × *E. nobilis* on the Dorrigo Plateau in northern NSW, where the majority of plantations were suffering damage of greater then 95% severity (Carnegie, 2007b). The affected plantations of *E. nitens* recovered poorly from damage and thus were susceptible to stem fungi, including *Holocryphia eucalypti*, resulting in top-death and tree mortality (Carnegie, 2007b). More recent surveys of plantations in Queensland have revealed severe outbreaks and damage by species of *Kirramyces* in plantations of *E. grandis* × *E. camaldulensis* in central Queensland. Due to the severity of damage, and symptoms observed, this disease was described as kirramyces leaf blight (KLB) (Carnegie *et al.*, 2008). The impact of this disease in the region has increased annually and whilst it was initially thought that older trees and progeny of hybrid crosses with *E. urophylla* or *E. pellita* parents were more resistant to KLB, it is now known that most eucalypt species and hybrids in trials to date, are susceptible.

Based on spore morphology and sequence data, the causal agent of KLB in Queensland was initially identified as *T. eucalypti*, a species first described from fading leaves of *Eucalyptus* sp. collected from Melbourne, Victoria, Australia, in 1884 (Cooke, 1889). The fungus was also found on *E. dalrympleana* and *E. viminalis* in NSW (Heather, 1961) and in plantations of *E. nitens* and

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E. globulus in southern NSW and Tasmania (Yuan *et al.*, 2000). *Teratosphaeria eucalypti* has been recorded in Queensland since 1971 (Australian Plant Pest Database), but it was not considered a pathogen of concern. However, the symptomatology and impact of the disease in Queensland differed to that observed for *T. eucalypti* elsewhere in Australia. In Queensland infection results in a leaf blight and total defoliation while elsewhere infection is characterized by discrete lesions and minimal leaf loss.

Outside Australia, *T. eucalypti* has been found only in New Zealand, where it is known to have been introduced with plantings of *E. nitens* from south-east Australia (Miller *et al.*, 1992) and was initially regarded as a minor pathogen (Dick, 1982; Gadgil & Dick, 1983). However, this situation has changed with the establishment of plantations of susceptible eucalypt species during the 1990s when *T. eucalypti* was found responsible for complete defoliation of juvenile leaves of *E. nitens* and became known as septoria leaf blight (Hood *et al.*, 2002a,b). This disease outbreak happened because the *E. nitens* plantation was established in a region with a climate favourable to *T. eucalypti* (Ridly, 2004).

The aim of the current study was to use a phylogeographic approach to construct multiple gene phylogenies to determine if KLD in Queensland is caused by *T. eucalypti* or a new sister species.

Materials and methods

Fungal isolates

Teratosphaeria eucalypti isolates were collected from several geographical regions where this pathogen is known to occur: central NSW (C-NSW), high-altitude northern NSW (HAN-NSW), northern NSW (N-NSW), south Queensland (S-QLD), central Queensland (C-QLD), far north Queensland (FNQ), Victoria (VIC), Tasmania (TAS) and New Zealand (NZ). *Teratosphaeria eucalypti* was isolated under a dissecting microscope as described previously (Andjic *et al.*, 2007c).

Cultures were maintained at 20°C on 2% malt extract agar (MEA; 20 g of malt extract and 20 g of agar in 1 L of distilled water). All isolates are maintained in the Murdoch University culture collection (MUCC) or in the collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Fifty-nine representative isolates from a range of plantations and hosts throughout Australia were used: 16 from NSW (three from C-NSW; seven from HAN-NSW; six from N-NSW), 21 from three regions in QLD (seven from FNQ; seven from C-QLD; seven from S-QLD), 12 from TAS, three from VIC and seven from NZ (Table 1).

DNA extraction, PCR amplification and sequencing

The isolates were grown on 2% MEA at 20°C for 4 weeks and the mycelium was harvested and placed in 1·5 mL sterile Eppendorf tubes. Harvested mycelium was frozen in liquid nitrogen, ground to a fine powder and genomic DNA extracted as described previously (Andjic *et al.*, 2007c).

This study included complete amplification of the mitochondrial ATPase protein gene (ATP-6), internal transcribed spacer region (ITS-2), part of the β-tubulin gene region (βT) and part of elongation factor 1α gene (EF-1α). Primers used for amplification of these regions are listed in Table 2 and the amplification protocol was according to Andjic *et al.* (2007a). For failed amplifications, the magnesium concentration was increased to 4 mM, and primer concentration to 0·9 pmol and the following PCR conditions were used: 7 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 45°C, 2 min at 72°C and a final elongation step of 10 min at 72°C. Amplicons were visualized and sequenced as described previously (Andjic *et al.*, 2007a).

Haplotype network estimation

Haplotype networks were generated using the statistical parsimony method in the TCS v. 1.21 software program (Clement *et al.*, 2000). The program collapses DNA sequences into haplotypes and calculates the frequencies of haplotypes in the sample, which are used to estimate haplotype out-group probabilities, that correlate with haplotype age (Donnelly & Tavaré, 1986; Castelloe & Templeton, 1994). It then calculates an absolute distance matrix from which it estimates phylogenetic networks using a probability of parsimony, until the probability exceeds 0·95 (Templeton *et al.*, 1992). The analysis was performed on the combined dataset of ATP-6, βT, EF-1α and ITS-2 DNA sequences.

Phylogenetic analysis

Phylogeny of *T. eucalypti* isolates were estimated using a combination of parsimony and maximum likelihood methods. For each locus, DNA sequence data were assembled using SEQUENCE NAVIGATOR v. 1.01 (Perkin Elmer) and aligned in CLUSTAL X (Thompson *et al.*, 1997) and manual adjustments were made visually where necessary. All sequences derived in this study were deposited in GenBank and accession numbers are shown in Table 1.

The initial analysis was performed on each dataset alone and subsequent analyses were performed on a combined dataset of βT, EF-1α and ITS-2 sequence, after a partition homogeneity test (PHT) had been performed in PAUP v. 4.0b10 (Swofford, 2000) to determine whether sequence data from the four separate gene regions were statistically congruent (Farris *et al.*, 1995; Huelsenbeck *et al.*, 1996). Parsimony analysis with heuristic search was performed using PAUP * and Bayesian analysis was conducted on the same aligned and combined dataset as described previously (Andjic *et al.*, 2007a). Trees were rooted to *Dothistroma septospora*.

