New cryptic species of *Teratosphaeria* on *Eucalyptus* in Australia

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**Abstract:** *Teratosphaeria destructans* and *T. viscida* are serious pathogens causing leaf, bud and shoot blight diseases of *Eucalyptus* plantations in the subtropics and tropics of South-East Asia (*T. destructans*) and North Queensland, Australia (*T. viscida*). During disease surveys in northern Western Australia and the Northern Territory of Australia, symptoms resembling those of *T. destructans* were observed on young and adult leaves of native and plantation *Eucalyptus* spp. and its hybrids. Phylogenetic studies revealed *Teratosphaeria* species associated with these symptoms are new taxonomic novelties described here as *T. novaehollandiae* and *T. tiwiana* spp. nov. Isolates from previous records of *T. destructans* recorded in Australia were re-examined and based upon the phylogenetic evidence are reassigned to these new taxa. We conclude that *T. destructans* is absent from Australia.

**Key words:** biosecurity DNA phylogeny *Kirramyces*

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**INTRODUCTION**

Teratosphaeria leaf diseases (TLD; Crous et al. 2006, Hunter *et al*. 2006, Crous 2009, Crous et al. 2009a) have emerged as significant foliar diseases impacting on the eucalypt plantation industry in subtropical and tropical areas of Australia (Carnegie *et al*. 2007a, b, c). *Teratosphaeria* species with kirramyces-like asexual morphs have emerged as the most significant foliar pathogens of this genus; namely *T. destructans*, *T. eucalypti*, *T. pseudoeucalypti*, *T. suttonii*, and *T. viscida* (Carnegie *et al*. 1996, Park *et al*. 2000, Carnegie 2007a, b, Andjic *et al*. 2010a). These five species cause a serious leaf blight disease, leading to premature defoliation and in some instances tree mortality (Andjic *et al*. 2007b, 2010a, Carnegie 2007a, b, c). Symptoms are similar and include brown to purple spots on leaves with diffuse border and red brown margin, necrotic lesions delimited by veins and presence of spore masses and conidia (Dick 1982, Walker *et al*. 1992, Wingfield *et al*. 1996, Burgess *et al*. 2006, Andjic *et al*. 2007a, b, c, Andjic *et al*. 2010a). Conidia of these species are all long, variously curved, subhyaline to pale brown, smooth to verruculose and are virtually indistinguishable by morphology (excluding *T. suttonii*), thus making diagnostics based on morphology impossible (Andjic *et al*. 2010b, Hunter *et al*. 2011).

*Teratosphaeria destructans* is an aggressive pathogen causing a leaf, bud and shoot blight disease (Wingfield *et al*. 1996). This pathogen was first discovered in Indonesia in 1996 and has since been detected in Thailand, China, Vietnam, and most recently South Africa (Burgess *et al*. 2006, Old *et al*. 2003a, b, Wingfield *et al*. 1996, Greyling *et al*. 2016). In Australia, *T. destructans* is only reported from Tiwi Island in the Northern Territory (on introduced plantation *Eucalyptus* hybrids) and Derby in Western Australia (on amenity *Eucalyptus* sp.) (Burgess *et al*. 2007). *Teratosphaeria eucalypti* is a leaf parasite of endemic *Eucalyptus* species (eastern Australia) but under favourable conditions can cause a serious leaf blight disease mostly infecting juvenile leaves of some *Eucalyptus* species in plantations (Carnegie 2007b). *Teratosphaeria eucalypti* is known to have been introduced with plantings of *E. nitens* from Australia into New Zealand, where it has resulted in complete defoliation of juvenile leaves of *E. nitens* (Dick 1982, Miller *et al*. 1992). *Teratosphaeria pseudoeucalypti* was first discovered on an unidentified *Eucalyptus* sp. and hybrids of *E. grandis × camaldulensis* in Central Queensland, where it caused severe outbreaks and damage (Andjic *et al*. 2010a). Since then the pathogen has been detected in Argentina (Ramos & Perez 2015), Brazil (de Souza *et al*. 2014), and Uruguay (Soria *et al*. 2014). *Teratosphaeria suttonii* is known from many countries (Park *et al*. 2000, Sankaran *et al*. 1995, Taole *et al*. 2015) and can cause severe damage in eucalypt plantations (Carnegie 2007b). *Teratosphaeria viscida* was first detected in 2005 causing leaf and shoot blight in *E. grandis* and complete defoliation of *E. grandis × camaldulensis* hybrids in Mareeba, North Queensland (Andjic *et al*. 2007b).

