Phylogenetic relationships and taxonomy of species in *Ceratocystis sensu lato*

by

Marelize van Wyk

Submitted in partial fulfilment of the requirements for the degree

Philosophiae Doctor

In the Faculty of Natural & Agricultural Sciences, Department of Genetics, University of Pretoria, Pretoria, South Africa

April 2013

Supervisor: Prof. Michael J. Wingfield
Co-supervisor: Prof. Brenda D. Wingfield

DECLARATION

I, Marelize van Wyk, declare that the dissertation, which I hereby submit for the degree *Philosophiae Doctor* in Genetics at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

_____

Marelize van Wyk

April 2013

I dedicate this thesis to my parents Barney and Greta van Wyk without whom this would not have been possible.

“I can almost see it, that dream I am dreaming. But there's a voice inside my head saying "You'll never reach it". Every step I'm taking every move I make feels lost with no direction, my faith is shaking. But I got to keep trying, got to keep my head held high. There's always going to be another mountain. I'm always going to want to make it move. Always going to be a uphill battle, sometimes I'm going to have to lose. Ain't about how fast I get there, ain't about what's waiting on the other side, it's the climb. The struggles I'm facing, the chances I'm taking, sometimes might knock me down, but no, I'm not breaking. I may not know it but these are the moments that I'm going to remember most. Just got to keep going, and I, I got to be strong, just keep pushing on.”

Jessi Alexander & Jon Mabe

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

This thesis would not have materialised without the support from a wide variety of people and I would like to thank the following people:

- My parents, Barney and Greta, they have supported me financially and with unconditional love and dedication towards finishing my studies.
- My study leaders, Mike and Brenda Wingfield, whom have taught me not only science but valuable life lessons as well.
- Jolanda Roux for her help in the field trials and support in meetings
- The FABI family, to work in such an environment has been an enormous privilege and I will truly miss it.
- The Tree Protection Co-operative Programme (TPCP), FABI, National Research Foundation (NRF) and the University of Pretoria for the magnificent facilities, bursaries and opportunities that have made this study possible.
- The administrators at the Natural Sciences Department, in particular Matilda Beresford and Cathy Barnard for their support
- The FABI administrators**;** Helen Doman, Rose Visser & Matha Mahlangu thank you for all the help and support you have given me
- I also would like to thank our Lord. None of this would have been possible if not for his grace.

PREFACE

Ceratocystis is a genus of Ascomycete fungi residing in the family Ceratocystidaceae (Order: Microascales; Class: Sordariomycetes). Species of *Ceratocystis* are best-known as wound-infecting fungi, which are mostly carried by insects. Little is known regarding the basis of the relationship between insects and *Ceratocystis* spp., but in some cases it might extend to levels of mutualism. The taxonomic history of the genus is complex and has, during the course of more than 100 years, been intertwined with several other genera. However, in recent years, the resolution arising from DNA sequence comparisons has resulted in the recognition of at least four major groups within *Ceratocystis.*

Ceratocystis species in the broad sense (including *Ambrosiella*) represent ecologically diverse assemblages, including four very clearly defined groups. One of these groups, which I refer to as the *C. coerulescens sensu lato* (*s.l.*) group, which mainly cause blue-stain in timber, is the only group that is known to have a very close association with conifer-infesting bark beetles. Perhaps the best-known group is the *C. fimbriata s.l.* assemblage of species, which includes a large number of serious pathogens of angiosperms, mostly trees. This group also includes non-pathogenic wound-infecting species. There are no specific insect vectors of these species but they produce fruity aromas, which attract a wide range of insects such as flies, ants, mites and nitidulid beetles that aid in their dissemination to fresh wounds. The majority of the species in the *C. fimbriata s.l.* group are primary pathogens causing cankers that can girdle and eventually kill the affected areas. The third group of fungi in the broadly defined *Ceratocystis s.l.* is the *C. moniliformis s.l.* group. This group includes only saprophytes that, similar to species in *C. fimbriata s.l.,* produce fruity odours and they are thus vectored by opportunistic insects including flies and nitidulid beetles. *Ambrosiella* spp. are broadly included in the assemblage accommodating *Ceratocystis* spp. These fungi have no known sexual state and they live in obligate symbioses with ambrosia beetles (*Scolytinae*).

Ceratocystis and its relatives have had a complex taxonomic history ever since the genus was first described by Halstead in 1890. Much of the confusion regarding their taxonomy has arisen from a dependence on morphology for classification and the fact that these and other fungi have undergone convergent evolution related to their association with insects. Until the late 1990s, genera and species in this group were described based only on morphology, although the

importance of various morphological features was strongly debated. However, between 1950-1999, a turning point arose in the way that these fungi were recognised. This change emerged first from the inclusion of various chemical approaches and then later the application of molecular techniques to identify them. The most dramatic changes have come with the widespread application of DNA sequence comparisons, which were first applied to the group in the early 1990's. This made it possible to define higher-order relationships and brought the first clear evidence that species in *Ceratocystis* and *Ophiostoma* were phylogenetically unrelated. A complete taxonomic history of this group is provided in the timeline presented in Table 1.

This thesis deals primarily with two groups in the broadly defined *Ceratocystis.* These are species in the *C. moniliformis s.l.* and the *C. fimbriata s.l.* complexes, but with a considerably greater focus on the *C. fimbriata s.l.* complex. Morphologically, species in the two complexes are easy to distinguish from each other even though they share some common characteristics. Species in both complexes have hat-shaped ascospores and both have the anamorphic characteristics typical of species in the genus *Thielaviopsis*. The ascomatal bases are mostly globose to sub-globose with elongated necks. There are two main features that species in the *C. moniliformis s.l.* complex have that are not observed in the *C. fimbriata s.l.* complex. One is a very distinct "collar-like" structure at the base of the ascomatal necks of species in the *C. moniliformis s.l.* complex. These "collar-like" structures result in the necks being fragile and they dislodge from the ascomatal bases very easily. The other distinct characteristic is that these fungi have conical spines on their ascomatal bases, giving the structures a textured appearance. This is in contrast to the ascomatal bases in the *C. fimbriata* complex that are smooth. Species in the *C. moniliformis s.l.* complex are non-pathogenic and cause only some sap-stain in the host tissues. The *C. fimbriata s.l.* complex includes a large number of primary pathogens, mainly of trees but also including root crops. A phylogenetic tree including all species in the *C. fimbriata s.l* and *C. moniliformis s.l.* complexes is presented in Figure 1-3. In generating this tree, three gene regions i.e. the Internal Transcribed Spacer Region including the 5.8S rRNA operon (ITS), part of the the Beta-Tubulin 1 (BT) gene and part of the Transcription Elongation Factor 1 alpha (TEF) gene have been combined to represent species in the two complexes. Both Bootstrap as well as Bayesian analyses were run to obtain confidence intervals. More detailed methods are presented in the legends to the trees.

Chapter one of this thesis presents a review of the groups in *Ceratocystis*. With the aid of DNA sequence comparisons, three phylogenetically distinct groups are identified. These groups are characterised by three well-known species each now defined as a species complex i.e. the *Ceratocystis coerulescens* complex, the *C. moniliformis s.l.* complex and the *C. fimbriata s.l.* complex. Species of *Ambrosiella* are also recognised as representing a discrete and related group. These three species complexes are not only differentiated based on DNA sequence comparison but have distinct morphological features that distinguish them from each other. They are also defined by having very distinct ecological roles in nature. Chapter 2 specifically reviews the key literature dealing with the taxonomy of species in the *C. fimbriata* complex. This group is defined by *Ceratocystis fimbriata s.s.*, which was first described as a pathogen causing black rot on sweet potato. Subsequent to the emergence of DNA sequencing techniques to define species, many new species in the *C. fimbriata s.l.* complex have been defined and described.

Chapters three and four of this thesis deal with a species of *Ceratocystis* collected from dying Mango trees in Oman. A very serious disease known as Mango Sudden Decline disease was first observed in Oman and has recently been subjected to intensive investigation. This led to a recognition that a species related to *C. fimbriata* might be responsible for the disease of *Mangifera indica.* As part of the process of identifying the causal agent of Mango Sudden Decline, two *Ceratocystis* spp. were described. One is the previously described and non-pathogenic *C. omanensis* residing in the *C. moniliformis s.l.* complex and the other is *C. manginecans*, a virulent pathogen residing in the *C. fimbriata s.l.* complex (Chapter 4).

Chapters five through eleven of this thesis include the descriptions of new species in the *C. fimbriata* complex that have emerged over a seven year period. These descriptions have all relied strongly on DNA sequence based phylogenetic inference as well as morphology. It is important to recognise, however, that the morphological features are very similar in most species and that differences rely on the size ranges of key features in this group.

The genus *Ceratocystis* represents a complex of many species and is set to be sub-divided into at least three discrete genera, not including *Ambrosiella*, which is also closely related and already well-defined. Studies in this thesis began at a time when it became possible to define species of

Ceratocystis based strongly on a phylogenetic concept. Thus, while morphological and ecological features were taken into account, there has been a heavy reliance on DNA sequence comparisons. It is likely that in coming years, new gene regions will be recognised that will allow a refinement of genus and species boundaries. Furthermore, having access to whole-genome sequencing will add deeply to our understanding of taxonomic relationships between these important and fascinating fungi. It is my hope that the foundation provided by the studies incorporated in this thesis will be useful as this new wave of study emerges.

REFERENCES (this reference list relates mostly to citations included in the Tables and Figures)

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.

Barr M.E. (1990) Prodromus to nonlichenized, pyrenomycetous members of class Hymenoascomycetes. *Mycotaxon*: **39**: 43-184.

Batista A.C. & Vital A.F. (1956) *Hughesiella* – novo e curioso genero de fungos dematiaceae. *Anais da Sociedade de Biologia de Pernambuco* **14**: 141-144.

Batra L.R. (1967) Ambrosia fungi: A taxonomic revision, and nutritional studies of some species. *Mycologia* **59**: 976-1017.

Benny G.L. & Kimbrough J.W. (1980) A synopsis of the orders and families of Plectomycetes with keys to genera. *Mycotaxon* **12**: 1-91.

Berbee M.L. & Taylor J.W. (1992) Fungal molecular evolution: gene trees and geologic time. The Mycota VII Part B. Systematics and Evolution **10**: 229-245.

Bessey E.A. (1950) Morphology and taxonomy of fungi. Blakiston, Philadelphia.

Blackwell M. & Jones K. (1997) Taxonomic diversity and interactions of insect-associated ascomycetes. *Biodiversity & Conservation* **6**: 689-699.

Brader L. (1964) Etude de la relation entre le Scolyte des rameaux de caféier, *Xyleborus compactus* Eichh. (*X. morstatti* Hag.), et sa plante-hôte. *Mededelingen Landbouwhogeschool Wageningen* **41**: 1-109.

Cassar S. & Blackwell M. (1996) Convergent origins of Ambrosia fungi. *Mycologia* **88**: 596-601.

Corda A.C.I. (1837) Icones Fungorum: Hucusque cognitorum. *Pragae*. **1**: 8.

Corda A.C.I. (1838) Icones Fungorum. *Pragae* **2**: 1-43.

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.

De Hoog G.S. (1974) The genera *Blastobotrys, Sporothrix, Calcarisporium* and *Calcarisporiella gen. nov*. *Studies in Mycology* **7**: 1-80.

De Hoog G.S. & Scheffer R.J. (1984) *Ceratocystis* versus *Ophiostoma*: A reappraisal. *Mycologia* **76**: 292-299.

Elliott J.A. (1923) The ascigerous stage of the sweet potato black-rot fungus. *Phytopathology* **13**: 56.

Fries E.M. (1815) *Observationes Mycologicae* Sumptibus gerhardi bonnier **1**: 187.

Fries, E.M. (1818) *Observationes Mycologicae* Sumptibus gerhardi bonnier **2**: 340.

Goidánich G. (1936) II genere de ascomiceti *Grosmannia* G. Goid. *Bollettino della R. Stazione di Patologia Vegetale Roma* 16: 26-60.

Griffin H.D. (1968) The genus *Ceratocystis* in Ontario. *Canadian Journal of Botany* **46**: 689-718.

Grosmann H. (1932) Über die systematischen beziehunger der gattung *Leptographium* Lagerberg et Melin zur Gattung *Ceratostomella* Sacc. *Nova Hedwigia* **72**: 180-198.

Halsted B.D. (1890) Some fungous disease of the sweet potato. *Agricultural College Experiment Station* Bulletin **76**: 1-32.

Harrington T.C. (1981) Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. *Mycologia* **73**: 1123-1129.

Harrington T.C. (2000) Host specialization and speciation in the American wilt pathogen *Ceratocystis fimbriata*. *Fitopatologia Brasileira* **25**: 262-263.

Harrington T.C., Aghayeva D.N. & Fraedrich S.W. (2010) New combinations in *Raffaelea*, *Ambrosiella*, and *Hyalorhinocladiella*, and four new species from the redbay ambrosia beetle, *Xyleborus glabratus*. *Mycotaxon* **111**: 337-361.

Hausner G., Reid J. & Klassen G.R. (1993a) On the subdivision of *Ceratocystis s.l.*, based on partial ribosomal DNA sequences. *Canadian Journal of Botany* **71**: 52-63.

Hausner G., Reid J. & Klassen G.R. (1993b) *Ceratocystis*: a reappraisal based on molecular criteria. *Mycological Research* **97**: 625-633.

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

Kirk P.M., Cannon P.F., David J.C. & Stalpers J.A. (Eds.) (2001) Dictionary of the Fungi, 9th edition. 41-44, 96, 319, 396, 439, 507. CABI Publishing.

Lagerberg T., Lundberg G. & Melin E. (1927). Biological and practical researches into blueing in pine and spruce. *Svenska Skogsvårdsföreningens Tidskrift* **25**: 145-272.

Lindau G. (1907) Kryptogamenflora vor Deutschland, Osterreich und der Schweiz. Fungi imperfecti: Hyphomycetes (erste Hälfte). Leipzig.

Locquin M.V. (1972) Synopsis generalis fungorum, excerpta ex libro De Taxia Fungorum. *Revue de Mycologie, Supplement.*

Luttrell E.S. (1951) Taxonomy of the pyrenomycetes. The university of Missouri studies **24**: 55-120.

Marias G.J., Wingfield M.J., Viljoen C.D. & Wingfield B.D. (1998) A new ophiostomatoid genus from *Protea* infructescences. *Mycologia* **90**: 136-141.

Melin E. & Nannfeldt J.A. (1934) Researches into the blueing of ground wood-pulp. *Svenska Skogvårdsföreningen Tidskrift* **32**: 397-616.

Műnch E. (1907). Die Blaufaule des Nadelholzes. I-II. *Naturwissenschaftliche Zeitschrift fur Land –und Forstwirtschaft* **5**: 531-573.

Műnch E. (1908). Die Blaufaule des Nadelholzes. I-II. *Naturwissenschaftliche Zeitschrift fur Land –und Forstwirtschaft* **6**: 297-323.

Nag Raj T.R. & Kendrick W.B. (1975) A Monograph of *Chalara* and Allied Genera. Wilfrid Laurier University Press Waterloo, Ontario, Canada.

Nannfeldt J.A. (1932) Studien über die morphologie und systematic der nicht-lichenisierten inoperculaten discomyceten. *Nova acta regiae societatis scientiarum upsaliensis* **8**: 28-53.

Olchowecki A. & Reid J. (1974) Taxonomy of the genus *Ceratocystis* in Manitoba. *Canadian Journal of Botany* **52**: 1675-1711.

Parker A.K. (1957) *Europhium*, a new genus of the ascomycetes with a *Leptographium* imperfect state. *Canadian Journal of Botany* **35**: 173-179.

Paulin-Mahady A.E. & Harrington T.C. (2002) Phylogenetic placement of anamorphic species of *Chalara* among *Ceratocystis* and other ascomycetes. *Studies in Mycology* **45**: 209-222.

Peyronel B. (1916) Una nova malattia del lupino prootta da *Chalaropsis thielavioides* Peyr. nov. gen. et nova sp. *Le Stazioni Sperimentali Agrarie Italiane* **49**: 583-595.

Rabenhorst L. (1844) Deutshlands kryptogamen-Flora. *Leipzig* **1**: 609.

Réblová M. (2006). Molecular systematics of *Ceratostomella sensu lato* and morphologically similar fungi. *Mycologia* **98**: 68-93.

Réblová M. Gams W., Seifert K.A. (2011) *Monilochetes* and allied genera of the *Glomerellales*, and a reconsideration of the families in the *Microascales*. *Studies in Mycology* **68**: 163-191.

Redhead S.A & Malloch D.W. (1977) The Endomycetaceae: new concepts, new taxa. *Canadian Journal of Botany* **55**: 1701-1711.

Saccardo P.A. (1876) Fungi Veneti novi vel critici. Series V. Nuovo *Giornale Botanico Italiano* **8**: 161-211.

Saccardo P.A. (ed.) (1878) Fungi Veneti novi vel critici. Series IX. In : *Michelia I* 361-445. Patavii, Typis Seminarii.

Saccardo P.A. (1886) Sylloge Fungorum omnium hucusque cognitorum. *Pavia* **4**: 333-336

Saccardo P.A. (1892) Sylloge Fungorum omnium hucusque cognitorum. *Supplementum universale*. **10**: 213-216.

Seeler E.V. (1943). Several fungicolous fungi. *Farlowia* **1**: 119-133.

Siemaszko W. (1939) Zespoly grybow towartzyszacych komikom polskim. *Planta Polonica* **7**: 1-52.

Smith M.J., Patik C.M. & Rosinski M.A. (1967) A comparison of cellulose production in the genus *Ceratocystis. Mycologia* **59**: 965-969.

Spatafora J.W. & Blackwell M. (1994) The polyphyletic origins of ophiostomatoid fungi. *Mycological Research* **98**: 1-9.

Sydow H. & Sydow P. (1919) Mycologische Mitteilungen. *Sydowia* **1**: 33-47. (Reprinted 1962).

Upadhyay H.P. (1978) Proposal for the conservation of the generic name *Ceratocystis* Ell. & Halst. (1890) against *Sphaeronaemella* Karsten (1884). *Mycotaxon* **27**: 553-554.

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

Upadhyay H.P. & Kendrick W.B. (1974) A new *Graphium*-like genus (conidial state of *Ceratocystis*). *Mycologia* **66**: 181-183.

Upadhyay H.P. & Kendrick W.B. (1975) Prodromus for a revision of *Ceratocystis* (Microascales, Ascomycetes) and its conidial states. *Mycologia* **67**: 798-805.

Van Wyk M., Wingfield B.D. & Wingfield M.J. (2013) *Ceratocystis* species in the *Ceratocystis fimbriata* complex. In: *Ophiostomatoid Fungi: Expanding Frontiers* (Seifert, K.A., Wingfield, M.J. eds). *CBS Biodiversity Series* **12**: 65-76.

Viljoen C.D., Wingfield M.J., Jacobs K. & Wingfield B.D. (2000) *Connuvesica*, a new genus to accommodate *Ceratocystiopsis falcate*. *Mycological Research* **104**: 365-367.

Von Arx J.A. (1952) Ueber die Ascomycetengattungen *Ceratostomella* Sacc., *Ophiostoma* Syd. Und *Rostrella* Zimmerman. *Antonie van Leeuwenhoek* **18**: 13-213.

Von Arx J.A. & Hennebert G.L. (1965) Deux champignons ambrosia. *Mycopathologia et mycologia applicata* **25**: 309-315.

Von Arx J.A. & Műller E. (1954) Die Gattungen der amerosporen Pyrenomyceten. *Beiträge zur Kryptogamenflora der Schweiz* **11**: 1-134.

Von Hönel F. (1902) Fragmente zur mykologie (I. Mitteilung). Sitzungsberichten der Kaiserliche Akademie der Wissenschaften in Wien. Mathhematische-Naturwissenschaftliche Klasse. **111**: 987-1056.

Von Hönel F. (1918) Mycologische Fragmente. *Annales Mycologici* **16**: 40-41. (Reprinted 1962).

Webster R.K. & Butler E.E. (1967) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45**: 1457-1468.

Weijman A.C.M. & De Hoog G.S. (1975) On the subdivision of the genus *Ceratocystis*. *Antonie van Leeuwenhoek* **41**: 353-360.

Went F.A.C.G. (1893) De anannasziekte van het suikerriet. *Archief voor de Java suikerindustrie* **1**: 121-129.

Wingfield M.J., De Beer C., Visser C. & Wingfield B.D. (1996) A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematic and Applied Microbiology* **19**: 191-202.

Wingfield B.D, Van Wyk M., Roos H. & Wingfield M.J. (2013) *Ceratocystis*: Emerging evidence for discrete generic boundaries. In: *Ophiostomatoid Fungi: Expanding Frontiers* (Seifert, K.A., Wingfield, M.J. eds). *CBS Biodiversity Series* **12**: 57-64.

Winter G. (1885) Rabenhort's Kryptogamen-Flora edition 2 **1**: 247.

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

Witthuhn R.C., Wingfield B.D., Wolfaart M. & Harrington T.C. (1998) Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. *Mycologia* **90**: 96-101.

Wright E.F. & Cain R.F (1961) New species of the genus *Ceratocystis*. *Canadian Journal of Botany* **39**: 1215-1230.

Zhang N., Castlebury L.A., Miller A.N., Huhndorf S.M., Schoch C.L., Seifert K.A, Rogers J.D, Kohlmeyer J., Volkmann-Kohlmeyer B. & Sung G. (2006) An overview of the systematics of the Sordariomycetes based on a four-gene phylogeny. *Mycologia* **98**: 1076-1087.

Zimmerman A. (1900). De Kanker (Rostrella-ziekte) van *Coffea arabica*. *Mededeelingen uit's Lands Plantentuin* **37**: 24-62.

Table 1. Taxonomic history of species related to *Ceratocystis*.

	Author
PERIOD 1800 – 1899	
Sphaeronema Fr. established for fungi with perithecia of various shapes, frequently stalked with spores forming a gelatinous mass at the apex of the necks. (Different spelling by Fries in 1823; ***Sphaeronaema*** – considered the correct)	Fries 1815
Ceratostoma Fr. established for fungi with perithecia having coloured ascospores and elongated beak-like ostioles	Fries 1818
Ceratostoma rejected and species transferred to *Melanospora* Corda	Corda 1837
Torula (Pers.) Link **subgen.** ***Chalara*** Corda established due to the conidia arising by fragmentation of the fertile hyphae	Corda 1838
The genus ***Chalara*** (Corda) Rabenh. established (monotypic) and used for asexual states of *Ceratocystis* spp. for many years	Rabenhorst 1844
Ceratostoma Sacc. re-established for fungi with dematiaceaous spores that are 2-celled	Saccardo 1876
Ceratostomella Sacc. established for fungi with hyaline ascospores, astromatic perithecia and persistent asci	Saccardo 1878
Family ***Ceratostomataceae*** G. Winter established for fungi with black perithecia and long necks	Winter 1885
Chalara ungeri Sacc. described and later (Münch 1907) shown to be the asexual state of *Ceratocystis coerulescens*	Saccardo 1886
Ceratocystis Ellis & Halst. established with *C. fimbriata* as type (monotypic) specifically as the cause of black rot of sweet potato	Halstead 1890
Ceratocystis transferred to ***Sphaeronaema*** based on the misinterpretation of the ascospores as conidia and the perithecia as pycnidia	Saccardo 1892
Thielaviopsis Went established as the causal agent of sugar cane disease ("Ananasziekte van het suikerriet") with *T. basicola* (Berk. & Broome) Ferraris as the type species (monotypic). This species was later (Paulin-Mahady *et al*. 2002) designated as the type of all *Ceratocystis* anamorphs.	Went 1893
During this time period the major deciding factor for establishing genera and species was their differences in morphology.	
PERIOD 1900 – 1949	
Rostrella coffeae Zimm. (monotypic) described as the causal agent of a disease of coffee plants known as Rostrella-ziekte	Zimmerman 1900
The anamorph genus ***Chalara*** sub-divided into three genera; ***Euchalara*** for species with exogenous, acrogenous and catenulate conidia; ***Endochalara*** for species with endogenous conidia that emerge in chains from the apex of the fertile hyphae; ***Synchalara*** for species with short fertile hyphae densely connate on a thin subiculum and the conidia the same as those in *Endochalara*	Von Höhnel 1902
The genus ***Euchalara*** rejected due to the fact that the exogenous conidia that is described for this genus would excluded them from *Chalara* and because *Chalara* included only species with endogenous conidia. Thus, all three genera established by Von Höhnel, ***Euchalara***, ***Endochalara*** and ***Synchalara***, were rejected	Lindau 1907

Ceratostomella *coerulescens* Műnch described as the sexual state of ***Chalara*** *ungeri*	Műnch 1907
Endoconidiophora Műnch established (monotypic) with *E. coerulescens* as type species based on its endoconidial asexual state	Műnch 1908
Chalaropsis Peyronel (anamorphic genus) established for species with aleuroconidia (chlamydospores) produced singly with *C. thielavioides* as type species	Peyronel 1916
Ceratostomella split into ***Ceratostomella*** Höhn. and ***Linostoma*** Höhn. *Ceratostomella* retained for species with persistent asci, hyaline ascospores and no ostiolar hyphae. *Linostoma* described for species with evanescent asci, hyaline ascospores with ostiolar hyphae present. *Ceratostomella pilifera* (Fr.) Wint. transferred to *Linostoma*	Von Höhnel 1918
Ophiostoma Syd. & P. Syd. established for species in ***Linostoma*** and 11 species both from *Linostoma* and *Ceratostomella* transferred to *Ophiostoma* because the genus name *Linostoma* was already in use as a homonym for a genus of flowering plants (*Thymeleaceae*)	Sydow & Sydow 1919
(Possibly unaware of the work done between 1893 and 1919) ***Sphaeronaema*** *fimbriatum* was transferred to *Ceratostomella* Sacc. as the pycnidia were observed to be perithecia and the conidia ascospores	Elliot 1923
Anamorphic genus ***Leptographium*** Lagerb. & Melin established (monotypic based on *L. lundbergii*) for fungi that cause blue-stain on timber in Europe	Lagerberg *et al*. 1927
Ceratocystis described as belonging to the order ***Plectascales*** due to globose, irregularly disposed evanescent asci	Nannfeldt 1932
The family ***Ophiostomataceae*** Nannf. established for ***Ophiostoma*** and ***Microascus*** Sacc.	Nannfeldt 1932
Ceratostomella Sacc. placed in the order ***Sphaeriales*** of ***Ascohymeniales***	Nannfeldt 1932
Ceratostomella penicillata Grosm. discovered to be the teleomorphic stage of ***Leptographium*** *penicillatum* Grosm.	Grosmann 1932
Endoconidiophora reduced to synonymy with ***Ophiostoma*** due to the fact that the morphological differences between *E. coerulescens* and the species in ***Ophiostoma*** were limited	Melin & Nannfeldt 1934
Ceratostomella *fimbriata*, *C. adiposa* and *C. paradoxa* also transferred to ***Ophiostoma*** based on their similarity in morphology to *O. coerulescens*	Melin & Nannfeldt 1934
Ceratostomella *fimbriata* transferred to ***Endoconidiophora*** based on the fact that endoconidia were present (In doing so, Davidson was probably unaware of the work between 1923 and 1934)	Davidson 1935
Grosmannia Goid. established for four species; *G. serpens*, *G. penicillata*, *G. pini* and *G. ips*; based on a common anamorphic state, ***Leptographium***	Goidánich 1936
Grosmannia reduced to synonymy with ***Ophiostoma*** because the former genus had been established only for species with a specific anamorph (*Leptographium*) while other anamorphic genera (*Chalara*, *Graphium*, *Cladosporium*, *Cephalosporium* and *Thielaviopsis*) also exists for species in *Ophiostoma*. Another reason for rejection was based on the species *Ophiostoma ips* as it has both *Leptographium* and *Graphium* anamorphic states	Siemaszko 1939
Sphaeronaemella Karsten emended to include species that have hyaline ascospores produced in evanescent asci, with orange to yellow perithecia produced on the fruiting bodies of rotting *Discomycetes*	Seeler 1943

The morphological species concept still played a role in this era however, the focus was more on the anamorphic or asexual differences than on the sexual or teleomorphic states. This approach to erect genera based on different anamorphic states caused considerable taxonomic confusion that has perpetuated up to the present time.