Table 1 *Teratosphaeria* and other isolates considered in this study

Fungus	Culture no.[a]	Host	Location	Collector	Haplotype	GenBank Accession no.			
						ATP-6	EF-1α	ß-tubulin	ITS-2
Teratosphaeria eucalypti	CMW 19453	*Eucalyptus nitens*	Settlement Rd, New Zealand	M Dick	KE1	EU101472	EU101585	EU101529	FJ793234
T. eucalypti	CMW 19455	*E. nitens*	Coxs, New Zealand	M Dick	KE4	EU101515	EU101628	EU101571	FJ793260
T. eucalypti	CMW 19456	*E. nitens*	Douthetts, New Zealand	M Dick	KE3	EU101474	EU101587	EU101531	FJ793236
T. eucalypti	CMW 19461	*E. nitens*	Sun Valley, New Zealand	M Dick	KE1	EU101470	EU101583	EU101527	FJ793232
T. eucalypti	CMW 19462	*E. nitens*	Sun Valley, New Zealand	M Dick	KE1	EU101473	EU101586	EU101530	FJ793235
T. eucalypti	CMW 19463	*E. nitens*	Sun Valley, New Zealand	M Dick	KE1	EU101471	EU101584	EU101528	FJ793233
T. eucalypti	CMW 19464	*E. nitens*	Sun Valley, New Zealand	M Dick	KE1	EU101475	EU101588	EU101532	FJ793237
T. eucalypti	CMW 19470	*E. nitens*	Kawerau, New Zealand	M Dick	KE1	EU101476	EU101589	EU101533	FJ793238
T. eucalypti	MUCC 635	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	KE2	EU101501	EU101614	EU101557	FJ793250
T. eucalypti	MUCC 636	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	KE1	EU101477	EU101590	EU101534	FJ793239
T. eucalypti	MUCC 637	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	KE1	EU101517	EU101630	EU101573	FJ793261
T. eucalypti	MUCC 638	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	KE1	EU101518	EU101631	EU101574	FJ793262
T. eucalypti	MUCC 639	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	KE1	EU101519	EU101632	EU101575	FJ793263
T. eucalypti	MUCC 640	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	KE1	EU101525	EU101638	EU101581	FJ793265
T. eucalypti	MUCC 641	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	KE1	EU101520	EU101633	EU101576	FJ793264
T. eucalypti	MUCC 642	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	KE1	EU101521	EU101634	EU101577	EU101659
T. eucalypti	MUCC 643	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	KE1	EU101522	EU101635	EU101578	EU101656
T. eucalypti	MUCC 644	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	KE1	EU101526	EU101639	EU101582	EU101661
T. eucalypti	MUCC 645	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	KE1	EU101523	EU101636	EU101579	EU101657
T. eucalypti	MUCC 646	*E. nitens*	Roses Tier, TAS, Australia	T Wardlaw	KE1	EU101524	EU101637	EU101580	EU101660
T. eucalypti	MUCC 632	*E. nitens*	Kinglake, VIC, Australia	PA Barber	KE3	EU101494	DQ632726	DQ632631	DQ632661
T. eucalypti	MUCC 633	*E. nitens*	Kinglake, VIC, Australia	PA Barber	KE3	EU101495	EU101608	EU101551	FJ793247
T. eucalypti	MUCC 634	*E. nitens*	Kinglake, VIC, Australia	PA Barber	KE3	EU101478	EU101591	EU101535	DQ632664
T. eucalypti	MUCC 616	*Eucalyptus sp.*	Lithgow, C-NSW, Australia	AJ Carnegie	KE1	EU101496	EU101609	EU101552	FJ793248
T. eucalypti	MUCC 617	*Eucalyptus sp.*	Lithgow, C-NSW, Australia	AJ Carnegie	KE1	EU101497	EU101610	EU101553	FJ793249
T. eucalypti	MUCC 618	*Eucalyptus sp.*	Lithgow, C-NSW, Australia	AJ Carnegie	KE1	EU101514	EU101627	EU101570	FJ793259
T. eucalypti	MUCC 619	*E. nitens*	Dorrigo, HAN-NSW, Australia	AJ Carnegie	KE1	EU101506	EU101619	EU101562	FJ793251
T. eucalypti	MUCC 620	*E. nitens*	Dorrigo, HAN-NSW, Australia	AJ Carnegie	KE1	EU101507	EU101620	EU101563	FJ793252
T. eucalypti	MUCC 621	*E. nitens*	Dorrigo, HAN-NSW, Australia	AJ Carnegie	KE1	EU101508	EU101621	EU101564	FJ793253
T. eucalypti	MUCC 622	*E. nitens*	Dorrigo, HAN-NSW, Australia	AJ Carnegie	KE3	EU101509	EU101622	EU101565	FJ793254
T. eucalypti	MUCC 623	*E. nitens*	Dorrigo,HAN-NSW, Australia	AJ Carnegie	KE1	EU101510	EU101623	EU101566	FJ793255
T. eucalypti	MUCC 624	*E. nitens*	Dorrigo,HAN-NSW, Australia	AJ Carnegie	KE1	EU101511	EU101624	EU101567	FJ793256
T. eucalypti	MUCC 625	*E. nitens*	Dorrigo, HAN-NSW, Australia	AJ Carnegie	KE3	EU101512	EU101625	EU101568	FJ793257
T. eucalypti	MUCC 626	*E. grandis* x *tereticornis*	Kyogle, N-NSW, Australia	AJ Carnegie	KE5	EU101489	EU101602	EU101546	FJ793241
T. eucalypti	MUCC 627	*E. grandis* x *tereticornis*	Kyogle, N-NSW, Australia	AJ Carnegie	KE1	EU101490	EU101603	EU101547	FJ793242
T. eucalypti	MUCC 628	*E. grandis* x *tereticornis*	Kyogle, N-NSW, Australia	AJ Carnegie	KE1	EU101491	EU101604	EU101548	FJ793243
T. eucalypti	MUCC 629	*E. grandis* x *tereticornis*	Kyogle, N-NSW, Australia	AJ Carnegie	KE3	EU101492	EU101605	EU101549	FJ793244
T. eucalypti	MUCC 630	*E. grandis* x *tereticornis*	Kyogle, N-NSW, Australia	AJ Carnegie	KE6	EU101493	EU101606	EU101550	FJ793245
T. eucalypti	MUCC 631	*E. grandis* x *tereticornis*	Kyogle, N-NSW, Australia	AJ Carnegie	KE7	EU101513	EU101626	EU101569	FJ793258
T. pseudoeucalypti	MUCC 598	*E. grandis* x *E. camaldulensis*	Harrisville,S-QLD, Australia	AJ Carnegie	KE8	EU101479	EU101592	EU101536	FJ793215
T. pseudoeucalypti	MUCC 599	*E. grandis* x *E. camaldulensis*	Harrisville, S-QLD, Australia	AJ Carnegie	KE8	EU101480	EU101593	EU101537	FJ793216