Whilst *T. eucalypti*, *T. pseudoeucalypti*, *T. suttonii*, and *T. viscida* are all native to Australia, the origin of *T. destructans* is still unclear (Andjic *et al*. 2011). Based on DNA sequence variation of Australian isolates, it was thought that *T. destructans* originated from Australia (Burgess *et al*. 2007). *Teratosphaeria destructans* was a high risk pathogen for Australia and was on the Northern Australia Quarantine Strategy (NAQS) biosecurity target list (as *Kirramyces*

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destructans), but later removed after being reported in northern Australia by Burgess et al. (2007) (Jane Ray pers. comm.). During surveys of native and plantation eucalypt species in northern Western Australia and the north-western Northern Territory over the years 2006–12, we observed leaves exhibiting symptoms similar to those of T. destructans. Samples were collected across several sites and preliminary examination revealed a fungus with a conidial morphology similar to that of T. destructans. This study describes two new species of Teratosphaeria with long conidia found in northern Western Australia and the Northern Territory that are distinct from T. destructans.

MATERIALS AND METHODS

Collection and isolation
Eucalypt leaves with symptoms resembling those of Teratosphaeria destructans were collected from several different locations in Australia: (a) adult mature trees of a Eucalyptus sp. in Derby, Western Australia; (b) juvenile leaves from Eucalyptus hybrids in plantations on Tiwi Island, Northern Territory; and (c) juvenile and adult foliage from eucalypt woodlands at several locations in northern, Western Australia and north-western parts of Northern Territory (Table 1, Fig. 1C). Isolations were made as described previously (Andjic et al. 2007 a). Isolates are maintained in culture collections at Murdoch University, Perth, Western Australia (MUCC) and the Department of Agriculture and Water Resources (AQISWA), Perth, Western Australia. Ex-type cultures and leaf material have been deposited in the fungal collection of Queensland Plant Pathology Herbarium (BRIP), Brisbane, Queensland, Australia, and the KNAW-CBS Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands.

Morphological identification and characterisation
Preliminary identification of the Teratosphaeria isolates was by microscopic examination and culturing. Plugs (2 mm diam) were cut from actively growing cultures and placed at the centres of Petri dishes (55 mm diam) containing 2 % Malt Extract Agar (MEA). After 30 d, cultures were assessed for growth-rate, by taking two measurements of colony diameter from the centres of Petri dishes (55 mm diam) were cut from actively growing cultures and placed at several locations in northern, Western Australia and north-western parts of Northern Territory (Table 1, Fig. 1C). Isolations were made as described previously (Andjic et al. 2007 a). Isolates are maintained in culture collections at Murdoch University, Perth, Western Australia (MUCC) and the Department of Agriculture and Water Resources (AQISWA), Perth, Western Australia. Ex-type cultures and leaf material have been deposited in the fungal collection of Queensland Plant Pathology Herbarium (BRIP), Brisbane, Queensland, Australia, and the KNAW-CBS Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands.

Squash mounts of sporangial structures were prepared, from hand sections of lesions and from culture, on slides in lacto-glycerol (1:1:1 v lactic acid: glycerol: water) and observed at 1000× magnification with Leica DM5000 light microscope. Morphological characters used in this study to distinguish Teratosphaeria species producing kiramycete-like long conidia included: conidial shape, size, pigmentation and number of septa. Wherever possible, 30 measurements of all potentially taxonomically relevant structures were recorded for each species and the extremes are presented in parentheses. Measurements of conidial size were obtained using image analysis software Leica Image Application Suite (LAS) and adjusted to the nearest 0.5 μm. Conidium lengths were recorded as straight-line (linear) length following the method of previous studies of Teratosphaeria (Wingfield et al. 1996). Data analyses were performed using descriptive statistics in Microsoft Excel.

DNA Extraction, PCR amplification and sequencing
Isolates were grown on 2 % MEA at 20 °C for 4 wk and the mycelium was harvested and placed in a 1.5 mL sterile Eppendorf® tube. Harvested mycelium was ground to a fine powder using cordless motor pellet pestle (Sigma-Aldrich) and genomic DNA was extracted using a DNeasy® Plant Mini Kit (Qiagen) following the manufacturer’s instructions. ITS2 and part of the 5.8S region of the DNA (ITS2), and two partial protein-coding genes, β-tubulin (tub2) and translation elongation factor (tef1), were sequenced for all isolates as described previously (Andjic et al. 2007a).