PERIOD 1950 – 1999

Ceratostomataceae transferred to ***Melanosporaceae*** Bessey based on the similar morphology of the perithecia of these two families	Bessey 1950
Ceratostomella retained for species with persistent asci and monostichous (forming one row) spores	Bakshi 1951
Rostrella and ***Endoconidophora*** reduced to synonymy with ***Ceratocystis*** due to the fact they all produce endoconidia in the anamorph state	Bakshi 1951
Grosmannia rejected due to the two anamorphic states (*Leptographium* & *Graphium*) present in *O. ips* and the several other known anamorphic states (*Chalara, Leptographium, Graphium Cladosporium* & *Cephalosporium*) known for *Ophiostoma* species	Bakshi 1951
Microascales erected for ***Microascaceae*** (accommodating ***Microascus***) and ***Ophiostomataceae***. The descriptions of these families were invalid and they were re-described by Benny and Kimbrough (1980)	Luttrell 1951
Rostrella, Endoconidiophora and ***Grosmannia*** reduced to synonymy with ***Ophiostoma*** because they all belong to the ***Plectascales*** as they all have asci with walls that deliquesce very early liberating masses of spores within the ascocarp	Von Arx 1952
Ceratostomella retained for species with ellipsoidal, brown ascospores with terminal germ pores, asci with dissolving stipes and perithecia with reduced stromatic tissue around the base of the neck. Based on these characteristics *Ceratostomella* is treated as a synonym of ***Endoxyla*** Fuckel in ***Diaporthaceae***	Von Arx 1952; Von Arx & Müller 1954
Ceratostoma Fuckel, ***Sphaeronaemella*** Karsten emend. Seeler ***Ceratostomella*** Sacc. emend. Von Höhnel excluded from *Ceratocystis* (this was in strong contrast to the view of Von Arx 1952)	Hunt 1956
Rostrella Zimmerman considered a homonym of *Rostrella* Fabre and ***Linostoma*** Von Höhnel is considered a homonym of *Linostoma* Wallich	Hunt 1956
Ceratostomella Sacc. restricted to species with regularly arranged persistent asci	Hunt 1956
Sphaeria Haller : Fries (in part), ***Endoconidiophora***, ***Ophiostoma***, ***Grosmannia*** and ***Ceratostomella*** *Sacc.* (Comprising of species with evanescent asci) reduced to synonymy with ***Ceratocystis***	Hunt 1956
Ceratocystis divided into sections; 1 – Species with endoconidial imperfect stage; 2 – Species with *Graphium* or *Leptographium* imperfect stages; 3 – Species with mycelial conidial stages	Hunt 1956
Hughesiella Bat. & A.F. Vital, established for fungi with hyaline conidiophores of two forms; short phialides with long catenule of ellipsoidal continuous brown-blackish spores, and simple elongate to branched hyphae producing unicellular and lenticular blackish spores with one equatorial sub-hyaline band, simple or catenulate	Batista & Vital 1956
Europhium A.K. Parker established as the sexual stage for ascomycetes with ***Leptographium*** anamorphs and perithecia without ostioles but with evanescent asci	Parker 1957
Ceratocystis reviewed and species described based mainly on ascospore morphology. However, they also emphasised that together with this the shape and size of the perithecium and the	Wright & Cain 1961

ascospore's gelatinous sheath when present as well as the anamorphic state and cultural characteristics are all considered important for species descriptions	
Ambrosiella Von Arx & Hennebert emend. Batra established for anamorphic fungi living in association with ambrosia beetles	Brader 1964
Raffaelea Arx & Hennebert established for asexual species with conidiophores that taper gradually towards the apex, bearing a series of cicatrical conidial scars and conidia	Von Arx & Hennebert 1965
Ambrosiella considered to represent a complex of eight genera; *Cephaloascus*, *Endomycopsis*, *Ascoidea*, *Dipodascus*, *Monacrosporium* & *Ambrosiella*	Batra 1967
Ceratocystis shown to have distinct cell wall compositions and suggestions that this might define ***Ophiostma*** vs ***Ceratocytis***	Smith *et al.* 1967
Ceratocystis *spp.* studied in Ontario and species described based on ascospore morphology. The sections established by Hunt (1956) were not followed	Griffin 1968
Ceratocystis *fimbriata* isolates shown to have differences in morphology, colony type, pathogenicity and growth rate and a suggestion that the fungus might reflect more than one taxon	Webster & Butler 1967
Ceratocystis proposed to reside in the ***Ceratocystidaceae*** as **"*Ceratocystaceae*"**	Locquin 1972
Ceratocystis and ***Ophiostoma*** separated based on the fact that they have morphologically different conidial stages. ***Ceratocystis*** included species with endoconidia and residing in the anamorph genera ***Chalara***, ***Chalaropsis*** and ***Thielaviopsis***. ***Ophiostoma*** defined based on the presence of exoconidial anamorphs and residing in ***Sporothrix***, ***Verticicladiella*** and ***Graphium***	De Hoog 1974
Phialographium established for species of ***Ceratocystis*** Ell. & Halst. emend. Bakshi with synnematal conidial states and phialides producing enteroblastic-phialidic conidia in mucilage	Upadhyay & Kendrick 1974
Ceratocystis divided into four groups based on ascospore morphology in agreement with Griffin (1968). The four groups were defined as the **Ips**- (Ascospores which are cylindrical with obtuse ends or that are dumbbell-shaped), **Fimbriata**- (Curved, lunate or orange-section-shaped ascospores with either a uniform hyaline gelatinous sheath or a sheath that appears half-moon-shaped, hat-shaped or cucullate in side-view), **Minuta**- (Elongated and usually curved ascospores with hyaline gelatinous sheaths that are attenuated at the ends) and **Pilifera**-group (Ascospores appear curved, ovoid or cylindrical excluding any sheath)	Olchowecki & Reid 1974
Ceratocystiopsis established for species of ***Ceratocystis*** with elongated, aseptate or 1-septate fusiform or clavate ascospores, with a hyaline gelatinous sheath and attenuated ends	Upadhyay & Kendrick 1975
Europhium reduced to synonymy with ***Ceratocystis*** because the lack of perithecial necks was not considered to be taxonomically relevant	Upadhyay & Kendrick 1975
Thielaviopsis, ***Chalaropsis*** reduced to synonymy with ***Chalara*** due to the morphological similarity of their anamorphs	Nag Raj & Kendrick 1975
Ceratocystis and ***Ophiostoma*** subdivided based on the difference in their endoconidial vs exoconidial anamorph states as well as the difference in the composition of their cell walls. ***Ophiostoma*** contains rhamnose and cellulose in their cell walls while ***Ceratocystis*** does not contain either of these components	Weijman & De Hoog 1975
Ophiostomataceae reduced to synonymy with the ***Endomycetaceae***; ***Ceratocystiopsis, Ceratocystis s.str., Europhium*** and ***Ophiostoma*** included in ***Endomycetaceae***	Redhead & Malloch 1977
Sphaeronaemella reduced to synonymy with ***Ceratocystis*** justified by the fact that they differed only in the colour of their ascocarps	Upadhyay 1978

Ophiostomatales erected for ***Ophiostoma*** based on the following features; ascospores not dextrinoid, without germ pores, centrum of pseudoparenchymatous cells, asci not catenulate, ascogenous hyphae arising from basal layers, central columns or tufts, ascocarps ostiolate or not, beaks when present composed of parallel hyphae usually relatively long	Benny & Kimbrough 1980
Microascales revised to include genera with ascospores dextrinoid when young, with one or two or no germ pores, asci usually catenulate, centrum initiated by paraphysoidal hyphae, ascocarps with or without beaks or ostioles, walls of beaked taxa cellular	Benny & Kimbrough 1980
Ceratocystis and ***Ophiostoma*** viewed as separate based on the sensitivity to cycloheximide in the former and not the latter genus	Harrington 1981
Ophiostoma, ***Sphaeronaemella***, ***Rostrella***, ***Endoconidiophora***, ***Linostoma***, ***Grosmannia*** and ***Europhium*** reduced to synonymy with ***Ceratocystis*** based on the fact that they all have short, not falcate ascospores, asci are spherical to sub-spherical, ascocarp necks usually cylindrical and ostiolar hyphae not conically arranged. However ***Ceratocystis*** divided into sections; 1 – ***Ophiostoma*** (Ascospores lacking a hyaline gelatinous sheath), 2 – ***Ips*** (Ascospores surrounded by a sheath making them appear rectangular, ossiform or pillow-shaped in side-view, never curved), 3 – ***Ceratocystis*** (Ascospores short, with sheath appearing irregularly curved, half moon-shaped, hat-shaped, or cucullate in side view), 4 – ***Endoconidiophora*** (Ascospores with sheath elongated or inequilateral)	Upadhyay 1981
Ceratocystiopsis treated as a separate genus because the ascospores are elongate, falcate with attenuated ends, asci are fusiform or clavate, ascocarp necks mostly tapered towards the apex with conically arranged ostiolar hyphae	Upadhyay 1981
Ophiostomataceae revised to include species with ascocarp bases sub-globose to flask-shaped, hyaline to dark brown or black, long ostiolar beak or lacking both beak and ostiole, asci irregularly distributed throughout centrum, without crosiers, clavate to sub-spherical or spherical or fusiform, evanescent, ascospores hyaline, aseptate or 1-septate, with or without a hyaline gelatinous sheath, lacking a germ pore, embedded in a mucilaginous matrix. ***Ceratocystis*** defined as the type species	Upadhyay 1981
Ceratocystis, ***Ophiostoma***, ***Ceratocystiopsis*** and ***Sphaeronaemella*** separated based on morphology and biochemistry. ***Ophiostoma*** have anamorphs other than ***Chalara***, ascospores hyaline variously shaped but not falcate without germ-slits, rhamnose and cellulose in their cell walls and are resistant to cycloheximide. ***Ceratocystis*** have ***Chalara*** anamorphs, ascospores hyaline variously shaped but not falcate without germ-slits, does not contain cellulose or rhamnose in cell walls and are sensitive to cycloheximide. ***Ceratocystiopsis*** anamorphs other than ***Chalara***, ascospores hyaline falcate without germ-slits, resistant to cycloheximide. ***Sphaeronaemella*** perithecia pink, ascospores brown, oblate with germ-slits	De Hoog & Scheffer 1984
Ceratocystis moved from the order ***Ophiostomatales*** based on the morphological differences between ***Ceratocystis*** and ***Ophiostoma***. ***Ceratocystis*** placed in ***Lasiosphaeriaceae*** (***Sordariales***). ***Ophiostoma*** placed in the ***Ophiostomataceae*** (***Microascales***)	Barr 1990
Sporothrix schenkii an anamorphic state linked with the teleomorphic state ***Ophiostoma*** with DNA sequencing	Berbee & Taylor 1992
Ceratocystiopsis reduced to synonymy with ***Ophiostoma*** based on analyses of partial rDNA sequences – the first study to show phylogenetic relationships with molecular tools	Hausner *et al.* 1993a
Sphaeronaemella treated as a separate genus based on lack of phylogenetic relatedness (partial rDNA sequences from both the large subunit and small subunit) to either ***Ceratocystis*** or ***Ophiostoma***	Hausner *et al.* 1993a

Ceratocystis and ***Ophiostoma*** separated based on partial rDNA sequence comparisons with species of ***Ceratocystis*** (***Chalara*** anamorphs) sensitive to cyclohexamide while those of ***Ophiostoma*** having ***Graphium, Leptographium, Sporothrix*** and ***Verticicladiella*** anamorphs resistant to cycloheximide and having rhamnose and cellulose in their cell walls	Hausner *et al*. 1993b
Ceratocystis placed in the ***Microascales*** based on rDNA sequence analyses (Small subunit)	Spatafora & Blackwell 1994
Ophiostoma placed into family ***Ophiostomatales*** based on rDNA sequence (Small subunit)	Spatafora & Blackwell 1994
Ceratocystis albifundus described as a separate entity and not ***C. fimbriata*** as previously described. This was the first species to be segregated for *C. fimbriata* and the start of the emergence of the *C. fimbriata* complex	Wingfield *et al.* 1996
Ophiostoma and ***Ceratocystis*** shown to be polyphyletic groups including ***Ambrosiella*** species in each of the groups. This suggested that *Ambrosiella* should be re-defined and separated into different genera	Cassar & Blackwell 1996; Blackwell & Jones 1997
Gondwanamyces G.J. Marais & M.J. Wingf. established for two species. The anamorphic state is known as ***Knoxdaviesia*** M.J. Wingf., P.S. van Wyk & Marasas and they are sensitive to cycloheximide	Marais *et al.* 1998
Ceratocystis recognised as representing two distinct groups; **Fimbriata** and **Coerulescens**. Species previously recognised as *C. coerulescens* shown to represent five taxa; *C. coerulecens, C. virescens, C. laricicola, C. polonica* and *C. pinicola* based on phylogenetic inference from DNA sequence comparisons	Witthuhn *et al.* 1998, 1999

At the start of this era genera and species were still being described based on morphology. Later the DNA sequence comparisons provided robust tools to define genera and species. This was the turning point for studies of fungi in this group as they could now be placed into families and orders with high certainty

PERIOD 2000 – 2011

Ceratocystis fimbriata based on DNA sequence data suggested to represent various species defined by geographical origin (Latin America & North America)	Harrington 2000
Cornuvesica C.D. Viljoen, M.J. Wingf. & K. Jacobs described as a new anamorph genus for *C. falcata* (monotypic) with falcate, sheathed and septate ascospores with a similar anamorph to ***Chalara*** but with obovoid conidia with one truncated end	Viljoen *et al.* 2000
The family ***Ceratocystidaceae*** erected in the order ***Microascales*** to accommodate the genus ***Ceratocystis*** and ***Gondwanamyces***	Kirk *et al.* 2001
Chalara with ***Ceratocystis*** teleomorphs transferred to ***Thielaviopsis*** due to the fact that ***Chalara*** is polyphyletic and that the type species of ***Chalara*** do not fall within this group. An alternative anamorph genus was thus required for species with ***Ceratocystis*** teleomorphs	Paulin-Mahady *et al.* 2002
Chalaropsis and ***Hughesiella*** reduced to synonymy with ***Thielaviopsis*** due to the fact that they are monophyletic and they have limited morphological characteristics that were used to distinguish them earlier	Paulin-Mahady *et al.* 2002
Ceratostomella revised for species with thick perithecial wall, clavate asci, broad-celled paraphyses, shallow indistinct apical annulus and suballantoid to reniform to irregularly ellipsoidal ascospores 2-3 seriate or in a fascicle within the ascus and *Ceratostomella rostrata* as the type species	Réblová 2006

Microascales suggested to be polyphyletic based on a phylogenetic analysis of sequences for the small subunit (SSU), large subunit (LSU), transcription elongation factor (TEF) and the polymerase II polypeptide B (RPB2) genes	Zhang *et al.* 2006
Ambrosiella species with ***Ophiostoma*** teleomorphs transferred to genus ***Raffaelea*** Arx & Hennebert	Harrington *et al*. 2010
Ceratocystidaceae, ***Gondwananmycetaceae***, ***Halosphaeriaceae*** & ***Microascaceae*** recognised in the ***Microascales***	Réblová et al. 2011
Ceratocystidaceae validated as a monophyletic group including species of ***Ceratocystis***, ***Cornuvesica***, ***Thielaviopsis*** and the type species of ***Ambrosiella***	Réblová *et al.* 2011
Ceratocystis hypothesised to include three genera; Coerulescens , Moniliformis, Fimbriata	Wingfield *et al.* 2013

The phylogenetic species concept was adopted in this era. The 100 year long debate regarding the placement of the genera in the fugal tree of life had largely ended. But much work remains to be done to define generic and species boundaries in these and other fungi.

Table 2. Isolates of *Ceratocystis* used to determine the phylogeny of *C. fimbriata* and *C. moniliformis.*

Species	Isolate no.	GenBank accession no.	Host	Area
C. fimbriata s.l.				
C. acaciivora	CMW22563	EU588656 EU588636 EU588646	*Acacia mangium*	Indonesia
C. acaciivora	CMW22562	EU588655 EU588635 EU588645	*Acacia mangium*	Indonesia
C. albifundus	CMW4068	DQ520638 EF070429 EF070400	*Acacia mearnsii*	RSA
C. albifundus	CMW5329	AF388947 DQ371649 EF070401	*Acacia mearnsii*	Uganda
C. atrox	CMW19383 CBS120517	EF070414 EF070430 EF070402	*Eucalyptus grandis*	Australia
C. atrox	CMW19385 CBS120518	EF070415 EF070431 EF070403	*Eucalyptus grandis*	Australia
C. cacaofunesta	CMW15051 CBS152.62	DQ520636 EF070427 EF070398	*Theobroma cacao*	Costa Rica
C. cacaofunesta	CMW14809 CBS115169	DQ520637 EF070428 EF070399	*Theobroma cacao*	Ecuador
C. caryae	CMW14793 CBS114716	EF070424 EF070439 EF070412	*Carya cordiformis*	USA
C. caryae	CMW14808 CBS115168	EF070423 EF070440 EF070411	*Carya ovata*	USA
C. colombiana	CMW9565 CBS121790	AY233864 AY233870 EU241487	Soil in coffee plantation	Colombia
C. colombiana	CMW5751 CBS121792	AY177233 AY177225 EU241493	*Coffea arabica*	Colombia
C. corymbiicola	CMW29354	HM071907 HM071919 HQ236455	*Corymbia variegata*	Australia
C. corymbiicola	CMW29120	HM071902 HM071914 HQ236453	*Corymbia variegata*	Australia
C. curvata	CMW22442 CBS122603	FJ151436 FJ151448 FJ151470	*Eucalyptus deglupta*	Colombia
C. curvata	CMW22433 CBS122513	FJ151438 FJ151450 FJ151472	*Eucalyptus deglupta*	Colombia
C. diversiconidia	CMW22447 CBS122818	FJ151442 FJ151454 FJ151476	*Terminalia ivorensis*	Colombia
C. diversiconidia	CMW22448 CBS122605	FJ151441FJ151453 FJ151475	*Terminalia ivorensis*	Colombia
C. ecuadoriana	CMW22092 CBS124020	FJ151432 FJ151444 FJ151466	*Eucalyptus deglupta*	Colombia
C. ecuadoriana	CMW22093 CBS124021	FJ151433 FJ151445 FJ151467	*Eucalyptus deglupta*	Colombia
C. eucalypticola	CMW12663	FJ236724 FJ236784 FJ236754	*Eucalyptus* sp.	South Africa
C. eucalypticola	CMW11536 CBS124016	FJ236723 FJ236783 FJ236753	*Eucalyptus* sp.	South Africa
C. fimbriata s.s	CMW15049 CBS141.37	DQ520629 EF070442 EF070394	*Ipomaea batatas*	USA
C. fimbriata s.s.	CMW1547 CBS123010	AF264904 EF070443 EF070395	*Ipomaea batatas*	Papua New Guinea
C. fimbriatomima	CMW24174 CBS121786	EF190963 EF190951 EF190957	*Eucalyptus* sp.	Venezuela

C. fimbriatomima	CMW24176 CBS121787	EF190964 EF190952 EF190958	*Eucalyptus* sp.	Venezuela
C. larium	CMW25434 CBS122512	EU881906 EU881894 EU881900	*Styrax benzoin*	Indonesia
C. larium	CMW25435 CBS122606	EU881907 EU881895 EU881901	*Styrax benzoin*	Indonesia
C. mangicola	CMW14797 CBS114721	AY953382 EF433307 EF433307	*Mangifera indica*	Brazil
C. mangicola	CMW27306	FJ200256 FJ200269 FJ200282	*Mangifera indica*	Brazil
C. manginecans	CMW13851 CBS121659	AY953383 EF433308 EF433317	*Mangifera indica*	Oman
C. manginecans	CMW13852 CBS121660	AY953384 EF433309 EF433318	*Hypocryphalus mangifera*	Oman
C. mangivora	CMW15052 CBS600.70	EF433298, EF4333306, EF433315	*Mangifera indica*	Brazil
C. mangivora	CMW27304	FJ200261 FJ200274 FJ200287	*Mangifera indica*	Brazil
C. neglecta	CMW11284 CBS121789	EF127988 EU881898 EU881904	*Eucalyptus* sp.	Colombia
C. neglecta	CMW11285 CBS121017	EF127989 EU881899 EU881905	*Eucalyptus* sp.	Colombia
C. obpyriformis	CMW23807 CBS122608	EU245004 EU244976 EU244936	*Acacia mearnsii*	South Africa
C. obpyriformis	CMW23808 CBS122511	EU245003 EU244975 EU244935	*Acacia mearnsii*	South Africa
C. papillata	CMW8587	AY233868 AY233878 EU241483	*Annana muriata*	Colombia
C. papillata	CMW8856 CBS121793	AY233867 AY233874 EU241484	*Citrus lemon*	Colombia
C. pirilliformis	CMW6569	AF427104 DQ371652 AY528982	*Eucalyptus nitens*	Australia
C. pirilliformis	CMW6579 CBS118128	AF427105 DQ371653 AY528983	*Eucalyptus nitens*	Australia
C. platani	CMW14802 CBS115162	DQ520630 EF070425 EF070396	*Platanus occidentalis*	USA
C. platani	CMW26383 CBS114724	EF070426 EF070397 EU426554	*Platanus* sp.	Greece
C. polychroma	CMW11424 CBS115778	AY528970 AY528966 AY528978	*Syzygium aromaticum*	Indonesia
C. polychroma	CMW11436 CBS115777	AY528971 AY528967 AY528979	*Syzygium aromaticum*	Indonesia
C. polyconidia	CMW23809 CBS122289	EU245006 EU244978 EU244938	*Acacia mearnsii*	South Africa
C. polyconidia	CMW23818 CBS122290	EU245007 EU244979 EU244939	*Acacia mearnsii*	South Africa
C. populicola	CMW14789 CBS119.78	EF070418 EF070434 EF070406	*Populus* sp.	Poland
C. populicola	CMW14819 CBS114725	EF070419 EF070435 EF070407	*Populus* sp.	USA
C. smalleyi	CMW14800 CBS114724	EF070420 EF070436 EF070408	*Carya cordiformis*	USA
C. smalleyi	CMW26383 CBS114724	EU426553 EU426555 EU426556	*Carya cordiformis*	USA
C. tanganyicensis	CMW15991 CBS122295	EU244997 EU244969 EU244929	*Acacia mearnsii*	Tanzania

C. tanganyicensis	CMW15999 CBS122294	EU244998 EU244970 EU244939	*Acacia mearnsii*	Tanzania
C. tsitsikammensis	CMW14276 CBS121018	EF408555 EF408569 EF408576	*Rapanea melanophloeos*	South Africa
C. tsitsikammensis	CMW14278 CBS121019	EF408556 EF408570 EF408577	*Rapanea melanophloeos*	South Africa
C. variospora	CMW20935 CBS114715	EF070421 EF070437 EF070409	*Quercus alba*	USA
C. variospora	CMW20936 CBS114714	EF070422 EF070438 EF070410	*Quercus robur*	USA
C. zombamontana	CMW15235	EU245002 EU244974 EU244934	*Eucalyptus* sp.	Malawi
C. zombamontana	CMW15236	EU245000 EU244972 EU244932	*Eucalyptus* sp.	Malawi
C. moniliformis s.l.				
C. bhutanensis	CMW8217 CBS114289	AY528957 AY528962 AY528952	*Picea spinulosa*	Bhutan
C. bhutanensis	CMW8242 CBS112907	AY528956 AY528961 AY528951	*Picea spinulosa*	Bhutan
C. ceramica	CMW15245 CBS122299	EU245022 EU244994 EU244926	*Eucalyptus grandis*	Malawi
C. ceramica	CMW15248 CBS122300	EU245024 EU244996 EU244928	*Eucalyptus grandis*	Malawi
C. inquinans	CMW21106	EU588587 EU588666 EU588674	*Acacia mangium*	Indonesia
C. inquinans	CMW21107 CBS124009	EU588588 EU588667 EU588675	*Acacia mangium*	Indonesia
C. microbasis	CMW21115 CBS124015	EU588592 EU588671 EU588679	*Acacia mangium*	Indonesia
C. microbasis	CMW21117 CBS124013	EU588593 EU588672 EU588680	*Acacia mangium*	Indonesia
C. monilifomis	CMW10134 CBS118127	FJ151422 FJ151456 FJ151478	*Eucalyptus grandis*	South Africa
C. monilifomis	CMW4114 CBS118151	AY528997 AY528986 AY529007	*Schizolobium parahyba*	Ecuador
C. moniliformopsis	CMW9986 CBS109441	AY528998 AY528987 AY529008	*Eucalyptus oblique*	Australia
C. moniliformopsis	CMW10214 CBS115792	AY528999 AY528988 AY529009	*Eucalyptus sieberi*	Australia
C. oblonga	CMW23802 CBS122820	EU245020 EU244992 EU244952	*Acacia mearnsii*	South Africa
C. oblonga	CMW23803 CBS122291	EU245019 EU244991 EU244951	*Acacia mearnsii*	South Africa
C. omanensis	CMW3800 CBS117839	DQ074743 DQ074733 DQ074738	*Mangifera indica*	Oman
C. omanensis	CMW11048 CBS115787	DQ074742 DQ074732 DQ074737	*Mangifera indica*	Oman
C. savannae	CMW17278 CBS121019	EF408553 EF408567 EF408574	*Eucalyptus macarthurii*	South Africa
C. savannae	CMW17300 CBS121151	EF408551 EF408565 EF408572	*Acacia nigrescens*	South Africa
C. sublaevis	CMW22415	FJ151428 FJ151462 FJ151484	*Eucalyptus deglupta*	Ecuador

C. sublaevis	CMW22422 CBS122516	FJ151429 FJ151463 FJ151485	*Eucalyptus deglupta*	Ecuador
C. sumatrana	CMW21109 CBS124011	EU588589 EU588668 EU588676	*Acacia mangium*	Indonesia
C. sumatrana	CMW21111 CBS124012	EU588590 EU588669 EU588677	*Acacia mangium*	Indonesia
C. tribiliformis	CMW13011 CBS115867	AY528991 AY529001 AY529012	*Pinus merkusii*	Indonesia
C. tribiliformis	CMW13012 CBS118242	AY528992 AY529002 AY529013	*Pinus merkusii*	Indonesia
C. tyalla	CMW28928 CBS128342	HM071898 HM071912 HQ236451	*Eucalyptus dunnii*	Australia
C. tyalla	CMW28932 CBS128703	HM071900 HM071913 HQ236452	*Eucalyptus dunnii*	Australia
Outgroup				
C. coerulescens	CMW26364	FJ411321 FJ411347 FJ411295	*Picea abies*	USA
C. coerulescens	CMW26365 CBS140.37	FJ411322 FJ411348 FJ411296	*Picea abies*	Germany

Figure 1. Phylogenetic tree with isolates representing species in the *C. fimbriata* and *C. moniliformis* complex. In the partition homogeneity test, the data set gave a P-value greater than the minimum required value of P = 0.05. Twelve trees were obtained with parsimony analyses of which one was selected to present here. The tree is described as follows; 2262 characters, 1045 are constant, 44 variable characters are parsimony uninformative, 1173 variable characters are parsimony informative, tree length is 2999 bases, Consistency Index = 0.7, Retention Index = 0.9, Rescaled Consistence = 0.7. For both ITS and BT datasets the GTR+G models were selected in MrModeltest2, while the GTR+I+G was selected for the EF dataset. Three thousand trees were discarder in the Bayesian analysis. Bootstrap values are indicated next to the branch while the Bayesian values are indicated in brackets. (*Ceratocystis ficola* was not included in the analyses as only ITS sequences are available for this species).
(Treebase reference: http://purl.org/phylo/treebase/phylows/study/TB2:S13766.)

