Molecular phylogeny of the *Ceratocystis moniliformis* complex and description of *C. tribiliformis* sp. nov.

Marelize van Wyk[1*], Jolanda Roux[1], Irene Barnes[2], Brenda D. Wingfield[2] and Michael J. Wingfield[1].

[1]Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, 0002
[2]Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, 0002

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Ceratocystis moniliformis is a colonist of fresh wounds on trees, mainly in the tropics. The fungus is not known to be a pathogen and has thus not been widely studied. *Ceratocystis moniliformis* has been reported on many taxonomically different plants and in many different climatic zones. It is thought to represent a complex of morphologically similar but physiologically and phylogenetically different species, some of which have recently been described. The aim of this study was to consider the phylogenetic relationship of *C. moniliformis* isolates from various hosts and origins, based on comparisons of DNA sequences of the Internal Transcribed Spacer (ITS) regions including the 5.8S gene of rDNA, β-tubulin and EF1-α genes. Four distinct clades were discernable in the phylogenetic tree. Two recently described species, *C. bhutanensis* and *C. moniliformopsis*, were confirmed to be phylogenetically distinct. *Ceratocystis moniliformis sensu stricto* isolated from Bhutan, Costa Rica, Ecuador and South Africa represented a well-resolved monophyletic group. A collection of isolates initially identified as *C. moniliformis*, from *Pinus merkusii* in Sumatra, Indonesia was found to reside in a distinct clade. *Ceratocystis tribiliformis* sp. nov. is described here to accommodate this group of isolates. Morphologically, *C. tribiliformis* differs from *C. moniliformis* in having obpyriform to globose ascomata and both smooth and granular hyphae. *Ceratocystis tribiliformis* also has distinct optimal growth at 20-25°C on malt extract agar.

Key words: DNA sequences, insect/fungus interactions, phylogenetics, phylogeography, taxonomy, *Thielaviopsis*

Introduction

Ceratocystis moniliformis Hedgcock is a cosmopolitan fungus that has been reported from many hosts and continents (Davidson, 1935; Bakshi, 1951; Hunt, 1956; Upadhyay, 1981) (Table 1). The fungus was first isolated from

*Corresponding author: Marelize van Wyk; e-mail: marelize.vanwyk@fabi.up.ac.za

Table 1. Distribution and host range of *Ceratocystis moniliformis* causing staining.

Host	Geographical distribution	Reference
Macaranga capensis	South Africa	Van Wyk *et al.*, 1991
Theobroma sp.	France	Paulin-Mahady *et al.*, 2002
Quercus robur	Poland	Kowalski and Butin, 1989
Liquidamber styraciflua	Texas, USA	Von Schrenk, 1903
Pinus ponderosa	Texas, USA	Hedgcock, 1906
Pinus palustris, *P. echinata*, *P. taeda*, *L. styraciflua*, *Liriodendron tulipifera*, *Nyssa aquatica*, *Fagus grandifolia*, *Magnolia* sp., *Quercus* sp.	USA	Davidson, 1935
F. crenata, *Q. glandulifera*, *M. hyplleuca*, *Kalopanax ricinifolius*, *Ptercarya rhoifolia*, *Cercidiphyllum japanicum*	Tokyo	Kitajima, 1936
Quercus sp.	Scotland	Bakshi, 1951
Pycnanthus kombo	Cameroon	Luc, 1952
Theobromae sp.	Madagascar	Luc, 1952
Calamus maximus, *Endospermum peltatum*, *Parkia javanica*	Philippines	Roldan, 1962
Hevea sp.	China	Witthuhn *et al.*, 1999
Erythrina sp.	South Africa	Witthuhn *et al.*, 1999

gumwood (*Liquidambar styraciflua* L.) in Texas (Von Schrenk, 1903) and was initially described as *Ceratostomella moniliformis* (Hedgcock, 1906). Moreau transferred the species to *Ceratocystis* in 1952 (Moreau, 1952).

Ceratocystis moniliformis is one of the six species belonging to this genus that has hat-shaped ascospores. It can, however, easily be distinguished from the other *Ceratocystis* spp. based on the presence of well-developed conical spines that cover the ascomatal bases (Hedgcock, 1906; Luc, 1952; Hunt, 1956; Upadhyay, 1981). Another distinguishing characteristic is the disc-formed bases of the ascomatal necks (Bakshi, 1951; Hunt, 1956). Two conidial forms; one cylindrical in shape and the other more barrel-shaped, are found in the *Thielaviopsis* Went anamorph of *C. moniliformis*. *Ceratocystis*

moniliformis is also one of the few *Ceratocystis* spp. not known to produce chlamydospores (Davidson, 1935; Paulin-Mahady *et al.*, 2002).

Descriptions for *C. moniliformis* have tended to be somewhat disparate. Some authors for example, failed to note the conical spines on the ascomatal bases (Kitajima, 1936; Luc, 1952; Roldan, 1962) but rather referred to the structures as hyphal ornamentation. The ascomatal bases have been described variably as globose (Hedgcock, 1906), elongate to pear-shaped (Davidson, 1935) and spherical or elongated (Luc, 1952). Dimensions for neck length and ascomatal base widths have also varied (Hedgcock, 1906; Kitajima, 1936; Bakshi, 1951; Hunt, 1956; Upadhyay, 1981) (Table 2). Likewise, there has been little agreement regarding the morphology of the conidiophores in various descriptions (Davidson, 1935; Kitajima, 1936; Bakshi, 1951; Hunt, 1956; Upadhyay, 1981) (Table 3).

Ceratocystis moniliformis commonly infects wounds on woody plants and especially trees (Grylls and Seifert, 1993; Kile, 1993). It is not considered to be a pathogen as it causes only sap stain. There is also no evidence of *C. moniliformis* imparting structural changes to infected wood, but discolouration can lead to a reduction in timber value (Davidson, 1935).

The wide host range and geographic distribution of *C. moniliformis* has led us to question whether this fungus might represent a species complex. This would be similar to the view that has emerged for the important tree pathogen, *C. fimbriata* Ell. & Halst. (Barnes, 2001a; Baker *et al.*, 2003; Van Wyk *et al.*, 2004b). The recent description of *C. moniliformopsis* Yuan & Mohammed (Yuan and Mohammed, 2002), a species morphologically very similar to *C. moniliformis*, provides the first support for the view that various species might have been aggregated with *C. moniliformis*. Recent phylogenetic studies have thus led us to describe a new species, *C. bhutanensis* M. van Wyk, M.J. Wingf. & T. Kirisits (Van Wyk *et al.*, 2004a) that is morphologically very similar to *C. moniliformis*.

