

# A new species of *Cryphonectria* from South Africa and Australia, pathogenic to *Eucalyptus*

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*Endothia gyrosa* causes cankers on several hardwood genera in North America, but occurs only on *Eucalyptus* spp. in South Africa and Australia. Previously, Australian and South African isolates of *E. gyrosa* were shown to be different from North American isolates. They were also closely related to *Cryphonectria parasitica* based on DNA sequences of the ITS1/2 regions of the ribosomal operon. The aim of the present study was to provide an appropriate name for the *Eucalyptus* fungus known as *E. gyrosa* from Australia and South Africa. Collections of *E. gyrosa* specimens from the different geographical areas were compared based on morphology and DNA sequence data. Sequences from both the  $\beta$ -tubulin and ITS1/2 regions confirmed that South African and Australian isolates of *E. gyrosa* group together with other *Cryphonectria* spp. and separately from *E. gyrosa* and *E. singularis*. Distinct morphological differences were observed between North American specimens and those from South Africa and Australia. Stromatal morphology for the South African and Australian specimens was more similar to that of *Cryphonectria* than to *Endothia*. The fungus on *Eucalyptus* in South Africa and Australia is thus a distinct species of *Cryphonectria* and is accordingly provided the new name *C. eucalypti*.

Keywords: *Cryphonectria eucalypti*, *Endothia gyrosa*, pathogen, *Eucalyptus*, systematics

*Endothia gyrosa* (Schw. : Fr.) Fr. is a fungal pathogen best known for its association with pin oak (*Quercus palustris* Muench.) blight in North America (Appel & Stipes, 1984, 1986; Roane & al., 1974; Stipes & Phipps, 1971). This fungus, apparently native to North America, also causes cankers on exotic Formosan sweetgum (*Liquidambar formosana* Hance.), *Liquidambar styraciflua* L. (Snow & al., 1974), several *Quercus* spp., *Acer saccharinum* L. (Roane & al., 1974), *Castanea* spp., *Ilex opaca* Aiton (Appel & Stipes, 1986), *Fagus sylvatica* L. (Shear & al., 1917), *F. grandiflora* Ehrh., *Prunus lauro-cerasus* L. (Roane, 1986a), *Ulmus*, *Corylus* and *Vitis* (Farr & al., 1989).

In the USA, *E. gyrosa* has been reported from various south-eastern states (Stevens, 1917), as well as isolated areas of Kansas (Shear & al., 1917; Stevens, 1917), Ohio (Appel & Stipes, 1986; Hunter & Stipes, 1978; Stevens, 1917), Michigan, Maryland, New Jersey, Connecticut, New York and California (Shear & al., 1917; Stevens, 1917). *Endothia gyrosa* has also been found in China on *Quercus* (Teng, 1934) and Spaulding (1961) reported the fungus in Portugal, Spain and Italy on *Quercus* spp. and *Fagus* spp. He, furthermore, noted that *E. gyrosa* occurs in Germany, western Europe, Sri Lanka, New Zealand and the Philippines, but no hosts were noted.

*Endothia gyrosa* is known as a pathogen of *Eucalyptus* from mainland Australia (Davison & Coates, 1991; Old & al., 1986, 1990; Walker & al., 1985), Tasmania (Old & al., 1986; Yuan & Mohammed, 1997, 1999; Wardlaw, 1999) and South Africa (Van der Westhuizen & al., 1993). There is also a single account of the fungus on *E. diversicolor* F. Muell. from Portugal (Spaulding, 1961). In southeastern and western Australia, *E. gyrosa* is found on numerous species of *Eucalyptus* (Davison & Coates, 1991; Old & al., 1986; Walker & al., 1985; White & Kile, 1993). The fungus was recently reported from South Africa associated with cankers on several species of *Eucalyptus*, including *E. grandis* W. Hill ex Maid., *E. nitens* (Deane & Maid.) Maid., *E. urophylla* R. T. Blake and hybrids of *E. grandis* with *E. camaldulensis* Dehnh. and *E. urophylla* (Van der Westhuizen & al., 1993).

*Endothia gyrosa* was known to occur in the USA from the first half of the nineteenth century (Barr, 1978; Shear & al., 1917; Stevens, 1917). Its recent discovery in Australia and South Africa on a completely different host to those known in North America, is unusual. The question of the similarities and differences between the North American fungus and Southern Hemisphere *Eucalyptus* fungus was raised by Walker & al. (1985), who noted morphological differences between those two groups of fungi. Those differences were mainly that the stromata of the Australian specimens were less well developed, and that the lower part or the entire perithecial body is seated in the bark and not in fungal tissue, as is observed in specimens from North America. Nonetheless, the North American and Australian specimens had similar perithecia, asci and ascospores, leading to the identification of the Australian fungus as *E. gyrosa*.

Venter & al. (2001) used PCR based RFLPs and DNA sequence comparisons to show that the South African and Australian fungus was different to *E. gyrosa* from North America. Furthermore, the South African and Australian isolates grouped more closely with *Cryphonectria parasitica* (Murr.) Barr, than with *E. gyrosa* from North America. The aim of the present study was to provide an

appropriate name for the *Eucalyptus* fungus from South Africa and Australia. This was achieved using phylogenetic comparisons of various species of *Endothia* and *Cryphonectria*, based on two regions of the genome, and morphological comparisons of specimens and cultures of *E. gyrosa* from different areas of the world.

## Materials and methods

### Source of isolates and specimens

Isolates of *E. gyrosa*, *E. singularis* (H. & B. Syd.) Shear & Stevens, *C. parasitica*, *C. radicalis* (Schw. ex Fr.) Barr and *C. macrospora* (Kobayashi & Ito) Barr from different areas of the world were used for the molecular characterization and are listed in Tab. 1. Isolates named *E. gyrosa* representing South Africa, Australia and the USA were also used to conduct a growth study in culture (Tab. 1). Isolates were maintained on malt extract agar MEA [20 g/l malt extract agar (Biolab, Merck, Midrand, South Africa)] at 5 °C. All isolates used in this study are preserved in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Bark samples from South Africa were collected from different localities in commercial plantations of *Eucalyptus*, from trees exhibiting typical canker symptoms (Tab. 2). Other specimens studied include samples from trees in North America (supplied by Dr. C. S. Hodges, Dept. Plant Pathology, NC State University, Raleigh, NC, USA), and specimens of the Australian fungus [deposited by Walker & al. (1985) in the Plant Pathology Herbarium, Orange Agricultural Institute (DAR), Australia] (Tab. 2). All new samples considered have been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM) (Tab. 2). The type specimen of *E. gyrosa*, which has a North American origin, was found not to be useful for study because the original specimen has been moved and redistributed, and parts of it that could be found only contained anamorph structures (Shear & al., 1917). The co-type as listed by Shear & al. (1917), could not be located despite attempts to do so. Specimens of *C. gyrosa* (Berk. & Br.) Sacc., the type species of *Cryphonectria*, were also examined to compare the morphology of the Australian and South African samples to that of *Cryphonectria* (Tab. 2).