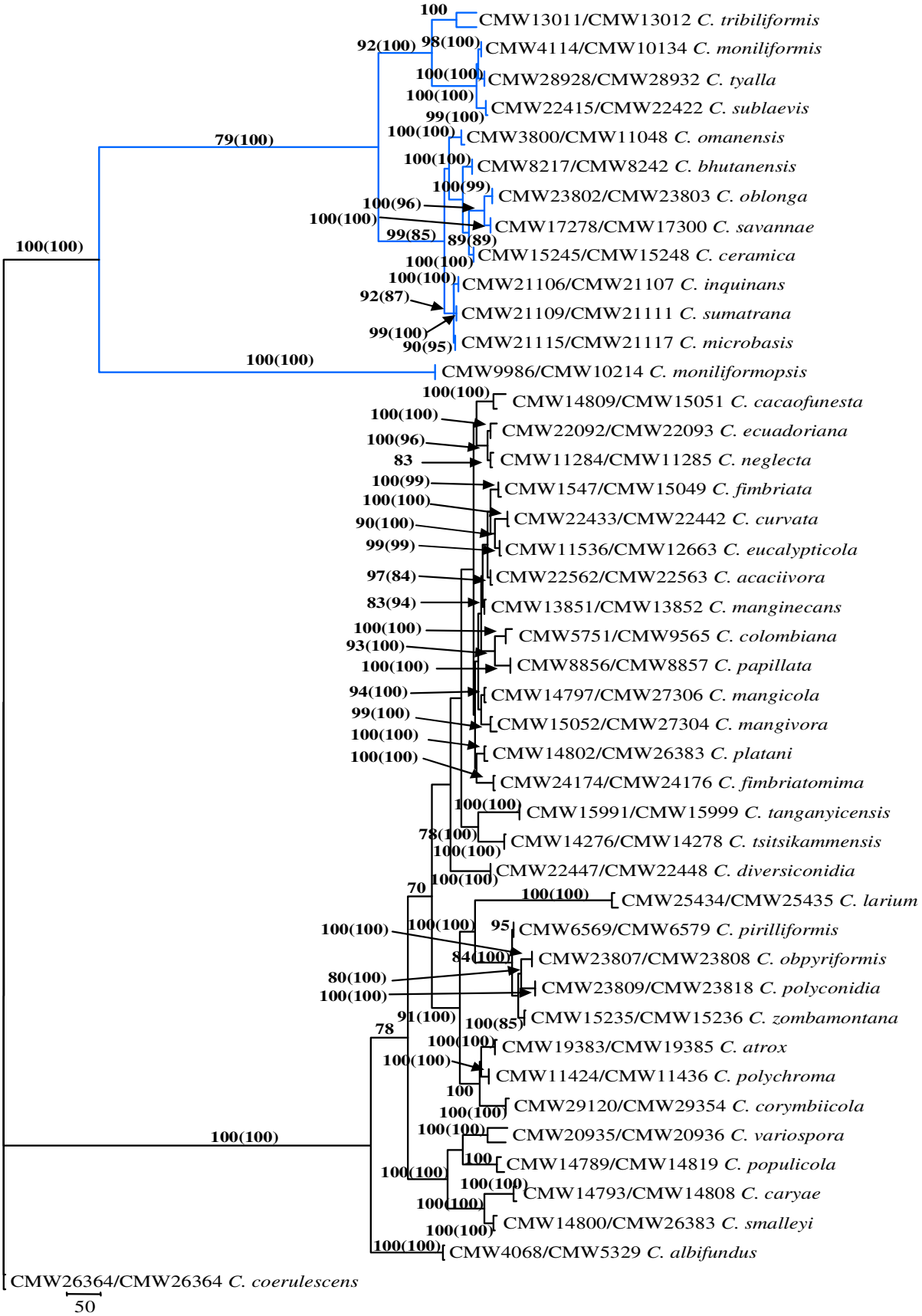


Figure 2. Phylogenetic tree with isolates representing species only in the *C. fimbriata* complex. The partition homogeneity test was congruent (P = 0.05). Four trees were obtained with parsimony analyses of which one was selected to present here. The tree is described as follows; 2048 characters, 1042 are constant, 45 variable characters are parsimony uninformative, 961 variable characters are parsimony informative, tree length is 2148 bases, Consistency Index = 0.7, Retention Index = 0.9, Rescaled Consistence = 0.6. For all three datasets (ITS, BT and EF) the GTR+G model was selected in MrModeltest 2. Bootstrap values are indicated next to the branch while the Bayesian values are indicated in brackets.
(Treebase reference: http://purl.org/phylo/treebase/phylows/study/TB2:S13766.)

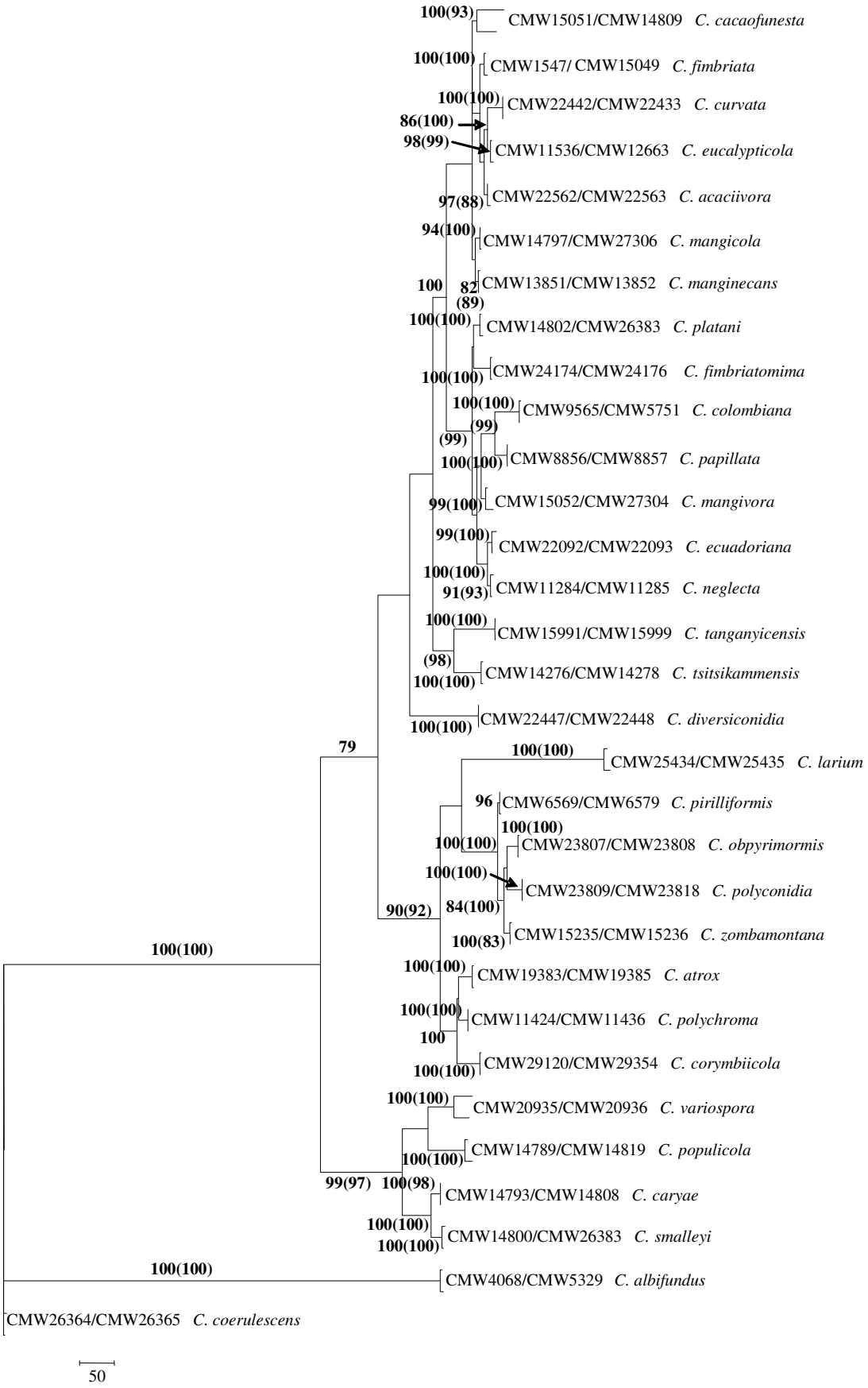
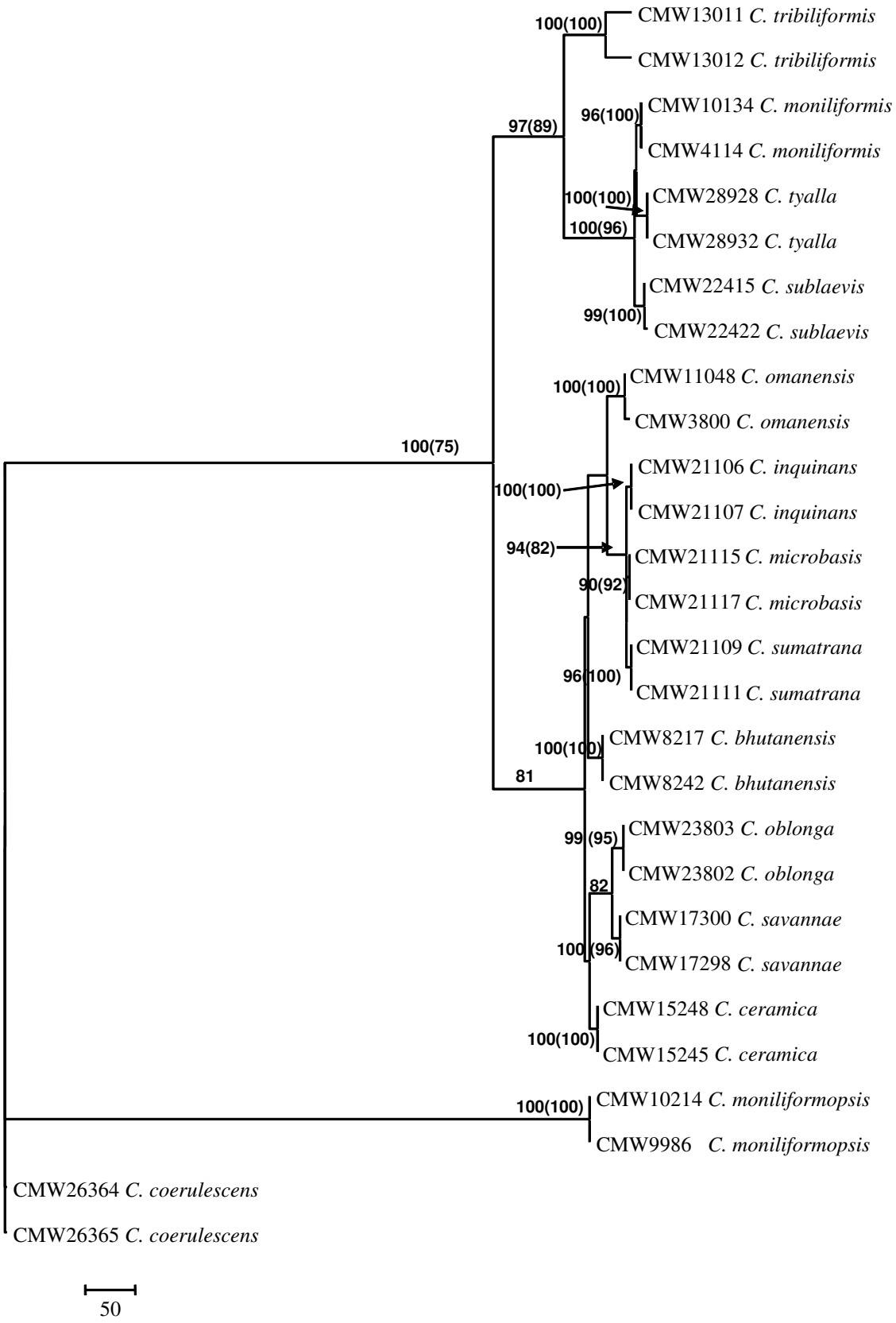


Figure 3. Phylogenetic tree with isolates representing species only in the *C. moniliformis* complex. The partition homogeneity test was congruent (P = 0.05). Two trees were obtained with parsimony analyses of which one was randomly selected to present here. The tree is described as follows; 1833 characters, 1169 are constant, 7 variable characters are parsimony uninformative, 657 variable characters are parsimony informative, tree length is 967 bases, Consistency Index = 0.9, Retention Index = 0.9, Rescaled Consistence = 0.8. For the ITS dataset the HKY+G model was selected in MrModeltest 2, for the BT dataset the HKY+I+G model was selected while the GTR+G model was selected for the EF dataset. Two thousand trees were discarded in the Bayesian analysis. Bootstrap values are indicated next to the branch while the Bayesian values are indicated in brackets.
(Treebase reference: http://purl.org/phylo/treebase/phylows/study/TB2:S13766.)



CHAPTER 1

Ceratocystis: Emerging evidence for discrete generic boundaries

Wingfield B.D., **Van Wyk M.**, Roos H. & Wingfield M.J. (2013) *Ceratocystis*: Emerging evidence for discrete generic boundaries. *Biodiversity Series* **12**: 57-64.

ABSTRACT

The genus *Ceratocystis* dates to 1890 and the description of the sweet potato black rot pathogen *Ceratocystis fimbriata*. Subsequently, *Ceratocystis* has a complicated taxonomic history involving substantial confusion with fungi now treated in *Ophiostoma*. Phylogenetic analyses based on DNA sequence data clearly show that *Ophiostoma* and *Ceratocystis* should be classified in different orders of fungi. DNA sequence data have enabled the recognition of distinct taxa amongst isolates previously treated as one species and have also assisted in the description of novel taxa from new collections. Consequently, the number of species treated in *Ceratocystis* has increased markedly, especially during the past decade. Analyses of DNA sequence data show clearly that *Ceratocystis* represents several distinct, well-defined phylogenetic lineages. The lineages correspond to the well-known species *Ceratocystis coerulescens, C. moniliformis* and *C. fimbriata*, each now known to represent a complex of species. Importantly, these groups can be characterized by distinct morphological characteristics and the species within the groups occupy defined ecological niches. Distinct generic status is currently being considered for these three groups.

INTRODUCTION

The genus *Ceratocystis* was established by Halsted (1890) to accommodate the causal agent of black rot of sweet potato. This fungus had black ascomata with long necks giving rise to slimy masses of ascospores. In this regard, it was similar to *Ophiostoma piliferum*, originally described as *Sphaeria pilifera* Fr. and first collected on wood in 1823 (Sydow & Sydow 1919). These two genera were considered distinct when first described; their relatedness became the subject of substantial taxonomic debate for almost 100 years. By the time of the first international workshop treating the Ophiostomatoid fungi in 1989, a term adopted to collectively accommodate *Ophiostoma* and *Ceratocystis* (Wingfield *et al.* 1993), it was generally accepted that the two genera represent distinct entities.

The first phylogenetic support for the separation of *Ophiostoma* and *Ceratocystis* was presented by Hausner *et al.* (1993a) using ~250 base pairs (bp) of large subunit ribosomal RNA gene (LSU) sequence. The conclusion was strengthened by the studies of Visser *et al.* (1995) based on 560 bp of ITS data and Jones and Blackwell (1998) who used 900 bp of small subunit ribosomal RNA (SSU) sequence. The two genera are now conclusively recognized as phylogenetically unrelated and classified in different taxonomic orders of the Sordariomycetes (Lutzoni *et al.* 2004). Anamorphs formerly assigned to *Chalara* are now classified in *Thielaviopsis,* with the former generic name now used for anamorphs in the *Helotiales*, *Leotiomycetes* (Paulin-Mahady *et al.* 2002). The hyphomycete genus *Ambrosiella* has also been connected phylogenetically to *Ceratocystis* using SSU (Cassar & Blackwell 1996) and LSU (Paulin-Mahady *et al.* 2002) analyses.

The first single-gene infrageneric nucleic acid analyses of *Ceratocystis* were reported by Wingfield *et al.* (1994), who showed that LSU phylogeny did not correlate with ascospores morphology. Witthuhn *et al.* (1998) used internal transcribed spacer (ITS) rDNA sequences to study species concepts in the *C. coerulescens* group, distinguishing *C. douglasii, C. rufipenni* and three forms of *C. coerulescens* referred to as species A, B and C; the latter were later named *C. pinicola, C. coerulescens* and *C. resinifera* (Harrington & Wingfield 1998). Three *Chalara* species (*Ch. australis, Ch. eucalypti, Ch. neocaledoniae*) were included in a former study and it was confirmed that these anamorph species were related to *Ceratocystis* as previously suggested. Witthuhn *et al.* (1999) subsequently presented the first phylogenetic treatment of a broader range

of *Ceratocystis* species, suggesting the existence of two major clades, the first including *C. adiposa, C. fagacearum, C. laricicola, C. moniliformis, C. paradoxa, C. pinicola, C. polonica, C. radicicola*, and *C. virescens,* and the second *C. albifundus* and *C. fimbriata,* based on ~1000 bp of the LSU.

During the last ten years, there has been a substantial increase in the number of species described in *Ceratocystis* (see references cited below). Many are morphologically cryptic and were discovered following phylogenetic analyses of strains previously considered conspecific. As with other fungi, the emergence of DNA sequence- based phylogenies allows much cleared species circumscription in *Ceratocystis*. More precise species concepts also allow a more refined interpretation of morphological characters and the ecological roles of these fungi. From these studies, three clear groups exist in *Ceratocystis*, which here we discuss as species complexes. The *C. coerulescens* complex includes at least eight distinct species. These include *C. fujiensis* and *C. polonica* in addition to the six listed above (Harrington & Wingfield 1998, Marin *et al.* 2005).

The complex of species related to *Ceratocystis fimbriata*, now clearly defined by Johnson *et al.* (2005), includes *C. acaciivora, C. albifundus, C. atrox, C. cacaofunesta*, *C. caryae, C. colombiana, C. fimbriata, C. fimbriatomima*, *C. larium*, *C. manginecans, C. papillata, C. neglecta, C. platani, C. pirilliformis, C. polychroma, C. obpyriformis, C. polyconidia C. populicola, C. smalleyi, C. tanganyicensis, C. tsitsikammensis, C. variospora and C. zombamontana* and some yet to be described new species (Barnes *et al.* 2003, Marin *et al.* 2003, Van Wyk *et al.* 2004b, 2007, 2009a, 2009b, 2010, Johnson *et al.* 2005, Engelbrecht *et al*. 2007, Kamgan *et al*. 2008, Rodas *et al*. 2008, Heath *et al*. 2009). The *C. moniliformis* complex includes numerous cryptic species now delimited as *C. bhutanensis, C. inquinans*, *C. omanensis*, *C. microbasis*, *C. moniliformis*, *C. moniliformopsis, C. oblonga, C. savannae*, *C. sumatrana, C. tribiliformis* (Yuan & Mohammed 2002, Al-Subhi *et al.* 2005, Van Wyk *et al.* 2006, Kamgan *et al*. 2008, Heath *et al*. 2009, Tarigan *et al.* 2010, 2011*)* and other undescribed species that are emerging from phylogenetic analyses of this group.

Single gene phylogenies provide a limited view of phylogenetic relationships. There is a general consensus that more than one gene region should be used for reliable assessment of phylogenetic relationships among and genetic boundaries between species (Baldauf 1999, Gill & Fast 2006, Schlegel 2003). Nuclear ribosomal regions, such as the LSU, SSU, and ITS were used

extensively for many ascomycete phylogenies (Lutzoni *et al.* 2004) and these regions are still useful. The ITS region is the region of choice for species differentiation within many genera, but it is problematic for some groups, for example *Fusarium,* where non-orthologous copies exist (O'Donnell & Cigelnik 1997). In addition, the ITS is insufficiently variable to allow clear distinctions among sibling species, for example *C. laricicola* and *C. polonica* (Witthuhn *et al.* 1999, Marin *et al.* 2003). Therefore, recent studies on *Ceratocystis* included sequences of several protein coding genes such as β-tubulin (*BENA*) and Translation Elongation Factor 1-α (*TEF1*-α) to provide sufficient resolution in phylogenetic analyses of closely related species (Barnes *et al.* 2003, Van Wyk *et al.* 2004a, 2004b, 2005, 2006, Marin *et al.* 2005). Although rRNA gene regions are not ideal for phylogenetic species recognition, phylogenies derived for *Ceratocystis* using these regions are congruent with those generated using protein coding genes (Barnes *et al.* 2003, Van Wyk *et al.* 2006). This provides confidence that *Ceratocystis* phylogenies generated using ribosomal genes are likely to accurately reflect whole genome relationships.

In this taxonomic and phylogenetic review of *Ceratocystis* at the generic level, we particularly emphasize developments during the past ten years. We especially attempt to provide an updated phylogeny for the genus using existing sequence data. Because protein coding gene sequence data are available only for the most recently described species, and given that there is sufficient congruency between the ribosomal sequences and protein coding genes phylogenies thus far generated for *Ceratocystis*, our focus is on the LSU and ITS ribosomal RNA sequences.

METHODS

All *Ceratocystis* ITS and LSU sequences used for phylogenetic reconstructions were obtained from GenBank (Table 1). Sequences were also obtained for species of the phylogenetically related anamorph genus *Thielaviopsis.* Because anamorphs of *Ceratocystis* were previously classified in *Chalara*, sequences attributed to species of this genus were evaluated using BLAST searches; those with significant similarity to *Ceratocystis* species were considered, and some are included in Table 1. Nine additional ITS sequences were newly generated for this study, as indicated in Table 1.

Petriella setifera was used as the outgroup for the LSU alignment. *Paecilomyces penicillatus* and *Lecythophora hoffmannii* were used as outgroups for the ITS sequences, after BLAST searches

demonstrated that they are the most closely related species to *Ceratocystis* for which ITS sequences are available on GenBank.

Sequences were aligned using the programme MAFFT version 5.8 (Katoh *et al.* 2002). Unique haplotypes were identified using Collapse Service (Villesen 2003) and duplicate haplotypes were removed from the data set. All regions containing gaps were replaced using codes generated using the GapCoder programme (Young & Healy 2003). Only parsimony-informative sites were used in the analyses. Phylogenetic relationships among species were determined using PAUP 4.0b10 (Swofford 2002), and trees were constructed using distance methods. Heuristic search options were used, with tree-bisection-reconnection. The model of evolution was determined using MODELTEST version 3.7 (Posada & Crandall 1998). Support for nodes was estimated using the bootstrap criterion (Felsenstein 1985) with 1000 replicates.