Table 1 *Continued.*

Fungus	Culture no.[a]	Host	Location	Collector	Haplotype	GenBank Accession no.			
						ATP-6	EF-1α	ß-tubulin	ITS-2
T. pseudoeucalypti	MUCC 600	*E. grandis* x *E. camaldulensis*	Harrisville, S-QLD, Australia	AJ Carnegie	KE8	EU101481	EU101594	EU101538	FJ793217
T. pseudoeucalypti	MUCC 601	*E. grandis* x *E. camaldulensis*	Harrisville, S-QLD, Australia	AJ Carnegie	KE8	EU101482	EU101595	EU101539	FJ793218
T. pseudoeucalypti	MUCC 602	*E. grandis* x *E. camaldulensis*	Harrisville, S-QLD, Australia	AJ Carnegie	KE8	EU101483	EU101596	EU101540	FJ793219
T. pseudoeucalypti	MUCC 604	*E. grandis* x *E. camaldulensis*	Harrisville, S-QLD, Australia	AJ Carnegie	KE8	EU101502	EU101615	EU101558	FJ793224
T. pseudoeucalypti	MUCC 605	*E. grandis* x *E. camaldulensis*	Harrisville, S-QLD, Australia	AJ Carnegie	KE8	EU101503	EU101616	EU101559	FJ793225
T. pseudoeucalypti	MUCC 606	*E. grandis* x *E. camaldulensis*	Miriam Vale, C-QLD,Australia	G Pegg	KE8	EU101516	EU101629	EU101572	FJ793226
T. pseudoeucalypti	MUCC 607	*E. grandis* x *E. camaldulensis*	Miriam Vale, C-QLD,Australia	G. Pegg	KE8	EU101485	EU101598	EU101542	FJ793220
T. pseudoeucalypti	MUCC 608	*E. grandis* x *E. camaldulensis*	Miriam Vale, C-QLD,Australia	G. Pegg	KE8	EU101504	EU101617	EU101560	FJ793227
T. pseudoeucalypti	MUCC 609	*E. grandis* x *E. camaldulensis*	Miriam Vale, C-QLD,Australia	G. Pegg	KE8	EU101505	EU101618	EU101561	FJ793228
T. pseudoeucalypti	MUCC 610	*E. grandis* x *E. camaldulensis*	Miriam Vale, C-QLD,Australia	G. Pegg	KE9	EU101486	EU101599	EU101543	FJ793221
T. pseudoeucalypti	MUCC 611	*E. grandis* x *E. camaldulensis*	Miriam Vale, C-QLD,Australia	G. Pegg	KE8	EU101487	EU101600	EU101544	FJ793222
T. pseudoeucalypti	MUCC 612	*E. grandis* x *E. camaldulensis*	Miriam Vale, C-QLD,Australia	G. Pegg	KE8	EU101488	EU101601	EU101545	FJ793223
T. pseudoeucalypti	MUCC 702	*Eucalyptus sp.*	FNQ, Australia	TI Burgess	KE8	FJ811971	FJ793203	FJ793207	FJ793211
T. pseudoeucalypti	MUCC 703	*Eucalyptus sp.*	FNQ, Australia	TI Burgess	KE8	FJ811972	FJ793204	FJ793208	FJ793212
T. pseudoeucalypti	MUCC 704	*Eucalyptus sp.*	FNQ, Australia	TI Burgess	KE10	FJ811973	FJ793205	FJ793209	FJ793213
T. pseudoeucalypti	MUCC 705	*Eucalyptus sp.*	FNQ, Australia	TI Burgess	KE11	FJ811974	FJ793206	FJ793210	FJ793214
T. pseudoeucalypti	MUCC 613	*Eucalyptus sp.*	Davies Creek, FNQ, Australia	TI Burgess	KE8	EU101498	EU101611	EU101554	FJ793229
T. pseudoeucalypti	MUCC 614	*Eucalyptus sp.*	Davies Creek, FNQ, Australia	TI Burgess	KE8	EU101499	EU101612	EU101555	FJ793230
T. pseudoeucalypti	MUCC 615	*Eucalyptus sp.*	Davies Creek, FNQ, Australia	TI Burgess	KE12	EU101500	EU101613	EU101556	FJ793231
T. cryptica	CBS110975	*E. globulus*	Australia	AJ Carnegie		NA	DQ235119	DQ658234	AY309623
T. destructans	CMW 19832	*E. grandis*	Sumatra, Indonesia	PA Barber		NA	DQ632665	DQ632623	DQ632665
T. destructans	CMW 17919	*E. urophylla*	Guangzhou, China	TI Burgess		NA	DQ632701	DQ632622	DQ632701
T. destructans	CMW 15089	*E. camaldulensis*	Vietnam	TI Burgess		NA	EF031465	EF031477	EF031465
T. destructans	CMW 16123	*E. camaldulensis*	Thailand	MJ Wingfield		NA	EF031468	EF031480	EF031468
T. molleriana	CBS117924	*Eucalyptus sp.*	Portugal	MJ Wingfield		NA	DQ239969	DQ240115	DQ239968
T. molleriana	CBS111164	*Eucalyptus sp.*	USA	MJ Wingfield		NA	DQ235104	AF309619	AF309620
T. nubilosa	CMW 11560	*E. globulus*	Tasmania	A Milgate		NA	DQ240176	DQ658236	DQ658232
T. suttonii	MUCC 425	*E. grandis*	New South Wales	TI Burgess		NA	DQ632713	DQ632613	DQ632655
T. suttonii	CMW 22484	*E. urophylla*	China	TI Burgess		NA	DQ632714	DQ632616	DQ632705
T. viscida	MUCC 452, CBS 121156	*E. grandis*	Mareeba, Australia	TI Burgess		NA	EF031495	EF031483	EF031471
T. viscida	MUCC 453, CBS 121157	*E. grandis*	Mareeba, Australia	TI Burgess		NA	EF031496	EF031484	EF031472
T. viscida	MUCC 454	*E. grandis*	Mareeba, Australia	TI Burgess		NA	EF031497	EF031485	EF031473
T. viscida	MUCC 455	*E. grandis*	Mareeba, Australia	TI Burgess		NA	EF031498	EF031486	EF031474
T. zuluensis	CBS117835	*E. grandis*	Mexico	MJ Wingfield		NA	DQ240161	DQ240108	DQ239987
T. zuluensis	CBS117262	*E. grandis*	South Africa	L Van Zyl		NA	DQ240155	DQ240102	DQ239976
Dothistroma septospora	CMW14822	*Pinus ponderosa*				NA	AY808265	AY80819	AY808300
D. septospora	CMW13122	*Pinus mugo*				NA	AY808260	AY808195	AY808295

[a]Designation of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; MUCC = Murdoch University Culture Collection, Perth, Western Australia.