### DNA isolation and amplification

DNA was isolated as described in Myburg & al. (1999). The ITS1, ITS2 and 5.8S rRNA gene of the ribosomal operon were amplified using primers ITS1 and ITS4 (White & al., 1990). Amplicons were

Tab. 1. – Isolates of *Endothia* spp. and *Cryphonectria* spp. used for the molecular comparison and growth study.

Culture number*	Taxon	Alternative designation <sup>†</sup>	Host	Origin	Collector	Genbank accession number <sup>‡</sup>
CMW7033	<i>C. eucalypti</i>	PREM56215 <sup>§</sup>	<i>Eucalyptus grandis</i>	KwaZulu/Natal, RSA	M. Venter	
CMW7034	<i>C. eucalypti</i>	PREM56211 <sup>§</sup>	<i>E. grandis</i> X <i>camaldulensis</i>	KwaZulu/Natal, RSA	M. Venter	
CMW7035	<i>C. eucalypti</i>	PREM56305 <sup>§</sup>	<i>E. saligna</i> Smith	KwaZulu/Natal, RSA	M. Venter	
CMW7036	<i>C. eucalypti</i>	CRY62*	<i>Eucalyptus</i> sp.	Mpumalanga, RSA	I. van der Westhuizen	AF232878 <sup>¶</sup> ; AF368341; AF368340
CMW7037	<i>C. eucalypti</i>	CRY45*	<i>E. delegatensis</i> R. T. Blake	NSW, Australia	K. Old	AF232880 <sup>¶</sup> ; AF368343; AF368342
CMW7038	<i>C. eucalypti</i>	CRY909*/ PREM56217 <sup>§</sup>	<i>E. globulus</i> Labill.	Western Australia, Aus.	M. Wingfield	AF232881 <sup>¶</sup> ; AF368345; AF368344
CMW7039	<i>E. gyrosa</i>	PREM56220 <sup>§</sup>	<i>Quercus falcata</i> Michx.	NC, USA	C. Vernia	
CMW7040	<i>E. gyrosa</i>	PREM56220 <sup>§</sup>	<i>Q. falcata</i>	NC, USA	C. Vernia	
CMW7041	<i>E. gyrosa</i>		<i>Q. borealis</i> Michx.	USA	S. Anagnostakis	
CMW7042	<i>E. gyrosa</i>	E2/ATCC48190	<i>Q. suber</i> L.	GA, USA	B. S. Crandall	-; AF368335; AF368334
CMW7043	<i>E. gyrosa</i>	E3/ATCC48191	<i>Q. marilandica</i> Muench.	VA, USA	J. D. Diller	AF368324; -; -
CMW2091	<i>E. gyrosa</i>	E13/ATCC48192	<i>Q. palustris</i> Muench.	VA, USA	R. J. Stipes	AF368325; AF368337; AF368336
CMW7045	<i>E. gyrosa</i>	E27	<i>Q. palustris</i>	VA, USA	R. J. Stipes	AF368326; AF368339; AF368338
CMW7046	<i>E. singularis</i>	E58	–	CO, USA	–	AF368323; AF368333; AF368332
CMW7047	<i>C. parasitica</i>	E5/ATCC48197	<i>Q. virginiana</i> Mill.	MS, USA	R. D. Wolfe	AF368329; AF273073; AF273469
CMW7048	<i>C. parasitica</i>	E9/ATCC48198	<i>Q. virginiana</i>	VA, USA	F. F. Lombard	AF368330; AF273076; AF273470

Tab. 1 (cont.). – Isolates of *Endothia* spp. and *Cryphonectria* spp. used for the molecular comparison and growth study.

Culture number*	Taxon	Alternative designation <sup>†</sup>	Host	Origin	Collector	Genbank accession number <sup>‡</sup>
CMW1652	<i>C. parasitica</i>	CRY44*		USA		AF046902 <sup>‡</sup> ; AF273075; AF273468
CMW1651	<i>C. parasitica</i>	CRY66*	<i>Castanea dentata</i> Borkh.	USA	P. J. Beeker	AF046901 <sup>‡</sup> ; AF273074; AF273467
CMW7051	<i>C. radicalis</i>	E76/CBS240.54	<i>C. sativa</i> Mill.	Italy	A. Biraghi	AF368328; AF368347; AF368346
CMW7052	<i>C. radicalis</i>	E83/CBS240.54	<i>C. sativa</i>	Italy	A. Biraghi	AF368327; AF368349; AF368348
CMW7053	<i>C. macrospora</i>	E54	<i>Castanopsis cuspidata</i> (Thunb.) Schottky	Japan	T. Kobayashi	AF368331; AF368351; AF368350
CMW2498	<i>Diaporthe ambigua</i>		<i>Malus sylvestris</i> Mill.	Netherlands	S. Truter	AF046906 <sup>‡</sup> ; AF273072; AF273471

\* Culture collection of the Forestry & Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, 0002. CMW and CRY refers to different subsets of the collection.

<sup>†</sup> PREM, National Collection of Fungi, Pretoria, South Africa; numbers preceded with E are designated numbers in the collection of R. J. Stipes, currently incorporated in the culture collection of FABI; American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209; Centraalbureau voor Schimmelcultures (CBS), Fungal and Yeast Collection, P. O. Box 273, 3240 AG, Baarn, Netherlands.

<sup>‡</sup> Vouchered specimens from which the particular isolates have been obtained.

<sup>§</sup> Given as sequences from the ITS region,  $\beta$ -tubulin 1a/1b and  $\beta$ -tubulin 2a/2b respectively.

<sup>¶</sup> Sequences obtained from Venter & al. (2001).

<sup>\*\*</sup> Sequences obtained from Myburg & al. (1999).

Tab. 2. – Specimens of *Endothia gyrosa*, *Cryphonectria eucalypti* and *C. gyrosa* used in morphological comparisons.