RESULTS

Sequences for 26 species of *Ceratocystis* were retrieved from GenBank (Table 1). Only ITS sequences were available for all described species. LSU sequences were available for only 13, and SSU for six species. Sequences for protein coding genes were available for some sets of species. *BenA* sequences were reported for 18 species, *TEF1-α* sequences were published for ten species, and MAT-2 HMG box sequences were available for 11 species. A limited number of other protein coding sequences exist for a few species (data not shown). Microsatellite primers were developed for several *Ceratocystis* species for population studies (Barnes *et al.* 2001, Geldenhuis *et al.* 2004, Steimel *et al.* 2004). These sequences were not considered further here because they are available only for a limited number of species, the primers are predominantly species-specific, and a genus-wide comparison was not feasible.

An LSU sequence of *Faurelina elongata* (DQ368625, based on CBS 126.78) has 100% identity with *Ceratocystis paradoxa* (Tang *et al.* 1997). Réblová *et al.* (2011) re-examined and resequenced this and a second strain identified as the same species from CBS and concluded that the sequence reported by Tang *et al.* (1997) was either based on a contaminant or mislabeling. Because of uncertainly about the identification of the strain, this sequence was not used in our phylogenetic analyses.

For *Thielaviopsis* anamorphs of *Ceratocystis,* SSU, LSU and ITS sequences were available for *T. australis, T. basicola, T. neocaledoniae, T. ovoidea, T. populi,* and *T. thielavioides*;. MAT 2 HMG box sequences were available for *T. australis* and *T. neocaledoniae*. ITS, TEF and β Tubulin were available for *T. ceramica*. SSU and LSU sequences were available for three species of *Ambrosiella*, namely *A. ferruginea, A. hartigii* and *A. xylebori.* The latter species was included in the multigene phylogeny by Spatafora *et al.* (2006), but none of the protein coding genes sequenced for *A. xylebori* are homologous to those sequenced for other *Ceratocystis* species. *Ambrosiella beaveri* is the most recently described *Ambrosiella* species and partial LSU and β Tublin sequence were provided in the description (Six *et al*. 2009). The lack of ITS sequence for *Ambrosiella* prevents us from placing the species within the context of our larger *Ceratocystis* phylogeny.

The ITS data set used in this study comprised 54 sequences, representing 26 *Ceratocystis* species and six *Thielaviopsis* species. The final data set had 615 characters of which 404 were uninformative, yielding 16796 most parsimonious trees with a heuristic search (tree length 615 steps, CI = 0.496, RI = 0.894, RC = 0.443, HI = 0.504). A representative tree based on these analyses is given in Figure 1. Sequence variation was observed within some species, suggesting that cryptic species may be present amongst isolates for which sequence data are available. One of these, *Ceratocystis fimbriata* f. sp. *platani* was already described recently as a distinct species (Engelbrecht & Harrington 2005).

For the LSU analysis, the data set comprised 587 characters, of which 358 were uninformative and excluded. Twenty most parsimonious trees were produced from a heuristic search and one is presented in Figure 2 (tree length 709 steps, CI = 0.539, RI = 0.729, RC = 0.393, HI = 0.461). Three main clades were distinguished in the LSU phylogeny, representing the *C. fimbriata* and *C. coerulescens* complexes (Clades 2 and 3) recognised in previous studies (Witthuhn *et al.* 1999) and Clade 1, which includes four *Thielaviopsis* spp. (*T. basicola, T. ovoidea, T. populi* and *T. thielaviopsis*). The remaining two *Thielaviopsis* species clustered with the *C. coerulescens* clade, and the residual *Ceratocystis* species did not group into any obvious clades in the LSU analysis.

The phylogenetic trees resulting from analysis of the ITS sequences (Figure 1) were largely congruent with the LSU phylogeny (Figure 2). There were three discrete clades (2, 3, and 4). Clades 2 and 3 comprised the *C. coerulescens* and *C. fimbriata* complexes, and clade 4 included

species resembling *C. moniliformis*. Because LSU sequences were only available for *C. moniliformis* and not other members of this species complex, this grouping is thus not reflected in the LSU phylogeny. Clade 1 from the LSU analysis, which included several *Thielaviopsis* species, was basal in the ITS tree and was not resolved as a monophyletic group but is indicated as clade 1 in the ITS figure for easy comparison with the LSU figure. This lack of monophyly possibly reflects the availability of only one sequence for each species, resulting in insufficient parsimoniously informative characters.

Ceratocystis paradoxa and *C. radicicola* grouped together in the ITS phylogeny, as did *C. fagacearum* and *C. adiposa.* Both of these smaller clades had strong bootstrap support. In the LSU phylogeny, there is support for a separation of *C. fagacearum* and *C. adiposa.*

DISCUSSION

DNA sequencing and phylogenetic analysis have provided researchers with new characters to separate closely related species of *Ceratocystis*. These analyses allow definition of important morphological characters for species delimitation, which correlate with phylogeny. This has resulted in a significant increase in the number of new *Ceratocystis* species being described.

There is a significant resource of DNA sequences currently available for *Ceratocystis* and on which to base a representative phylogeny, as reflected by the largely congruent gene trees for the ITS and LSU generated for this review. However, the absence of LSU sequences for about half the 26 *Ceratocystis* species considered resulted in some gaps in this phylogeny. Likewise, the paucity of ITS sequences for *Thielaviopsis* species resulted in inadequate placement in the ITS phylogeny.

Protein coding DNA sequences are available for about half the described *Ceratocystis* species, predominantly those described recently. A complete phylogeny for *Ceratocystis* based on protein coding genes is not yet possible, but we anticipate that it would be similar to the ITS phylogeny.

Despite these shortcomings, various patterns are obvious from the phylogenetic analyses provided here. It is clear that there are significant phylogenetic divisions within the current structure of the genus *Ceratocystis*. Three well supported groups are obvious in the ITS

phylogeny. These groups include species that morphologically closely resemble *C. fimbriata, C. coerulescens* and *C. moniliformis.* Although not fully resolved in the ITS tree, a fourth group emerges in the LSU phylogeny, including four of the six *Thielaviopsis* species sampled. The lack of support for this group probably reflects insufficient sampling, or it may also be possible that the group is an artefact of the LSU phylogeny.

The three major groups exemplified by *C. fimbriata*, *C. moniliformis* and *C. coerulescens* are supported by robust morphological characteristics. Species within these groups also share a high degree of ecological similarity. Species in the *Ceratocystis fimbriata* complex have distinctive ascomata with round bases lacking spines, relatively long necks with either convergent or divergent ostiolar hyphae and ascospores with hat-shaped sheaths (Hunt 1956, Upadhyay 1981, Engelbrecht & Harrington 2005, Van Wyk *et al.* 2012). Most of these species are important pathogens of woody angiosperms, but some, such as *C. fimbriata,* are pathogens of herbaceous root crops such as sweet potato and taro (Halstead 1980, Halstead & Fairchild 1981, Harrington *et al.* 2005). They lack specific associations with insects and are carried by insects such as flies and nitidulid beetles, with which they have only a casual association. All species studied have a unidirectional mating type switching system (Harrington & McNew 1997, 1998, Witthuhn *et al.* 2000).

Species in the *C. moniliformis* complex are superficially similar to those in the *C. fimbriata* complex. Ascospore morphology for species in the two groups is similar, but the bases of the ascomata in the species belonging to the *C. moniliformis* complex are typically covered in well-developed spines (Hunt 1956, Van Wyk *et al.* 2004a, 2006). In addition, all species have ascomatal necks that are attached to the ascomatal base by a disc-like structure. None of the species are primary pathogens and they appear to be mostly saprophytic. They also appear to lack specific associations with insect vectors. In this regard, *C. bhutanensis* is unusual in occurring in the galleries of the bark beetle *Ips schmutzenhoferi* (Van Wyk *et al.* 2004a, Kirisits *et al.* 2012). Although there are no published studies on mating systems in this clade, some species are similar to species in the *C. fimbriata* complex, undergoing unidirectional mating type switching (unpublished data).

Species in the *C. coerulescens* complex have round ascomatal bases lacking spines. Ascospores in this group are never hat-shaped like those of the *C. fimbriata* and *C. moniliformis* complexes

and are instead are typically oval, tending to have uniform sheaths. Ascospores of *C. polonica*, *C. laricicola* and *C. fujiensis* are smallest and appear to have one flat surface (Redfern *et al.* 1987, Harrington & Wingfield 1998, Marin *et al.* 2005). Most species occur on conifers, either as saprophytes causing blue stain or as pathogenic associates of bark beetles (Hunt 1956, Upadhyay 1981, Redfern *et al.* 1987). A few species also occur on hardwoods (Davidson 1944, Harrington *et al*. 1996, Kile *et al*. 1996, Upadhyay 1981).

In addition to these three obvious and well-supported monophyletic lineages in the ITS phylogeny, two smaller groups can be distinguished. These accommodate *C. adiposa /C. fagacearum* and *C. radicicola /C. paradoxa*. These two groups might reflect sampling limitations and it seems unwarranted to interpret their significance at this time. Witthuhn *et al.* (1999) previously remarked that *C. radicicola* and *C. paradoxa* were morphologically similar and pathogenic only to specific monocotyledonous hosts. It will likely be impossible to understand this part of the phylogeny until additional related species are sequenced.

Results of this and other studies on *Ceratocystis* species during the past decade provide support for the fact that the genus is polyphyletic. Phylogenetic data supported by morphological characters and habitat, strongly suggests that species in the *C. fimbriata*, *C. moniliformis* and *C. coerulescens* species complexes represent distinct monophyletic groups. The fact that a small number of species lie outside the three major groups for *Ceratocystis,* provides a taxonomic conundrum. The genus *Ceratocystis* must be reserved for species in the *C. fimbriata* complex. A new genus would then be proposed for species in the *C. moniliformis* complex, and *Endoconidiophora* removed from synonymy with *Ceratocystis* to accommodate the *C. coerulescens* complex. Further collections and analyses are clearly needed before generic placements could be reasonably suggested.

REFERENCES

Al-Subhi A.M, Al-Adawi A.O., Van Wyk M., Deadman M. & Wingfield M.J. (2005) *Ceratocystis omanensis*, a new species from diseased mango trees in Oman. *Mycological Research* **110**: 237–245.

Baldauf S.L. (1999) A search for the origins of animals and fungi: Comparing and combining molecular data. *American Naturalist* **154**: S178–S188.

Barnes I., Roux J., Wingfield B.D., Dudzinski M.J., Old K.M. & Wingfield M.J. (2003) *Ceratocystis pirilliformis*, a new species from *Eucalyptus nitens* in Australia. *Mycologia* **95**: 865–871.

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

Cassar S.C. & Blackwell M. (1996) Convergent origins of ambrosia fungi. *Mycologia* **88**: 596–601.

Davidson R.W. (1944) Two American hardwood species of *Endoconidiophora* described as new. *Mycologia* **36**: 300–306.

Engelbrecht C.J.B. & Harrington T.C. (2005) Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. *Mycologia* **97**: 57–69.

Engelbrecht C.J.B., Harrington T.C., Alfenas A. & Suarez C. (2007) Genetic variation in populations of the cacao wilt pathogen, *Ceratocystis cacaofunesta*. *Plant Pathology* **56**: 923-933.

Felsenstein J. (1985) Confidence intervals on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783–791.

Geldenhuis M.M., Roux J., Wingfield M.J. & Wingfield B.D. (2004) Development of polymorphic markers for the root pathogen *Thielaviopsis basicola* using ISSR-PCR. *Molecular Ecology Notes* **4**: 547–550.

Gill E.E. & Fast N.M. (2006) Assessing the microsporidia-fungi relationship: Combined phylogenetic analysis of eight genes. *Gene* **375**: 103–109.

Halsted B.D. (1890) Some fungous disease of sweet potato. *New Jersey Agricultural College Experiment Station Bulletin* **76**: 1–32.

Halsted B.D. & Fairchild D.G. (1891) Sweet-potato black rot. *Journal of Mycology* **VII**: 1–11.

Harrington T.C., Steimel J.P., Wingfield M.J. & Kile G.A. (1996) Isozyme variation and species delimitation in the *Ceratocystis coerulescens* complex. *Mycologia* **88**: 104-113.

Harrington T.C. & Wingfield M.J. (1998) The *Ceratocystis* species on conifers. *Canadian Journal of Botany* **76**: 1446–1447.

Harrington T.C. & McNew D.L. (1997) Self-fertility and unidirectional mating type switching in *Ceratocystis coerulescens*, a filamentous ascomycete. *Current Genetics* **32**: 52–59.

Harrington T.C. & McNew D.L. (1998) Partial interfertility among *Ceratocystis* species on conifers *Fungal Genetics and Biology* **25**: 44–53.

Harrington T.C., Thorpe D.J. Marinho V.L.A. & Furtado E.L. (2005) First report of black rot of *Colocasia esculenta* caused by *Ceratocystis fimbriata* in Brazil. *Fitopathology of Brasil* **30**: 88–89.

Hausner G., Reid J. & Klassen G.R. (1993a) Grouping of isolates and species of *Ceratocystis sensu lato* on the basis of molecular and morphological characters. In *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology and Pathogenicity. (Wingfield, M.J., Seifert, K.A. & Webber, J.F. Eds). APS Press, St. Paul. pp. 93–104.

Hausner G., Reid J. & Klassen G.R. (1993b) On the phylogeny of *Ophiostoma*, *Ceratocystis* s.s., *Microascus*, and relationships within *Ophiostoma* based on partial ribosomal DNA sequences. *Canadian Journal of Botany* **71**: 1249–1265.

Heath R.N., Wingfield M.J., Wingfield B.D., Meke G., Mbaga A. & Roux J. (2009) *Ceratocystis* species on *Acacia mearnsii* and *Eucalyptus* spp. in eastern and southern Africa including six new species. *Fungal Diversity* **34**: 41-67.

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

Johnson J.A., Harrington T.C. & Engelbrecht C.J.B. (2005) Phylogeny and taxonomy of the North American clade of the *Ceratocystis fimbriata* complex. *Mycologia* **97**: 1067–1092.

Jones K.G. & Blackwell M. (1998) Phylogenetic analysis of ambrosial species in the genus *Raffaelea* based on 18S rDNA sequences. *Mycological Research* **102**: 661–665.

Kamgan N.G., Jacobs K., De Beer Z.W., Wingfield M.J. & Roux J. (2008) *Ceratocystis* and *Ophiostoma* species including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* **29**: 37-59.

Katoh K., Misawa K., Kuma K. & Miyata T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**: 3059–3066.

Kile G.A., Harrington T.C., Yuan Z.Q., Dudzinski M.J. & Old K.M. (1996) *Ceratocystis eucalypti* sp nov, a vascular stain fungus from eucalypts in Australia. *Mycological Research* **100**: 571-579.

Lutzoni F., Kauff F., Cox C.J., McLaughlin D., Celio G., Dentinger B., Padamsee M., Hibbett D., James T.Y., Baloch E., Grube M., Reeb V., Hofstetter V., Schoch C., Arnold A.E., Miadlikowska J., Spatafora J., Johnson D., Hambleton S., Crockett M., Shoemaker R., Sung G.H., Lucking R., Lumbsch T., O'Donnell K., Binder M., Diederich P., Ertz D., Gueidan C., Hansen K., Harris R.C., Hosaka K., Lim Y.W., Matheny B., Nishida H., Pfister D., Rogers J., Rossman A., Schmitt I., Sipman H., Stone J., Sugiyama J., Yahr R., Vilgalys R. (2004) Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. *American Journal of Botany* **91**: 1446–1480.

Marin M., Castro B., Gaitan A., Preisig O., Wingfield B.D. & Wingfield M.J. (2003) Relationships of *Ceratocystis fimbriata* isolates from Colombian coffee-growing regions based on molecular data and pathogenicity *Journal of Phytopathology* **151**: 395–405.

Marin M., Preisig O., Wingfield B.D., Kirisits T., Yamaoka Y. & Wingfield M.J. (2005) Phenotypic and DNA sequence data comparisons reveal three discrete species in the *Ceratocystis polonica* species complex. *Mycological Research* **109**: 1137–1148.

O'Donnell K. & Cigelnik E. (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular. Phylogenetics and Evolution.* **7**: 103–116.

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

Posada D. & Crandall K.A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.

Redfern D.B., Stoakley J.T., Steele H. & Minter D.W. (1987) Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae*. *Plant Pathology* **36**: 467–480.

Rodas C.A., Roux J., Van Wyk M., Wingfield B.D. & Wingfield M.J. (2008) *Ceratocystis neglecta* sp. nov., infecting *Eucalyptus* trees in Colombia. *Fungal Diversity* **28**: 73-84.

Schlegel, M. (2003) Phylogeny of Eukaryotes recovered with molecular data: highlights and pitfalls. *European Journal of Protistology* **39**: 113–122.

Six D.L., Stone W.D., De Beer Z.W. & Woolfolk S.W. (2009) *Ambrosiella beaveri*, sp. nov., associated with an exotic ambrosia beetle, *Xylosandrus mutilatus* (Coleoptera: Curculionidae, Scolytinae), in Mississippi, USA. *Antonie Van Leeuwenhoek* **96**: 17-29.

Spatafora J.W., Sung G.H., Johnson D., Hesse C., O'Rourke B., Serdani M., Spotts R., Lutzoni F., Hofstetter V., Miadlikowska J., Reeb V., Gueidan C., Fraker E., Lumbsch T., Lucking R., Schmitt I., Hosaka K., Aptroot A., Roux C., Miller A.N., Geiser D.M., Hafellner J., Hestmark G., Arnold A.E., Budel B., Rauhut A., Hewitt D., Untereiner W.A., Cole M.S., Scheidegger C., Schultz M., Sipman H. & Schoch C.L. (2006) A five-gene phylogeny of Pezizomycotina. *Mycologia* **98**: 1018-1028.

Steimel J., Engelbrecht C.J.B. & Harrington T.C. (2004) Development and characterization of microsatellite markers for the fungus *Ceratocystis fimbriata*. *Molecular Ecology Notes* **4**: 215–218.

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

Sydow H. & Sydow P. (1919) Mykologische Mitteilungen. *Annales Mycologici* 17: 33–47.

Tang A.M.C., Jeewon R. & Hyde K.D. (1997) Phylogenetic utility of protein (RPB2, *β*-tubulin) and ribosomal (LSU, SSU) gene sequences in the systematics of Sordariomycetes (Ascomycota, Fungi) *Antonie van Leeuwenhoek* **91**: 327-349.

Tarigan M., Van Wyk M., Roux J., Tjahjono B. & Wingfield M.J. (2010) Three new *Ceratocystis* spp. in the *Ceratocystis moniliformis* complex from wounds on *Acacia mangium* and *A. Crassicarpa*. *Mycoscience* **51**: 53-67.

Tarigan M., Van Wyk M., Roux J., Tjahjono B. & Wingfield M.J. (2011) A new wilt and die-back disease of *Acacia mangium* associated with *Ceratocystis manginecans* and *C. acaciivora sp. nov*. in Indonesia. *South African Journal of Botany* **77**: 292-304.

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. & Wingfield M.J. (2004a) *Ceratocystis bhutanensis* sp nov., associated with the bark beetle *Ips schmutzenhoferi* on *Picea spinulosa* in Bhutan. *Studies in Mycology* **50**: 356–379.

Van Wyk M., Roux J., Barnes I., Wingfield B.D., Liew E.D., Assa B., Summerell B.A. & Wingfield M.J. (2004b) *Ceratocystis polychroma* sp nov., a new species from *Syzygium aromaticum* in Sulawesi. *Studies in Mycology* **50**: 273–282.

Van Wyk M., Al-Adawi A.O., Wingfield B.D., Al-Subhi A.M., Deadman M.L. & Wingfield M.J. (2005) DNA based characterization of *Ceratocystis fimbriata* isolates associated with mango decline in Oman. *Australasian Plant Pathology* **34**: 587–590.

Van Wyk M., Roux, J. Barnes I, Wingfield B.D. & Wingfield M.J. (2006) Molecular phylogeny of the *Ceratocystis moniliformis* complex and description of *C. tribiliformis sp nov*. *Fungal Diversity* **21**: 181–201.

Van Wyk M., Al Adawi A.O., Khan I.A., Deadman M.L., Al Jahwari A.A., Wingfield B.D., Ploetz R. & Wingfield

M.J. (2007) *Ceratocystis manginecans* sp. nov., causal agent of a destructive mango wilt disease in Oman and Pakistan. *Fungal Diversity* **27**: 213-230.

Van Wyk M., Wingfield B.D., Mohali S. & Wingfield M.J. (2009a) *Ceratocystis fimbriatomima,* a new species in the *C. fimbriata sensu lato* complex isolated from *Eucalyptus* trees in Venezuela. *Fungal Diversity* **34**: 175-185.

Van Wyk M., Wingfield B.D., Clegg P.A. & Wingfield M.J. (2009b) *Ceratocystis larium* sp. nov., a new species from *Styrax benzoin* wounds associated with incense harvesting in Indonesia. *Persoonia* **22**: 75-82.

Van Wyk M., Wingfield B.D., Marin M. & Wingfield M.J. (2010) New *Ceratocystis* species infecting coffee, cacao, citrus and native trees in Colombia. *Fungal Diversity* **40**: 103-117.

Villesen P. (2003) Collapse Service Available at: http://www.daimi.au.dk/~biopv/research/bioinformatics/collapse.php

Visser C., Wingfield M.J., Wingfield B.D. & Yamaoka Y. (1995) *Ophiostoma polonicum* is a species of *Ceratocystis sensu stricto*. *Systematic and Applied Microbiology* **18**: 403–409.

Wingfield M.J., Harrington T.C. & Solheim H. (1997) Two species in the *Ceratocystis coerulescens* complex from conifers in western North America. *Canadian Journal of Botany* **75**: 827–834.

Wingfield M.J., Seifert K.A. & Webber J. (1993) *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity.* American Phytopathological Society, St. Paul, Minnesota.

Wingfield B.D., Grant W.S., Wolfaardt J.F. & Wingfield M.J. (1994) rRNA sequence phylogeny is not congruent with ascospore morphology among species of *Ceratocystis sensu stricto*. *Molecular Biology and Evolution* **11**: 376–383.

Witthuhn R.C., Harrington T.C., Wingfield B.D., Steimel J.P. & Wingfield M.J. (2000) Deletion of the *MAT-2* mating type gene during uni-directional mating type switching in *Ceratocystis*. *Current Genetics* **38**: 48–52.

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

Witthuhn R.C., Wingfield B.D., Wingfield M.J., Wolfaardt M. & Harrington T.C. (1998) Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. *Mycologia* **90**: 96–101.

Young N.D. & Healy J. (2003) GapCoder automates the use of indel characters in phylogenetic analysis. *BMC Bioinformatics*. **4**: 6.

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

Table 1. GenBank and isolate/strain numbers of *Ceratocystis* (and anamorphs) ITS (internal transcribed spacer region) and LSU (large subunit) unique haplotypes used in the phylogenetic analyses.

Species	[a]Isolate/strain number	ITS GenBank number	LSU GenBank number
Ceratocystis adiposa	CMW1622	AF043606	AF043606
	C998, CBS600.74	AF275545	AF222481
C. albifundus	CMW247, PREM51641	AF043605	AF043605
	CMW17275	DQ250384	-
	C1042, CMW4062	-	AF275500
Thielaviopsis australis	C448	-	AF222450
T. basicola	C1515	DQ059579	-
	CBS414.52	-	AF222459
C. bhutanensis	CMW8394	AY528960	-
	CMW8399	AY528959	-
C. caryae	C1829	AY907035	-
	C1827	AY907034	-
C. coerulescens	CL8-44	AY214001	AY214001
	CL13-12	AY214000	-
C. douglasii	C324	U75626	-
C. eucalypti	C639	U75627	-
	C457	-	AF222482
C. fagacearum	CMW2651	AF043598	AF043598
	C1305	-	AF222483
C. fimbriata	CMW2220, PREM51644	AF043604	AF043604
C. fimbriata	*CMW14805, CBS115165, C1780	AY526297	-
C. cacaofunesta	*CMW15051	DQ520636	-
C. populicola	*CMW14798	EF070418	-
C. fimbriata	*CMW10844	AY177238	-
C. fimbriata	*CMW14797, C854	AY953382	-
		AF007749	-
C. fimbriata	* CMW15052	EF433298	-
C. fimbriata	* CMW5748	AY177237	-
C. populicola	C685, CBS11561	AY907028	AF275512
C. fujiensis	CMW1952	AY233924	-
C. laricicola	CMW1016	AF043600	AF043600

C. moniliformis	CMW8240	AY529000	-
	CMW3782	AF043597	AF043597
	C1007, CBS204.90	-	AF222487
C. moniliformopsis	CMW10214	AY528999	-
C. omanensis	CMW3800	DQ074743	-
	CMW11048	DQ074742	-
C. paradoxa	CMW1546	AF043607	AF043607
	C907, CMW1546	U75630	-
	C1001, CBS601.70	-	AF275498
C. pinicola	CMW1323, CMW490	AF043602	AF043602
	C488, CMW1311	-	AF275511
C. pirilliformis	CMW9043	AY428866	-
C. platani	UASWS0218	DQ399853	-
C. polonica	CMW7151	AY233896	-
	CMWC322, CMW0672, CBS133.38	-	AF043601
C. polychroma	*CMW11424	AY528966	-
	CMW11455	AY528973	-
	*CMW11436	AY528967	-
C. populicola	C995	AY907029	-
	C685	AY907028	-
C. radicicola	C1631	AF275492	-
C. resinifera	C666	U75618	-
C. rufipenni	C612	U75621	-
C. smalleyi	C1410	AY907031	-
C. tribiliformis	CMW13015	AY529004	-
C. variospora	C1965	AY907045	-
C. virescens	C1954	AY907043	-
	CMW3276	DQ061281	-
	C74	-	AF043603
	C69	-	U47824
T. neocaledoniae	CBS149.83	-	AF222471
T. ovoidea	C1376	AF275484	-
	C1375, CBS354.76	-	AF275502
T. populi	C2049	AY423551	-
	C1369, CBS486.71	-	AF275501
T. thielavioides	C1630	AF275491	-
	C1509, ICMP11355	-	AF275504

	C1630, CBS543.69	-	AF275507
Paecilomyces penicillatus	CBS448.69	AY624194	-
Lecythophora hoffmannii	DUMC134.97	AY853253	-
Petriella setifera	C42314	-	AF043596

* Sequences generated for this study.