The aim of this study was to compare a collection of *C. moniliformis sensu lato* isolates based on phylogenetic analysis of multiple gene sequences. Morphology and physiological growth at different temperatures of these *Ceratocystis* isolates were also studied.

Materials and methods

Isolates

Isolates collected for this study were identified as *C. moniliformis* based on morphological characteristics such as the presence of spines on the

Table 2. Morphology of the teleomorph of *Ceratocystis moniliformis* as described by various authors[a].

References/ Character	Hedgcock 1906	Davidson 1935	Kitajima 1936	Bakshi 1951	Luc 1952[b]	Luc 1952[b]	Hunt 1956	Roldan 1962	Upadhyay 1981
Ascomatal base									
Colour	Brown/ black	Black	-	Transparent green tinge	Brown/ Black	Dark brown	Black	Black	Brown/ black
Diameter	90-180	160-235	104 -244	190-245	210-270	135-190	120 -160	150-250	90-210
Ornamentation	Conical spines (sparse), 12-16 × 6 (base)	Brown bristles, 18-60 × 3-4	Brown bristles (numerous), 85-124 long	2 types: (1) 65 × 1µm (2) 11-36 × 7-15 (Base) 2-3 (Tip)	Dark brown conical spines, 7-20 × 6-12 (Base)	Elongated, Setae, straight, 12-57 × 6-11 (Base), 2-4 (Tip)	Brown, short, conical spines, 30 × 8	Dark, hyaline tip, 10-65 × 2-3	Brown conical spines, 12-45 × 3-8 (Base)
Shape	Globose	Elongate/ pear-shaped	Flask-shaped/ spherical	Round/ elongate	Sub-spherical/ Oval	Spherical/ Elongated	Globose/ pear shaped	Globose/ Pear shaped	Globose/ Pear shaped
Ascomatal neck									
Colour	-	Black	Black	Black	-	-	Black	Black	Black
Length	-	550-1000	305-609	731-896	600-900	500-700	900	920	550 -1000
Width: base	-	30-36	-	39-52	29-42	21-30	20-30	20-45	20-35
Width: tip	-	14-15	-	14	16-19	10-13	10-15	10-14	11-15

Table 2 continued. Morphology of the teleomorph of *Ceratocystis moniliformis* as described by various authors[a].

References/ Character	Hedgcock 1906	Davidson 1935	Kitajima 1936	Bakshi 1951	Luc 1952[b]	Luc 1952[b]	Hunt 1956	Roldan 1962	Upadhyay 1981
Ostiolar hyphae	Brown-black, short, thick, 12-18 × 2	8-12 hyphae, hyaline, filaments 15-25	7-12 hyphae, hyaline, filaments 11-63	2-14 hyphae, hyaline, 34-41 × 2-3 (Base)	8-15 hyphae, hyaline setae, 19-46 × 2	7-12 hyphae, 12-21	8-10 hyphae, hyaline, 25 × 2	10-16 hyphae, hyaline, bent, 10-30 × 2	1-25 hyphae, hyaline, divergent, 2-3
Ascus									
Colour	Hyaline	-	-	-	Barely visible	Poorly visible	Not seen	Evanescent	Not seen
Ascospores									
Colour	Hyaline	-	Hyaline	Hyaline	Hyaline/ yellowish	Hyaline/ yellowish	-	-	Hyaline
Shape: side view	Oval, one side flat	Broad ovoid (hat)	Kidney Shaped	Oval, brim (hat)	Oval, Flattened	Oval, Flattened	Hat-shaped	Hat-shaped	Oblong reniform
Length	4 -5	4-5	2-4	6-8	4-6	4-5	3-6	4-6	-
Width	3-4	2-3	4-5	3-4	3-5	3-5	2 - 3	3 - 4	-
Texture	Long, slimy, grey mass	Gelatinous sheath	Mucilaginous substance	Oval globule, mucilaginous	Pinkish yellow in mass	Pinkish yellow in mass	Gelatinous sheath	Gelatinous sheath	Gelatinous sheath

[a]All measurements are presented in microns (μ).
[b]Luc described two forms of *C. moniliformis; C. pycnanthi* (left column) and *C. theobromae* (right column).

Table 3. Morphology of the anamorph state of *Ceratocystis moniliformis* as described by various authors[a].

Character	Hedgcock 1906	Davidson 1935	Kitajima 1936	Bakshi 1951	Luc 1952 [b]	Luc 1952 [b]	Hunt 1956	Roldan 1962	Upadhyay 1981
Conidiophores									
Length	-	(1) 3 (2) 5-6	(1) 28-30 (2) 63-70	7-14	18-52	33-65	60 35	100	18-77
Width	-	-	(1) 5 (2) 7-8	(1) 4-9 (2) 5	7-8	4-5	(1) 4-6 (2) 6	4-6	2-3
Conidia									
Shape	Cylindrical	(1) Cylindrical (2) Barrel	(1) Cylindrical (2) Barrel	Cylindrical	Cylindrical to barrel	Cylindrical	(1) Cylindrical (2) Barrel	Cylindrical	(1) Cylindrical (2) Shorter
Length	6-8	(1) 6-10 (2) 5-7	(1) 7-8 (2) 8-14	4-16	5-8	6-20	(1) 6-19 (2) 6-9	6-20	(1) 16-20 (2) 6-9
Width	2	(1) 3 (2) 5-6	(1) 1-2 (2) 7-10	1-2	2-4	2-4	(1) 2-3 (2) 4-6	2-4	(1) 2-3 (2) 4-6
Culture									
Growth rate	-	38 mm in 5 days	-	60 mm in 10 days	-	-	45 mm in 5 days	25 mm in 5 days	70 mm in 12 days
Colour	Hyaline / grey /black	Hyaline / light brown	Hyaline / light brown	Hyaline / light brown	Hyaline / brown	-	White / grey / brown	White / brown	Hyaline / pale brown
Odour	-	Banana oil	-	Pear-drops	-	Ethyl acetate	Banana oil	Banana oil	Banana oil
Mycelium width	2-8	-	2-3	2-5	3-7	2-4	2-8	2-4	2-8