Table 2 Primer sets and annealing temperature used to amplify *Teratosphaeria spp.*

Region	Oligos	Oligo Sequence (5′–3′)	Amplicon size (bp)	AT (°C)	Reference
ATP-6	ATP6-1	ATTAATTSWCCWTTAGAWCAATT	600	45	(Kretzer & Bruns, 1999)
	ATP6-2	TAATTCTANWGCATCTTTAATRTA			
β-tubulin	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	680	45–58	(Glass & Donaldson, 1995)
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC			
EF-1α	EF1-728F	CATCGAGAAGTTCGAGAAGG	350	45–55	(Carbone & Kohn, 1999)
	EF1-986R	TACTTGAAGGAACCCTTACC			
ITS-2	ITS-3	GTATCGATGAAGAACGCAGC	300	50	(Gardes & Bruns, 1993)
	ITS-4	TCCTCCGCTTATTGATATGC			

Morphology and cultural characteristics

Representative isolates of *T. eucalypti* considered in this study were compared *in vivo* and *in vitro,* including herbarium specimens of *T. eucalypti* from QLD obtained from Plant Pathology Herbarium, Department of Primary Industries and Fisheries Brisbane, Queensland, Australia (BRIP), and previous observations from published literature. Plugs (2 mm diameter.) were cut from actively growing cultures and placed at the centres of Petri dishes (55 mm) containing one of four different nutrient media. Three replicates of each representative isolate (nine isolates in total) were grown on 2% MEA, oatmeal agar (OMA; 30 g of oats and 15 g of agar in 1 L of distilled water), potato dextrose agar (PDA, Biolab) and sterilized eucalypt leaves placed on the surface of tap water agar (TWA; sterilized eucalyptus leaves, 15 g of agar in 1 L of tap water) at 20 and 30°C in the dark. After 30 days, cultures were assessed for growth and photographed. Squash mounts of fruiting structures were prepared on slides in lacto-glycerol (1:1:1 volume of lactic acid, glycerol and water) and observed at 1000× magnification with an Olympus BH2 light microscope. The growth of cultures was determined by taking two measurements of colony diameter perpendicular to each other. Each isolate was assessed for conidial size, shape, pigmentation and number of septa. Wherever possible, 30 measurements (×1000 magnification) of all taxonomically relevant structures were recorded for each species and the extremes presented in parentheses. Colony colour was described using notations in the Munsell®Soil Color Charts (Gretag Macbeth, revised 2000). Measurements of conidial size were obtained using the image analysis software OLYSIA BIOREPORT 3·2 software imaging system. Data analyses were performed using descriptive statistics in MICROSOFT EXCEL.

Herbarium specimens examined in this study were: BRIP-8734a, BRIP-13248a, BRIP-2574a, BRIP-43738a, BRIP-5465a, BRIP-11345a, BRIP-40158a and BRIP-5464a.

Fructification rating

Parallel to morphological characterization, an independent experiment was set up in order to compare the estimated number of fruiting bodies (pycnidia) produced by each isolate grown on the different nutrient media MEA, OMA and TWA. Six representative isolates (two isolates from each location) were used for this study. Initially, isolates were grown on 2% MEA at 20°C in the dark. After 30 days pycnidia were harvested and a spore suspension made. Two hundred microlitres of each suspension was then spread on agar plates and placed in an incubator at 20°C. After 90 days, isolates were rated for sporulation. Scale rating was scored from 0 to 6 where 0 = no fruiting bodies produced, 6 = maximum fruiting bodies produced.

Results

Haplotype network

Haplotype network constructed in TCS software resulted in 12 haplotypes among the *Teratosphaeria* isolates from *Eucalyptus* spp. (Fig. 1). Haplotype KE-1 was represented by six isolates from NZ, 11 from TAS, three from C-NSW, five from HAN-NSW and two from N-NSW; haplotype KE-2 was represented by one isolate from TAS; haplotype KE-3 was represented by one isolate from NZ, three isolates from VIC, two isolates from HAN-NSW and one isolate from N-NSW; haplotype KE-4 was represented by only one isolate from NZ; haplotypes KE-5, KE-6 and KE-7 were each represented by one isolate from N-NSW; haplotype KE-8 was represented by seven isolates from S-QLD, six from C-QLD and four from FNQ; haplotype KE-9 was represented by only one isolate from C-QLD; haplotypes KE-10, KE-11 and KE-12 were each represented by one isolate from FNQ.

Regions from Queensland shared one common haplotype (KE-8), but did not share any haplotypes with isolates obtained elsewhere (Fig. 1). Five different haplotypes were observed in the population from NSW, of which two were shared with isolates from NZ, and one each with isolates from VIC and TAS. Three other haplotypes (KE-5, 6 and KE-7) were only present in N-NSW.

Phylogenetic analysis

Parsimony and Bayesian analysis of aligned data sets containing a representative of each haplotype (KE1–12) were performed on each dataset alone and in combination.

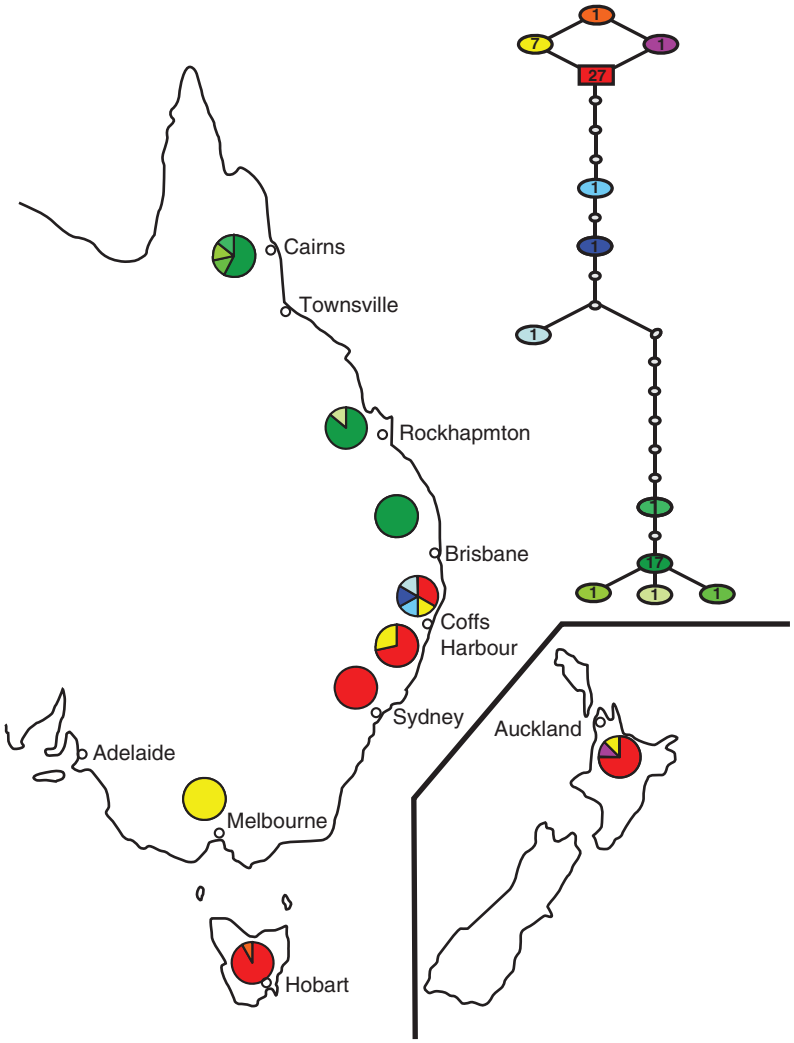

Figure 1 Distribution and proportion of the 12 detected *Teratosphaeria* haplotypes in eastern Australia estimated by TCS 1·21 software. Also shown is a haplotype network, with haplotype identity indicated by colours. Red = KE-1, Orange = KE-2, Yellow = KE-3, Purple = KE-4, Blue = KE-5, Dark Blue = KE-6, Light Blue = KE-7, Dark Green = KE-8, Light Green = KE-9, Lime = KE-10, Olive Green = KE-11, Green = KE-12.