Phylogenetic analysis
The phylogeny of the new Teratosphaeria isolates was estimated using parsimony and maximum likelihood methods. In order to compare Teratosphaeria species used in this study with other closely related species, additional ITS2, tub2 and tef1 sequences were obtained from GenBank (Table 1). Sequence data were assembled and aligned using the CLUSTALW algorithm implemented in Geneious R7 v. 7.0.4 (Biomatters). Adjustments to the alignments were made manually by inserting gaps where necessary.

Maximum parsimony analyses were performed on individual (data not shown) and combined data sets in PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2003) after a partition homogeneity test (PHT) of the combined ITS2, tub2 and tef1 alignments was conducted in PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2003) to test pairwise congruence between the sequence data sets.

The equally most parsimonious trees were obtained using heuristic searches with random stepwise taxon additions in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option off. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally most parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (Hillis & Huelsenbeck 1992). Branch and branch node support was determined using 1000 bootstrap replicates (Felsenstein 1985). Trees were rooted to Teratosphaeria nubilosa (CBS 116005).

The same aligned datasets were used for the Bayesian analysis, which was performed with MrBayes v. 3.2.6 (Ronquist & Huelsenbeck 2003) as implemented as Geneious plug-in after MrModeltest v. 3.5 (Nylander 2004) was used to determine the best nucleotide substitution model per gene region. For all gene regions the Hasegawa, Kishino and Yano (HKY85) nucleotide substitution model with gamma (G) and proportion of invariable site (I) parameters was the best model. Two independent runs of Markov Chain Monte Carlo (MCMC) were run over 1 100 000 generations. The heating parameter was set at 0.2 and trees were saved each 1 000 generations, resulting in 1 100 trees. Burn-in was set
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Fig. 1. A. Bayesian phylogram obtained from the combined ITS2, translation elongation factor 1-α and the β-tubulin sequence alignment. Bootstrap support based on parsimony analysis and posterior probabilities of the branch nodes based on Bayesian analysis (italics) are given at the nodes. All trees are rooted to Teratosphaeria nubilosa. B. Haplotype network based on sequence data from ITS2, tub2 and tef1 gene regions. Colours following the blocks in Fig. 1. A indicate the isolates and the localities in Fig. 1. C. Red=T. destructans; Green=T. tiwiana; Orange=T. novaehollandiae; Brown=T. viscida C. Map showing collection localities of species described with colour coding aligned to the phylogram (Fig. 1A) and haplotype network (Fig. 1 B).
| Fungus | Culture no. | Host | Location | Collector | GenBank accession no. |
|--------|-------------|------|----------|-----------|----------------------|
|        |             |      |          |           | tef1                  |
| T. eucalypti | CMW19416 |  E. nitens | Sun Valley, New Zealand | M Dick | EU010583 EU010527 FJ793232 |
| T. eucalypti | CMW19453 |  E. nitens | Settlement Rd, New Zealand | M Dick | EU010585 EU010529 FJ793234 |
| T. eucalypti | MUCC 623 |  E. nitens | Dorrigo, HAN-NSW, Australia | A.J. Carnegie | EU010623 EU010566 FJ793255 |
| T. eucalypti | MUCC 643 |  E. nitens | Roses Tier, TAS, Australia | T. Wardlaw | EU010635 EU010578 EU010666 |
| T. novaehollandiae | AQISWA 201513 | Eucalyptus sp. | Derby, WA, Australia | M.J. Wingfield | KT972355 KT972323 KT972291 |
| T. novaehollandiae | AQISWA 201514 | Eucalyptus sp. | Derby, WA, Australia | M.J. Wingfield | KT972356 KT972324 KT972292 |
| T. novaehollandiae | AQISWA 201515 | Eucalyptus sp. | Derby, WA, Australia | M.J. Wingfield | KT972357 KT972325 KT972293 |
| T. novaehollandiae | AQISWA 201516 | Eucalyptus sp. | Derby, WA, Australia | M.J. Wingfield | KT972358 KT972326 KT972294 |
| T. novaehollandiae | BRIP59486 |  E. camaldulensis | Kununurra, WA, Australia | A. Maxwell | KT972345 KT972313 KT972281 |
| T. novaehollandiae | BRIP59487 |  E. camaldulensis | Kununurra, WA, Australia | A. Maxwell | KT972346 KT972314 KT972282 |
| T. novaehollandiae | BRIP59488, CBS 141552 |  E. camaldulensis | Kununurra, WA, Australia | A. Maxwell | KT972347 KT972315 KT972283 |
| T. novaehollandiae | BRIP59490 |  E. camaldulensis | NS, Australia | V. Andic | KT972349 KT972317 KT972285 |