Taxon	State*	Host	Origin	Collector	Date	Herbarium allocation**
<i>E. gyrosa</i>	A, T	<i>Quercus phellos</i> L.	Raleigh, USA	L. Grand	1997	PREM56218
<i>C. eucalypti</i> (holotype)	T	<i>Eucalyptus grandis</i> X <i>camaldulensis</i>	Nyalazi, SA	M. Venter	1998	PREM56211
<i>C. eucalypti</i> (paratype)	T	<i>E. grandis</i>	Sabie, SA	J. Roux	1998	PREM56212
<i>C. eucalypti</i> (paratype)	T	<i>E. saligna</i> Smith	Tzaneen, SA	M. Venter	1999	PREM56305
<i>C. eucalypti</i> (paratype)	A	<i>E. grandis</i>	Dukuduku, SA	M. Venter	1998	PREM56214
<i>C. eucalypti</i> (paratype)	A	<i>E. grandis</i>	Amangwe, SA	M. Venter	1998	PREM56215
<i>C. eucalypti</i> (paratype)	A	<i>E. grandis</i>	Dukuduku, SA	M. Venter	1998	PREM56216
<i>C. eucalypti</i>	A	<i>E. globulus</i>	Perth, Australia	M. Wingfield	1997	PREM56217
<i>C. gyrosa</i>	T	<i>Elaeocarpus dentatus</i> Vahl.	Omahuta Forest, New Zealand	S. J. Hughes	1964	DAR14534 DAOM93506A
<i>C. gyrosa</i>	T	Dead twigs	Olinda pipe line, Maui, USA	C. L. Shear	1927	DAR49895
<i>E. gyrosa</i>	T	<i>E. saligna</i>	Termeil, Australia	K. Old	1983	DAR49904
<i>E. gyrosa</i>	T	<i>E. saligna</i>	Currowan, Clyde Mountain, Australia	K. Old	1984	DAR49905
<i>E. gyrosa</i>	T	<i>E. saligna</i>	Currowan, Clyde Mountain, Australia	K. Old	1983	DAR49906
<i>E. gyrosa</i>	T	<i>E. saligna</i>	Currowan, Clyde Mountain, Australia	K. Old	1983	DAR49907
<i>E. gyrosa</i>	T	<i>E. saligna</i>	Termeil, Australia	K. Old	1983	DAR49909
<i>E. gyrosa</i>	T	<i>Liquidambar</i> sp.	Grenada, USA	C. L. Shear & N. E. Stevens	1914	DAR49897

\* T: Teleomorph, A: Anamorph.

\*\* PREM, National Collection of Fungi, Pretoria, South Africa; DAR, Plant Pathology Herbarium, Orange Agricultural Institute, Forest Road, Orange, N. S. W. 2800, Australia; DAOM, National Mycological Herbarium, Eastern Cereal and Oilseed Center (ECORC), Agriculture and Agri-Food Canada, Edifice Wm. Saunders Bldg. #49, Ottawa, Ontario, Canada, K1A 0C6.

also obtained from the  $\beta$ -tubulin genes with primer pairs Bt1a/Bt1b and Bt2a/Bt2b (Glass & Donaldson, 1995).

Each 50  $\mu$ l PCR reaction for the ITS1/ITS2 region consisted of 1 mM dNTPs (0.25 mM of each), 1 x reaction mix (supplied with the enzyme), 2.5 mM MgCl<sub>2</sub>, 0.1  $\mu$ M of each primer, 5 units of *Taq* Polymerase (Roche Biochemicals, Mannheim, Germany) and DNA template. The only difference between the PCR reaction mix for the amplification of the  $\beta$ -tubulin gene was that Expand *Taq* Polymerase (Roche Biochemicals, Germany) was used. Amplification reactions were performed on a Perkin Elmer GeneAmp PCR System 9700 thermocycler (Perkin-Elmer Applied Biosystems, Inc., Foster City, California). The ITS1/ITS2 region was amplified using the same conditions as described by Myburg & al. (1999). The  $\beta$ -tubulin gene was amplified using an initial denaturing step (94°C, 1 min.), followed by 30 cycles of denaturing (94°C, 1 min.), primer annealing (Bt1a/1b = 55°C, Bt2a/2b 55°C – 68°C depending on the isolate, 1 min) and chain elongation (72°C, 1 min.). A final chain elongation step (72°C, 5 min.) followed the 30 amplification cycles. PCR products were run on 1% agarose gels containing ethidium bromide and were visualized under an UV light source.

#### Sequencing and analysis of sequence data

Amplification products were purified using a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany). Primers used in the PCR reaction were used to sequence the DNA fragments in both directions. The ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin-Elmer, Warrington, UK) was used to sequence the fragments. DNA sequences were obtained with an ABI PRISM 377™ automated DNA sequencer (Perkin Elmer, UK).

The DNA sequences were verified with Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems, Inc., California) and aligned with each other using PAUP\* (Phylogenetic Analysis Using Parsimony) version 4.0b (Swofford, 1998). Sequences of the ITS1/2 regions for isolates CMW7036, CMW7037 and CMW7038 (Venter & al., 2001) and the ITS1/2 sequences for isolates CMW1651, CMW1654 and CMW2498 (Myburg & al., 1999) were also included in the phylogenetic analyses. Phylogenetic analyses were executed using PAUP\*. The tree bisection-reconnection (TBR) swapping option of the heuristic search algorithm [save multiple trees (MulTrees) option effective, swap on best trees only] was used to obtain the most parsimonious tree. Characters were unordered and equally weighted. Gaps were treated as a fifth character (Newstate). The confidence levels of the branch points were determined with a Bootstrap analyses (1000 replications). *Diaporthe ambigua* Nits., which also belongs to the Valsaceae

(Hawksworth & al., 1996), was defined as a monophyletic outgroup taxon to root the phylogenetic tree.

### Morphological comparisons

Stromata together with surrounding bark tissue were removed from all bark samples using a scalpel, sectioned vertically with a razor blade, and mounted on microscope slides in lactophenol. Fruiting structures were also sectioned with a cryostat. Structures were cut out of the bark and rehydrated, according to a modified method of Huhndorf (1991), by steaming for 5 min. The structures were subsequently mounted in Leica mountant (Setpoint Premier, Johannesburg, South Africa) on the grid. Sections, 14–20  $\mu\text{m}$  thick, were obtained with a Reichert HistoSTAT cryostat at  $-20^{\circ}\text{C}$ . The sections were mounted in lactophenol on glass slides.

Fifty measurements were made of all structures believed to be of taxonomic significance, including perithecia, asci, ascospores, pycnidial locules, conidiogenous cells and conidia. Due to the limited occurrence, only 30 stromata were measured for the specimens selected to represent the holotype of the new taxon. The means, standard deviations and ranges of all measurements were computed and are presented as (min-)(x-sd) – (x+sd)(-max). The colour designations of Rayner (1970) were used to assign standard colours to structures.