[a]All measurements are presented in microns (µ).
[b]Luc described two forms of *C. moniliformis; C. pycnanthi* (left column) and *C. theobromae* (right column).

ascomatal bases. Isolates were collected over a seven-year period from various tree species in South Africa, Bhutan, Ecuador, Costa Rica and Sumatra (Indonesia) (Table 4). The closely related species, *C. moniliformopsis* and *C. bhutanensis*, originally thought to represent *C. moniliformis* due to their morphological similarities were also included (Table 4). All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative isolates have also been deposited with the Centraalbureau voor Schimmelcutures (CBS), Utrecht, The Netherlands. Holotype material of the *Ceratocystis* sp. from Sumatra, including dried cultures on malt extract agar (MEA) has been deposited in the National Fungal Herbarium, Pretoria, South Africa (PREM) (Table 4).

DNA extraction

Cultures for DNA extraction were grown on 2% MEA, (Biolab, Midrand, South Africa) at 25 °C for two weeks. Masses of fungal mycelium and ascomata were scraped directly from the actively growing cultures, transferred to Eppendorf tubes and lyophilised for two days. The lyophilised mycelium was placed in liquid nitrogen, ground to a fine powder and DNA was extracted using the method described by Barnes *et al.* (2001b).

PCR amplification

Three gene regions were amplified using the Polymerase Chain Reaction (PCR). The Internal Transcribed Spacer regions (ITS1 and ITS2) and the 5.8S gene of the ribosomal DNA (rDNA) operon were amplified using the primers ITS1 and ITS4 (White *et al.*, 1990). A region of the β-tubulin gene was amplified using the primers βt1a and βt1b (Glass and Donaldson, 1995) and a portion of the EF1-α gene was amplified using the primers EF1-728F and EF1-986R (Carbone and Kohn, 1999).

PCR reaction mixtures, for all three gene regions, consisted of 200 ηM of the forward and reverse primers, 200 μM of each dNTP, Expand High Fidelity PCR System enzyme mix (1.75 U) (Roche Diagnostics, Mannheim, Germany), 1 × Expand HF Buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme) and 2-10 ng DNA. Reaction volumes were adjusted to 25 μL with sterile water. The PCR programme was set at 96°C for 2 min for DNA denaturation, followed by 10 cycles at 94°C for 20 s, 55°C for 40 s for annealing and 72°C for 45 s for elongation. A further 30 cycles at 94°C for 20 s, 55°C for 40 s

Table 4. *Ceratocystis* isolates used in this study[a].

Fungus	Isolate no.[b]	Alternative numbers	GenBank accession nr.	Date of isolation	Host	Geographical origin	Associated insect	Collector(s)
C. moniliformis	CMW 13011[#]	PREM 57825	AY528991 AY529001 AY529012	1996	*Pinus merkusii*	Sumatra, Indonesia	None	M.J. Wingfield
"	CMW 13012[#*]	PREM 57826	AY528992 AY529002 AY529013	"	"	"	"	"
"	CMW 13013[#*+]	PREM 57827	AY528993 AY529003 AY529014	"	"	"	"	"
"	CMW 13015[#*]	PREM 57828	AY528994 AY529004 AY529015	"	"	"	"	"
"	CMW 9590[#+]		AY528985 AY528996 AY529006	2002	*Eucalyptus grandis*	Mpumalanga, South Africa	"	J. Roux
"	CMW 10134[#+]		N/A	"	"	"	"	M. van Wyk
"	CMW 4114[#]		AY528986 AY528997 AY529007	1997	*Schizolobium parahybum*	Ecuador, South America	"	M. J. Wingfield

[a] Isolates marked with # were sequenced in this study, those followed by * were used for morphological descriptions and those marked with + were included in the growth studies.

[b] CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. CBS to the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, and PREM to the National Fungal Herbarium (PREM), Pretoria, South Africa.

Table 4 continued. *Ceratocystis* isolates used in this study.

Fungus	Isolate no.[b]	Alternative numbers	GenBank accession nr.	Date of isolation	Host	Geographical origin	Associated insect	Collector(s)
C. moniliformis	CMW 9990[#]	CBS 155.62	N/A	1962	*Theobroma cacao*	Costa Rica	"	A. J. Hansen
"	CMW 8379[#]		AY528995 AY529005 AY529016	2001	*Cassia fistula*	Punaka, Bhutan	"	M.J. Wingfield, T. Kirisits & D.B. Chhetri
"	CMW 8240[#]		AY528989 AY529000 AY529010	"	"	Wangdi, Bhutan	"	"
"	CMW 8238[+]		N/A	"	"	"	"	"
C. bhutanensis	CMW 8242	CBS 112907 PREM 57809	AY528956 AY528961 AY528951	"	"	"	*Ips schmutzenhoferi*	"
"	CMW 8217	CBS 114289 PREM 57807	AY528957 AY528962 AY528952	"	"	"	"	"
"	CMW 8244	CBS 114287 PREM 57811	N/A	"	"	"	"	"
"	CMW 8241	CBS 115773	N/A	"	"	"	"	"
C. moniliformopsis	CMW 9986[#+]	CBS 109441	AY528987 AY528998 AY529008	1999	*Eucalyptus obliqua*	Tasmania, Australia	None	Z.Q. Yuan

Table 4 continued. *Ceratocystis* isolates used in this study.

Fungus	Isolate no.[b]	Alternative numbers	GenBank accession nr.	Date of isolation	Host	Geographical origin	Associated insect	Collector(s)
C. moniliformopsis	CMW 10215[a]	CBS 115793	N/A	1999	*Eucalyptus obliqua*	Tasmania, Australia	None	Z.Q. Yuan
C. virescens	CMW 3276[a]		AY528984 AY528990 AY529011	1963	*Quercus*	Warrenber, U.S.A.	"	T. Hinds

with a 5 s extension step, after each cycle was included, followed by 72°C for 45 s. A final step of 10 min at 72 °C completed the programme. Amplification of the respective genes was confirmed under UV illumination using 2% agarose (Roche diagnostics, Mannheim, Germany) gel electrophoresis in the presence of ethidium bromide. After amplification, amplicons were purified using Sephadex G-50 columns (1 g in 15 ml H_2O, SIGMA, Steinheim, Germany).