| T. novaehollandiae | BRIP59481 |  E. camaldulensis | NS, Australia | V. Andic | KT972350 KT972318 KT972286 |
| T. novaehollandiae | BRIP63522 |  E. victix | Pilbara, WA, Australia | G. Hardy | KT972352 KT972320 KT972288 |
| T. novaehollandiae | BRIP63523, CBS 141554 |  E. victix | Pilbara, WA, Australia | G. Hardy | KT972351 KT972319 KT972287 |
| T. novaehollandiae | AQISWA 201403 |  E. victix | Pilbara, WA, Australia | G. Hardy | KT972353 KT972321 KT972289 |
| T. novaehollandiae | AQISWA 201404 |  E. victix | Pilbara, WA, Australia | G. Hardy | KT972354 KT972322 KT972290 |
| T. novaehollandiae | MUCC 599 |  E. grandis × camaldulensis | Harrisville, S-QLD, Australia | A.J. Carnegie | EU010593 EU010537 FJ793216 |
| T. novaehollandiae | MUCC 605 |  E. grandis × camaldulensis | Harrisville, S-QLD, Australia | A.J. Carnegie | EU010616 EU010559 FJ793225 |
| T. novaehollandiae | MUCC 607 |  E. grandis × camaldulensis | Miriam Vale, C-QLD, Australia | G. Pegg | EU010598 EU010542 FJ793220 |
| T. novaehollandiae | MUCC 612 |  E. grandis × camaldulensis | Miriam Vale, C-QLD, Australia | G. Pegg | EU010601 EU010545 FJ793223 |
| T. tiwiana | MUCC463 |  E. grandis × camaldulensis | Tiwi Island, Australia | M.J. Wingfield | EU009640 EU009649 EU009631 |
| T. tiwiana | MUCC465 |  E. grandis × camaldulensis | Tiwi Island, Australia | M.J. Wingfield | EU009641 EU009650 EU009632 |
| T. tiwiana | AQISWA 201501, CBS 141547 |  E. grandis × urophylla | Tiwi Island, Australia | M.J. Wingfield | KT972361 KT972329 KT972298 |
| T. tiwiana | BRIP63496, CBS 141549 |  E. urophylla hybrids | Tiwi Island, Australia | M.J. Wingfield | KT972362 KT972330 KT972298 |
| Culture no.          | Host          | Accession no. | Location                        | Collector       |
|----------------------|---------------|---------------|---------------------------------|-----------------|
| AQISWA 201503        | E. unrophylla | KT972299      | Tiwi Island, Australia TI Burgess | KT972363       |
| BRIP63492, CBS 141553| E. unrophylla | KT972365      | Tiwi Island, Australia TI Burgess | KT972365       |
| BRIP63497, CBS 141550| E. unrophylla | KT972365      | Tiwi Island, Australia TI Burgess | KT972365       |
| BRIP63494, CBS 141552| E. unrophylla | KT972365      | Tiwi Island, Australia TI Burgess | KT972365       |
| BRIP63524             | E. unrophylla | KT972365      | Tiwi Island, Australia TI Burgess | KT972365       |
| MUCC 456, CBS 121156  | E. grandis    | EF031498      | Mareeba, Australia              | EF031498        |
| MUCC 452, CBS 121157  | E. grandis    | EF031498      | Mareeba, Australia              | EF031498        |
| MUCC 457, CBS 121157  | E. grandis    | EF031498      | Mareeba, Australia              | EF031498        |
| MUCC 458              | E. grandis    | EF031498      | Mareeba, Australia              | EF031498        |
| MUCC 459              | E. grandis    | EF031498      | Mareeba, Australia              | EF031498        |

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Table 1. Continuation:

| Culture no.          | Host          | Accession no. | Location                        | Collector       |
|----------------------|---------------|---------------|---------------------------------|-----------------|
| BRIP63492, CBS 141553| E. unrophylla | KT972365      | Tiwi Island, Australia TI Burgess | KT972365       |
| BRIP63497, CBS 141550| E. unrophylla | KT972365      | Tiwi Island, Australia TI Burgess | KT972365       |
| BRIP63494, CBS 141552| E. unrophylla | KT972365      | Tiwi Island, Australia TI Burgess | KT972365       |
| BRIP63524             | E. unrophylla | KT972365      | Tiwi Island, Australia TI Burgess | KT972365       |
| MUCC 462, CBS 121156  | E. grandis    | EF031498      | Mareeba, Australia              | EF031498        |
| MUCC 463, CBS 121157  | E. grandis    | EF031498      | Mareeba, Australia              | EF031498        |
| MUCC 465              | E. grandis    | EF031498      | Mareeba, Australia              | EF031498        |

**Haplotype network estimation**

Haplotype networks were used to compare isolates in order to infer which isolates were most closely related to one another. Haplotype networks were generated using the statistical parsimony method in the TCS v. 1.21 software programme (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 & Tavare 1986, Castelloe & Tempelton 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 ITS2, tub1 and tef1 DNA sequences.