### Growth in culture

The growth in culture of isolates CMW7033, CMW7034, CMW7035 (South Africa), CMW7037, CMW7038 (Australia) and CMW7041, CMW7039, CMW7040 (North America) (Tab. 1), were compared. These studies were conducted on MEA [20 g/l malt extract agar (Biolab, Merck, South Africa)] and PDA [50 g/l (Difco Laboratories, Detroit, USA)]. A disc 6 mm in diameter, taken from the edge of actively growing colonies, was placed in the center of each of four 90 mm Petri dishes per isolate. Growth studies were conducted in the dark from 10 to 30  $^{\circ}\text{C}$ , with 5  $^{\circ}\text{C}$  intervals. Two measurements, perpendicular to each other, of the colony diameter (mm) on each plate were taken daily from the third day of the experiment until the first isolates completely covered the plates after nine days. Colony diameter of each isolate was compared as an average of the eight readings per isolate.

## Results

### Sequencing and analysis of sequencing data

The aligned sequences of the ITS region resulted in a data set of 593 characters, of which 334 characters were constant, 95 characters

were parsimony-uninformative and 164 were parsimony-informative. Two most parsimonious trees were obtained. The general topology of the trees remained the same and the trees only differed in the internal grouping of the isolates. One of them was chosen for presentation (Fig. 1a). The trees had a length of 419 steps, consistency index/CI of 0.7828 and retention index/RI of 0.8392. The phylogenetic signal was significant as indicated by the *g*1 value of -0.861901 (Hillis & Huelsenbeck, 1992).

The aligned sequences of the  $\beta$ -tubulin data set amounted to 1104 characters, of which 119 were parsimony-uninformative, 600 were constant and 385 were parsimony-informative. Four trees were generated due to internal base pair differences between the *C. parasitica* isolates, and the *E. gyrosa* isolates from South Africa and Australia, respectively. Again, the general topology of the trees remained the same, with the only differences occurring in the internal nodes of the *C. parasitica* isolates and *E. gyrosa* isolates. One of the trees was chosen for presentation (Fig. 1b). The tree had a length of 958 steps, CI of 0.7568 and RI of 0.8278. The phylogenetic signal was also significant (*g*1 value = -1.136463) (Hillis & Huelsenbeck,

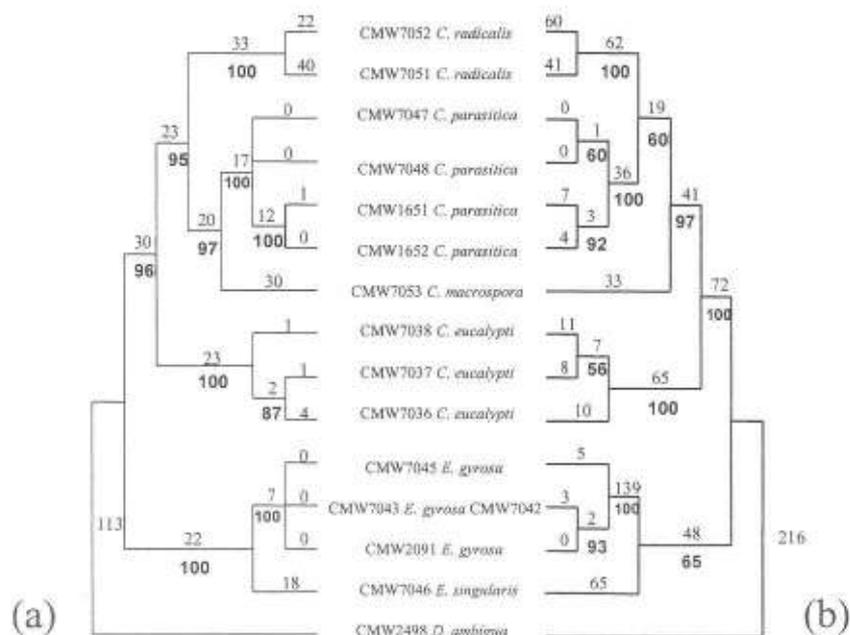


Fig. 1. - The most parsimonious trees obtained from sequences of the a) ITS1, 5.8S rRNA gene and ITS2 regions of the ribosomal operon, and b)  $\beta$ -tubulin genes. Both trees were produced with PAUP\* 4.0b using the TBR option of the heuristic search algorithm, with gaps treated as newstate (fifth character). The number of steps is indicated above the branches, and the bootstrap confidence levels are indicated in bold below the branches. *Diaporthe ambigua* was defined as outgroup.

1992). The data partitions ITS1, 5.8S, ITS2, Bt1a/1b and Bt2a/2b were not compatible with each other as indicated by the partition homogeneity test ( $P$  value = 0.01).

The trees obtained from the ITS and  $\beta$ -tubulin regions both indicated that the isolates from *Eucalyptus* in Australia and South Africa, previously known as *E. gyrosa*, do not belong in *Endothia*. Instead, these isolates grouped more closely together with the other *Cryphonectria* isolates in both the ITS-derived tree (Fig. 1a) and the  $\beta$ -tubulin-derived tree (Fig. 1b) (96% confidence and 100% confidence respectively). In both trees, isolates of *E. gyrosa* and *E. singularis* formed a clade separate to the South African and Australian isolates, and other *Cryphonectria* species (100% confidence for the ITS-derived tree, 65% confidence for the  $\beta$ -tubulin-derived tree, respectively).

### Morphological comparisons

The fungi from the southern hemisphere and North America, previously referred to as *E. gyrosa*, were found to be distinctly different from each other, although they were similar in terms of ascus and ascospore morphology. The South African and Australian specimens from *Eucalyptus*, however, were morphologically indistinguishable from each other. The stromata of the North American specimens were much larger [(911–1562(–1800)  $\mu\text{m}$  high, (1250–)1296–1881(–2050)  $\mu\text{m}$  in diameter] than those of the South African specimens [(202–)389–741(–940)  $\mu\text{m}$  high, (186–)343–704(–868)  $\mu\text{m}$  in diameter]. The stromata of the North American specimens were also superficial and subclavate (Fig. 2a, 2b) compared with the South African specimens, where stromata were usually semi-immersed (although fully immersed and superficial stromata also occurred occasionally) (Fig. 3a, 3d, 4a, 4d). The ratio of the parts of the stromata that were erumpent, to the entire length of the stromata, was 1:2 for the South African and Australian fungus (sometimes as great as 1:4). The same ratio for the North American fungus was 1:1, since the stromata of the North American fungus were almost completely erumpent.