Sequencing and analysis

PCR amplicons were sequenced in both directions using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California). The same primers as those used in the PCR reactions were used for sequencing. Sequencing reactions were run on an ABI PRISM™ 3100 Autosequencer (Applied BioSystems, Foster City, California, USA) and sequences were analysed using Sequence Navigator version 1.0.1 (Applied BioSystems, Foster City, California).

Sequences were manually aligned and analysed using PAUP version 4.0b10* (Swofford, 2002). A partition homogeneity test (Swofford, 2002) was used to determine whether the sequence data sets for the three different gene regions could be combined. Gaps were treated as "newstate" and trees were obtained via stepwise addition of 1,000 replicates with the Mulpar option effective. The heuristic search option based on parsimony with tree bisection reconnection (TBR) was used to obtain the phylogram. Confidence intervals using 1000 bootstrap replicates were calculated. *Ceratocystis virescens* (Davidson) Moreau was designated as the out-group taxon. All sequences derived from this study have been deposited in GenBank (Table 4).

The Markov Chain Monte Carlo (MCMC) method (Larget and Simon, 1999), with a Bayesian framework was used to estimate the posterior probability of nodes in the phylogenetic tree. One hundred thousand random trees were generated using the MCMC procedure, sampling every 100^{th} tree and saving every 10^{th} tree. To avoid including trees sampled before convergence of the Markov chain, the first 4,700 trees were discarded. For the combined dataset of the three gene sequences, gamma rate heterogeneity was set, and no codon specific sites were included for the ITS gene. For β-tubulin and EF1-α sequences, codon specific sites were specified with a site-specific substitution rate and the site partition was treated as a by-codon.

Cultural characteristics

Growth of three selected isolates for each species was compared on MEA at different temperatures (Table 5). Inocula for the growth study were prepared by growing the fungi on 2% MEA. After a two-week incubation period at 25°C, mycelial plugs were taken from the margins of the actively growing cultures using a 5 mm diameter cork borer, and transferred to the centres of 90 mm Petri dishes containing 2% MEA. Five plates of each isolate were incubated at 4°C, 10°C, 15°C, 20°C, 25°C, 30°C and 35°C respectively. Growth was assessed by taking two measurements of the colony diameter at right angles to each other, every day for 3 days. Averages and standard deviations were computed for all growth measurements. The entire experiment was repeated once. Colony colour of the isolates was determined using the colour tables of Rayner (1970) and colony textures were noted.

Morphology

Morphological characteristics of the isolates from Sumatra (Indonesia) were described from 10-day-old cultures on 2% MEA. Sexual and asexual states of the fungus were mounted in lactophenol on glass slides and examined under a Zeiss light microscope. Fifty measurements were taken for each taxonomically relevant structure of isolate CMW 13013, which was chosen to represent the group of isolates from Sumatra. In addition, ten measurements were made for *Ceratocystis moniliformis sensu stricto*, *C. bhutanensis* and *C. moniliformopsis*.

Results

Phylogenetic analysis

PCR amplification of the ITS regions (including the 5.8S gene) and β-tubulin gene resulted in fragments of ~500 base pairs (bp) while the amplification of the EF1-α gene resulted in fragments of ~300 bp. All three-gene regions were successfully amplified for all isolates.

The partition homogeneity test showed that the sequence data sets for β-tubulin, ITS and EF1-α could be combined ($P = 0.05$). The combined data set resulted in 1 396 characters, of which 873 characters were constant, 353 were parsimony-uninformative and 170 were parsimony informative. Analysis of the data set resulted in one most parsimonious tree (Fig. 1), with a consistency

index (CI) of 0.941, homoplasy index (HI) of 0.059, retention index (RI) of 0.889 and a rescaled consistency index (RC) of 0.837.

Four distinct clades, representing four different phylogenetic species, were observed in the combined analysis, each with a strong bootstrap support (77-100%) (Fig. 1). The *C. moniliformis* group was separated into two distinct clades. One clade included only isolates from Sumatra, which were from cut pine timber, while the second clade represented all the other *C. moniliformis* isolates from Bhutan, South Africa, Ecuador and Costa Rica. The other two clades represented the two closely related but distinct species, *C. moniliformopis* and *C. bhutanensis*. The posterior probability of the branch nodes of the combined tree, generated using Bayesian inference, supported the bootstrap values (Fig. 1).

Cultural characteristics

There were distinct differences in the growth and culture morphology for the *C. moniliformis* isolates studied. No growth was observed for the isolates from Sumatra at 4°C, 10°C, 15°C, 30°C or at 35°C and they grew only at 20°C and 25°C. The isolates from Sumatra grew more slowly than the *C. moniliformis* isolates from South Africa, Bhutan, Ecuador and Costa Rica. The *C. moniliformis* isolates grew at 15°C and above but not at 35°C and they grew rapidly, reaching the edges of the Petri dishes (90 mm) in 72 hours at 30°C (Table 5). Isolates of *C. moniliformopsis* grew slowly compared to those of *C. moniliformis*. They displayed optimal growth between 15°C and 20°C and did not grow at temperatures above 20°C (Table 5). Isolates of *C. bhutanensis* grew well at most temperatures and had an optimum growth between 20°C to 25°C.

Ceratocystis moniliformopsis cultures had buff-yellow (19d) aerial mycelium and Isabella colour (19"i) submerged mycelium, older cultures have fawn (13"") aerial and submerged mycelium, with few observed ascomata. The submerged mycelium of *C. bhutanensis* was umber (15 m) with the well-developed aerial mycelium being ecru-drab (13""""d). The *C. moniliformis* isolates could be separated into two groups that were consistent with those resulting from the phylogenetic comparisons. Isolates from Sumatra differed from others in having virtually no aerial mycelium and they had masses of ascomata covering the plates. The remaining *C. moniliformis* isolates had abundant buff-yellow (19d) aerial mycelium with ascomata tending to occur below the mycelium, consistent with the description of this species (Hedgcock, 1906; Upadhyay, 1981).