**RESULTS**

**Morphological identification**

The fungal isolates obtained in this study were characterised as slow-growing cultures on MEA. Morphological characteristics of the conidia of the *Teratosphaeria* isolates were similar in pigmentation, length, size, shape, and septa number (Table 2).

Conidia were hyaline, subhyaline to pale brown, straight to variously curved, with 0–3 septa, and ranging from 30–50 × 2–3.5 μm (*in vivo*). These characteristics are typical for all *Teratosphaeria* species with kirramyces-like long conidia isolated from *Eucalyptus*. Morphological features of *Teratosphaeria* asexual morphs with long and short conidia are variable and not reliable for species separation, therefore the identification of those species relies on DNA sequencing (Andjic et al. 2007c, 2010b).

**Molecular identification and phylogenetic analysis**

A BLASTn search was conducted in GenBank to compare the ITS2 sequences of the *Teratosphaeria* isolates being examined in this study with those already there. The returned sequences were most similar to *T. destructans* (for isolates from Tiwi Island) and *T. viscida* (for isolates from Western Australia, WA, and Northern Territories, NT) and these and other less related species (*T. eucalypti* and *T. 
pseudoeucalypti) were used in the phylogenetic analyses. The aligned combined data set of ITS2, tub2, and tef1 consisted of 870 characters, of which 72 were parsimony-informative and were used in the analysis. The partition homogeneity test showed no significant difference (P > 0.01; P = 0.32) between data from different gene regions, and so these data were combined. These data contained significant phylogenetic signal (P < 0.01; gl = -0.95). Heuristic searches of unweighted characters in PAUP resulted in 18 equally most parsimonious trees of 91 steps (CI=0.89, RI=0.98; TreeBASE S18826; Fig. 1). The Bayesian analysis resulted in a tree with the same topology and clades as those revealed in the parsimony analysis and presented as Fig. 1A (TreeBASE S18826; Fig. 1). In the Bayesian analysis, the tub2, tef1 and ITS2 regions consisted of 47, 55 and 31 unique site patterns respectively.

The phylogeny generated from the combined alignment (Fig. 1A) resulted in three major clades: the first major clade comprising *T. destructans* and isolates from Tiwi Island with 93% bootstrap support and a Bayesian posterior probability of 1.0; the second comprising isolates from WA and the NT with 100% bootstrap support and a Bayesian posterior probability of 1.0; and the third containing isolates of *T. viscida* with 100% bootstrap support and a Bayesian posterior probability of 1.0. Furthermore, the first major clade was subdivided in two sub-clades, one containing isolates of *T. destructans* and the second containing *Teratosphaeria* isolates from Tiwi Island. The *T. destructans* sub-clade was well supported with 78% bootstrap support and a Bayesian posterior probability of 1.0. The Tiwi Island isolates subclade was supported with 68% bootstrap support and a strong Bayesian posterior probability of 0.93. Although the bootstrap support for isolates from Tiwi Island was relatively low, posterior probability was strong for that node and the tree topology was consistent in all 18 equally most parsimonious trees. *Teratosphaeria* isolates from Tiwi Island were monophyletic in all 18 equally most parsimonious trees and consistently separated from *T. destructans* (data not shown).

The second clade containing isolates from WA and NT was well supported with a 100% bootstrap value and a posterior probability of 1.0. The unnamed *Teratosphaeria* from this clade was closely related to, but phylogenetically distinct from *T. viscida* (Fig 1A). Isolates of both *T. viscida* and the undescribed *Teratosphaeria* were monophyletic with some sequence variation observed amongst new *Teratosphaeria* isolates. There were 12 fixed polymorphic sites distinguishing *T. viscida* from the new *Teratosphaeria* across the three gene regions indicating that isolates from this clade represent a new taxon (Table 3).