Stromata from *Eucalyptus* were more variable in shape than those from trees in North America. Furthermore, the lower halves of the stromata for the North American specimens had a typical pseudostromatic appearance with host cells distributed throughout the stromatal tissue (Fig. 2a, 2b). The ectostromatic and entostromatic layers were also continuous with each other, but they were predominantly entostromatic. The lower parts of the stromata of the South African and Australian specimens consisted predominantly of host tissue, while the upper parts of the stromata in these specimens were made up of primarily fungal tissue (Fig. 3a, 3d, 4a, 4d). The

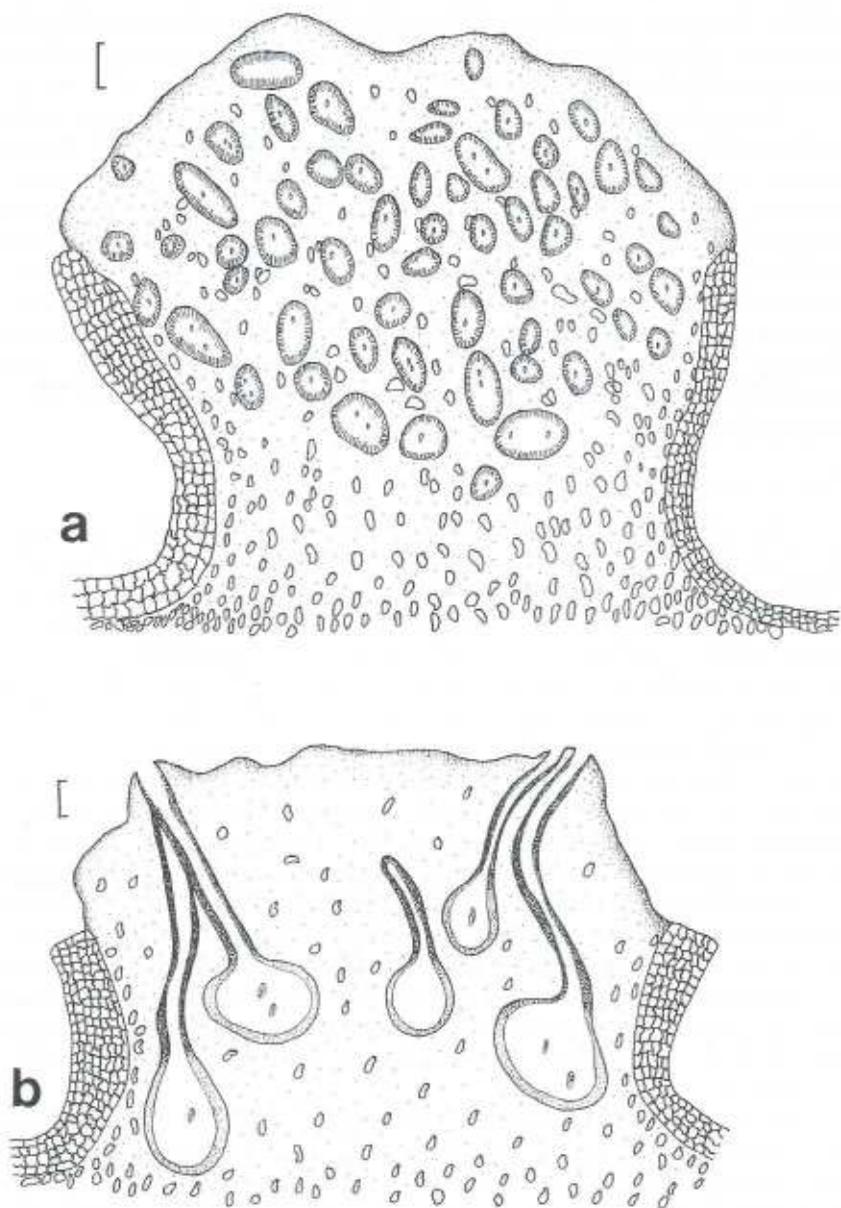


Fig. 2. - Anamorph and teleomorph states of *Endothia gyrosa*. - a. Anamorphic stroma. - b. Teleomorphic stroma. - Bars: a, d = 100  $\mu$ m.

stromata in the South African and Australian fungus thus had typical ectostromatic discs forming the erumpent parts, and entostromatic discs containing the perithecia.

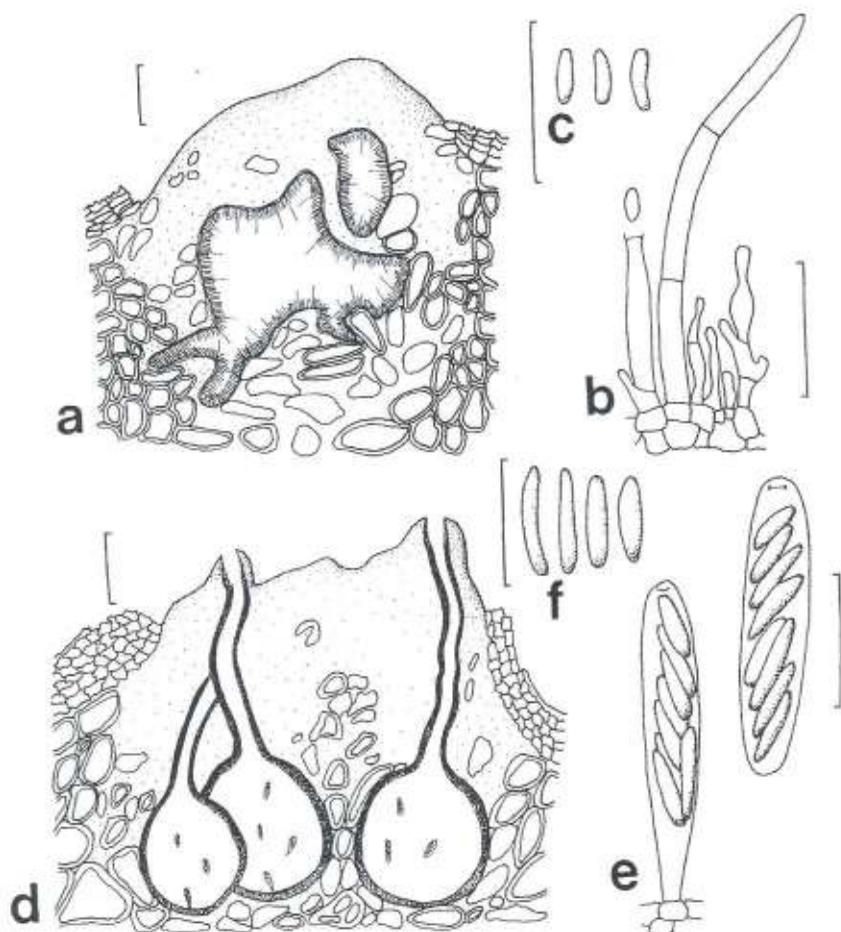


Fig. 3. - Anamorph and teleomorph states of *Cryphonectria eucalypti*. - a. Anamorphic stroma. - b. Conidiogenous cells and paraphysis. - c. Conidia. - d. Teleomorphic stroma. - e. Asci. - f. Ascospores. - Bars: a, d = 100  $\mu$ m; b, c, e, f = 10  $\mu$ m.