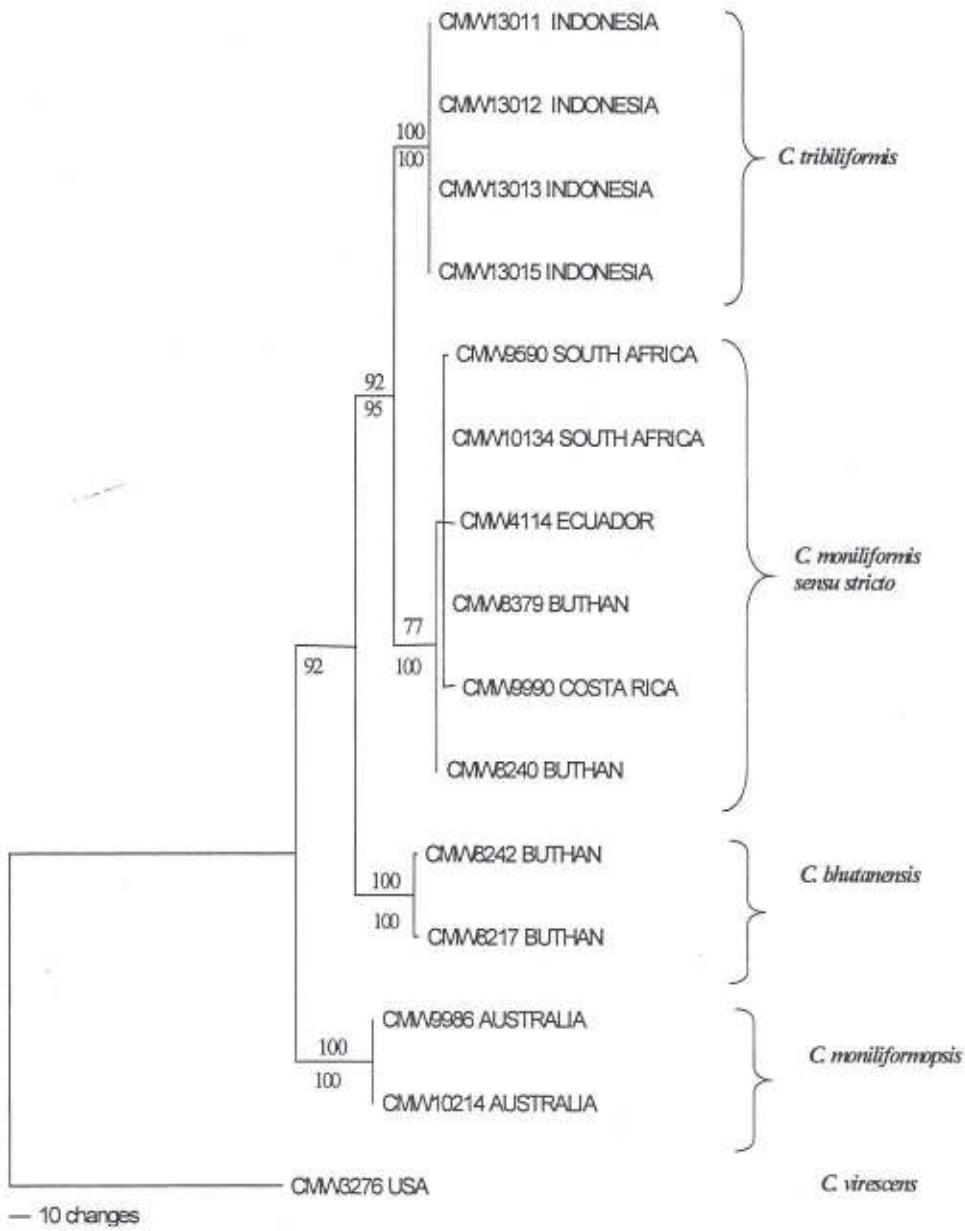

Fig. 1. A phylogenetic tree based on the combined sequence data from three gene regions; ITS, β-tubulin and EF1-α, showing the monophyletic nature of *C. moniliformis sensu lato*. The phylogram was obtained using the heuristic search option based on parsimony. Bootstrap values are indicated above the branches and Bayesian values are indicated below the branches. *Ceratocystis virescens* was treated as the out-group.

Table 5. Comparison of growth of *Ceratocystis moniliformis sensu stricto*, *C. bhutanensis*, *C. moniliformopsis* and isolates from Sumatra[ab].

Species	Isolate number	Growth (mm) at different temperatures (°C)						
		4	10	15	20	25	30	35
C. moniliformis sensu stricto	CMW 9590	0.0	11.3(3.2)	11.3(3.0)	64.0(1.9)	72.3(0.8)	90.0(0.0)	0.0
	CMW 8238	0.0	0.0	0.0	51.3(2.4)	66.9(1.4)	62.6(2.9)	0.0
	CMW 10134	0.0	0.0	0.0	19.0(4.0)	17.9(5.0)	14.0(1.4)	0.0
	CMW 13014	0.0	0.0	0.0	52.8(3.0)	37.9(2.1)	0.0	0.0
Isolates from Sumatra	CMW 13013	0.0	0.0	0.0	45.1(1.9)	37.7(2.7)	0.0	0.0
	CMW 13015	0.0	0.0	0.0	45.0(1.7)	37.5(2.4)	0.0	0.0
	CMW 8217	16.8(1.3)	17.6(0.5)	30.1(0.5)	60.0(3.9)	60.0(3.7)	11.8(2.0)	0.0
	CMW 8244	18.6(1.1)	16.8(0.6)	31.6(0.6)	64.6(7.3)	51.0(3.2)	0.0	0.0
C. bhutanensis	CMW 8241	16.3(0.6)	13.9(0.8)	28.3(0.8)	70.0(5.0)	44.1(4.1)	0.0	0.0
	CMW 9986	0.0	0.0	18.5(1.8)	36.6(1.1)	0.0	0.0	0.0
	CMW 10214	0.0	0.0	16.2(6.6)	50.1(0.7)	0.0	0.0	0.0
C. moniliformopsis	CMW 10215	0.0	0.0	12.6(1.2)	27.7(5.8)	0.0	0.0	0.0

[a]/Growth assessed as average colony diameter for five cultures per isolate on 2% MEA after 3 d.
[b]/Standard deviations are presented in parentheses.