Analysis and resultant trees for individual βT, ITS-2 and EF-1α datasets are given in TreeBase S10492. As there were only two polymorphic sites in the ATP-6 dataset the analysis was not performed. In all three analyses, *Teratosphaeria* isolates from QLD (KE9–12) were closely related to, but phylogenetically distinct from *T. eucalypti* from elsewhere (KE1–8). The aligned data set for the combined βT, ITS-2 and EF-1α sequences consisted of 990 characters of which 446 were parsimony informative and used in the analysis. The partition homogeneity test showed no significant difference ($P > 0{\cdot}01$; $P = 0{\cdot}33$) between data from the different gene regions (sum of lengths of original partition was 1020, range for 1000 randomizations was 1013–1027) thus data were combined. The combined data set contained significant ($P < 0{\cdot}01$; gl = −2·25) phylogenetic signal compared to 1000 random trees. Heuristic searches of unweighted characters in PAUP resulted in one most parsimonious trees of 858 steps (CI = 0·793, RI = 0·876) (Fig. 2). Bayesian analysis resulted in a tree with identical topology and clades as those revealed in the parsimony tree (TreeBase S10492-21664, Fig. 2).

Phylogeny generated from the combined data (Fig. 2) recognized two major clades. One comprised *Teratosphaeria* isolates from QLD and the second, isolates of *T. eucalypti* from elsewhere. The second clade was subdivided into two sub-clades. The three isolates from N-NSW were clearly separated from two other sub-clades with 65% bootstrap support and 1·00 Bayesian posterior probability. The two major clades were strongly supported with both Bayesian and parsimony analysis.

There were 16 polymorphic sites across the four sequenced gene regions among *T. eucalypti* isolates. Two

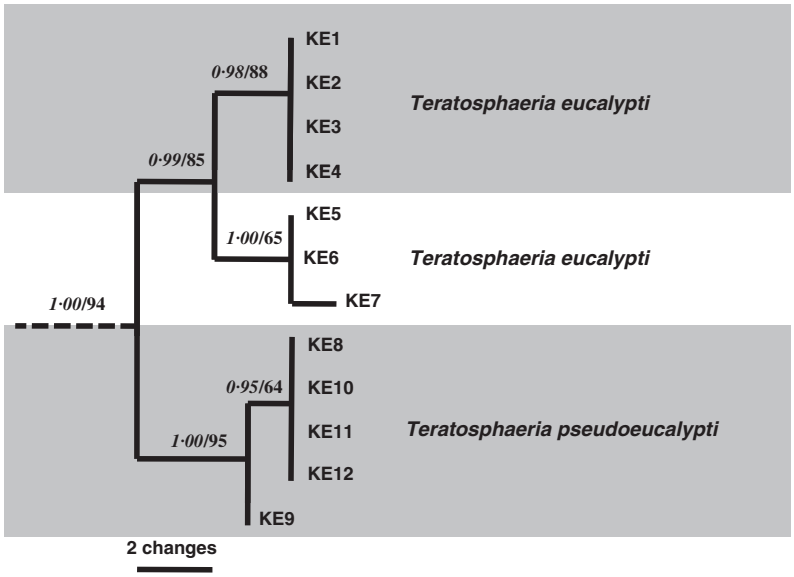

Figure 2 Part of a phylogram of the most parsimonious tree of 858 steps inferred from the combined datasets of βT, EF-1α and ITS-2 (for complete analysis see TreeBASE SN4360). Bootstrap support based on parsimony analysis and posterior probabilities of the branch nodes based on Bayesian analysis (italics) are given above the line. *Teratosphaeria pseudoeucalypti* resides in a strongly supported clade close to *T. eucalypti*.

polymorphic sites were detected in the ATP-6 region, seven in the βT region, four in the EF-1α and three in ITS-2 region (Table 3). Of the 16 polymorphic sites, nine fixed sites separated isolates from QLD with those from elsewhere.

Morphological characterization

Morphological examination of conidia of all *Teratosphaeria* isolates showed similar pigmentation and overlapping measurements for length, width and septa number. Conidia were hyaline to pale brown, (0–)1(–2) (rarely 3)-septate, slightly verruculose, straight to variously curved with high levels of length variability, depending on the origin of the specimen, ranging from 30 to 48·5 μm (Table 4, Fig. 3). High level of variability in conidia length had been previously observed amongst *T. eucalypti* from New Zealand (30–60 μm) (Gadgil & Dick, 1983), New South Wales (24–57 μm) (Heather, 1961) and Victoria (35–50 μm) (Walker

Table 3 Haplotypes of *Teratosphaeria* considered in the phylogenetic study. Positions of polymorphic nucleotides from aligned sequence data of ITS-2, ATP-6, β-tubulin and EF-1α gene regions showing the variation between haplotypes. Only parsimony informative nucleotides (=characters) are shown. For comparison purposes polymorphisms shared with the first haplotype are highlighted

	EF-1 α				β-tubulin							ATP-6		ITS-2		
Haplotype	31	34	52	143	73	91	93	98	201	209	236	236	295	146	193	249
Teratosphaeria eucalypti																
KE1	C	C	C	C	T	G	A	A	T	G	C	T	A	C	T	C
KE 2	C	G	C	C	T	G	A	A	T	G	C	G	A	C	T	C
KE 3	C	G	C	C	T	G	A	A	T	G	C	T	A	C	T	C
KE 4	C	C	C	C	T	G	A	A	T	G	C	G	A	C	T	C
Teratosphaeria eucalypti																
KE 5	C	C	C	C	T	C	G	A	C	A	C	T	A	C	T	C
KE 6	C	G	C	T	T	C	G	A	C	A	C	T	A	C	T	C
KE 7	C	G	C	T	T	C	G	A	C	A	C	T	A	C	T	T
Teratosphaeria pseudoeucalypti																
KE 8	T	C	T	T	C	G	A	G	C	A	T	G	A	T	G	T
KE 9	T	C	T	T	C	G	A	A	C	A	T	G	A	T	G	T
KE 10	T	C	T	T	C	G	A	G	C	A	T	G	A	C	T	T
KE 11	T	C	T	T	C	G	A	G	C	A	T	T	A	T	G	T
KE 12	T	C	T	T	C	G	A	G	C	A	T	G	T	T	G	T

Table 4 Morphological features of conidia of *Teratosphaeria* isolates from eucalypts recorded in published literature and in the present study