The pycnidial locules in the South African and Australian specimens were usually less than 10 in number per stroma, irregular and convoluted in shape (Fig. 3a, 4a). In contrast, each pycnidial stroma of the North American specimens had more than 30 discrete locules, and the locules were not convoluted (Fig. 2a). In most cases, the pycnidial locules in the South African specimens were large [(56-)104-368(-558)  $\mu$ m long, (50-)82-230(-350)  $\mu$ m wide] relative to the stromata, with some smaller locules also being present. The locules in the North American specimens [(47-)67-163(-279)  $\mu$ m long, (35-)51-107(-175)  $\mu$ m wide] were small relative to the stromata, and were dispersed throughout the stromata (Fig. 2a). Conidia in the

specimens from North America exuded from pycnidia in large orange (15) droplets becoming sienna (15i) in colour when older. Conidia of the South African and Australian specimens were exuded either as brilliantly orange (15) or brilliantly luteous (19) spore tendrils or in orange droplets.

The conidiogenous cells in the South African specimens were longer [(4-)8-17.5(-27)  $\mu\text{m}$  long, 0.5-1.5(-2)  $\mu\text{m}$  wide; mean length/width ratio 13:1] (Fig. 3b, 4b) than those of the North American specimens [(4-)5.5-9.5(-12)  $\mu\text{m}$  long, 1-1.5(-2)  $\mu\text{m}$  wide; mean length/width ratio 7.5:1]. Furthermore, hyphae longer than the conidiogenous cells occurred between the conidiogenous cells in the South African specimens (Fig. 3b, 4c), but not in the North American specimens. These long hyphae were never seen to produce conidia and were probably sterile. Such long hyphae were also noted by Walker & al. (1985) and have been reported in *C. parasitica* (Shear & al., 1917), although not in any other species of this group (Kobayashi, 1970; Roane, 1986a; Shear & al., 1917). These hyphae are similar to structures observed in *Phomopsis longiparaphysata* Uecker & Kuo that have been called paraphyses (Uecker & Kuo, 1992).

The perithecial bases in the South African and Australian specimens were generally dark with dark necks (Fig. 3d, 4d), while the bases of the young perithecia were usually light coloured. The perithecial bases in the North American specimens were not always darkened, or only had darkened areas around the bases of the necks (Fig. 2b), similar to young perithecia in the South African and Australian samples. The perithecial bases of the North American specimens were also situated in the stromatal tissue above the level of the bark surface (Fig. 2b). This is in contrast to those of the South African and Australian perithecia that generally occurred below the level of the bark surface, frequently surrounded with bark tissue, and not exclusively surrounded with fungal tissue (Fig. 3d, 4d). Furthermore, in the North American specimens, perithecia often occurred together with active pycnidial locules. Perithecia were seldom found together with pycnidial locules in the South African specimens. When they did occur together, the pycnidial locules were barren.

Lactophenol always became orange-coloured when mounting *E. gyrosa* specimens from North America in this mounting medium. In contrast, South African and Australian specimens did not discolour lactophenol. This discolouration of lactophenol was previously noted for *Endothia* and *Cryphonectria* and is due to the bisanthraquinone pigments, skyrin, oxyskyrin, skyrinol and regulosin, found in these genera (Roane & Stipes, 1978; Roane, 1986b).

## Growth in culture

Clear differences in colony morphology were observed between the South African, Australian and North American isolates of *E. gyrosa*. Colonies of isolates from North America were fuscous black (13''m) to buff (19''f) to cinnamon (15'') and had either a uniformly fluffy or sectored appearance with either smooth or crenate margins. Colonies of the South African and Australian isolates were white and fluffy, with the Australian isolates slightly different in texture with a denser mycelium. Colony margins were generally smooth. Similar differences were observed on both MEA and PDA. Although not produced during the growth study, South African and Australian isolates usually produce orange anamorph structures in culture, while North American isolates seldom produce anamorphic fruiting structures. *Endothia gyrosa* is also known to colour the growth medium purple due to the production of a compound known as endothine red, or to produce red crystals of this substance in the mycelium (Roane & Stipes, 1978; Micales & Stipes, 1986; Roane, 1986b). No such discoloration or crystals was observed for the Australian and South African isolates.

## Discussion

In this study we have shown conclusively that the fungus known as *E. gyrosa* from *Eucalyptus* in South Africa and Australia is different to *E. gyrosa* from North America. This is in terms of phylogenetic studies based on DNA sequences, morphology of structures on host tissue, culture morphology and the production of pigments. These morphological observations support previously presented DNA sequence and RFLP data showing that South African and Australian isolates are distinct from North American isolates (Venter & al., 2001).

We have confirmed that the South African and Australian fungus is more closely related to *C. parasitica* than to *E. gyrosa* as proposed by Venter & al. (2001). This was achieved by including additional species of *Endothia* and *Cryphonectria* in the phylogenetic comparison, and with sequence for an additional region of the genome. Data clearly show that *Endothia* and *Cryphonectria* form two distinct groups and that the fungus from *Eucalyptus* in Australia and South Africa, although grouping more distantly from other species of *Cryphonectria*, represents a unique species of *Cryphonectria*.

Morphological observations support DNA-based results showing that the South African and Australian fungus is a species of *Cryphonectria*. *Endothia* species have strongly developed, widely erumpent, sub-globose stromata consisting primarily of pseudoparenchyma and continuous entostroma and ectostroma. The perithecia

generally occur above the bark surface in a diatrypoid configuration. In contrast, *Cryphonectria* species, as well as the South African and Australian *Eucalyptus* fungus, have semi-immersed stromata consisting primarily of prosenchyma and with distinct ectostromatal and entostromatal discs. The perithecia occur underneath the bark surface and have a valsoid appearance near the periphery of the stroma, when the stroma is weakly developed. This supports previous observations where similar morphological distinctions were observed, which distinguished between *Endothia* and *Cryphonectria* (Micales & Stipes, 1987). The latter specimens also had stromata that were similar to those in specimens of *C. gyrosa*, which is the type species of *Cryphonectria*.