Morphology

The ascomatal bases for the Sumatra isolates were black and obpyriform (Fig. 2.1) and the ostiolar hyphae were divergent (Fig. 2.2). The bases of the necks at the points of attachment resembled those of *C. moniliformis* (Fig. 2.3). The ascomatal bases also resembled those of typical *C. moniliformis*, which were covered in short conical spines (Fig. 2.4). Masses of hat-shaped

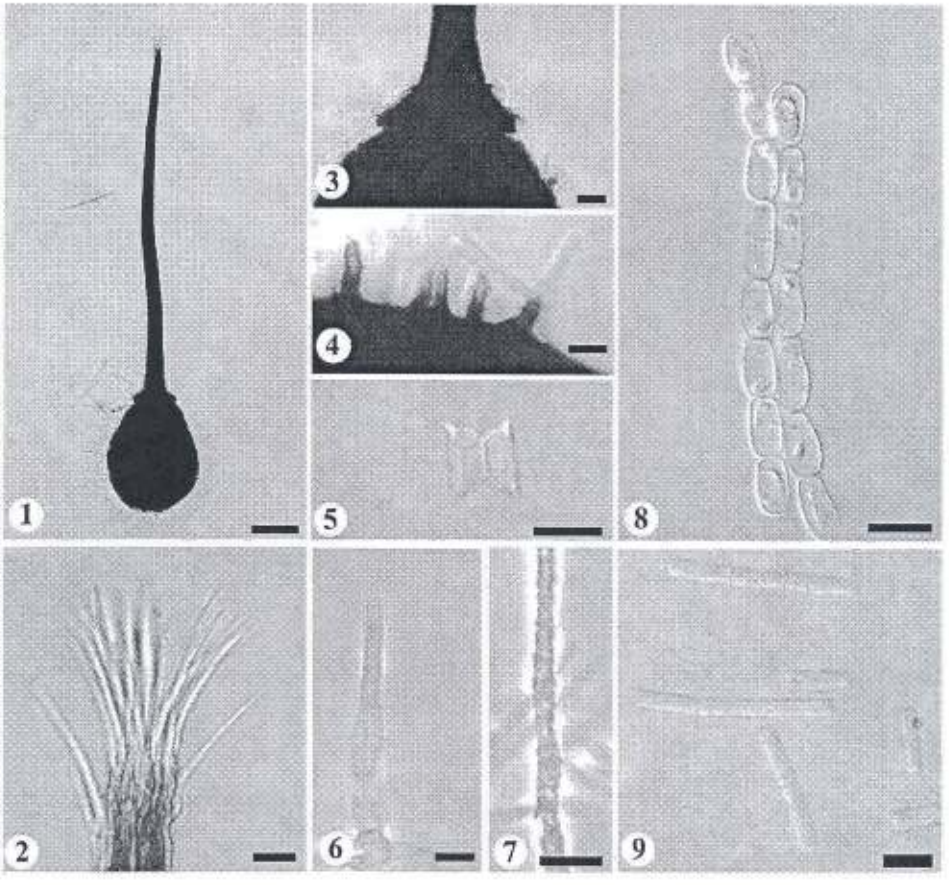

Fig. 3. *Ceratocystis tribiliformis* (from holotype) (CMW 13015). **1.** Obpyriform ascoma. **2.** Divergent ostiolar hyphae. **3.** Ascomatal neck disc-shaped at base. **4.** Ascomatal base with short, conical spines and hyphal ornamentation. **5.** Hat-shaped ascospore in side view, oval in face view. **6.** Phialidic conidiogenous cell. **7.** Hyphae with rough edges. **8.** Barrel-shaped conidia. **9.** Cylindrical conidia. Bars: 1 = 100 µm; 2 = 10 µm; 3 = 10 µm; 4 = 10 µm; 5 = 10 µm; 6 = 5 µm; 7 = 5 µm; 8 = 10 µm; 9 = 10 µm.

ascospores were observed in both groups of isolates (Fig. 2.5). The anamorph was typical of *Thielaviopsis*, with phialidic conidiogenous cells (Fig. 2.6). Both smooth-walled hyphae and those having a granular appearance were observed (Fig. 2.7) and both barrel-shaped (Fig. 2.8) and cylindrical conidia were present (Fig. 2.9).

Taxonomy

DNA sequence analyses, growth comparisons, cultural characteristics and fruit-body morphology provided support for the view that the isolates thought to be *C. moniliformis* from Sumatra, represent a previously undescribed species of *Ceratocystis*. The fungus is therefore described as follows:

Ceratocystis tribiliformis M. van Wyk & M.J. Wingf., **sp. nov.** (Fig. 2)

Etymology: Shape of the ascomata similar to the fruit of the plant *Tribulus terrestris* L., known in the Afrikaans language as the "dubbeltjie".

Coloniae albae. *Mycelium* rarum, praecipue in medio immersum. *Temperatura faustissima* 20-25°C, supra 30°C. *Hyphae* laeves vel granulatae, in septis non-constrictae, 2-4 µm latae. *Bases ascomatum* atrobrunneae vel nigrae, obpyriformes, spinis hyphibusque ornatae, spinis atrobrunneis vel nigris, 4-12 µm ($\overline{x}$ = 6-10 µm), bases 196-264 µm ($\overline{x}$ = 203-249 µm) diametro. *Colla ascomatum* atrobrunnea vel nigra, 741-1047 × 43-53 µm ($\overline{x}$ = 782-986 × 44-50 µm basi discoidea) 741-1047 × 13-20 ($\overline{x}$ = 782-986 × 14-18 µm apice). *Hyphae ostiolares* divergentes, hyalinae, 22-32 µm ($\overline{x}$ = 25-31 µm) longae. *Asci* non visi. *Ascosporae* lateraliter visae cucullatae, aseptatae, hyalinae, in vagina investitae, cum vagina 5-6 × 2-3 µm, sine illa 4-5 × 2-3 µm. Ascosporae in massis bubalino-luteis mucilaginis in apicibus collorum ascomatum convenientes. *Anamorpha Thielaviopsis*: conidiophora singula in mycelio crescentia, apicem versus angustata, 21-46 × 3-4 µm ($\overline{x}$ = 22-40 µm × 3-4 µm) (longa, basi), 21-46 × 1-3 µm ($\overline{x}$ = 22-40 µm × 1-3 µm) (apice). Evolutio *conidii* phialidici per parietes annulares faciendas, *conidia* biformiia: conidia primaria hyalina, aseptata, cylindrica 7-9 × 2 µm, conidia secondaria hyalina, aseptata, doliiformia, 7-9 × 3-4 µm.