Fungus	Specimen number	Pigmentation	Conidial length (*in vivo*)[a] µm	Conidial width (*in vivo*) µm	Conidial length (*in vitro*)[b] µm	Conidial width (*in vitro*) µm	Number of septa
T. eucalypti (NSW)							
HAN-NSW	MURU449	Hyaline to sub-hyaline	34–41	2·5–3	n/a[c]	n/a	0–1
C-NSW	MURU451	Hyaline to sub-hyaline	39–47	2–3	n/a	n/a	0–1
N-NSW	MURU424	Sub-hyaline to pale brown	38–48·5	2·5–3	n/a	n/a	0–1
T. eucalypti (VIC) (Walker *et al.*, 1992)	K(M) 39487	Pale brown	35–50	3–4	n/a	n/a	0–2
Septoria normae (Heather, 1961)	DAR 65742	Hyaline, yellow to light brown	24–57	3–3·5	n/a	n/a	1–2
Septoria pulcherrima (Gadgil & Dick, 1983)	PDD 42838	Hyaline to pale brown	30–60	3–4	n/a	n/a	0–2
T. eucalypti (TAS) TAS	MURU452	Hyaline to sub-hyaline	30–40·5	2–3	33–41	2·5–3	0–1
T. pseudoeucalypti (QLD) BRIP (this study)	BRIP(average)	Sub-hyaline to pale brown	34–45	1·5–2·5	n/a	n/a	n/a
T. pseudoeucalypti (QLD)							
S-QLD	MURU448	Sub-hyaline to pale brown	31·5–39·5	2–2·5	n/a	n/a	0–2
C-QLD	MURU450	Sub-hyaline to pale	33–40	2–2·5	31·5–37·5	2–3	0–2
FNQ	MURU446	Brown Pale brown	n/a	n/a	31–39	2–3	0–3

[a]*In vivo* = herbarium specimens.
[b]*In vitro* = isolates from culture.
[c]n/a = not applicable (the isolates did not produce conidia in culture or were not available).

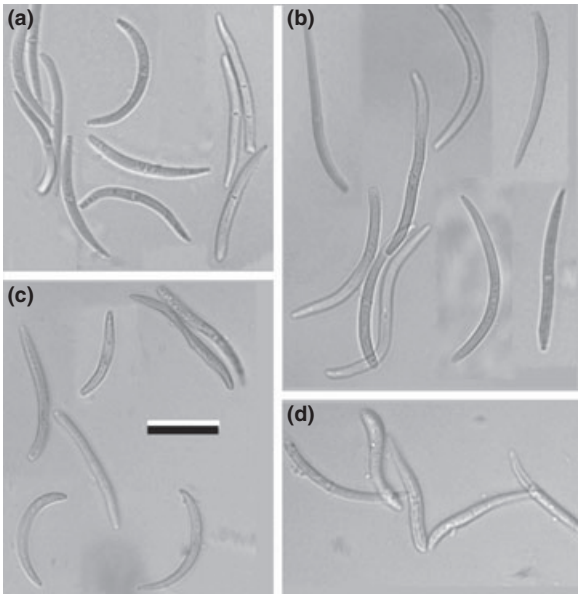

Figure 3 Conidia *in vivo* of (a) *Teratosphaeria pseudoeucalypti* specimen, MURU 450; (b) *T. eucalypti* specimen MURU 451. Conidia *in vitro* of (c) *T. pseudoeucalypti* isolate MUCC 607; (d) *T. eucalypti* isolate MUCC 631. Bar = 20 µm.

et al., 1992). Isolates from QLD were on average slightly shorter and less variable than those from elsewhere (Table 4).

In this study, the conidia of the specimens of *T. eucalypti* from HAN-NSW (39–47 µm) and N-NSW (38–48·5 µm) were slightly longer than the conidia of specimens collected from elsewhere including BRIP herbarium specimens (34–45 µm). The pycnidia of specimens collected from FNQ were immature therefore conidia could not be measured.

Conidia of C-QLD isolates produced in culture were slightly shorter (31·5–37·5 µm) than conidia observed from leaf material (33–40 µm). This was also true for isolates of *T. eucalypti* from VIC where conidia produced in culture were shorter (25–35 µm) than conidia produced on leaf material (35–50 µm). No variation in conidia length was found between culture and leaf material among isolates from TAS. Isolates from NSW, S-QLD and FNQ did not produce spores in culture.

Cultural characteristics and fructification

No significant effect of temperature (20 and 30°C) on colony morphology was observed among all isolates across the four tested media and thus cultural characteristics are reported only for isolates maintained at 20°C. In general isolates from QLD had a slower growth rate and were more olive green in colour than isolates from elsewhere (Table 5).

On average, isolates of *Teratosphaeria* from QLD produced fewer fruiting bodies than isolates from elsewhere across all media (rate 0·5–2) (Table 5). Isolates from S-QLD did not sporulate on any tested media.

Table 5 Comparison between colony diameter (mm) and morphology after 30 days at 20°C on four media for isolates of *Teratosphaeria eucalypti* and *T. pseudoeucalypti*

	Teratosphaeria pseudoeucalypti		*Teratosphaeria eucalypti*	
Media	Colony diameter	Colony characteristics	Colony diameter	Colony characteristics
MEA	S-QLD 18 mm C-QLD 11–30 mm FNQ 9–26 mm (Fig. 4m,n,o)	*Margins* irregular, sometimes lobed, sometimes smooth and pink *Upper surface* white to pinkish white with black pycnidia when present *Reverse* light reddish brown *Fructification rating* 2·72 ± 0·21	NSW 12–16 mm TAS 7–10 mm VIC 13–15 mm (Fig. 4p,q,r)	*Margins* irregular *Upper surface* pinkish white with olive green aerial mycelium *Reverse* olive brown *Fructification rating* 2·07 ± 0·17
OMA	S-QLD 10–13 mm C-QLD 7–16 mm FNQ 11–14 mm	*Margins* irregular, sometimes light olive brown *Upper surface* pink, sometimes with a smooth surface and white aerial mycelium *Reverse* pink to light red *Fructification rating* 3·17 ± 0·17	NSW 7–10 mm TAS 5–7 mm VIC 8–11 mm	*Margins* irregular *Upper surface* pinkish white with olive green outer zone *Reverse* olive green *Fructification rating* 0·94 ± 0·18
TWA	S-QLD 5–11 mm C-QLD 7–16 mm FNQ 7–11 mm	*Margins* regular to irregular *Upper surface* white, sometimes with a smooth light olive brown surface *Reverse* light olive brown *Fructification rating* 2·44 ± 0·23	NSW 2–7 mm TAS 2–7 mm VIC 2–7 mm	*Margins* irregular *Upper surface* black with pinkish white aerial mycelium *Reverse* olive brown *Fructification rating* 0·61 ± 0·16
½ PDA	S-QLD 17–21 mm C-QLD 12–23 mm FNQ 8–13 mm	*Margins* irregular, lobed, *Upper surface* pink with black spore masses *Reverse* light red to red and black at the point of inoculation	NSW 17–18 mm TAS 14–16 mm VIC 19–20 mm	*Margins* regular sometimes lobed *Upper surface* pale red with pinkish aerial mycelium *Reverse* pink red and black at inoculation point

Taxonomy

Although morphological characteristics showed no major differences amongst *Teratosphaeria* isolates, phylogenetic inference and cultural characteristics and sporulation have provided robust evidence to show that the causal agent of a serious leaf disease on *Eucalyptus* hybrids in Queensland represents a unique taxon. The fungus is thus described as a new cryptic species as follows:

Teratosphaeria pseudoeucalypti Andjic, T.I. Burgess sp. nov (Figs 3a,c and 4m,n,o)

Mycobank no MB 514057

Teleomorph: ***Teratosphaeria*** sp. (based on phylogenetic inferences, but not seen)

Etymology: Named after its sister species, *T. eucalypti*.