[a] CMW – Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), South Africa; C – Culture collection of T. C. Harrington; CBS – CBS Fungal Diversity Centre, Netherlands; PREM – National Collection of Fungi, South Africa; ICMP - International Collection Of Micro-organisms From Plants, New Zealand; DUMC – Duke University Medical Centre, USA

Figure 1. A representative most parsimonious tree produced as the result of a heuristic search analysis using the available LSU DNA sequence data from *Ceratocystis* and related species. Bootstrap values over 60 are given above the branch nodes. The three clades (1, 2 and 3) are demarcated and labelled.

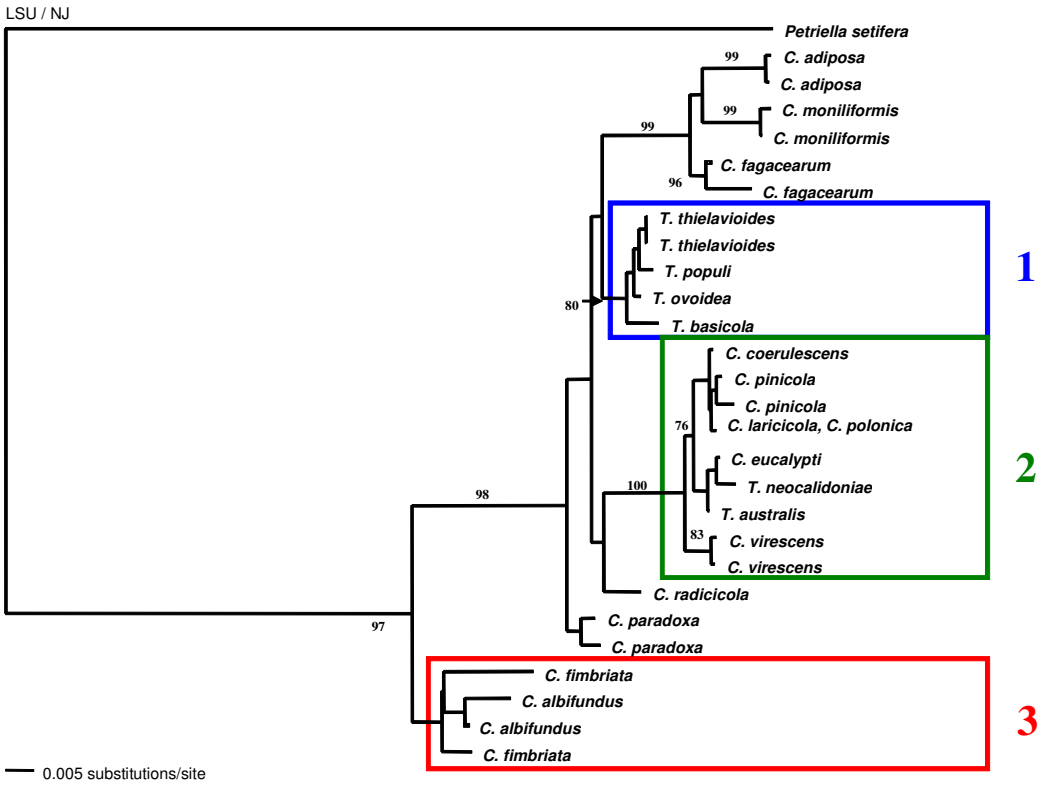
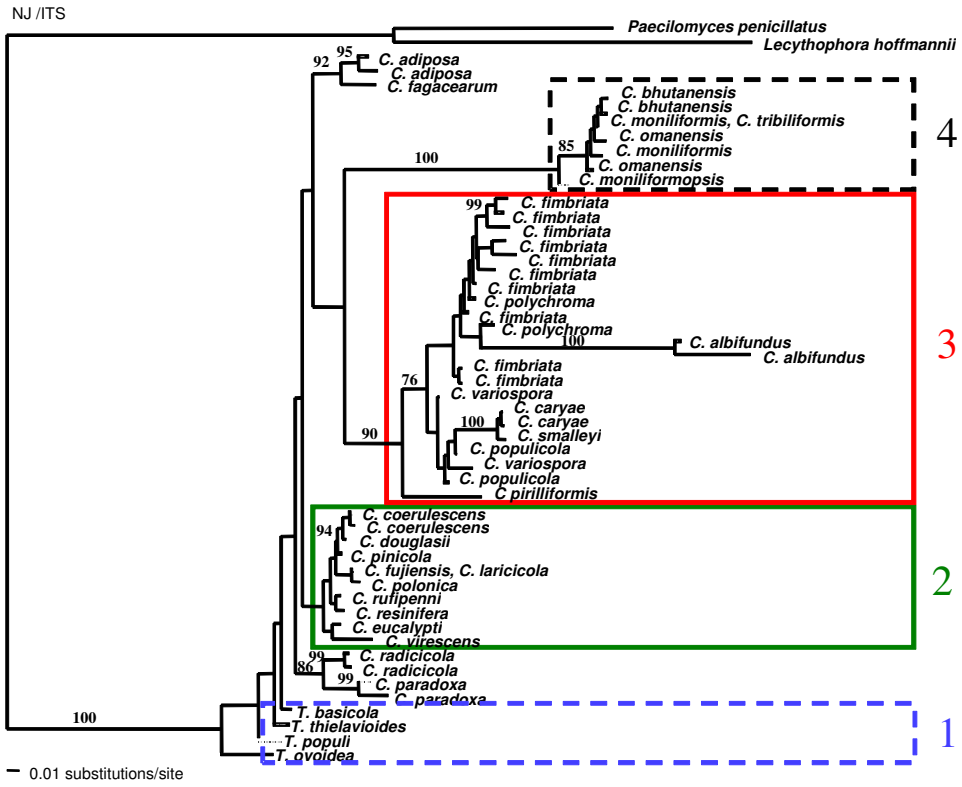


Figure 2. One of a number of most parsimonious trees generated from ITS DNA sequence data obtained from *Ceratocystis* and related species. Boot strap values are stated above the branch nodes. The four clades (1, 2, 3 and 4) are demarcated and labelled. Clades1, 2 and 3 are equivalent to the clades so numbered in Figure 1.



CHAPTER 2

Ceratocystis species in the *Ceratocystis fimbriata* complex

Van Wyk M., Wingfield B.D. & Wingfield M.J. (2012). *Ceratocystis* species in the *Ceratocystis fimbriata* complex. *Biodiversity Series* **12**: 65-76.

ABSTRACT

Ceratocystis fimbriata was described in 1891 from sweet potato (*Ipomoea batatas*) in the USA, and subsequently was recorded from many plant host genera and geographic regions. The first suggestion that *C. fimbriata* represents a species complex was raised in the 1960's but it was only in the 1990's, when DNA sequence comparisons became available, that discrete groupings in the *C. fimbriata* complex emerged. The first segregate species was *C. albifundus*, a serious pathogen of *Acacia mearnsii* in South Africa, distinguished primarily by DNA sequence comparisons, but also on morphological characters. Recently, numerous other cryptic species have been described in the complex. *Ceratocystis fimbriata sensu stricto* specifically represents the sweet potato pathogen from the USA and isolates phylogenetically related to it. Some of the newly described species are host specific while others have broad host ranges; the specificity of others has not yet been considered. Large numbers of strains reside in distinct phylogenetic clades for which names have yet to be assigned. Presently, the *C. fimbriata* complex is defined based on phylogenetic inference and is distinct from at least two other major lineages in *Ceratocystis* based on morphology and ITS sequence comparisons. Most species in the *C. fimbriata* complex are difficult to distinguish based on morphology, making DNA sequence comparisons essential for reliable identification.

INTRODUCTION

Ceratocystis fimbriata was described by Halsted (1890) from *Ipomoea batatas* as the type of its genus. In the original description, the ascospores were confused with conidia, and the perithecia were interpreted as pycnidia. This error was corrected by Höhnel (1918) and independently by Elliot (1923). Subsequently, the taxonomy of *Ceratocystis* was regularly intermingled with the genus *Ophiostoma*, with some authors treating the genera as distinct and others interpreting them collectively (Hunt 1956, Upadhyay 1981, Samuels 1993).

The taxonomy of the so-called Ophiostomatoid fungi was the main focus of the international workshop on *Ceratocystis* and *Ophiostoma* in 1990 (Wingfield *et al.* 1993). Although there was general consensus that *Ceratocystis* and *Ophiostoma* represented discrete genera, it was later that DNA sequence data provided evidence to support this view. Hausner *et al*. (1992, 1993a, b) and Spatafora & Blackwell (1994) showed that these two genera are distantly related and should be classified in different orders of Ascomycota. As summarized by B. Wingfield *et al.* (2012), subsequent studies added support to this view. Subsequent to the discovery of *C. fimbriata*, fungi identified as that species were isolated from numerous hosts from six different continents (Kile 1993). Webster & Butler (1967) provided the first evidence that this taxon might represent an aggregate of cryptic species. Despite their insightful studies, approximately two decades passed before a distinctive pathogen of *Acacia mearnsii* in South Africa, initially treated as *C. fimbriata* (Morris *et al*. 1993), was segregated as *C. albifundus* (Morris *et al.* 1993, Wingfield *et al*. 1996).

Witthuhn *et al.* (1999) provided the first evidence that *Ceratocystis* is comprised of discrete taxonomic lineages. Recent research (Barnes *et al*. 2003, Van Wyk *et al*. 2004, Engelbrecht & Harrington 2005, Johnson *et al*. 2005, Marin *et al*. 2005, Heath *et al*. 2009, Tarigan *et al*. 2010, Van Wyk *et al*. 2010, 2011) add substance to this view and it is probable that *Ceratocystis* as delimited for the past 20 years (here referred to as *sensu lato* = *s.l.*) will be sub-divided shortly into segregate genera (Wingfield *et al*. 2012).

One of the obvious monophyletic lineages within *Ceratocystis* accommodates species of the *C. fimbriata* complex (Wingfield *et al.* 2012). Most of these species are important pathogens of angiosperm plants (Figure 1-6) and especially trees (Kile 1993). They are characterised by ascomata with smooth bases and necks lacking a collar. Ascospores have hat-shaped sheaths

(Hunt 1956, Upadhyay 1981, Van Wyk *et al*. 1991) and anamorphs classified in *Thielaviopsis* Went (Paulin-Mahady *et al.* 2002). These anamorphs have conidia that can be either cylindrical, barrel-shaped or both and most produce dark chlamydospores (Paulin-Mahady *et al*. 2002). Unlike some other species of *Ceratocystis*, members of the *C. fimbriata* complex lack specific insect associates and most are casually vectored by insects such as flies (*Diptera*), picnic beetles (*Nitidulidae*) or non-specific ambrosia beetles (Himelick & Curl 1958, Moller & De Vay 1968, Juzwick & French 1983, Kirschner 2001, Kirisits 2004). These insects are hypothesised to be attracted to the sweet aromas that these species have (Lanza *et al.* 1976, 1977).

With the common use of phylogenetic inference to define species boundaries, numerous cryptic species that would have been assigned previously to *C. fimbriata* have been described. Thus, Barnes *et al*. (2003) described *C. pirilliformis* from wounds on *Eucalyptus* in Australia. Van Wyk *et al*. (2004) described *C. polychroma* associated with dying *Syzygium aromaticum* and damage by *Hexamitodera semivelutina* (*Coleoptera: Cerambycidae*) in Sulawesi, Indonesia. Engelbrecht & Harrington (2005) described *C. cacaofunesta* as a serious pathogen of cacao trees in Brazil. These authors also elevated *C. fimbriata f. sp. platani,* the causal agent of canker stain of *Platanus* in the United States and Europe, to species status as *C. platani* (Engelbrecht & Harrington 2005). In the same study, they restricted *C. fimbriata sensu stricto* (*s.s.*) specifically to the sweet potato pathogen. Johnson *et al*. (2005) described *C. smalleyi* and *C. caryae* as pathogens of *Carya* in the USA. *Ceratocystis populicola* was described as a pathogen of *Populus* in the USA and Poland while *C. variospora,* was reinstated as a distinct species (Johnson *et al.* 2005) on *Quercus* in the USA.

A serious pathogen of mango trees in Oman first identified as *C. fimbriata* (Van Wyk *et al.* 2005, Al-Adawi *et al*. 2006) was recognised as the discrete species *C manginecans*, and was also found in Pakistan (Van Wyk *et al*. 2007a). Another recently recognised species is *C. atrox*, found on *Eucalyptus* trees damaged by the bulls-eye borer (*Phoracantha acanthocera*; *Coleoptera*: *Cerambicydae*) in Australia (Van Wyk *et al*. 2007b). Rodas *et al*. (2008) described *C. neglecta* associated with wounds on *Eucalyptus* in Colombia. Likewise, a pathogen of *Rapanea melanophloeos*, *C. tsitsikammensis*, was described from South Africa (Kamgan *et al*. 2008) and *C. fimbriatomima* was identified as a vascular staining fungus on *Eucalyptus* in Venezuela (Van Wyk *et al*. 2009). During a disease survey in Africa Heath *et al.* (2009) evaluated *Ceratocystis* spp. infecting wounds on plantation-grown non-native *A. mearnsii* and *Eucalyptus* spp. They

reported four new new species in the *C. fimbriata* species complex and described them as *C. obpyriformis*, *C. polyconidia*, *C. tanganyicensis*, and *C. zombamontana* (Heath *et al.* 2009).

A serious wilt disease of *Acacia mangium* was in Indonesia was inspected to assess the probable causal agent (Tarigan *et al*. 2010); a previously unknown species of *Ceratocystis* was described as *C. acaciivora* and noted to be a close relative of *C. manginecans*, which kills mango in Oman and Pakistan (Tarigan *et al*. 2010). Recently, a disease survey of *Eucalyptus* trees in Ecuador resulted in the discovery of three new species complex, which were described as *C. curvata*, *C. ecuadoriana* and *C. diversiconidia* (Van Wyk *et al*. 2011).

Species of the *C. fimbriata* complex are currently recognised based primarily on DNA sequence comparisons. Morphological support for some cryptic species exists, but this is becoming increasingly difficult. Mating tests and the biological species approach have also been useful to distinguish among closely related species (Engelbrecht & Harrington 2005, Johnson *et al*. 2005). Some species apparently have high host specificity and this can be useful in defining or identifying species. The following sections treat these four approaches to define species in the *C. fimbriata* complex.

MORPHOLOGICAL CHARACTERS

All species of *Ceratocystis* have teleomorph and anamorph structures that are morphologically similar. Their ascomata have globose bases giving rise to necks of variable length, terminating in ostiolar hyphae. Asci are consistently evanescent and generally are not seen. The anamorphs of *Ceratocystis* species were traditionally treated in *Chalara* but are now accommodated in *Thielaviopsis* (Paulin-Mahady *et al.* 2002). *Thielaviopsis* anamorphs are characterised by two kinds of conidia, one produced by phialides, and the second pigmented chlamydospores produced in chains at the tips of specialized hyphae (Paulin-Mahady *et al.* 2002).

Species of *Ceratocystis* can be divided into three well-defined groups based on morphological features, i.e. the species complexes around *C. fimbriata*, *C. moniliformis* and *C. coerulescens* (B. Wingfield *et al*. this volume). The main morphological character that distinguishes members of the *C. moniliformis* complex from members of the *C. fimbriata* complex is conical spines on the ascomatal bases (Figure 7–9); they also have distinctive ‘collars’ at the bases of the ascomatal

necks (Figure 8). Species in the *C. fimbriata* complex have dark brown chlamydospores with thick walls, structures that are absent in anamorphs of the *C. moniliformis* complex (Figure 10). Species in the *C. fimbriata* complex have ascospores with hat-shaped sheaths (Figure 11), which differ from the elongate or orange section-shaped ascospores (Figure 12) of the *C. coerulescens* complex.

Within the *C. fimbriata* complex, there are minor morphological differences between species (Van Wyk *et al*. 2004, Engelbrecht & Harrington 2005). Species such as *C. pirilliformis*, *C. polychroma*, *C. platani, C. cacaofunesta, C. variospora*, *C. populicola*, *C. smalleyi* and *C. caryae* differ in dimensions of key morphological structures such as perithecial bases, necks, phialides and conidia (Barnes *et al*. 2003, Van Wyk *et al*. 2004, Engelbrecht & Harrington 2005, Johnson *et al*. 2005). Differences in the shapes of the phialides and the presence or absence of various forms of conidia and chlamydospores also help to define species (Barnes *et al*. 2003, Van Wyk *et al*. 2004, Engelbrecht & Harrington 2005, Johnson *et al*. 2005, Van Wyk *et al*. 2005, 2006, 2007, 2008, 2009, 2010, 2011). The differences between these character states are relatively minor and would be difficult for those inexperienced with these fungi to interpret. As additional species are recognized, the morphological differences between them are also becoming increasingly inconspicuous.

PHYLOGENETIC INFERENCE

The use of DNA sequence data to distinguish species in the *C. fimbriata* complex dates to the study by Wingfield *et al.* (1996), who showed that a fungus previously identified as *C. fimbriata* displayed several distinct morphological features. It also differed from *C. fimbriata* based on sequences of the internal transcribed spacer (ITS, including the 5.8S rDNA). The fungus, an important pathogen of *A. mearnsii* in South Africa (Morris *et al.* 1993, Roux *et al.* 2012) was subsequently described as *C. albifundus* (Wingfield *et al.* 1996).

The ITS has emerged as the most useful region for rapid and reliable identification of species within the *C. fimbriata* complex. For example, *C. pirilliformis*, another species initially confused with *C. fimbriata,* was recognized as distinct based on distinct ITS region sequences (Barnes *et al*. 2003). Similarly, Johnson *et al*. (2005) used the ITS gene to distinguish among strains previously regarded as *C. fimbriata,* describing them as *C. variospora*, *C. populicola*, *C. smalleyi*

and *C. caryae*. Since this first application of the ITS gene for species recognition, at least 25 species (Table 1) have been described in the *C. fimbriata* complex.

As of January 2011, 236 database entries were found in GenBank specifically for fungi treated as *C. fimbriata*. One hundred and sixteen (49%) of these sequences are for the ITS. Other accessions are for Translation Elongation Factor 1-α (*TEF1-α;* 3%), part of the β-tubulin gene (*BenA;* 5%), the mating type gene (MAT; 9%), microsatellite regions (22%), ribosomal large subunit (LSU; 4%), ribosomal small subunit (SSU 4%) and other gene regions (4%). A complicating factor in dealing with these accessions is that many sequences represent the *C. fimbriata* species complex in the broad sense and do not reflect the recent recognition of cryptic species in the group.

Phylogenetic analyses of the ITS data for the *C. fimbriata* complex available in GenBank generated 1475 most parsimonious phylogenetic trees, of which one was selected for presentation (Figure 13). A total of 114 isolates were included and *C. virescens* (Davidson) Moreau was selected as the out group. With this tree, we could identify species within the *C. fimbriata* complex that have not yet been described (Figure 3). Furthermore, at least two major lineages in the *C. fimbriata* complex clade were observed.

Harrington (2000) recognised three lineages in the *C. fimbriata* complex and defined them as the Asian, Latin American and North American clades. The Asian clade consisted of isolates from *Ficus* (fig), *Colocasia* (taro), namely *C. pirilliformis* and *C. polychroma* (Johnson *et al.* 2005). The North American clade included the tree pathogens *C. populicola, C. variospora, C. smalleyi* and *C. caryae* (Johnson *et al.* 2005). The Latin American clade has been the most extensively studied and included *C. fimbriata s.s., C. platani, C. cacaofunesta* and a *Xhanthosoma* pathogen (Johnson *et al.* 2005).

The biogeographical interpretation of the clades and species as defined by Harrington (2000) is open to debate. In the analysis presented here, it is not possible to make a clear distinction among these three clades. In our analyses, the bootstrap support for the Asian and North American clades as described by Harrington (2000) is low (Figure 13). We thus prefer to treat the so-called Asian and North American clades as one group. One of the two clades that emerged from our analysis included the newly described species *C. albifundus, C. atrox*, *C. caryae, C. larium*, *C. obpyriformis*, *C. pirilliformis*, *C. polyconidia*, *C. polychroma, C. populicola*, *C. smalleyi*, *C.*

variospora and *C. zombamontana* that include isolates from China, Cuba, Hawaii and Fiji. It would be misleading to suggest that it has an American phylogenetic base. *Ceratocystis albifundus* is probably native to southern Africa (Barnes *et al*. 2005, Roux *et al*. 2007), and it is puzzling to suggest that species in this clade have an Asian origin. The second clade in our study included species such as *C. acaciivora*, *C. colombiana*, *C. curvata*, *C. diversiconidia, C. fimbriata s.s., C. cacaofunesta, C. ecuadoriana*, *C. fimbriatomima*, *C. manginecans*, *C. neglecta, C. papillata*, *C. platani*, *C. tanganyicensis*, *C. tsitsikammensis*. This clade included isolates from countries other than Latin America, including; Oman, China, Switzerland, France, Greece, South Africa, Uganda, Congo and Hawaii (Table 1, Figure 13) and it is illogical to refer to it as having a predominately Latin American connection.

Single gene region phylogenetic analyses are increasingly questioned and often regarded as insufficient (Barrett *et al.* 1991, Rokas *et al*. 2003, Ciccarelli *et al*. 2006, Robbertse *et al*. 2006). For *Ceratocystis*, there are several examples where the ITS region alone has failed to distinguish among closely related species. For example, *C. laricicola* Redfern & Minter and *C. polonica* (Siemaszko) Moreau could not be distinguished using this region (Witthuhn *et al*. 1998, 1999). Similarly, for the *C. moniliformis* complex, there are essentially no differences in ITS sequences among the described species (Van Wyk *et al*. 2006).

Multigene analyses have recently been applied to taxonomic questions of species delimitation with regards to the phylogenetic species concept in *Ceratocystis* (Marin *et al*. 2005, Van Wyk *et al*. 2004a, 2006, 2007a,b, 2010, 2011). Amongst the regions used are the *BenA*, TEF1-α, and the mating type idiomorphs. Multigene phylogenetic analyses have shown that *Thielaviopsis* is the appropriate genus for anamorphs of *Ceratocystis* (Paulin-Mahady *et al*. 2002). Our view is that multiple gene analyses should routinely be used to delimit species of *Ceratocystis,* although for the *C. fimbriata* complex the ITS region provides the most useful insight into species boundaries. Despite this, there is a need to seek new markers to improve resolution of cryptic taxa in this group. These will most likely be derived from whole genome sequencing, such as the effort for *C. fimbriata s.s.* that has recently been completed (Wilken *et al.* 2010).

MATING COMPATIBILITY

Species in the *C. fimbriata* complex appear to share the unusual behaviour of uni-directional mating type switching (Webster & Butler 1967, Harrington & McNew 1997, Witthuhn *et al.* 2000). In many ascomycetes, there are two idiomorphs responsible for sexual compatibility, the MAT-1 and MAT-2 alleles, located at a single locus. Strains containing both idiomorphs are self-fertile. However, nearly 50% of single ascospore isolates from such self-fertile isolates are self-sterile. This phenomenon is known as uni-directional mating type switching (Mathieson 1952, Perkins 1987). Strains that have lost the MAT-2 idiomorph are referred to as MAT-1 and are self-sterile. Self-sterile MAT-1 strains are observed commonly in the laboratory and can only reproduce sexually with strains containing the MAT-2 idiomorph. Occasionally self-sterile MAT-2 strains are found but the genetic basis for this loss of fertility is unknown. They can be recognised by their ability to mate successfully with MAT-1 self-sterile isolates.

Application of mating experiments to determine species boundaries can be a difficult and lengthy process. In theory, there is a 50% chance of obtaining self-sterile MAT-1 isolates from a fertile perithecium and this is easily achieved. To obtain self-sterile MAT-2 isolates, is more challenging as it requires a mutation or deletion in the MAT-1 gene. These isolates can be recognized by sectoring in vegetatively growing cultures (Harrington & McNew 1997). The search for self-sterile MAT-2 isolates is of variable complexity for different *Ceratocystis* species.

Interferility between strains observed in the laboratory might not reflect actual mating in nature (Harrington & Rizzo 1999). Mating recognition between closely related fungal species is not uncommon and makes such crosses difficult to interpret. In some cases, while interspecies mating occurs, the resulting hybrid offspring are less fit than the parents. This may be reflected by reduced viability of the ascospores, which can be measured. In other cases, the F1 backcrosses are viable but infertile (Harrington & McNew 1997). Hybrid breakdown can also occur when the F1 is fertile for one generation but then their viability reduces (Harrington *et al.* 2002).

Interspecies mating was used effectively in taxonomic studies to distinguish *C. lariciola* and *C. polonica*. These are two very closely related species with an identical ITS region (Witthuhn *et al.* 1998), which were mated to obtain clarity regarding their species boundaries (Harrington & McNew 1998, Harrington *et al.* 2002). Some strains of these two species mated and produced

perithecia with viable ascospores, thus demonstrating interfertility. However, the perithecia produced low numbers of ascospores and cultures from of F1 progeny grew slower and produced few perithecia. The conclusion was that these two species are closely related, but distinct based on physiological specialization to their hosts and adaptation to their different bark beetle vectors (Harrington *et al*. 2002).

Johnson *et al*. (2005) attempted to use mating studies to support their view that isolates within the *C. fimbriata* complex from *Carya* trees in the USA are genetically different. The two groups of strains were morphologically and phylogenetically distinct, but produced fertile F1 progeny. Nonetheless, the authors designated the two groups as distinct species, *C. caryae* and *C. smalleyi*. They hypothesised that *C. smalleyi* was in the process of diverging from *C. caryae* (Johnson *et al.* 2005).

Ferreira *et al.* (2010) used mating studies as well as microsatellite markers to show that the species within the *C. fimbriata s.l.* complex, specifically from Brazil, represent populations rather than distinct species. They mated isolates from Brazil with each other and with *C. fimbriata s.s.* and obtained some fertile offspring and thus argued not to elevate the isolates to species level.