### Haplotype network

Haplotype networks constructed in TCS software resulted in ten haplotypes (H-1–H-10) amongst the isolates used in this study (Fig. 1B): *Teratosphaeria destructans* was represented by one haplotype, H-1 (six isolates from Asia, AAA); *T. tiwiana* from Tiwi Islands was represented by three haplotypes (H-2, two isolates, ABB; H-3, six isolates, BBB; and H-4, six isolates BBC); the new *Teratosphaeria* isolates were represented by five haplotypes: H-5 (two isolates from the Kimberley region in WA and one from the NT, CDD); H-6 (one isolate from the NT, CCE); H-7 (two isolates from the NT, CDD); H-8 (four isolates from the Pilbara, WA, CED);
and H-9 (four isolates from Derby, WA, DFD); and *T. viscid* was represented by one haplotype, H-10 (four isolates from Queensland, EGF).

Three different haplotypes (H-2, H-3, H-4) were observed in the population from Tiwi Island, but none of them were shared with the phylogenetically closely related *T. destructans* (H-1).

Five haplotypes were detected in the population from WA and NT. Only one haplotype was shared among isolates from WA and the NT (H-5), and none of the haplotypes were shared with the closely related *T. viscid* (H-10).

Morphological examination did not show any major differences between the *Teratosphaeria* isolates obtained in this study. This situation is common in species lacking a known sexual morph. However, the combination of phylogenetic inference and haplotype analysis provides robust evidence that isolates from Tiwi Island, NT and WA are distinct from both *T. destructans* and *T. viscid*. They are therefore described as new species here.

**Fig. 2.** Morphological features of *Teratosphaeria destructans*, *T. tiwiana*, *T. novaehollandiae*, and *T. viscid* from eucalypts. A–D. *T. destructans* specimen PREM 59261 (CMW 17919). A. Leaf symptoms. B. Culture morphology on MEA. C. Conidia morphology. D. Conidiogenous cells and conidiogenesis. E–H. *T. tiwiana* holotype specimen and ex type culture BRIP 63496 (CBS 141549). E. Leaf symptoms. F. Culture morphology on MEA. G. Conidia morphology. H. Conidiogenous cells and conidiogenesis. I–L. *T. novaehollandiae* holotypespecimen and ex type culture BRIP 59486. I. Leaf symptoms. J. Culture morphology on MEA. K. Conidia morphology. L. Conidiogenous cells and conidiogenesis. M–P. *T. viscid* specimen BRIP 49804 CBS 121156). M. Leaf symptoms. N. Culture morphology on MEA. O. Conidia morphology. P. Conidiogenous cells and conidiogenesis. Bars = 10 µm.
### TAXONOMY

**Teratosphaeria novaehollandiae** V. Andjic, T.I. Burgess, A. Maxwell, *sp. nov.*

MycoBank MB815681 (Fig. 2I–L)

**Etymology:** Name refers to original Dutch name for the geographic western half of Australia, where the fungus was collected.

**Diagnosis:** Distinguished from *T. viscida* (cfr. Figs. 2 I–L and 2 M–P) in not producing highly hydrophobic and viscous spore masses. *In vivo,* *T. novaehollandiae* produces shorter conidia (33–40 μm) than those of *T. viscida* (47–60 μm). *In vitro,* *T. novaehollandiae* produce shorter conidia (27–31 μm) than *T. viscida* (35–40 μm). Unlike *T. viscida,* *T. novaehollandiae* does not produce a synasexual morph with chlamydospore-like structures in culture. Based on phylogenetic analyses of sequence data obtained for the ITS2, *tef1* and *tub2* gene regions, *T. novaehollandiae* has 12 fixed polymorphic sites across three gene regions which distinguish it from the closely related *T. viscida* (Table 3).

**Type:** Australia: Western Australia: Kununurra, isolated from leaves of *Eucalyptus camaldulensis*, Apr. 2012, A. Maxwell & V. Andjic (BRIP 59487, BRIP 59488 = CBS 141552, BRIP 59490, and BRIP 59481); Western Australia: Pilbara isolated from *Eucalyptus victoriae*, Aug. 2013, G. Hardy (BRIP 63522, BRIP 63523 = CBS 141545, AQISWA 201403, AQISWA 201404); Derby, isolated from leaves of *Eucalyptus sp.*., July 2006, T.I. Burgess & M.J. Wingfield (BRIP 64754, culture not viable).