Different hosts, bark types and environments could influence the stromatal morphology of *Endothia* and *Cryphonectria*. More specifically, the number of layers of pycnidia and perithecia can be influenced by the bark of the host, while the sequence of formation of perithecia and pycnidia appears to be influenced by climatic factors (Shear & al., 1917). Moreover, the degree to which perithecia are positioned to assume a diatrypoid or valsoid configuration, appears to depend on the degree of stromatal development (Cannon, 1988). The size, structure and degree to which stromata are embedded, depends on the bark, host and external factors, such as moisture (Cannon, 1988; Fernández & Hanlin, 1996; Hodges & al., 1986; Kobayashi, 1970; Micales & Stipes, 1987; Roane, 1986a; Shear & al., 1917). This variability due to external factors and host, was the primary argument provided by Walker & al. (1985) to explain the differences between Australian and North American specimens. The magnitude of differences between these fungi, is, however, great and we are convinced that the differences are not due to host or environmental factors. The fact that molecular data support our morphological observations, further indicates that the South African and Australian fungus represents a distinct taxon.

Ascospores of the South African and Australian specimens are clearly non-septate and cylindrical to allantoid. This is typical of the genus *Endothia* (Roane, 1986a; Shear & al., 1917). However, stromatal morphology in this fungus closely resembles that of *Cryphonectria* as described by Micales & Stipes (1987) and is the distinguishing character that supports the phylogenetic distinction between the North American isolates and South African and Australian isolates. Stromatal structure is thus the more useful characteristic to separate the genera, being more conserved than ascospore morphology, which has been used in the past to differentiate between these two genera. Molecular studies done in parallel with morphological comparisons of available stromatal collections and herbarium specimens, should be carried out to redefine taxonomic relationships between these two genera.

The fungus from *Eucalyptus* in South Africa and Australia represents a new species that resides in *Cryphonectria*, and not in *Endothia*. The following description is thus provided for the new taxon.

*Cryphonectria eucalypti* M. Venter & M. J. Wingfield, sp. nov. – Figs. 3, 4.

Stromata in cortice semi-immersa, aliquando erumpentia vel omnino immersa, (202-)389-741(-940)  $\mu\text{m}$  alta, (186-)343-704(-868)  $\mu\text{m}$  lata, aurantiaca (15), parte superiore eustomatica, pseudoparenchymatica, parte inferiore pseudostromatica, in centro cum prosenchymate basibus peritheciarum vel loculis pycnidiorum in textura hospitis formato. Perithecia sub corticis superficie in basibus stromatorum inclusa (105-)161-245(-288)  $\mu\text{m}$  longa, (82-)157-291(-257)  $\mu\text{m}$  latae, parietibus fuscis, 1-12 in quoque stromate. Colla (65-)118-385(-630)  $\mu\text{m}$  longa,

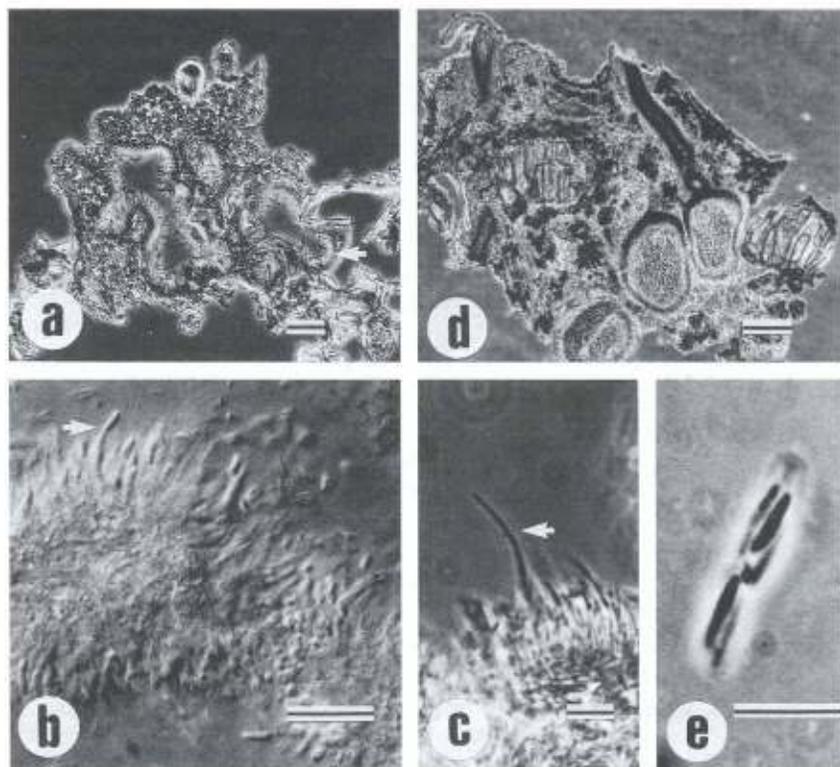


Fig. 4. – Light micrographs of the anamorph and teleomorph states of *Cryphonectria eucalypti*. – a. Anamorph stroma containing locules (indicated with arrow) lined with conidiogenous cells. – b. Conidiogenous cells producing conidia (indicated with arrow). c. – Paraphysis (indicated with arrow). d. – Teleomorphic stroma containing perithecia. – e. Ascus containing ascospores. – Bars: a, d = 100  $\mu\text{m}$ ; b, c, e = 10  $\mu\text{m}$ .