HOST SPECIFICITY

Host specificity can provide a useful indication of species boundaries in plant pathogenic fungi. The *C. fimbriata* complex includes numerous important plant pathogens (Kile 1993, Baker *et al*. 2003, Johnson *et al*. 2005, Van Wyk *et al*. 2007a, Tarigan *et al.* 2010). However, relatively little research has been conducted on host specificity for the *C. fimbriata* complex although some species are reported to have high specificity. One of the best examples is *C. platani*, which occurs only on *Platanus* (plane) trees in the USA (Engelbrecht & Harrington 2005) where it is considered native and in Europe as an introduced, invasive pathogen (Ocasio-Morales *et al.* 2007). Another example is *C. cacaofunesta*, known only as a pathogen of cacao trees (Baker *et al*. 2003). In countries where *C. cacaofunesta* occurs, other *Ceratocystis* species are also common, however they occur on hosts such as coffee and citrus but not on cacao trees, and *C. cacaofunesta* has not been isolated from these other hosts (Baker *et al*. 2003, Marin 2004).

Other species, including some important pathogens, have broad host ranges. *Ceratocystis albifundus* has been isolated from eight different host genera in Africa, but is known as a pathogen only on *Acacia mearnsii*, a non-native in the area where the fungus occurs naturally (Roux *et al*. 2007, this volume). Another example is groups of isolates first discovered as pathogens of coffee in Colombia (Marin *et al*. 2003) and that have been described recently as *C. colombiana* and *C. papillata*. These species were were subsequently isolated from other host species, including native trees in Colombia (Marin 2004, Van Wyk *et al*. 2010).

Although host specificity of some strains was sometimes reported when they were isolated, this phenomenon was generally ignored as all fungi resembling *C. fimbriata* were treated as a single species (Webster & Butler 1967). Many of the newly described species have not been tested for host specificity. For example, although *C. pirilliformis* and *C. atrox* have been isolated only from *Eucalyptus* trees, this does not necessarily mean they occur only on this host. Clearly, more work is needed to understand the host specificity of most species in the *C. fimbriata* complex.

CONCLUSIONS

A contemporary view of *C. fimbriata* is that it represents a complex of species. Only a small number have been described and many remain to be or discovered or delimited. A firm foundation for the recognition of new and cryptic taxa is provided by the restriction of *C. fimbriata s.s.* to the sweet potato black rot pathogen.

Species of the *C. fimbriata* complex are most easily recognised by phylogenetic inference. While morphological characters can be found to recognise species, these are not always obvious. As additional species are described, it will become increasingly difficult to identify species based on morphology. Thus, reliance on DNA sequence comparisons will be increasingly strong.

Most studies on the taxonomy of species in the *C. fimbriata* complex have relied on ITS data to distinguish species, but this region fails in some cases. Sequences of additional genes, such as β-tubulin, *TEF1-α* and the MAT-2 gene, will be useful in delimiting taxa in this group. Furthermore, whole genome sequences such as that recently completed for *C. fimbriata s.s.* will identify new and informative gene regions that will improve the resolution of phylogenetic analyses in the future.

Interfertility studies are useful to define species boundaries for some species in the *C. fimbriata* complex. However, this approach is laborious, time intensive and the results do not always correlate with phylogenetic and morphological conclusions. Taxonomic conclusions based on these tests should be viewed with circumspection.

Host specificity provides useful information supporting the delimitation of some species of *Ceratocystis*. However, there are species with apparently broad host ranges and others that occupy a common ecological niche. The natural hosts of many species of the *C. fimbriata* complex are unknown, and several species have apparently undergone host shifts from native to non-native hosts.

Some species in the *C. fimbriata* complex have insect associations but little is known about their specificity. It is generally accepted that these fungi are carried by a wide range of generalist insects attracted to the sweet aromas that the fungi produce but this has not been tested in all species. However, the assumption that the vectors are non-specific might be incorrect for some species.

The phylogenetic structure of the *C. fimbriata* complex is being clarified by ITS sequences, revealing terminal clades that may represent undescribed taxa and larger, more inclusive clades that might eventually be treated at the generic level.

Many species in the *C. fimbriata* complex are important pathogens are easily moved between different geographic regions. A robust, accurate and reliable system for identification of species in this group is enormously important for quarantine purposes and fundamental important to global food and plant security.

REFERENCES

Al Adawi A.O., Deadman M.L., Al Rawahi A.K., Al Maqbali Y.M., Al Jahwari A.A., Al Saadi B.A., Al Amri I.S. & Wingfield M.J. (2006) Aetiology and causal agents of mango sudden decline disease in the Sultanate of Oman. *European Journal of Plant Pathology* **116**: 247–254.

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

Barnes I., Nakabonge G., Roux J., Wingfield B.D. & Wingfield M.J. (2005) Comparison of populations of the wilt pathogen *Ceratocystis albifundus* in South Africa and Uganda. *Plant Pathology* **54**: 189–195.

Barnes I., Roux J., Wingfield M.J., Old K.M. & Dudzinski M. (2003) *Ceratocystis pirilliformis*, a new species from *Eucalyptus nitens* in Australia. *Mycologia* **95**: 865–871.

Barrett M., Donoghue M.J. & Sober E. (1991) Against consensus. *Systematic Zoology* **40**: 486–493.

Ciccarelli F.D., Doerks T., Von Mering C., Creevey C.J., Snel B. & Bork P (2006) Toward automatic reconstruction of a highly resolved tree of life. *Science* **311**: 1283–1287.

Elliott J.A. (1923) The ascigerous stage of the sweet potato black-rot fungus. *Phytopathology* **13**: 56.

Engelbrecht C.J.B. & Harrington T.C. (2005) Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. *Mycologia* **97**: 57–69.

Ferreira E.M., Harrington T.C., Thorpe D.J. & Alfenas A.C. (2010) Genetic diversity and interfertility among highly differentiated populations of *Ceratocystis fimbriata* in Brazil. *Plant Pathology* **59**:721–735.

Halsted B.D. (1890) Some fungous disease of the sweet potato. *Agricultural College Experiment Station Bulletin* **76**: 1–32.

Harrington T.C. (2000) Host specialization and speciation in the American wilt pathogen *Ceratocystis fimbriata*. *Fitopatologia Brasileira* **25**: 262–263.

Harrington T.C. & McNew D.L. (1997) Self-fertility and uni-directional mating type switching in *Ceratocystis coerulescens*, a filamentous ascomycete. *Current Genetics* **32**: 52–59.

Harrington T.C. & McNew D.L. (1998) Partial interfertility among the *Ceratocystis* species on Conifers. *Fungal Genetics and Biology* **25**: 44–53.

Harrington T.C., Pashenova N.V., McNew D.L., Steimel J. & Konstantinov M.Y. (2002) Species delimination and host specialization of *Ceratocystis laricicola* and *C. polonica* to larch and spruce. *Plant Disease* **86**: 418–422.

Harrington T.C. & Rizzo D.M. (1999) Defining species in fungi. In: *Structure and dynamics of fungal populations* (Worrall JJ, ed). Kluwer Press, Dordrecht, The Netherlands: 43–71.

Hausner G., Reid J. & Klassen G.R. (1992) Do galeate-ascospore members of the *Cephaloascaceae*, *Endomycetaceae* and *Ophiostomataceae* share a common phylogeny? *Mycologia* **84**: 870–881.

Hausner G., Reid J & Klassen G.R. (1993a) On the subdivision of *Ceratocystis s.l.*, based on partial ribosomal DNA sequences. *Canadian Journal of Botany* **71**: 52–63.

Hausner G., Reid J. & Klassen G.R. (1993b) Grouping of isolates and species of *Ceratocystis sensu lato* on the basis of molecular and morphological characters. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology, and Pathogenicity* (Wingfield MJ, Seifert KA, Webber JF, eds). APS Press, St. Paul, Minnesota: 93–104.

Heath R.N., Wingfield M.J., Wingfield B.D., Meke G., Mbaga A. & Roux J. (2009) *Ceratocystis* species on *Acacia mearnsii* and *Eucalyptus* spp. in eastern and southern Africa including six new species. *Fungal Diversity* **34**: 41–67.

Himelick E.B. & Curl E.A. (1958) Transmission of *Ceratocystis fagacearum* by insects and mites. *Plant Disease Reporter* **42**: 538–545.

Höhnel F. (1918) Mycologische Fragmente. *Annales Mycologici* **16**: 40–41. (Reprinted 1962).

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

Johnson J.A., Harrington T.C. & Engelbrecht C.J.B. (2005) Phylogeny and taxonomy of the North American clade of the *Ceratocystis fimbriata* complex. *Mycologia* **97**: 1067–1092.

Juzwik J. & French D.W. (1983) *Ceratocystis fagacearum* and *C. piceae* on the surface of free-flying and fungus-mat-inhabiting nitidulids. *Phytopathology* **73**: 1164–1168.

Kamgan N.G., Jacobs K., De Beer Z.W., Wingfield M.J. & Roux J. (2008) *Ceratocystis* and *Ophiostoma* species, including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* **29**: 37–59.

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

Kirisits T. (2004) Fungal associates of European bark beetles with special emphasis on the ophiostomatoid fungi. In: *Bark and wood boring insects in living trees in Europe, A Synthesis* (Lieutier F, Day KR, Battisti A, Grégoire JC, Evans H, eds). Dordrecht, Kluwer: 185–223.

Kirschner R. (2001) Diversity of filamentous fungi in bark beetles in central Europe. In: *Trichomycetes and other fungal groups* (Roberts W Lichtwardt Commemoration Volume. (Misra JK, Horn BW, eds). Science Publishers, Inc., Enfield, Plymouth.

Lanza E., Ko K.H. & Palmer J.K. (1976) Aroma production by cultures of *Ceratocystis moniliformis*. *Journal of Agriculture, Food and Chemistry* **24**: 1247-1250.

Lanza E. & Palmer J.K. (1977) Biosynthesis of monoterpenes by *Ceratocystis moniliformis*. *Phytochemistry* **16**: 1555-1560.

Marin M. (2004) Phylogenetic and molecular population biology studies on *Ceratocystis* spp. associated with conifer and coffee diseases. PhD thesis, Department of Plant Pathology and Microbiology, University of Pretoria, South Africa.

Marin M., Castro B., Gaitan A., Preisig O., Wingfield B.D. & Wingfield M.J. (2003) Relationships of *Ceratocystis fimbriata* isolates from Colombian coffee-growing regions based on molecular data and pathogenicity. *Journal of Phytopathology* **151**: 395–405.

Marin M., Preisig O., Wingfield B.D., Kirisits T., Yamaoka Y. & Wingfield M.J. (2005) Phenotypic and DNA sequence data comparisons reveal three discrete species in the *Ceratocystis polonica* species complex. *Mycological Research* **109**: 1137–1148.

Mathieson M. (1952) Ascospore dimorphism and mating-type in *Chromocrea spinulosa* (Fuckel) Petch n. comb. *Annals of Botany* **16**: 449–467.

Moller W.J. & De Vay J.E. (1968) Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* **58**: 1499–1507.

Morris M.J., Wingfield M.J. & De Beer C. (1993) Gummosis and wilt of *Acacia mearnsii* in South Africa caused *Ceratocystis fimbriata*. *Plant pathology* **42**: 814–817.

Ocasio-Morales R.G., Tsopelas P. & Harrington T.C. (2007) Origin of *Ceratocystis platani* on native *Platanus orientalis* in Greece and its impact on natural forests. *Plant Disease* **91**: 901–904.

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

Perkins D. (1987) Mating-type switching in filamentous ascomycetes. *Genetics* **115**: 215–216.

Robbertse B., Reeves J.B., Schoch C.L., Spatafora J.W. (2006) A phylogenomic analysis of the Ascomycota. *Fungal Genetics in Biology* **43**: 715–725.

Rodas C., Roux J., Van Wyk M., Wingfield B.D. & Wingfield M.J. (2007) *Ceratocystis neglecta* sp. nov., infecting *Eucalyptus* trees in Colombia. *Fungal Diversity* **28**: 73–84.

Rokas A., Williams B.L., King N. & Carroll S.B. (2003) Genome-scale approaches to resolving incongruence in molecular phylogenies. *Nature.* **425**: 798–804.

Roux J., Heath R.N., Labuschagne L., Kamgan N.G. & Wingfield M.J. (2007) Occurrence of the wattle wilt pathogen, *Ceratocystis albifundus* on native South African trees. *Forest Pathology* **37**: 1–11.

Samuels G.J. (1993) The case for distinguishing *Ceratocystis* and *Ophiostoma*. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology, and Pathogenicity* (Wingfield MJ, Seifert KA, Webber JF, eds). APS Press, St. Paul, Minnesota: 15–20.

Spatafora J.W. & Blackwell M. (1994) The polyphyletic origins of ophiostomatoid fungi. *Mycological Research* **98**: 1–9.

Tarigan M., Roux J., Van Wyk M., Tjahjono B. & Wingfield M.J. (2010) A new wilt and die-back disease of *Acacia mangium* associated with *Ceratocystis manginecans* and *C. acaciivora* sp. nov. in Indonesia. *South African Journal of Botany* **77**: 292-304.

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

Van Wyk M., Al-Adawi A.O., Khan I.A., Deadman M.L., Al-Jahwari A., Wingfield B.D., Ploetz R. & Wingfield M.J. (2007a) *Ceratocystis manginecans* sp. nov., causal agent of a destructive mango wilt disease in Oman and Pakistan. *Fungal Diversity* **27**: 213–230.

Van Wyk M., Al Adawi A.O., Wingfield B.D., Al Subhi A.M., Deadman M.L. & Wingfield M.J. (2005) DNA based characterization of *Ceratocystis fimbriata* isolates associated with mango decline in Oman. *Australasian Plant Pathology* **34**: 587–590.

Van Wyk M, Pegg G., Lawson S. & Wingfield M.J. (2007b) *Ceratocystis atrox* sp. nov associated with *Phoracanthta acanthocera* infestations on *Eucalyptus* in Australia. *Australian Journal of Plant Pathology* **36**: 407–414.

Van Wyk M., Roux J., Barnes I., Wingfield B.D., Liew E.C.Y. Assa, B. Summerell B.A. & Wingfield M.J. (2004) *Ceratocystis polychroma* sp. nov., a new species from *Syzygium aromaticum* in Sulawesi. *Studies in Mycology* **50**: 273–282.

Van Wyk M., Roux J., Barnes I., Wingfield B.D. & Wingfield M.J. (2006) Molecular phylogeny of the *Ceratocystis moniliformis* complex and description of *C. tribiliformis* sp. nov. *Fungal Diversity* **21**: 181–201.

Van Wyk M., Wingfield B.D., Marin M. & Wingfield M.J. (2010) New *Ceratocystis* species infecting coffee, cacao, citrus and native trees in Colombia. *Fungal Diversity* **40**: 103-117.

Van Wyk M., Wingfield B.D., Mohali S. & Wingfield M.J. (2009) *Ceratocystis fimbriatomima,* a new species in the *C. fimbriata sensu lato* complex isolated from *Eucalyptus* trees in Venezuela. *Fungal Diversity* **34**: 173–183.

Van Wyk M., Wingfield B.D. & Wingfield M.J. (2011) Four new *Ceratocystis* spp. associated with wounds on *Eucalyptus*, *Schizolobium* and *Terminalia* trees in Ecuador. *Fungal Diversity* **46**: 111-131.

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

Webster R.K. & Bulter E.E. (1967) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45**: 1457–1467.

Wilken P.M., De Beer Z.W., Steenkamp E.T., Wingfield M.J. & Wingfield B.D. (2010) Full genome sequencing contributes to an understanding of mating in *Ceratocystis fimbriata. South African Society for Plant Pathology* (Abstract).

Wingfield M.J., Seifert K.A. & Webber J.F. (eds.) (1993). *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology, and Pathogenicity APS Press, St. Paul, Minnesota.

Wingfield M.J., De Beer C., Visser C. & Wingfield B.D. (1996) A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematic and Applied Microbiology* **19**: 191–202.

Witthuhn R.C., Harrington T.C., Wingfield B.D., Steimel J.P. & Wingfield M.J. (2000) Deletion of the MAT-2 mating-type gene during uni-directional mating-type switching in *Ceratocystis. Current Genetics* **38**: 48–52.

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

Witthuhn R.C., Wingfield B.D., Wolfaart M. & Harrington T.C. (1998) Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. *Mycologia* **90**: 96–101.

Table 1. Members of the *C. fimbriata* complex described as distinct species with the aid of phylogenetic data.

Species	Authority	Host	Geographical area	Reference
C. acaciivora	M Tarigan, M van Wyk & MJ Wingf.	*Acacia*	Indonesia	Tarigan *et al*. 2010
C. albifundus	MJ Wingf., Morris & De Beer	*Acacia*	Africa	Wingfield *et al*. 1996
C. atrox	M van Wyk & MJ Wingf.	*Eucalyptus*	Australia	Van Wyk *et al*. 2007b
C. cacaofunesta	Engelbrecht & Harrington	*Theobroma*	Brazil	Baker *et al.* 2005
C. caryae	JA Johnson & Harrington	*Carya*	USA	Jonhson *et al*. 2005
C. colombiana	M van Wyk & MJ Wingf.	*Coffea*	Colombia	Van Wyk *et al.* 2010
C. curvata	M van Wyk & MJ Wingf.	*Eucalyptus*	Ecuador	Van Wyk *et al.* 2011
C. diversiconidia	M van Wyk & MJ Wingf.	*Terminalia*	Ecuador	Van Wyk *et al.* 2011
C. ecuadoriana	M van Wyk & MJ Wingf.	*Eucalytus*	Ecuador	Van Wyk *et al.* 2011
C. fimbriata s.s.	Ellis & Halsted	*Ipomoea*	USA	Baker *et al.* 2005
C. fimbriatatomima	M van Wyk & MJ Wingf.	*Eucalyptus*	Venezuela	Van Wyk *et al.* 2008
C. larium	M van Wyk & MJ Wingf.	*Styrax*	Indonesia	Van Wyk *et al*. 2009
C. manginecans	M van Wyk, A Al Adawi & MJ Wingf.	*Mangifera*	Oman	Van Wyk *et al*. 2007a
C. neglecta	M van Wyk, Jol Roux & C Rodas	*Eucalyptus*	Colombia	Rodas *et al*. 2008
C. obpyriformis	RN Heath & Jol. Roux	*Acacia*	South Africa	Heath *et al*. 2009
C. papillata	M van Wyk & MJ Wingf.	*Coffea*	Colombia	Van Wyk *et al.* 2010
C. pirilliformis	I Barnes & MJ Wingf.	*Eucalyptus*	Australia	Barnes *et al*. 2003
C. platani	(Walter) Engelbrecht & Harrington	*Platanus*	USA	Baker *et al.* 2005

C. polychroma	M van Wyk, MJ Wingf. & ECY Liew	*Syzygium*	Indonesia	Van Wyk *et al*. 2004
C. polyconidia	RN Heath & Jol. Roux	*Acacia*	South Africa	Heath *et al*. 2009
C. populicola	JA Johnson & Harrington	*Populus*	Canada	Jonhson *et al*. 2005
C. smalleyi	JA Johnson & Harrington	*Carya*	USA	Jonhson *et al*. 2005
C. tanganyicensis	RN Heath & Jol. Roux	*Acacia*	Tanzania	Heath *et al*. 2009
C. tsitsikammensis	Kamgan & Jol Roux	*Rapanea*	South Africa	Kamgan *et al*. 2008
C. variospora	(RW Davidson) C Moreau	*Quercus*	USA	Jonhson *et al*. 2005
C. zombamontana	RN Heath & Jol. Roux	*Eucalyptus*	Malawi	Heath *et al*. 2009

Figure 1. Symptoms of diseases caused by species in the *C. fimbriata* group. 1.1) Dying and dead *Syzygium aromaticum* trees in Sulawesi 1.2) Vascular staining within the *S. aromaticum* trees caused by *C. polychroma*. 1.3) Dying and dead *Acacia mearnsii* trees in Malawi 1.4) Sexual structures of *C. albifundus* as seen on the dying *A. mearnsii* trees. 1.5) Dying *Mangifera indica* tree in Oman. 1.6) Vascular staining caused by *C. manginecans* on the *M. indica* tree.


1


2


3


4


5


6

Figure 2. Critical morphological characters for distinguishing species complexes in *Ceratocystis*. 2.1) Ascomatal bases of the *C. fimbriata* group and the *C. coerulescens* group lack disk-shaped collar at the bottom of the necks. 2.2) Distinct "collar" at the bottom of the ascomatal necks characterizing the *C. moniliformis* complex. 2.3) Conical spines on the ascomatal base characterizing the *C. moniliformis* complex. 2.4) Dark brown, thick-walled chlamydospores characterizing the *C. fimbriata* and *C. coerulescens* complexes. 2.5"Hat-shaped" ascospores typical of the *C. fimbriata* and *C. moniliformis* complexes. 2.6) Orange-sectioned shaped ascospores characterizing the *C. coerulescens* group.

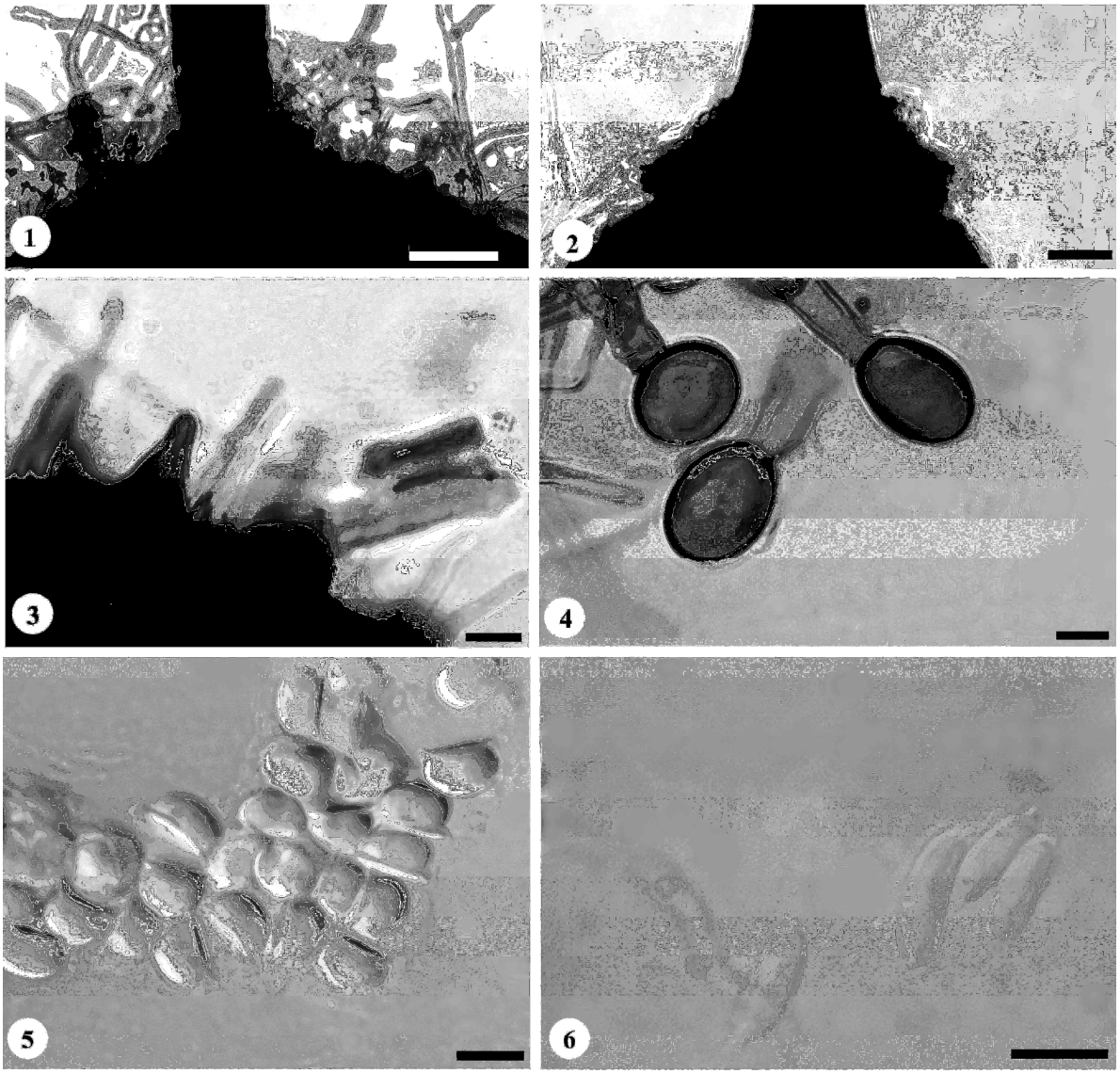
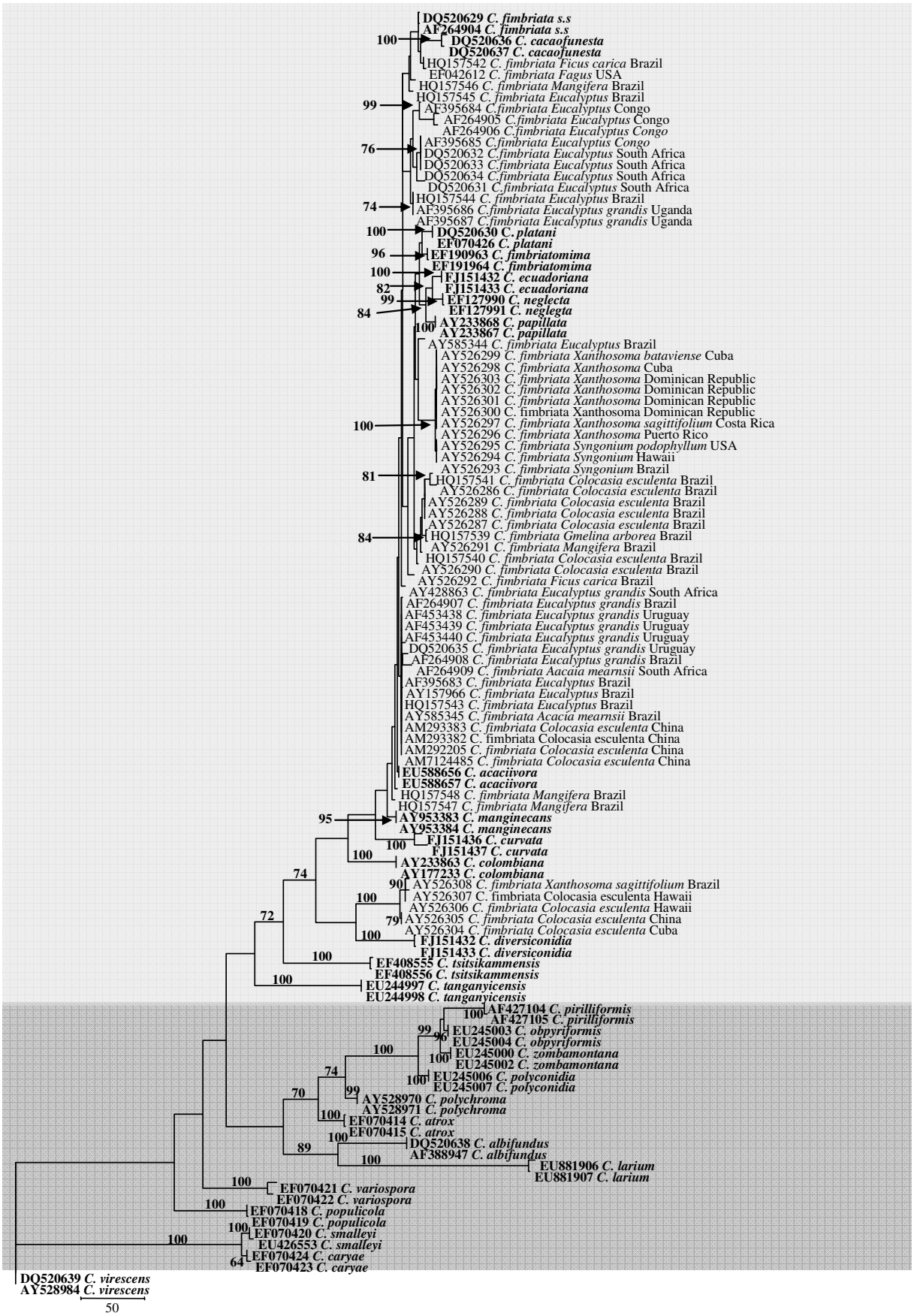


Figure 3. Selected most parsimonious phylogenetic tree based on a heuristic search of aligned ITS sequences of the *C. fimbriata* complex, with *C. virescens* designated as out-group. Bootstrap values above 70% are given based on 1000 replicates (tree length 1425 steps, CI = 0.6, RI = 0.9, RC = 0.5, 384 of 625 characters parsimony informative with gaps treated as fifth character, trees obtained by stepwise addition with mulpars in effect).