**Teratosphaeria tiwiana** V. Andjic, T.I. Burgess, A. Maxwell, *sp. nov.*

MycoBank MB815680 (Fig. 2 E–H)

**Etymology:** Named after Tiwi Island, the type locality.

**Diagnosis:** Distinguished from *T. destructans* (cfr. Fig. 2 E–H v. A–D) by producing slightly shorter conidia and in septa number. *In vivo,* *T. tiwiana* produces shorter and less curved conidia (35–40 μm) than those of *T. destructans* (38–65 μm). In contrast to *T. destructans,* whose conidia is 1–3-septate, *T. tiwiana* are 1–2-septate (Fig. 2C, G). Based on multi-gene phylogeny *T. tiwiana* can be distinguished from *T. destructans* with 6 bp differences across three gene regions.

**Type:** Australia: Northern Territory: Tiwi Island, isolated from leaves of *Eucalyptus hybrids* *E. grandis* × *E. urophylla*, Aug. 2007, T.I. Burgess (BRIP 63496– holotype; BRIP 63496 = CBS 141549– ex-type cultures).

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### Table 3. Polymorphic nucleotides from sequence data of ITS2, *tef1* and *tub2* gene regions showing the variation between isolates of *Teratosphaeria viscida* and *T. novaehollandiae*. Ex-type cultures are indicated in bold face.

|   | tub2 | tef1 | ITS2 |
|---|------|------|------|
| **Teratosphaeria viscida** |      |      |      |
| CBS 121156   | G A G T | T G A A G G | T G |
| CBS 121157   | G A G T | T G A A G G | T G |
| MUCC 456     | G A G T | T G A A G G | T G |
| MUCC 455     | G A G T | T G A A G G | T G |
| **Teratosphaeria novaehollandiae** |      |      |      |
| BRIP59486    | A G T C | C A C T A A | C C |
| BRIP59488    | A G T C | C A C T A A | C C |
| BRIP63523    | A G T C | C A C T A A | C C |
| BRIP63523    | A G T C | C A C T A A | C C |
| AQISWA201513 | A G T C | C A C T A A | C C |
Description: Leaf spots circular to irregular, 3–20 mm diam, single to confluent, pale to medium brown with red brown border on the top surface, light brown below. Conidiomata pycnidial, hypophyllous, single, dark brown. Conidiophores reduced to conidiogenous cells. Conidia solitary, 1–2-septate, predominantly with 1-septum, pale brown, slightly verruculose, cylindrical, straight to variously curved, base truncate, sometimes with marginal flange, apex obtuse, (25.5–)35–40(–44.5) × (2–)2.5–3.0(–3.5) (mean = 35 × 2.8 μm).

Culture characteristics: Colonies 25 × 25 mm after 1 mo at 25 °C in the dark on MEA, white 5YR 8/1 to pink 5YR 8/4 on the upper surface, olive grey 5YR 7/1 on reverse. Mycelium subhyaline to pale brown, septate, branched. Conidiomata, if present, pycnidial, single, dark brown to black, globose to subglobose, unilocular: wall of textura angularis. Conidiogenous cells not seen in culture. Conidia solitary, 0–1–septate, subhyaline to pale brown, slightly verruculose, cylindrical, straight to variously curved (25–)35–40(–56.5) × (2–)2.5–3(–3.5) (mean = 38 × 3.0 μm), lateral branches occasionally present as secondary conidia.

Additional specimens examined: Australia: Northern Territory: Tiwi Island, isolated from E. grandis and E. urophylla hybrids, Aug. 2007, T. Burgess (BRIP 63492 = CBS 141553, BRIP 63491 = CBS 141551, BRIP 63493, BRIP 63494, BRIP 63497 = CBS 141550, BRIP 63495 = CBS 141548, BRIP 63524).

DISCUSSION

We describe two new cryptic Teratosphaeria species isolated from Eucalyptus in northern Australia: T. tiwiana and T. novaehollandiae. Australian isolates previously described as T. destructans were re-examined and are here assigned to these two new taxa. Teratosphaeria destructans s. str., therefore, has not been correctly recorded in Australia, and remains restricted to South-East Asia and Africa.

The two new Teratosphaeria species could not be morphologically distinguished, thus the description was based on data inferred from multi-gene phylogeny: applying the Genealogical Concordance for Phylogenetic Species Recognition (GCPSPR; Taylor et al. 2000) criteria, and noting the haplotype analysis of combined sequence data for the ITS2, tub2 and tef1 gene regions. The GCPSPR concept uses the phylogenetic concordance of multiple unlinked genes to indicate a lack of genetic exchange and thus evolutionary independence of lineages (Geiser et al. 1998, Taylor et al. 2000, Starkey et al. 2007, Cai et al. 2011). It is a useful criterion for the discrimination of species when other species recognition criteria (morphological, physiological, reproduction, host specificity) fail (Cai et al. 2011). GCPSPR has already proved to be a valuable tool for recognising cryptic species in Colletotrichum, Diaporthe, Phyllosticta, and Fusarium species complexes (Glience et al. 2011, Damm et al. 2012, Shivas & Cai 2012, Gomes et al. 2013, Hansen & Oliariaga 2015).