Conidiomata pycnidialia, hypophylla, singularia, atrobrunnea ad atra. Conidiophori redigent ad cellulas conidiogenas. *Conidia* singularia, 0–3-septata, subhyalina et pallide brunnea, parum verruculosa, cylindracea, recta ad varie curvata, cum parietibus crassis, ad basim truncata, interdum cum margine fimbriato, apex obtusus, (26·0—)31·5–40·0(—58·0) × (1·7—)2·0–2·5(3·5—).

Leaf spots: subcircular to irregular, 2–15 mm diameter, single to confluent, often blighting on *E. grandis* hybrids, initially pale green, turning chlorotic before becoming necrotic, light to medium brown with red-purple margin on the upper and lower surface. *Conidiomata*: pycnidial, hypophyllous, single, black. Conidiophores reduced to conidiogenous cells. *Conidia*: solitary, 0–3 septate, subhyaline to pale brown, slightly verruculose, cylindrical, straight to variously curved, thick-walled, base truncate sometimes with marginal frill, apex obtuse, (26·0—)31·5–40·0(—58·0) × (1·7—)2·0–2·5(3·5—) (mean = 35 × 2·2μm).

Cultures: Colonies 9–29 mm after 1 month at 20°C in the dark on MEA, margins irregular, sometimes lobed; the upper surface white 5YR 8/1 to pinkish white 5YR 8/2 with black pycnidia, margin pink and smooth; the lower surface light reddish brown 5YR 6/4. *Conidiomata* if present, pycnidial, single, black. *Conidiogenous cells*: not seen in culture. *Conidia*: solitary, 0–3-septate, subhyaline to pale brown, smooth to slightly verruculose, cylindrical, straight to variously curved (27·0—)31·0–39·0(—43·0) × (1·5—)2·0–3·0(—3·0) (mean = 35·0 × 2·5 μm).

Holotype: on leaves of *E. grandis* × *E. camaldulensis* Miriam Vale, Queensland, Australia, G. Pegg, August 2005 (HOLOTYPE MURU450; culture ex-type MUCC607, CBS 124577).

Hosts: Eucalyptus sp., *E. grandis* × *E. camaldulensis*

Geographic distribution: Queensland.