CHAPTER 3

First report on *C. fimbriata* in Oman on mango

Van Wyk M., Al-Adawi A.O., Wingfield B.D., Al-Subhi A.M., Deadman M.L. & Wingfield M.J. (2005) DNA based characterization of *Ceratocystis fimbriata* isolates associated with mango decline in Oman. *Australasian Plant Pathology* **34**: 587–590.

ABSTRACT

A serious mango decline disease has recently been reported from the Sultanate of Oman. Based on morphological characteristics, the pathogen responsible for the disease was previously identified as *Ceratocystis fimbriata*. *Ceratocystis fimbriata* is one of the most important pathogens of mango in Brazil and its appearance in Oman is of great concern. Recent phylogenetic studies have shown that *C. fimbriata* most likely represents a species complex. The aim of this study was to confirm the identity of the mango pathogen in Oman based on DNA sequences. Sequence data were obtained for the ITS 1 and 2 regions and the 5.8S rRNA gene regions and these were compared with sequence data of *C. fimbriata* from a number of hosts and geographic areas. The isolates from Oman where shown to represent *C. fimbriata sensu lato* and also to be most closely related to an isolate from Brazil, from mango in that country. This provides some evidence that the mango pathogen in Oman may have originated in Brazil.

INTRODUCTION

Ceratocystis fimbriata Ell. & Halst. is an important canker and wilt pathogen on a wide range of hosts. These include many fruit and plantation trees as well as a number of root crops (Kile 1993). *Ceratocystis fimbriata* is a soil borne pathogen and it also produces a fruity odor facilitating dispersal by casual insects such as flies (Diptera) and picnic beetles (Coleoptera: Nitidulidae) (Himelick & Curl 1958, Upadhyay 1981, Kile 1993). The pathogen typically infects wounds on plants either through soil–borne structures or via sexual or asexual spores carried to these wounds by insects.

Mango decline caused by *C. fimbriata* is one of the most serious diseases affecting production of *Mangifera indica* L. (mango trees) in Brazil (Batista 1960). In that country *C. fimbriata* causes mango blight also known as “seca” or “murcha” (Viegas 1960, De Toledo Piza 1966, Ribiero 1980). Symptoms are identical to those of Recife sickness, caused by *Diplodia recifensis* Bat. (Ploetz 2003). A Scolytid beetle, the *Cryphalus mangifera* (Coleoptera: Scolytidae) was identified by Ribiero (1980) to be the primary species responsible for the dissemination of *C. fimbriata* in Brazil. Until recently, mango disease caused by *C. fimbriata* was known only in Brazil.

Ceratocystis fimbriata, possibly in association with *Diplodia theobromae* (Pat.) W. Nowell, has recently been reported to cause mango decline in the Sultanate of Oman. In this situation, the disease is associated with the beetle, *Cryphalus scabrecollis* Eichhoff (Coleoptera: Scolytidae) (Al-Adawi *et al.* 2003). Symptoms include dark staining of the wood that spreads from the points of infection, gum exudation from the trunks, wilting and browning of leaves on single branches, and eventually tree death. The disease severely threatens mango production in Oman and studies are currently underway to reduce its impact.

Ceratocystis fimbriata has a wide range of hosts and it has long been recognized as probably encompassing more than one taxonomic entity (Webster & Butler 1967). Contemporary DNA based studies have reinforced this view, with some species initially described as *C. fimbriata*, now clearly recognized as discrete taxa (Barnes *et al.* 2003, Van Wyk *et al.* 2004). The wilt pathogen of the forest plantation tree *Acacia mearnsii* De Wild., *C. albifundus* M.J. Wingf., De Beer & M.J. Morris provides a relevant example (Wingfield *et al.* 1996). Contemporary DNA-

based studies have shown that *C. fimbriata* represents discrete groups based on host specificity as well as geographical areas (Harrington 2000, Barnes *et al*. 2001, Baker *et al.* 2003).

Identification of *C. fimbriata* associated with mango decline in Oman was based solely on morphological characteristics (Al-Adawi *et al*. 2003). Although the morphological structures defining this species are reasonably well defined, these characteristics are insufficient to recognize emergent groupings within *C. fimbriata*. The aim of this study was to confirm the identity of *C. fimbriata* isolates from mango in Oman and to compare DNA sequences of the ITS 1 and 2 regions and the 5.8S rRNA gene for these isolates with those from other hosts and geographic areas.

MATERIALS & METHODS

Isolates

Isolates of *C. fimbriata* were obtained from wood taken from infected mango trees in the Al-Batinah region of Oman. Perithecia from primary isolations were induced by incubating infected tissue in moist chambers or by baiting through placing tissue between two slices of carrot (Moller & De Vay 1968). Pure cultures were obtained by lifting ascospore masses from the apices of perithecia developing on infected wood after incubation in moist chambers and transferring these to 2% Malt Extract Agar (MEA) (20 g/L Malt, 20 g/L Agar) (Biolab, Midrand, South Africa) and maintained at 25 °C. Cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Phylogeny

DNA was extracted using a modified version of the technique described by Raeder and Broda (1985). PCR amplifications were conducted with the primer pairs, ITS1 and ITS4 (White *et al.* 1990) for the gene region ITS1, 5.8S, ITS2. The final reaction volumes of the PCR were adjusted to 25 µL with sterile water. The PCR mixture consisted of 5–10 ng of genomic DNA, 0.2 mM of dNTP, 0.2 µM of each primers, 1.75 U Expand High Fidelity PCR System enzyme mix (Roche Diagnostics, Mannheim, Germany) and 1 × Expand HF Buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme). Amplifications were performed in a Mastercycler gradient thermal cycler (Eppendorf, Germany) using the following parameters: a 2 min step at 96 ºC, followed by 10 cycles of 20 s at 94 ºC, 40 s at 55 ºC and 45 s at 72 ºC. The last three temperature intervals

were repeated for another 30 cycles with a 5 s increase per cycle for the elongation step at 72 ºC, and then a final 10 min at 72 ºC. Products were resolved by electrophoresis in a 2% agarose gel (Roche diagnostics, Mannheim, Germany), stained with ethidium bromide. The PCR products were purified using Sephadex columns, 1g in 15ml sterile water (SIGMA, Steinheim, Germany).

For sequencing, the same primers were used as for the generation of the PCR products. Sequences were determined using an ABI PRISM™ 3100 Autosequencer (Applied BioSystems, Foster City, California, U.S.A.) and sequence data were analysed using Sequence Navigator version 1.0.1 (Applied BioSystems, Foster City, California). The sequences of the ITS region for the *Ceratocystis* spp. from mango trees were compared with those of morphologically similar *Ceratocystis* spp. obtained from GenBank (Figure 1). Sequences were aligned manually and analysed using PAUP version 4.0b10* (Swofford 2002). The heuristic search was performed with 100 random addition sequence replications. Gaps were treated as a fifth character "newstate". Confidence intervals of branching points were determined using 1000 bootstrap replicates. The tree was rooted using *C. albifundus* as the out-group taxon.

RESULTS & DISCUSSION

Phylogeny

The DNA sequence data for the ITS region provided support for the identification of *C. fimbriata* isolated from diseased mango trees in Oman. This is important as the fungus could easily be mistaken for morphologically similar sibling species such as *C. polychroma* M. van Wyk, M.J. Wingf. & E.C.Y. Liew (Van Wyk *et al*. 2004) or *C. pirilliformis* Barnes & M.J. Wingf. (Barnes *et al*. 2003). The three isolates from Oman (CMW 13851 / AY953383, CMW 13852 / AY953384 & CMW 13854 / AY953385) grouped together in a clade with a bootstrap support of 91%.

The *C. fimbriata* isolates from diseased mango trees in Oman grouped most closely (bootstrap support, 55%) to an isolate from Brazil (CMW 14797 / AY953382) that was also isolated from mango trees. Although a comparison with a greater number of isolates from mango would be desired, this does suggest that the fungus on mango in Oman and Brazil represent the same form of *C. fimbriata*. It is possible that the fungus was introduced into the Sultanate of Oman from Brazil by a route that has yet to be determined. More detailed studies of larger numbers of isolates employing microsatellite markers are currently underway to consider this hypothesis.

Isolate CMW 15052 (AY157964), obtained from mango in Brazil grouped separately from the other isolates from mango used in this study. This isolate clustered together with isolate CMW 14812 (AY953386) from cacao in Brazil (90% bootstrap support). With the exception of the clade containing isolates CMW 15052 and CMW 14812 all other clades in the phylogenetic tree of *C. fimbriata* reflected either host specificity of isolates or geographical area of isolation, or both (Figure 1). This supports the view that *C. fimbriata* is both host and geographically restricted (Harrington 2000). Isolates from coffee in Colombia (CMW 10844 / AY177238, CMW 5746 / AY953388 & CMW 5747 / AY9533898, CMW 9555 / AY177232) resided in two distinct clades as previously shown by Marin *et al*. 2003 and Barnes *et al*. 2002. These groups of isolates are not necessarily strictly host related, implying strong host specificity in some cases and not in others. This might also explain why one mango isolate from Brazil in this study was more closely related to an isolate from cacao than to others from Mango.

The disease of mango in Oman that is apparently caused by *C. fimbriata* is extremely severe and has a very significant impact on mango production in the country. This study provides some evidence that the pathogen has been introduced into the country from Brazil, where it is also a serious pathogen. In Brazil, infections by *C. fimbriata* occur in association with the insect *Cryphalus mangifera*. In Oman, the disease is also closely associated with an insect, the *Cryphalus scabrecollis.* These insects appear to have a similar biology and their association with *C. fimbriata* has clearly given rise to serious disease problems.

Management of the mango disease associated with *C. fimbriata* in Oman is receiving attention. The best possible option to reduce the impact of the disease will be through planting disease resistant tress. In this regard, an understanding of the population biology of the pathogen in Oman will be important and research on the origin and population biology of *C. fimbriata* in this country is currently underway.

REFERENCES

Al-Adawi A.O., Deadman M.L., Al Rawahi A.K., Khan A.J. & Al Maqbali Y.M. (2003) *Diplodia theobromae* associated with sudden decline of mango in the Sultanate of Oman. *Plant Pathology* **52**: 419.

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

Barnes I., Guar A., Burgess T., Roux J., Wingfield B.D. & Wingfield M.J. (2001) 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., Wingfield M.J., Old K.M. & Dudzinski M. (2003) *Ceratocystis pirilliformis*, a new species from *Eucalyptus nitens* in Australia. *Mycologia* **95**: 865–871.

Barnes I., Roux J., Wingfield M.J. & Wingfield B.D. (2002) Population structure of *Ceratocystis fimbriata* from Congo, Colombia and Uruguay, determined using microsatellite markers. 7th Meeting of the International Mycological Society, August 2002, Oslo, Norway.

Batista A.C. (1960) *Ceratocystis fimbriata* Ell. & Halst. sôbre *Mangifera indica* L. Publicação 244, Instituto de Micologia da Universidade do Recife. pp. 1–46.

De Toleda Piza C. (ed.) (1966) Anais do Simposio Sobre a Seca da Mangueira. (abstacted in *Review of Applied Mycology* **46**: 378, 1967).

Harrington T. (2000) Host specialization and speciation in the American wilt pathogen *Ceratocystis fimbriata*. *Fitopatologia Brazileira* **25**: 262–263.

Himelick E.B. & Curl E.A. (1958) Transmission of *Ceratocystis fagacearum* by insects and mites. *Plant Disease Reporter* **42**: 538–545.

Kile G.A. (1993) Plant disease caused by species of *Ceratocystis sensu stricto* and *Chalara.* In "*Ceratocystis and Ophiostoma,* Taxonomy, Ecology, and Pathogenicity". (Eds. MJ Wingfield, KA Seifert, JF Webber). APS press:" St. Paul, Minnesota. pp. 173–183.

Marin M., Castro B., Gaitan A., Preisig O., Wingfield B.D. & Wingfield M.J. (2003) Relationships of *Ceratocystis fimbriata* isolates from Colombian coffee-growing regions based on molecular data and pathogenicity. *Journal of Phytopathology* **151**: 395–405.

Moller W., De Vay J. (1968) Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* **58**: 1499–1508.

Ploetz, R.C. (Ed) (2003) Diseases of tropical fruit crops. CABI Publishing. CAB International, Wallingford, UK, pp 327–365.

Raeder U., Broda P. (1985) Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**: 17–20.

Ribiero I.J.A. (1980) Seca de manguera. Agentes causais e estudio da molesta. In: *Anais do I Simposio Brasiliero Sobre a Cultura de Mangueira*. Sociedad Brasileira de Fruticultura, Jacoticobal, November 24–28, 1980, pp. 123–130.

Swofford D.L. (2002) PAUP* Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Upadhyay H.P. (1981) A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press. Athens, pp 51–52

Van Wyk M., Roux J., Barnes I., Wingfield B.D., Liew E.C.Y., Assa B., Summerell B.A., Wingfield M.J. (2004) *Ceratocystis polychroma* sp. nov., a new species from *Syzygium aromaticum* in Sulawesi. *Studies in Mycology* **50**: 273–282.

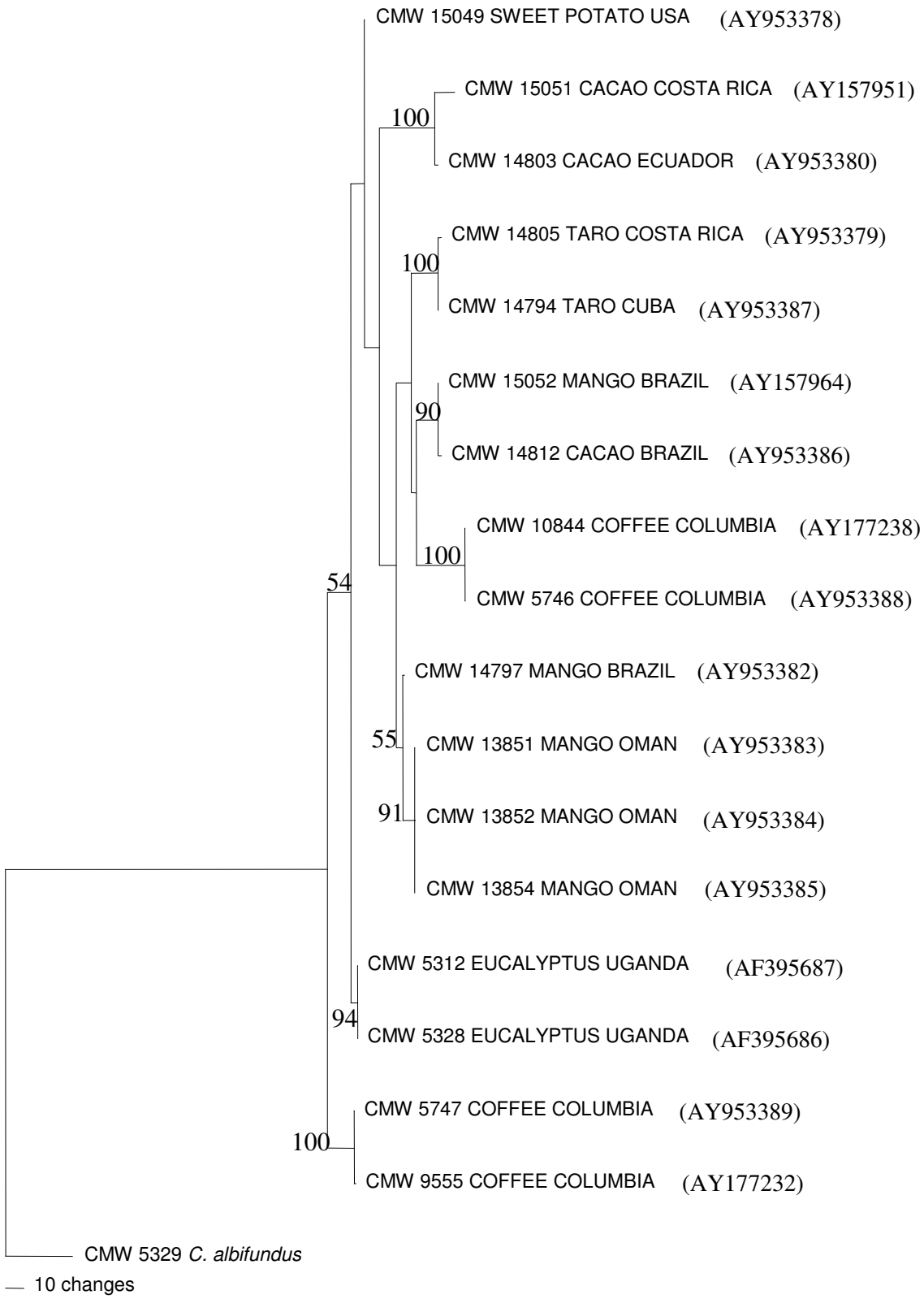
Viegas A.P. (1960) Mango blight. *Bragantia* **19**, 163–182 (abstracted in *Review of Applied Mycology* **42**, 696, 1963)

Webster R., Butler E. (1967) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45**: 1457–1468.

White T.J., Bruns T., Lee S., 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. Innis MA, Gelfand DH, Sninsky JJ, White TJ) pp. 315–322. Academic Press, San Diego.

Wingfield M.J., De Beer C., Visser C., Wingfield B.D. (1996) A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematic and Applied Microbiology* **19**: 191–202.

Figure 1. Phylogenetic tree based on parsimony analysis of the ITS1-5.8S rRNA gene-ITS2 region of *Ceratosystis fimbriata*. Bootstrap values are indicated above the branches. *Ceratocystis albifundus* is used as the out-group taxon. GenBank accession numbers are indicated in brackets. The Scale bar indicates 10 nucleotide changes



CHAPTER 4

Ceratocystis manginecans sp. nov., causal agent of the destructive mango wilt disease in Oman and Pakistan

Van Wyk M., Al Adawi A.O., Khan I.A., Deadman M.L. Al Jahwari A.A., Wingfield B.D., Ploetz R. & Wingfield M.J. (2007) *Ceratocystis manginecans* sp. nov., causal agent of the destructive mango wilt disease in Oman and Pakistan. *Fungal Diversity* **27**: 213-230.

ABSTRACT

A serious wilt disease of mango trees (*Mangifera indica*) has recently appeared in Oman and Pakistan. Symptoms on affected trees resemble those of the mango disease known as "seca" in Brazil and include discolouration of the vascular tissue, gum exudation, galleries of the putative beetle vector of the fungal pathogen, wilting and rapid death. In both countries, the disease has been attributed to *Ceratocystis fimbriata*. This fungus is recognised as a complex of species and *C. fimbriata sensu stricto* is unlikely to be an appropriate name for the causal agent. We, therefore, considered the identity of Pakistan and Oman isolates using comparisons of combined DNA sequence data for partial ITS, *β*-tubulin and EF-1α gene regions. These comparisons were supported with morphological characteristics. Results indicated that isolates from mango in Pakistan and Oman represent a species, distinct from other species in the *C. fimbriata sensu lato* species complex. The name *Ceratocystis manginecans sp. nov*. is provided for the fungus, which is also shown to be closely related to available isolates from mango in Brazil.

INTRODUCTION

The genus *Ceratocystis* includes many plant and particularly tree pathogens (Upadhyay 1993, Kile 1993). Species of *Ceratocystis* are responsible for a wide range of disease symptoms including staining of the vascular tissue, cankers wilting and root disease. *Ceratocystis fimbriata* Ell. & Halst. was the first species to be described after it was found associated with black rot of sweet potato (Halsted & Fairchild, 1891). This fungus has subsequently been reported from six continents on a great number of hosts and with varying levels of pathogenicity.

The variability of isolates representing *C. fimbriata*, its wide host range and its extensive geographic distribution gave rise to the view that the fungus might represent a species complex (Webster & Butler 1967a, b). More recently, DNA-based techniques have made it possible to recognise distinct taxa that might otherwise have been assigned to *C. fimbriata*. The first example emerged with the description of *C. albifundus* De Beer, Wingfield and Morris, a fungus causing a serious wilt disease of *Acacia mearnsii* de Wildt. in South Africa, which had initially been identified as *C. fimbriata* (Morris *et al*. 1993, Wingfield *et al*. 1996). Likewise, *C. pirilliformis* Barnes and M.J. Wingf. from *Eucalyptus* in Australia (Barnes *et al*. 2003) represents a species described in this complex. Recently, Baker-Engelbrecht and Harrington (2005) provided a re-description of *C. fimbriata sensu stricto* (*s.s.*) and defined this fungus as specifically representing the causal agent of sweet potato (*Ipomoea batatas* L.) black rot. Names have also been applied to isolates representing other cryptic species residing in various monophyletic lineages in the *C. fimbriata sensu lato* (*s.l.*) species complex (Van Wyk *et al*. 2004, Baker-Engelbrecht & Harrington 2005, Johnson *et al*. 2005, Van Wyk *et al*. 2007).

Ceratocystis fimbriata, is known to be an important pathogen of mango in Brazil (Ploetz 2003). The fungus was first recorded on these trees in the 1930's, where it caused a serious die-back disease known as “seca” or “murcha” disease (Ploetz 2003). This is an important disease of mango in Brazil and it is closely associated with infestations of the wood-boring scolytid *Hypocryphalus mangiferae* Stebbing (Coleoptera: Scolytidae), which is also thought to spread the pathogen (Ribeiro 1980, Yamashiro & Myazaki 1985, Ploetz 2003).

Severely diseased mango trees were observed in Oman early in 1998. Symptoms on these trees included dark staining of the affected wood and insect galleries, exudation of gum, leaf wilting

and ultimately tree death (Al Adawi *et al*. 2003). Two *Ceratocystis* spp. were isolated from these trees. One of these is related to *C. moniliformis* Hedgc. and it was subsequently described as *C. omanensis* Al Subhi, M.J. Wingf. M. van Wyk and Deadman (Al Subhi *et al*. 2006). The other fungus was morphologically similar to *C. fimbriata s.l.* and a detailed study incorporating morphological characteristics and DNA sequence comparisons, confirmed that it belonged to this species complex (Van Wyk *et al*. 2005). Al Adawi *et al.* (2006) provided details of the disease in Oman including proof of pathogenicity of *C. fimbriata s.l* and they also showed that *H. mangiferae*, the same scolytid beetle associated with the fungus in Brazil, is closely linked to the disease in Oman.

A wilt disease of mango similar to that observed in Oman was reported from Pakistan in 2005 (Malik *et al*. 2005). Although little detailed evidence was provided to support this view, it was suggested that the disease is caused by *C. fimbriata*. Symptoms observed in Pakistan included gum exudation from the stems, vascular discolouration, branch death and holes in the bark as a result of insect infestation. The bark beetle *H. mangifera* (Florida Department of Agriculture, identification reference E2006-7581-701) was also found associated with this disease (Al Adawi unpublished).

The pathogen responsible for mango wilt disease in Oman has been identified as *C. fimbriata s.l* which resides in the broad phylogenetic assemblage that includes *C. fimbriata s.s.*, various other sibling species that have been provided with names, and a relatively large number that await taxonomic treatment (Van Wyk *et al*. 2005). The aim of this study was to provide an appropriate taxonomic placement for mango pathogen in Oman and Pakistan, based on isolates from mango trees and *H. mangifera* in both countries.

MATERIALS & METHODS

Isolates

Isolates from mango in Oman used in this study were the same as those used in previous studies by Van Wyk *et al*. (2005). In the case of Pakistan, several mango farms in Faisalabad and Multan were visited in June 2005 and May 2006 to examine symptoms of mango decline disease. These (Figure 1) were generally the same as those described from Oman (Van Wyk *et al*. 2005) including rapid wilting of the leaves on infected parts of the trees, gum exuding from cracks in

the bark, a streaked patter of vascular discoloration and the presence of infestation by the *H. mangifera*. In both regions, longitudinal cuts were made on 20 grafted mango trees affected by the disease at the junction of the rootstock with the scion. This was done to determine whether vascular discolouration was present on the scion and/or rootstock. Forty samples including discoloured xylem were collected from diseased mango trees in Pakistan. Scolytid beetles found under the bark of affected trees were also collected and sent to the Division of Plant Industry, Florida Department of Agriculture & Consumer Services, Department of Agriculture (Gainesville, FL. USA) for identification.