Colony white on malt extract agar. *Mycelium* sparse mostly submerged in medium. *Optimal temperature* range for growth 20-25°C, no growth above 30°C. *Hyphae* smooth or granulated, not constricted at septa, 2-4 µm wide. *Ascomatal bases* dark brown to black, globose to obpyriform, ornamented with spines and hyphae, spines dark brown to black, 4-12 µm ($\overline{x}$ = 6-10 µm) long, bases 196-264 µm ($\overline{x}$ = 203-249 µm) in diam. *Ascomatal necks* dark brown to black, 741-1047 × 43-53 µm ($\overline{x}$ = 782-986 × 44-50 µm wide at base) 741-1047 × 13-20 ($\overline{x}$ = 782-986 × 14-18 µm wide at the apex), with a disc-like base. *Ostiolar hyphae* divergent, hyaline, 22-32 µm ($\overline{x}$ = 25-31 µm) long. *Asci* not observed. *Ascospores* hat-shaped in side view, aseptate, hyaline, invested in sheath, 5-6 × 2-3 µm with sheath, 4-5 × 2-3 µm without sheath. Ascospores accumulating in buff-yellow (19d) mucilaginous masses on the apices of

ascomatal necks. *Thielaviopsis anamorph*: conidiophores occurring singly on mycelium, hyaline, swollen at the base, tapering towards the apex, 21-46 × 3-4 µm ($\bar{x}$ = 22-40 µm × 3-4 µm) (at base), 21-46 × 1-3 µm ($\bar{x}$ = 22-40 µm × 1-3 µm) (at the apices). *Conidium development* through ring wall building, *conidia* of two types: primary conidia hyaline, aseptate, cylindrical 7-9 × 2 µm, secondary conidia hyaline, aseptate, barrel-shaped 7-9 × 3-4 µm.

Habitat: Wood of *Pinus merkusii*.

Known distribution: Indonesia, Sumatra.

Material examined: Indonesia: Sumatra isolated from wood of *Pinus merkusii*, 1996, M.J. Wingfield (PREM 57827 - holotypus, living culture: CMW 13013).

Additional specimens examined (paratypes): Indonesia: Sumatra, isolated from wood of *Pinus merkusii*, 1996, M.J. Wingfield (PREM 57825, living culture CMW 13011); same collection data (PREM 57826, living culture CMW 13012/CBS 118242); same collection data (PREM 57828, living culture CMW 13015/CBS 115949).

Discussion

In this study, we considered the taxonomy and relationships of a collection of *Ceratocystis moniliformis sensu lato* isolates from a wide range of hosts and geographic areas. These included isolates from Sumatra tentatively identified as *C. moniliformis* and the two closely related species, *C. bhutanensis* and *C. moniliformopsis*. Comparisons were based on DNA sequences, morphology and growth characteristics in culture. Our results showed that the isolates identified as *C. moniliformis* represent two discrete phylogenetic lineages. One of the clades, consisting of isolates from a wide geographic and host range represents *C. moniliformis sensu stricto*. The second group of isolates from Sumatra represents an undescribed species, described here as *C. tribiliformis*. Data emerging from this study also provide additional support for the separation of *C. bhutanensis* and *C. moniliformopsis*, the two species most closely related to *C. moniliformis*. We consider these species to form part of the larger *C. moniliformis sensu lato* complex, characterised by the formation of hat-shaped ascospores, disc-shaped bases to the ascomatal necks and short conical spines on their ascomatal bases.

Ceratocystis tribiliformis is morphologically very similar to *C. moniliformis*. Small but distinct differences in the morphology of the fruiting structures can be used to distinguish between them. The ascomatal bases of *C. tribiliformis* are characteristically obpyriform to globose while *C. moniliformis*, *C. bhutanensis* and *C. moniliformopsis* have distinctly globose bases. *Ceratocystis tribiliformis* and *C. bhutanensis* both have smooth hyphae as observed in *C. moniliformis* and *C. moniliformopsis*, and hyphae with granular surfaces. These fungi can also easily be distinguished from each other based on

growth characteristics in culture. In culture, *C. tribiliformis*, has very little, if any, aerial mycelium, while all the other species in the *C. moniliformis sensu lato* complex produce prolific aerial mycelium (Hedgcock, 1906; Davidson, 1935; Yuan and Mohammed, 2002; Van Wyk *et al.*, 2004a). Ascomata cover the agar in cultures of *C. tribiliformis*, while isolates of *C. moniliformis* sporulate less prolifically and generally require the addition of thiamine to enhance the production of ascomata (Robbins and Ma, 1942; Hawker, 1966; Upadhyay, 1981).

Analysis of sequence data for the ITS regions alone did not provide convincing separation between isolates of *C. bhutanensis*, *C. moniliformis*, *C. moniliformopsis*, and *C. tribiliformis*. However, addition of sequences for the β-tubulin and EF1-α gene areas provided clear resolution of these four species into distinct clades with robust bootstrap and Bayesian support. This supports the morphological and cultural differences observed in these species and emphasises the importance of considering multiple gene regions in both taxonomic and phylogenetic studies.

Results of this study provide the first phylogenetic comparison for a collection of isolates, many of which have previously been referred to as *C. moniliformis*. Clearly, *C. moniliformis sensu stricto* has a wider geographic distribution than previously thought. Other apparently cryptic species that have emerged from this and other recent studies appear, however, to be restricted to specific geographical areas. *Ceratocystis moniliformopsis* is known only from Australia (Yuan and Mohammed, 2002); *C. bhutanensis* is associated with the scolytine bark beetle, *Ips schmutzenhoferi* Holzschuh and found only in Bhutan (Van Wyk *et al.*, 2004a); and *C. tribiliformis* described in this study has, to date, been found only on the island of Sumatra in Indonesia. The known distribution for some of these species is likely to expand if additional collections are undertaken. However, some species such as *C. bhutanensis*, appear to be ecologically adapted to their areas of origin and hosts.