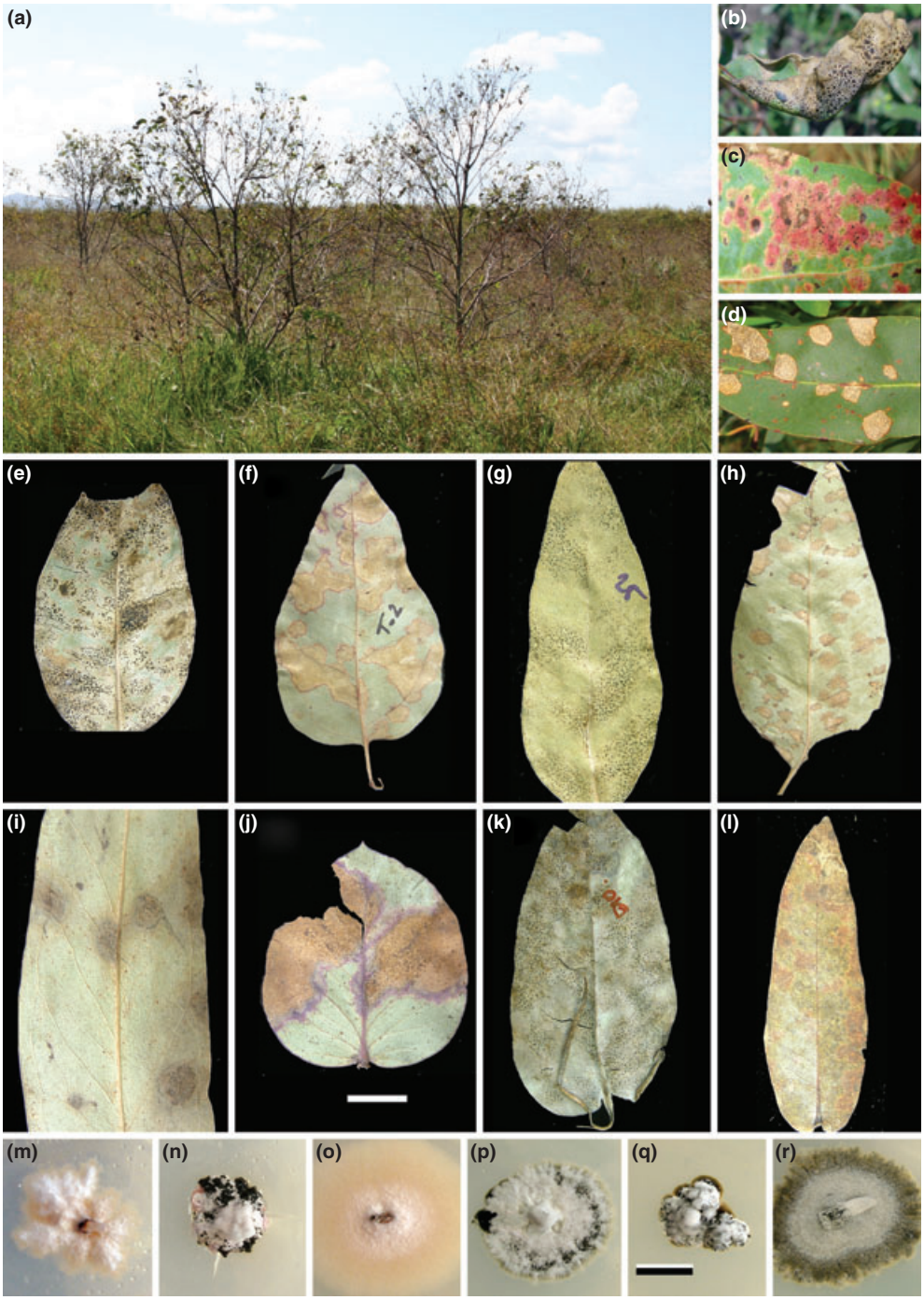

Figure 4 A comparison between the foliar disease symptoms and cultural morphology of *Teratosphaeria eucalypti* and *T. pseudoeucalypti*. *Teratosphaeria pseudoeucalypti*: (a) defoliated *Eucalyptus* hybrid in C-QLD; (b, e) leaf infection of *E. grandis* × *E. camaldulensis,* C-QLD; (f) leaf infection of *Eucalyptus* sp., FNQ; (g) leaf infection of *E. grandis* × *E. camaldulensis* hybrids, S-QLD. Colony morphology on MEA at 20°C of (m) MUCC600 from S-QLD; (n) MUCC613 from C-QLD; (o) MUCC607 from FNQ. *Teratosphaeria eucalypti*: (c, l) leaf infection of *E. nitens,* Tasmania; (d, h) leaf infection of *E. grandis* × *E. tereticornis* hybrids, Kyogle, N-NSW; (i) leaf infection of *Eucalyptus,* Victoria; (j) leaf infection of *E. nitens,* HNA-NSW; (k) leaf infection of *E. nitens,* New Zealand. Colony morphology on MEA at 20°C of (p) MUCC632 from VIC; (q) MUCC635 from TAS; (r) MUCC626 from N-NSW. Bar = 10 mm.

Additional specimens examined: T. pseudoeucalypti on *E. grandis* × *E. tereticornis*, Harrisville, Queensland, Australia, A.J. Carnegie, G. Pegg, August 2005 (MURU 448; culture ex-isotypes, MUCC600) and *Eucalyptus* sp., North Queensland, Australia, T.I. Burgess, August 2006 (MURU447; culture ex-isotypes MUCC614).

Comparison of distribution, impact and symptoms of *T. pseudoeucalypti* and *T. eucalypti*

The disease caused by *T. pseudoeucalypti* was first detected in August 2005 causing leaf blight to *E. grandis* × *E. camaldulensis* hybrids at Harrisville, S-QLD and Miriam Vale, C-QLD, and based on symptoms and the blighting nature of damage resembled *T. destructans* (Fig. 4a). However, at the time the causal agent was identified as *T. eucalypti* based on conidia size and morphology. Results of collections reveal that whilst the major damage to plantations in NSW is caused by *T. eucalypti*, in Queensland, the major damage is caused by *T. pseudoeucalypti*. The current geographical distribution of *T. pseudoeucalypti* is unknown, but the results of this study suggest that this pathogen is limited to regions with sub-tropical and tropical climate, whilst *T. eucalypti* is found in both temperate and sub-tropical areas. *Teratosphaeria pseudoeucalypti* has recently also been found and confirmed from a production nursery in central NSW on *E. grandis* × *E. camaldulensis* material derived from Queensland.

Symptoms on leaves caused by *T. pseudoeucalypti* are variable and similar to those caused by both *T. eucalypti* and *T. destructans* depending on host and potentially maturity of leaves at time of infection. On *E. grandis* × *E. camaldulensis* hybrids symptoms were typically similar to *T. destructans*, with large blights that crinkled leaves (Fig. 4b,e,g), while on *E. camaldulensis*, and in some cases older leaves of *E. grandis* × *E. tereticornis*, symptoms were more commonly individual necrotic leaf spots.

Discussion

The genetic diversity of the leaf pathogen, *T. eucalypti*, was examined using nucleotide sequence variation of four gene regions. Nine fixed polymorphic sites were found in three genomic and one mitochondrial gene (1496 bp of sequence) distinguishing isolates of *Teratosphaeria* from *Eucalyptus* spp. in Queensland, Australia, from *T. eucalypti* found elsewhere. The fungal isolates from Queensland represent a new cryptic species and has been described as *T. pseudoeucalypti*.

In general, boundaries of fungal species are recognized using a simple approach by fulfilling either of two criteria: (i) genealogical concordance, to identify independent evolutionary lineages and phylogenetic species from multiple gene genealogies, a clade must be present in the majority of the single locus genealogies; (ii) genealogical nondiscordance; recognizes a clade as an independent evolutionary lineage if it is well supported by at least one single locus genealogies by both bootstrap and posterior probabilities values above 70% and 0·95 respectively, and if it is not contradicted by any other single locus genealogies determined by the same methods (Dettman *et al.*, 2003). In the present study, phylogenetic analyses based on multiple gene phylogeny strongly support the existence of an independent evolutionary lineage of isolates from Queensland, now designated as *T. pseudoeucalypti*, by fulfilling both the aforementioned criteria. Data obtained by haplotype networking also distinguished *T. pseudoeucalypti* from *T. eucalypti*. Furthermore, there were no shared haplotypes between isolates from Queensland and those from elsewhere.

Apart from cultural characteristics and higher sporulation rate of *T. eucalypti*, *T. eucalypti* and *T. pseudoeucalypti* are morphologically similar and this differentiation has been based principally on DNA sequence comparisons. However, this is not surprising as *Teratosphaeria* anamorphs (*Kirramyces*-like) from eucalypts are often morphologically similar, thus relying heavily on DNA sequence comparison for differentiation (Andjic *et al.*, 2007a,b,c).

Cryptic speciation has been seen in *Paracoccidioides brasiliensis*, an important human pathogen, endemic to Latin America (Restrepo, 2003). Whilst considered to be a clonal species by mycological criteria, this assumption was not supported by multiple gene phylogenies. As a result *P. brasiliensis* was divided into three distinct species (Matute *et al.*, 2006). A similar situation has been seen with species of *Teratosphaeria cryptica* and *T. pseudocryptica*, *T. endophytica* and *T. pseudoendophytica* (Crous *et al.*, 2006), *T. vespa* and *T. pseudovespa* (Carnegie *et al.*, 2007c) and *T. destructans* and *T. viscida* (Andjic *et al.*, 2007b; Burgess *et al.*, 2007).

Teratosphaeria eucalypti isolates were collected from three geographical regions in NSW: C-NSW, HAN-NSW and N-NSW. The DNA sequence of isolates collected from N-NSW was more variable than that of isolates collected from C-NSW. Phylogenetic analysis using multiple genes has separated three isolates from N-NSW in one sub-clade which was strongly supported by Bayesian analysis. The three isolates from N-NSW were from a taxa trial (at Kyogle) where severe defoliation eventually resulted in the death of many trees. Interestingly, this trial location is less than 60 km south of where *T. pseudoeucalypti* was first collected (Harrisville, Queensland) and while phylogenetically close to *T. eucalypti*, could represent a new cryptic species or a hybrid with *T. pseudoeucalypti*.

The impact of this disease in central Queensland has increased annually and while it was initially thought that older trees and some hybrid crosses were more resistant to KLB it is now known that if the inoculum load is high most eucalypt species and hybrids trialled to date in sub-tropical Australia are susceptible. The confirmation of *T. pseudoeucalypti* from a production nursery in central NSW on material derived from Queensland is of concern as it appears to be a more significant pathogen than other *Teratosphaeria* species already established in NSW.

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