Teratosphaeria tiwiana, a cryptic species similar to T. destructans, was isolated from non-endemic juvenile eucalypt leaves from a clonal taxa trial from Tiwi Island, NT, Australia. Previously, based on symptoms, conidial morphology and multilocus sequence data, the isolates from Tiwi Island had been identified as T. destructans although they grouped separately from T. destructans from Asia (Burgess et al. 2007). As a consequence, T. destructans was removed from the NAQS target list for exotic invasive plant pathogens. This study included more isolates from Tiwi Island than the initial study, and re-evaluated the relationship between Australian and Asian isolates using multi-locus sequence data and haplotype analysis. The DNA sequence analysis obtained in this study was in agreement with the findings in Burgess et al. (2007); there were 6 bp differences amongst isolates across three gene regions. In that previous study, it was thought that 6 bp difference was within the normal limits of infraspecific variation and the isolates from Australia were identified as T. destructans However, multigene phylogeny and haplotype analysis obtained in the present study provided sufficient evidence to support the separation of Australian isolates as a separate species described here as T. tiwiana. According to the Genealogical Concordance Concept of Dettman et al. (2003), a clade is recognised as an independent evolutionary lineage if the clade was present in the majority of the single locus genealogies and the clade is identified from the majority rule consensus tree regardless of its bootstrap or posterior probability support. In this study, the T. tiwiana clade was recovered in all three single strict consensus trees satisfying the criterion of genealogical concordance (data not shown); the clade was monophyletic in the phylogenetic trees inferred from single gene regions and from the combined dataset; and the lineage was supported by Bayesian analyses. Furthermore, T. tiwiana isolates showed sequence variation and were split in three haplotypes, while the sequences of T. destructans isolates were identical and contained only one haplotype. The haplotypes were not shared between these two species. This suggests that T. tiwiana is an endemic Australian cryptic species.

Teratosphaeria novaehollandiae was found on amenity plantings of an unidentified native Eucalyptus species in Derby, endemic E. camaldulensis woodlands in northern WA and the NT, and on E. victrix in the Pilbara, WA. These isolates were collected from adult and juvenile eucalypt foliage across an extensive area of northern Australia and where present caused minor to moderate levels of damage to Eucalyptus leaves. The sequences of T. novaehollandiae were variable and split into five haplotypes. Two haplotypes contained isolates from northern WA, one haplotype contained isolates from NT, one contained isolates from Pilbara and one contained isolates from Derby, WA. Despite having unique haplotypes, isolates from across northern WA and the NT could not be consistently split into separate phylogenetic species.

In a previous study, based on conidial morphology and phylogenetic analysis, isolates from Derby (WA) were assigned to T. destructans (Burgess et al. 2007). We have now re-evaluated the taxonomic position of the Derby isolates using multigene sequence data including haplotype analysis. The results obtained in this study have demonstrated that all isolates from Derby grouped together and were separated from Asian and Australian isolates previously named as T. destructans (i.e. T. tiwiana). The Derby isolates clustered
within the *T. novaehollandiae* clade, which was well supported (1,00 Posterior probabilities) and was distinct from both *T. destructans* and *T. viscida*. The Derby isolates were not consistently separated from other isolates within *T. novaehollandiae* and are therefore here recognised as the new cryptic species *T. novaehollandiae*.

Sequence data and ex-type cultures are now available for for eight *Teratosphaeria* species described from eucalypts and for which a kirramyces-like asexual morph is known. Conidia range in size from the shortest *T. novaehollandiae* (33–40 µm) to the longest, *T. destructans* (50–65 µm), pigmentation and septation also varies, but generally conidia are sepatate. These species produce lesions on leaves with various symptoms. All except *T. destructans* have been reported in Australia. The expansion of eucalypt plantation forestry into the subtropics of Australia has led to the discovery of many new *Teratosphaeria* species, and it appears to be the dominant fungal genus on subtropical eucalypt leaves. Currently, the two species newly described in this study are not causing any significant damage to Australian eucalypt plantations, but the threat they may pose to the forestry industry is unknown.

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