Very little is known regarding the ecology of the species in the *C. moniliformis sensu lato* complex. In pathogenicity trials (Davidson, 1935), it has been concluded that the fungus is a wound inhabiting saprophyte. With the recognition of more species, additional pathogenicity tests should be carried out, on their hosts of origin and in the areas from which they have originated. These tests will expand our understanding of the *C. moniliformis* species complex and thereby possibly, lead to the discovery of new pathogens.

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References

Baker, C.J., Harrington, T.C., Krauss, U. and Alfenas, A.C. (2003). Genetic variability and host specialization in the Latin American clade of *Ceratocystis fimbriata*. Phytopathology 93: 1274-1284.

Bakshi, B.K. (1951). Studies on four species of *Ceratocystis*, with a discussion of fungi causing sap-stain in Britain. Mycological Paper 35: 1-16.

Barnes, I., Guar, A., Burgess, T., Roux, J., Wingfield, B.D. and Wingfield, M.J. (2001a). Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt pathogen, *Ceratocystis fimbriata*. Molecular Plant Pathology 2: 319-325.

Barnes, I., Roux, J., Coetzee M.P.A. and Wingfield, M.J. (2001b). Characterization of *Seiridium* spp. associated with cypress canker based on β-tubulin and histone sequences. Plant Disease 85: 317-321.

Carbone, I. and Kohn, L.M. (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91: 553-556.

Davidson, R.W. (1935). Fungi causing stain in logs and lumber in the Southern states, including five new species. Journal of Agricultural Research 50: 789-807.

Glass, N.L. and Donaldson, G.C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Applied and Environmental Microbiology 61: 1323-1330.

Grylls, B.T. and Seifert, K.A. (1993). A synoptic key to species of *Ophiostoma*, *Ceratocystis* and *Ceratocystiopsis*. In: *Ceratocystis and Ophiostoma*: *Taxonomy, Ecology, and Pathogenicity* (eds. M.J. Wingfield, K.A. Seifert and J.F. Webber). APS Press, St. Paul, Minnesota: 261-268.

Hedgcock, G.G. (1906). Studies upon some chromogenic fungi which discolor wood. Missouri Botanical Garden 17: 59-124.

Hawker, E.L. (1966). Environmental influences on reproduction. In: *The Fungi*: *An Advanced Treatise*. *Volume II, The Fungal Organism* (eds. G.C. Ainsworth and A.S. Sussman) Academic Press. New York. London: 435-469.

Hunt, J. (1956). Taxonomy of the genus *Ceratocystis*. Lloydia 19: 1-58.

Kile, G.A. (1993). Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In: *Ceratocystis and Ophiostoma*: *Taxonomy, Ecology, and Pathogenicity* (eds. M.J. Wingfield, K.A. Seifert and J.F. Webber). APS Press, St. Paul, Minnesota: 173-183.

Kitajima, K. (1936). Researches on the discolourations of logs of *Fagus crenata* Blume caused by *Endoconidiophora Bunae*, n. sp. and on its preventive method. Bulletin of Imperial Forest Experiments station, Meguro, Tokyo 35: 1-134.

Kowalski, T. and Butin, H. (1989). Taxonomie bekannter und neuer *Ceratocystis*-Arten an Eiche (*Quercus robur* L.). Phytopathology 124: 236-248.

Larget, B. and Simon, D.L. (1999). Markov Chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. Molecular Biology and Evolution 16: 750-759.

Luc, M. (1952). *Ophiostoma moniliforme* (Hedgc.) H. et P. Syd. and its various forms. Reviews in Mycology 17: 10-16.

Moreau, C. (1952). Coexistence des formes *Thielaviopsis* et *Graphium* chez une souche de *Ceratocystis major* (van Beyma) nov. comb. *Revue de Mycologie*. (Paris) Supplement Colonial 17: 17-22.

Paulin-Mahady, A.E., Harrington, T.C. and McNew, D.L. (2002). Phylogenetic and taxonomic evaluation of *Chalara*, *Chalaropsis*, and *Thielaviopsis* anamorphs associated with *Ceratocystis*. Mycologia 94: 62-72.

Rayner, R.W. (1970). A mycological colour chart. Commonwealth Mycological Institute and British Mycological Society, Kew, Surrey.

Robbins, W.J. and Ma, R. (1942). Vitamin deficiencies of *Ceratostomella* and related fungi. American Journal of Botany 29: 835-843.

Roldan, E.F. (1962). Species of *Ceratocystis* (*Ceratostomella*) causing stain in rattan. The Philippine Journal of Science 91: 415-423.

Swofford, D.L. (2002). PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4.0b10. Sunderland, Massachusetts: Sinauer Associates.

Upadhyay, H.P. (1981). *A monograph of Ceratocystis and Ceratocystiopsis*. University of Georgia Press. Athens.

Van Wyk, M., Roux, J., Barnes, I., Wingfield, B.D., Chhetri, D.B., Kirisits, T. and Wingfield, M.J. (2004a). *Ceratocystis bhutanensis sp. nov.*, associated with the bark beetle *Ips schmutzenhoferi* on *Picea spinulosa* in Bhutan. Studies in Mycology 50: 365-379.

Van Wyk, M., Roux, J., Barnes, I., Wingfield, B.D., Liew, E.C.Y., Assa, B., Summerell, B.A. and Wingfield, M.J. (2004b). *Ceratocystis polychroma sp. nov.*, a new species from *Syzygium aromaticum* in Sulawesi. Studies in Mycology 50: 272-282.

Van Wyk, P.W.J., Wingfield, M.J. and Van Wyk, P.S. (1991). Ascospore development in *Ceratocystis moniliformis*. Mycological Research 95: 96-103.

Von Schrenk, H. (1903). The "bluing" and the "red-hot" of the western yellow pine, with special reference to the Black Hills Forest Reserve. U.S. Department of Agriculture. Bureau of Plant Industry Bulletin 36: 1-46.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A sequencing guide to methods and applications*. (eds. M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White). Academic Press, San Diego: 315-322.

Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J. and Harrington, T.C. (1999). PCR-based identification and phylogeny of species of *Ceratocystis sensu stricto*. Mycological Research 103: 743-749.

Yuan, Z.Q. and Mohammed, C. (2002). *Ceratocystis moniliformopsis sp. nov.*, an early colonizer of *Eucalyptus obliqua* logs in Tasmania, Australia. Australian Systematic Botany 15: 125-133.

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