(31-)33-53(-65)  $\mu\text{m}$  lata, fusca, gracilia, periphysata, in base peritheci centralia, colla ad superficiem stromatis ut ostiola, papillae vel rostra longa cum textura stromatis aurantiaca tecta erumpentia, usque ad 170  $\mu\text{m}$  supra superficiem stromatis extensa. Asci (12-)17.5-26.5(-34)  $\mu\text{m}$  longi, 4.5-7(-9)  $\mu\text{m}$  lati, cylindrici vel fusiformes, diffluentes, dum immaturi stipitati, in quoqueasco 8 ascosporis. Ascosporae (4.5-)6-9(-12.5)  $\mu\text{m}$  longae, (0.5-)1-1.5(-2)  $\mu\text{m}$  latae, cylindricae vel fusiformes, allantoidae vel in uno extremitate sublaaxatae, hyalinae, non-septatae. Stromata multilocularia, usque 10 loculi pycnidiorum in quoque stromate, (56-)104-368(-558)  $\mu\text{m}$  longi, (50-)82-230(-350)  $\mu\text{m}$  lati, statura variabili forma et directione irregulari, saepe convoluti, non ostiolati. Parietes loculorum 1-3 stratis cellularum compositus, (5-)6-14(-20)  $\mu\text{m}$  crassus, intus cellulis conidiogenis vestitus. Cellulae conidiogenae (4-)8-17.5(-27)  $\mu\text{m}$  longae, 0.5-1.5(-2)  $\mu\text{m}$  latae, hyalinae, phialides, septatae, ramosae. Paraphyses (22-)28.5-50(-71.5)  $\mu\text{m}$  longae, 1-1.5  $\mu\text{m}$  latae, hyalinae, inter cellula conidiogena, septatae. Conidia (2.5-)3-4(-5)  $\mu\text{m}$  longae, 0.5-1  $\mu\text{m}$  latae, oblongo-cylindricae, allantoidae, oblongae vel ovoideae, hyalinae, amerosporae.

Holotypus. - AFRICA AUSTRALIS, Provincia "KwaZulu-Natal" septentrionalis, in regione "Nyalazi" dicta, in cortice *Eucalypti* clonis GC747, 25 Februarii 1998, M. Venter (PREM56211; cultura viva CMW7034).

Stromata on host in papulose areas either gregarious or individually, sometimes confluent; semi-immersed in bark, occasionally erumpent or totally immersed, (202-)389-741(-940)  $\mu\text{m}$  high, (186-)343-704(-868)  $\mu\text{m}$  wide (Figs. 3a, 3d, 4a, 4d), orange (15), upper region eustromatic, pseudoparenchymatous; lower region pseudostromatic with prosenchyma in centre, composed mainly of perithecial bases or pycnidial locules within host tissue. - Perithecia embedded beneath surface of bark at base of stromata, (105-)161-245(-288)  $\mu\text{m}$  long, (82-)157-291(-257)  $\mu\text{m}$  wide, globose to sub-globose to triangular, dark-walled, 1-12 per stroma (Figs. 3d, 4d), basal cells with *textura epidermoidea* in surface view, perithecial walls (8-)13-22(-31)  $\mu\text{m}$  diam. - Necks (65-)118-385(-630)  $\mu\text{m}$  long, (31-)33-53(-65)  $\mu\text{m}$  wide, length depending on depth of perithecium in stroma, slender, dark, periphysate, with a central position on base of perithecium; ostiolar canal 20-26(-28)  $\mu\text{m}$  wide, neck wall (10-)11-14(-15)  $\mu\text{m}$  diam., neck cells with *textura intricata* to *textura porrecta* in surface view, with *textura porrecta* at apex of neck, necks wind through stromatic tissue and emerge at stromatal surface as ostioles, papillae or long beaks covered with orange stromatal tissue, extending up to 170  $\mu\text{m}$  above stromatal surface. - Asci (12-)17.5-26.5(-34)  $\mu\text{m}$  long, 4.5-7(-9)  $\mu\text{m}$  wide, mean length/width ratio 4:1, cylindrical to fusiform, numerous, evanescent, floating freely in perithecial cavity, stipitate when immature, unitunicate with non-amyloid, refractive apical rings; asci with 8 ascospores (Fig. 3e, 4e). - Ascospores (4.5-)6-9(-12.5)  $\mu\text{m}$  long, (0.5-)1-1.5(-2)  $\mu\text{m}$  wide, mean ascospore length/width ratio 7.5:1, cylindrical to fusiform, allantoid or slightly wider at one end, ends rounded or slightly tapered, hyaline with greenish tint, aseptate (Figs. 3f, 4e).

Anamorphic stroma multilocular, less than 10 pycnidial locules per stroma, variable in size, (56-)104-368(-558)  $\mu\text{m}$  long, (50-)82-230(-350)  $\mu\text{m}$  wide, shape and orientation irregular, often convoluted, non-ostiolate (Fig. 3a, 4a). Cells in locule walls 1-3 layers thick, (5-)6-14(-20)  $\mu\text{m}$ , hyaline with *textura globulosa*. - Conidiogenous cells (4-)8-17.5(-27)  $\mu\text{m}$  long, 0.5-1.5(-2)  $\mu\text{m}$  wide, mean conidiogenous cell length/width ratio 13:1, cylindrical, usually slightly tapered towards apex, some with inflated bases, hyaline, phialidic, septate, branched (Fig. 3b, 4b). - Paraphyses amongst conidiogenous cells, (22-)28.5-50(-71.5)  $\mu\text{m}$  long, 1-1.5  $\mu\text{m}$  wide, cylindrical, slightly tapered towards apex, septate (Fig. 3b, 4c). - Conidia (2.5-)3-4(-5)  $\mu\text{m}$  long, 0.5-1  $\mu\text{m}$  wide, hyaline, mean length/width ratio 3.5:1, oblong-cylindrical, allantoid, or oblong to obovoid, hyaline, aseptate (Fig. 3c, 4b).

Cultures on MEA and PDA white, fluffy with a smooth margin, sometimes with straw yellow (21'd) patches, fast growing, covering a 90 mm plate after minimum of nine days, optimum temperature 25-30 °C.

Specimens examined. - Holotype. SOUTH AFRICA. Nyalazi: Northern KwaZulu/Natal, bark of GC747 clone of *Eucalyptus*, 25 February 1998, M. Venter (PREM56211; living culture CMW7034). - Paratypes. SOUTH AFRICA. Sabie: Mpumalanga, bark of *E. grandis*, August 1998, J. Roux (PREM56212); Tzaneen: Mpumalanga, bark of *E. saligna*, 6 February 1999, M. Venter (PREM56305; living culture CMW7035); Dukuduku: Northern KwaZulu/Natal, bark of *E. grandis*, October 1998, M. Venter (PREM56214); Amangwe: Northern KwaZulu/Natal, bark of *E. grandis*, October 1998, M. Venter (PREM56215; living culture CMW7033); Dukuduku: Northern KwaZulu/Natal, bark of *E. grandis*, October 1998, M. Venter (PREM56216).

Host. - *Eucalyptus* spp.

Etymology. - "eucalypti" refers to the occurrence of this fungus primarily on *Eucalyptus* spp.

Distribution. - South Africa, Tasmania and mainland Australia.

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