Primary isolations from samples with vascular discolouration were made by incubating pieces of wood in moist chambers or, when fungal structures were not observed on the surface of the wood, by placing them between two slices of carrot (Moller & De Vay 1968). Insects collected from under the bark of diseased trees were crushed and placed between carrot slices and incubated at room temperature for seven to 10 days. Ascospore masses from the apices of ascomata developing on infected wood or on carrot slices were transferred to 2% malt extract agar (MEA) (20 g/L) (Biolab, Midrand, South Africa) and maintained at room temperature (~25°C). All isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and representative isolates have been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Representative cultures were dried on 30% glycerol and have been deposited with the National Herbarium of South Africa (PREM).

Phylogeny

Four isolates (CMW23628, CMW23634, CMW23641 & CMW23643) from Pakistan and three isolates (CMW13851, CMW13852 & CMW13854) from Oman were selected for phylogenetic studies. The isolates from Oman were those used in a previous study (Van Wyk *et al*. 2005). DNA from the seven isolates was extracted as described by Van Wyk *et al*. (2007). Polymerase Chain Reactions for three gene regions, the Internal Transcription Spacer (ITS) region including the 5.8S rDNA operon (White *et al*. 1990), Beta-tubulin (βt) (Glass & Donaldson 1995) and Transcription Elongation Factor-1α (EF-1α) (Jacobs *et al*. 2004) were performed. The PCR conditions were as described by Van Wyk *et al*. (2007). The amplified products were purified using 6% Sephadex G-50 columns (Steinheim, Germany). PCR amplicons were sequenced in both directions using the ABI PRISM^{TM} Big DYE Terminator Cycle Sequencing Ready Reaction

Kit (Applied BioSystems, Foster City, California), with the same primers as those used for DNA amplification. Sequencing reactions were run on an ABI PRISM^{TM} 3100 Autosequencer (Applied BioSystems, Foster City, California, USA).

Sequences were analysed using Sequence Navigator version 1.0.1 (Applied BioSystems, Foster City, California). Sequence data for closely related *Ceratocystis* spp. were obtained from GenBank for comparative purposes. Sequences were aligned using MAFFT (http://timpani.genome.ad.jp/%7emafft/server/) (Katoh *et al*. 2002) and confirmed manually. The dataset was analysed using PAUP version 4.0b10* (Swofford 2002). A partition homogeneity test (Swofford 2002) was used to determine whether the three datasets could be combined. Gaps were treated as a fifth character and trees were obtained via stepwise addition of 1000 replicates with the Mulpar option in effect. The heuristic search option based on parsimony with stepwise addition 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 were deposited in GenBank (Table 1).

Morphology

Two isolates, one from Oman and the other from Pakistan, (CMW23641 and CMW13854 respectively) were selected for growth studies in culture. These isolates were grown for 14 d on 2% MEA, after which a 5mm plug was transferred to the centres of 90mm Petri dishes containing 2% MEA. Five replicates of each isolate were used for each of seven different incubation temperatures ranging from 5°C to 35°C at five degree intervals. The plates were incubated in the dark for seven days, after which two measurements of colony diameter were made at right angles to each other and averages were computed for each temperature. Colour designations were made for the cultures using the colour charts of Rayner (1970).

Morphological characteristics were assessed using 10-d-old cultures and structures were mounted in lactophenol on glass slides. Fifty measurements of each relevant taxonomic structure was made for a single isolate. Ten additional measurements of the relevant structures were made from two other randomly selected isolates. Averages (mean), standard deviation (stdv) and minimum (min) and maximum (max) measurements are presented for each structure as follows: (min-) mean minus stdv – mean plus stdv (-max).

RESULTS

Isolates

Isolations from symptomatic xylem tissue from 40 mango trees, in Pakistan, yielded 43 isolates. Ten of these isolates were retrieved from adult *H. mangifera* beetles in Pakistan. Cultures generally resembled those of *Ceratocystis* spp. in the *C. fimbriata* species complex, having characteristic dark green colonies and the typical banana fruit odour.

Phylogeny

The combined dataset of sequences for the three gene regions (1978 characters) produced four most parsimonious trees of which one is presented (Figure 2). The tree had a length of 1556 bases of which 1176 characters were constant, 64 characters are parsimony uninformative and 738 characters were parsimony informative. The tree had a consistency index of 0.7333, a retention index of 0.8564 and a rescaled consistency of 0.6280. All the isolates from mango in Oman and Pakistan resided in a tightly nested clade (bootstrap = 88%), separate from other species in the *C. fimbriata* species complex. The isolates from mango in Pakistan and Oman were phylogenetically most closely related to each other. The isolates most closely related to those from Pakistan and Oman were those from mango trees in Brazil. These isolates, however, did not fall within the clade representing the Pakistan and Oman isolates and the branches were not well supported (Bootstrap = 66%) (Figure 2). The species of *Ceratocystis* phylogenetically most closely related to those in the "mango" clade are *C. cacaofunesta* Engelbrecht and Harrington, *C. platani* Engelbrecht and Harrington and *C. fimbriata s.s.* They, however, resided in strongly supported groups, raising the view that the *Ceratocystis* sp. from mango in Oman and Pakistan represents a distinct and undescribed taxon.

Morphology

Isolates CMW13854 and CMW23634 from Oman and Pakistan, respectively, displayed similar growth patterns in culture and grew optimally between 20-25°C. No growth was observed at 5°C, 10°C and 35°C. After 7 days, both cultures reached an average of 27mm and 29mm at 15°C and 30°C, respectively. At 20°C both isolates had reached an average of 43mm and at 25°C an average diameter of 45mm was reached.

Isolates of the *Ceratocystis* sp. from Pakistan and Oman were morphologically indistinguishable. The fungus could be easily distinguished from *C. fimbriata s.s.* by its segmented hyphae, and production of both secondary and primary conidiophores and cylindrical and barrel-shaped conidia. *Ceratocystis fimbriata s.s.* does not have secondary conidiophores that produce barrel-shaped conidia. The *Ceratocystis* sp. from Pakistan and Oman is morphologically similar to *C. platani*. In this respect, both fungi produce primary and secondary conidiophore and the two conidial forms. They could, however, be distinguished from each other by the fact that *C. platani* has longer ascomatal necks (535-835 µm vs 557-635 µm), longer ostiolar hyphae (20-90 µm vs 45-59 µm) and both the conidiophore forms are shorter than those from mango in Pakistan and Oman (24-90 µm vs 81-109 µm and 35-50 µm vs 65-77 µm). And the isolates from mango have segmented hyphae, whereas *C. platani* has unsegmented hyphae.

TAXONOMY

The *Ceratocystis* sp. from mango in Oman and Pakistan resides in a phylogenetic group, distinct from other species in the *C. fimbriata* species complex. Although all of these fungi are morphologically very similar, the mango taxon from Oman and Pakistan can be distinguished from its closest phylogenetic relatives based on morphological characteristics. The fungus is consequently described as a new taxon as follows.

Ceratocystis manginecans M. van Wyk, A Adawi & M.J. Wingf. ***sp. nov.*** (Figure 3a-g)
MB510851

Etymology: The name of this fungus is derived from the Latin word *neco* "to kill, slay or put to death", which refers to the fact the fungus is responsible for a serious disease of mango trees.

Hyphae laeves segmentatae. *Bases ascomatum* nigrae, globosae. *Colla ascomatum* basi atro-brunnea, apicem versus pallescentia. *Hyphae ostiolares* divergentes, hyalinae. *Ascosporae* in massa globosa alba vel fulva in apicibus collorum ascomatum crescunt; lateraliter visa cucullatae, non septatae, hyalinae. *Anamorpha Thielaviopsi*s: conidiophorae biformes, primariae hyalinae, secondariae hyalinae. Evolutio *conidiorum* phialidicorum per faciendo parietum annularium; conidia biformia singula vel catenata. *Conidia primaria*; *secondaria. Chlamydosporae* coffeinae vel umbrinae, globosae vel subglobosae.

Colonies grayish olive (21"") in colour on 2% MEA. *Odour* banana. *Hyphae* smooth and segmented. *Ascomatal bases* globose, black, (153-)192–254(-281) µm in diam. *Ascomatal necks* dark brown becoming lighter towards apices (514-)557–635(-673) µm long, (25-)32–42(-48) µm wide at base, (14-)16–22(-26) µm wide at tip. *Ostiolar hyphae* hyaline, divergent, (42-)45–59(-69) µm long. *Asci* evanescent, not seen. *Ascospores* hyaline, hat-shaped, 3–4 µm in length, 4–5 µm wide excluding sheath, 7–8 µm wide including sheath. *Thielaviopsis anamorph*: *Conidiophores* of two morphological forms. *Primary conidiophores* phialidic, lageniform, hyaline, (72-)81–109(-144) µm long, 5–7(-9) µm wide, 6–8(-9) µm wide at broadest point, 3–6 µm wide at tips. *Secondary conidiophores*, tube-like, flaring at mouths, short, hyaline, (59-)65–77(-84) µm long, 5–8 µm wide at bases and (5-)6–8 µm wide at tips. *Conidia* of two types. *Primary conidia*, hyaline, cylindrical, (15-)23–29(-33) µm in length, 3–6 µm wide. *Secondary conidia*, hyaline, barrel-shaped, (8-)9–11(-12) µm in length, 5–7(-8) µm wide. *Chlamydospores* brown, thick-walled, globose to sub-globose, (11-)12–14 µm in length by 9–11(-12) µm wide.

Habitat: Diseased *Mangifera indica*
Known distribution: Oman & Pakistan
Material examined: **Oman**, from diseased *Mangifera indica* trees, A.O. Al Adawi, holotype PREM59612, CMW13851, CBS121659 (2002). **Oman**, from diseased *Mangifera indica* trees, A.O. Al Adawi, PREM59613, CMW13852, CBS121660 (2002). **Pakistan**, from bark beetle, isolated A.O. Al Adawi, PREM59614, CMW23634,CBS121661 (2002).

DISCUSSION

Ceratocystis manginecans described in this study represents a growing number of cryptic species, which in the past would have been assigned to the well-known plant pathogen, *C. fimbriata*. These cryptic species have only become obvious in recent years and subsequent to the ability to distinguish them based on phylogenetic inference. Results of this study have shown that the newly described *C. manginecans* from mango in Oman and Pakistan, can easily be distinguished from *C. fimbriata s.s.* and other species in the *C. fimbriata s.l.* species complex, using DNA sequence comparisons. Although most of these species are morphologically very similar, accounting for the fact that they were treated as a single taxon in the past, *C. manginecans* can be morphologically distinguished from its closest phylogenetic relatives. *Ceratocystis manginecans*

also occurs in an ecological niche very different to any other species in the *C. fimbriata* species complex and it is unlikely to be confused with other species.

Phylogenetic comparisons based on three gene regions were used in this study, to distinguish between *C. manginecans* and other species in the *C. fimbriata sensu lato* complex. In this respect, it is possible to distinguish the fungus based on sequences of the ITS region of the rDNA operon and various other species in the group have been recognised at this level (Wingfield *et al*. 1996, Barnes *et al*. 2003, Baker-Engelbrecht & Harrington 2005). However, there are many situations where single gene regions are insufficient to distinguish species as is for example evident for most species in the *C. moniliformis* species complex (Van Wyk *et al*. 2006, Al Subhi *et al*. 2006). The fact that sequence comparisons for three gene regions distinguish *C. manginecan*s from its closest relatives provides confidence that this is a unique taxon.

Ceratocystis manginecans is the cause of a disease that seriously threatens the mango industry of Oman (Al Adawi *et al*. 2006). The disease has resulted in the death of a great number of trees in that country and it has recently been recognised as a serious constraint to mango cultivation in Pakistan (Malik *et al*. 2005). Accurate recognition of the pathogen responsible for this disease will hopefully contribute to measures that will reduce its impact. Certainly, recognizing that *C. manginecans* differs from *C. fimbriata s.s.* will enable, promote and improve understanding of the disease and possibly its origin.

The serious mango disease caused by the fungus that has been referred to as *C. fimbriata s.l.* in Brazil, has symptoms very similar to those observed in Oman and Pakistan (Viegas 1960, Ribeiro 1980, Ploetz 2003, Al Adawi *et al*. 2006). Clearly, the disease in Brazil is not caused by *C. fimbriata s.s.,* but to a sibling species in this complex. The small number of isolates from mango in Brazil that were included in this study, are phylogenetically similar to those from Oman and Pakistan. The fungus in Brazil might, therefore, represent the same species. However, a larger number of isolates from Brazil should be compared with *C. manginecans*, before this question is answered definitively.

Inoculation tests with *C. manginecans* in Oman have shown that the fungus is a virulent pathogen, able to kill mango trees rapidly (Al Adawi *et al*. 2006). Although we are not aware of similar tests in Pakistan, it is likely that the same will be true in that country. The fungus

represents a serious threat to the mango growing community in these countries, many of which are dependant on this crop for sustainability.

The origin of *C. manginecans* in Oman and Pakistan is unknown. It has, however, been suggested that it was introduced into Oman, originally from South America (Van Wyk *et al*. 2005). *Ceratocystis manginecans* could easily have been introduced on soil associated with this material as this fungus is well-adapted to a soil-borne habitat. There have been anecdotal suggestions that a producer from Pakistan imported planting stock in from Brazil and that this was also established at a production site in Oman. This could explain the original outbreak in Oman and then only later in Pakistan.

An intriguing similarity between the mango disease caused by *C. manginecans* in Oman and Pakistan and the disease in Brazil, is that the scolytid beetle *H. mangifera,* is associated with the disease in all three areas (Al Adawi *et al*. 2006, Van Wyk *et al*. 2006, Ploetz 2003). *Ceratocystis manginecans* can easily be isolated from this insect in both Oman and Pakistan as has been found in the present and previous studies (Al Adawi *et al*. 2006, Van Wyk *et al*. 2006). In Oman and Pakistan, trees are commonly found with insect probing damage, before the onset of infection. In this respect, the insect appears to play an important role in disease spread and development. This has also been suggested for the disease in Brazil (Ribeiro 1980).

Hypocryphalus mangifera is thought to be native to southern Asia, in areas such as India (Wood 1982, Butani 1993, Atkinson & Peck 1994) and Pakistan where mango is also native (Wood 1982, Mukherjee 1997). If this is true, the insect would have been introduced into Brazil. It is intriguing to speculate that *C. manginecans* could have been introduced into Oman and Pakistan from Brazil where it has established a relationship with an insect, native in the area. In Brazil, the situation would then be opposite, with the insect vector being introduced and having become associated with a native fungus. Studies to determine the origin of *C. manginecans* are planned and should help to resolve these questions.

The mango disease caused by *C. manginecans* seriously threatens the future of mango cultivation in Oman and Pakistan. It could also threaten mango cultivation in other parts of that region such as India, which is the world's most important producer. Important sources of mango germplasm (monoembryonic *M. indica* evolved in India and polyembryonic *M. indica* in Southeast Asia),

and other species of *Mangifera* which are most diverse in Southeast Asia could also be threatened. Every effort must thus be made to prevent the introduction of *C. manginecans* and its *H. mangifera* vector into new environments. Understanding its mode of spread and promoting quarantine procedures to limit its movement should be a high priority.

REFERENCES

Al Adawi A.O., Deadman M.L., Al Rawahi A.K., Khan A.J. & Al Maqbali Y.M. (2003) *Diplodia theobromae* associated with sudden decline of mango in the Sultanate of Oman. *Plant Pathology* **52**: 419.

Al Adawi, A.O., Deadman M.L., Al Rawahi A.K., Al Maqbali Y.M., Al Jahwari A.A., Al Saadi B.A., Al Amri I.S. & Wingfield, M.J. (2006) Aetiology and causal agents of mango sudden decline disease in the Sultanate of Oman. *European Journal of Plant Pathology* **116**: 247–254.

Al Subhi A.M., Al Adawi A.O., Van Wyk M., Deadman M.L. & Wingfield M.J. (2006) *Ceratocystis omanensis*, a new species from diseased mango trees in Oman. *Mycological Research* **110**: 237–245.

Atkinson T.H. & Peck S.B. (1994) Annotated checklist of the bark and ambrosia beetles (Coleoptera: Platypodidae and Scolytidae) of tropical Southern Florida. *Florida Entomologist Online* **77**: 313–329.

Baker-Engelbrecht C.J. & Harrington, T.C. (2005) Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. *Mycologia* **97**: 57–69.

Barnes I., Roux J., Wingfield M.J., Old K.M. & Dudzinski M. (2003) *Ceratocystis pirilliformis*, a new species from *Eucalyptus nitens* in Australia. *Mycologia* **95**: 865–871.

Butani D.K. (1993) *Mango: Pest Problems.* Periodical Expert Book. Agency D-42, Vivek Vihar, Delhi, India.

Glass N.L. & 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.

Halsted B.D. & Fairchild D.G. (1891) Sweet-potato black rot. *Journal of Mycology* **7**: 1-11.

Jacobs K., Bergdahl D.R., Wingfield M.J., Halik S., Seifert K.A., Bright D.E. & Wingfield B.D. (2004) *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* 108: 411–418**.**

Johnson J.A., Harrington T.C. & Engelbrecht C.J.B. (2005) Phylogeny and taxonomy of the North American clade of the *Ceratocystis fimbriata* complex. *Mycologia* **97**: 1067–1092.

Katoh K., Misawa K., Kuma K. & Miyata T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**: 3059–3066.

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.

Malik M.T., Khan S.M., Dasti A.A. & Kazmi, M.R. (2005) First record of *Ceratocystis fimbriata*, causal organism of mango sudden death in Pakistan. *Pakistan Journal of Phytopathology* **17**: 187–191.

Moller W. & DeVay J. (1968) Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* **58**: 1499–1508.

Morris M.J., Wingfield M.J. & De Beer C. (1993) Gummosis and wilt of *Acacia mearnsii* in South Africa caused *Ceratocystis fimbriata*. *Plant pathology* **42**: 814–817.

Mukherjee S.K. (1997) Introduction: Botany and Importance. In: *The Mango: Botany, Production and Uses* (ed. R.E. Litz). CAB International, Wallingford, UK: 1–19.

Ploetz R.C. (2003) Diseases of mango. In: *Diseases of tropical fruit crops* (ed. R.C. Ploetz). CABI Publishing, Wallingford, Oxford: **15**:1–35.

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

Ribeiro I.J.A. (1980) Seca de manguera. Agentes causais e studio da molesta. *In: Anais do I Simposio Brasiliero Sobre a Cultura de Mangeura*. Sociedad Brasileira de Fruticultura, Jacoticobal, November 24-28, 1980, 123–130.

Ribeiro I.J.A., Rossetto C.J., Donadio L.C., Sabino J.C., Martins A.L.M. & Gallo P.B. (1995) Mango wilt. XIV Selection of mango (*Mangifera indica* L.) rootstocks resistant to the mango wilt fungus *Ceratocystis fimbriata* Ell. & Halst. *Acta Horticulturae* **370**: 159–161.

Rossetto C.J. & Ribeiro I.J.A. (1990) Mango wilt. XII. Recommendations for control. Revista de Agricultura. *Piracicaba* **65**: 173–180.

Upadhyay H.P. (1993) Classification of the ophiostomatoid fungi. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology, and Pathogenicity* (eds. M.J. Wingfield, K.A. Seifert and J.F. Webber). APS Press, St. Paul, Minnesota: 269–287.

Swofford D.L. (2002) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Van Wyk, M., Al Adawi A.O., Wingfield B.D., Al Subhi A.M., Deadman M.L. & Wingfield M.J. (2005) DNA based characterization of *Ceratocystis fimbriata* isolates associated with mango decline in Oman. *Australasian Plant Pathology* **34**: 587–590.

Van Wyk M., Pegg G., Lawson S. & Wingfield M.J. (2007) *Ceratocystis atrox* sp. nov associated with *Phoracanthta acanthocera* infestations on *Eucalyptus* in Australia. *Australian Journal of Plant Pathology* (In Press).

Van Wyk, M., Roux, J., Barnes, I., Wingfield, B.D., Liew, E.C.Y., Assa, B., Summerell, B.A. and Wingfield, M.J. (2004). *Ceratocystis polychroma* sp. nov., a new species from *Syzygium aromaticum* in Sulawesi. *Studies in Mycology* **50**: 273–282.

Van Wyk M., Roux J., Barnes I., Wingfield B.D. & Wingfield M.J. (2006) Molecular phylogeny of the *Ceratocystis moniliformis* complex and description of *C. tribiliformis* sp. nov. *Fungal Diversity* **21**: 181–201.

Viegas A.P. (1960) Mango blight. *Bragantia* 19: 163-182 (abstracted in *Review of Applied Mycology* **42**: 696, 1963)

Webster R.K. & Butler E.E. (1967a) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45**: 1457–1468.

Webster R.K. & Butler E.E. (1967b) The origin of self-sterile, cross-fertile strains and culture sterility in *Ceratocystis fimbriata*. *Mycologia* **59**: 212–221.

White T.J., Bruns T., Lee S. & 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.

Wingfield M.J., De Beer C., Visser C. & Wingfield B.D. (1996) A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematic and Applied Microbiology* **19**: 191–202.

Wood S.L. (1982) The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. *Great Basin Naturalist, Memoirs* **6**: 1–1356.

Yamashiro T. & Myazaki I. (1985) Principal pests and diseases of mango - *Mangifera indica* L. - in the State of Sao Paulo and updated control methods. *Biológico* **51**:41–50.

Table 1. *Ceratocystis spp*. for which isolates or sequences were used in this study[a].

Species	Isolate no.	GenBank Accession no.	Host	Geographical origin
C. albifundus	CMW4068	DQ520638 EF070429 EF070400	*Acacia mearnsii*	RSA
C. albifundus	CMW5329	AF388947 DQ371649 EF070401	*Acacia mearnsii*	Uganda
C. atrox	CMW19383 CBS120517	EF070414 EF070430 EF070402	*Eucalyptus grandis*	Australia
C. atrox	CMW19385 CBS120518	EF070415 EF070431 EF070403	*Eucalyptus grandis*	Australia
C. cacaofunesta	CMW15051 CBS152.62	DQ520636 EF070427 EF070398	*Theobroma cacao*	Costa Rica
C. cacaofunesta	CMW14809 CBS115169	DQ520637 EF070428 EF070399	*Theobroma cacao*	Ecuador
C. caryae	CMW14793 CBS114716	EF070424 EF070439 EF070412	*Carya cordiformis*	USA
C. caryae	CMW14808 CBS115168	EF070423 EF070440 EF070411	*Carya ovata*	USA
C. fimbriata s.s	CMW15049 CBS141.37	DQ520629 EF070442 EF070394	*Ipomaea batatas*	USA
C. fimbriata s.s	CMW1547	AF264904 EF070443 EF070395	*Ipomaea batatas*	Papua New Guinea
C. fimbriata s.l	CMW14797 CBS114721	AY953382 EF433307 EF433316	*Mangifera indica*	Brazil
C. fimbriata s.l	CMW15052 CBS600.7	EF433298 EF433306 EF433315	*Mangifera indica*	Brazil
C. manginecans	**CMW13851 CBS1211659**	**AY953383 EF433308 EF433317**	***Mangifera indica***	**Oman**
C. manginecans	**CMW13852 CBS121660**	**AY953384 EF433309 EF433318**	***Hypocryphalus mangifera***	**Oman**
C. manginecans	**CMW13854**	**AY953385 EF433310 EF433319**	***Mangifera indica***	**Oman**
C. manginecans	**CMW23634 CBS121661**	**EF433302 EF433311 EF433320**	***Hypocryphalus mangifera***	**Pakistan**
C. manginecans	**CMW23628**	**EF433303 EF433312 EF433321**	***Hypocryphalus mangifera***	**Pakistan**

Species	Isolate no.	GenBank Accession no.	Host	Geographical origin
C. manginecans	**CMW23643**	**EF433304** **EF433313** **EF433322**	***Mangifera indica***	**Pakistan**
C. manginecans	**CMW23641**	**EF433305** **EF433314** **EF433323**	***Mangifera indica***	**Pakistan**
C. pirilliformis	CMW6569	AF427104 DQ371652 AY528982	*Eucalyptus nitens*	Australia
C. pirilliformis	CMW6579 CBS118128	AF427105 DQ371653 AY528983	*Eucalyptus nitens*	Australia
C. platani	CMW14802 CBS115162	DQ520630 EF070425 EF070396	*Platanus occidentalis*	USA
C. platani	CMW23918	EF070426 EF070397	*Platanus* sp.	Greece
C. polychroma	CMW11424 CBS115778	AY528970 AY528966 AY528978	*Syzygium aromaticum*	Indonesia
C. polychroma	CMW11436 CBS115777	AY528971 AY528967 AY528979	*Syzygium aromaticum*	Indonesia
C. populicola	CMW14789 CBS119.78	EF070418 EF070434 EF070406	*Populus* sp.	Poland
C. populicola	CMW14819 CBS114725	EF070419 EF070435 EF070407	*Populus* sp.	USA
C. smalleyi	CMW14800 CBS114724	EF070420 EF070436 EF070408	*Carya cordiformis*	USA
C. variospora	CMW20935 CBS114715	EF070421 EF070437 EF070409	*Quercus alba*	USA
C. variospora	CMW20936 CBS114714	EF070422 EF070438 EF070410	*Quercus robur*	USA
C. virescens	CMW11164	DQ520639 EF070441 EF070413	*Fagus americanum*	USA
C. virescens	CMW3276	AY528984 AY528990 AY528991	*Quercus* sp.	USA

[a] / Isolates of *C. manginecans* were sequenced in this study. Other sequences for phylogenetic comparison where obtained from Genbank.

Figure 1. Wilting and internal symptoms observed on mango trees in Pakistan. (a). Tree displaying rapid wilt in part of the crown. (b). Gum exuding from a crack in the bark of an infected stem. (c, d). Stem lesions showing typically streaked (d) vascular discoloration. (e). Entrance hole of *H. mangifera* and adult insect (white arrow) on infected stem.

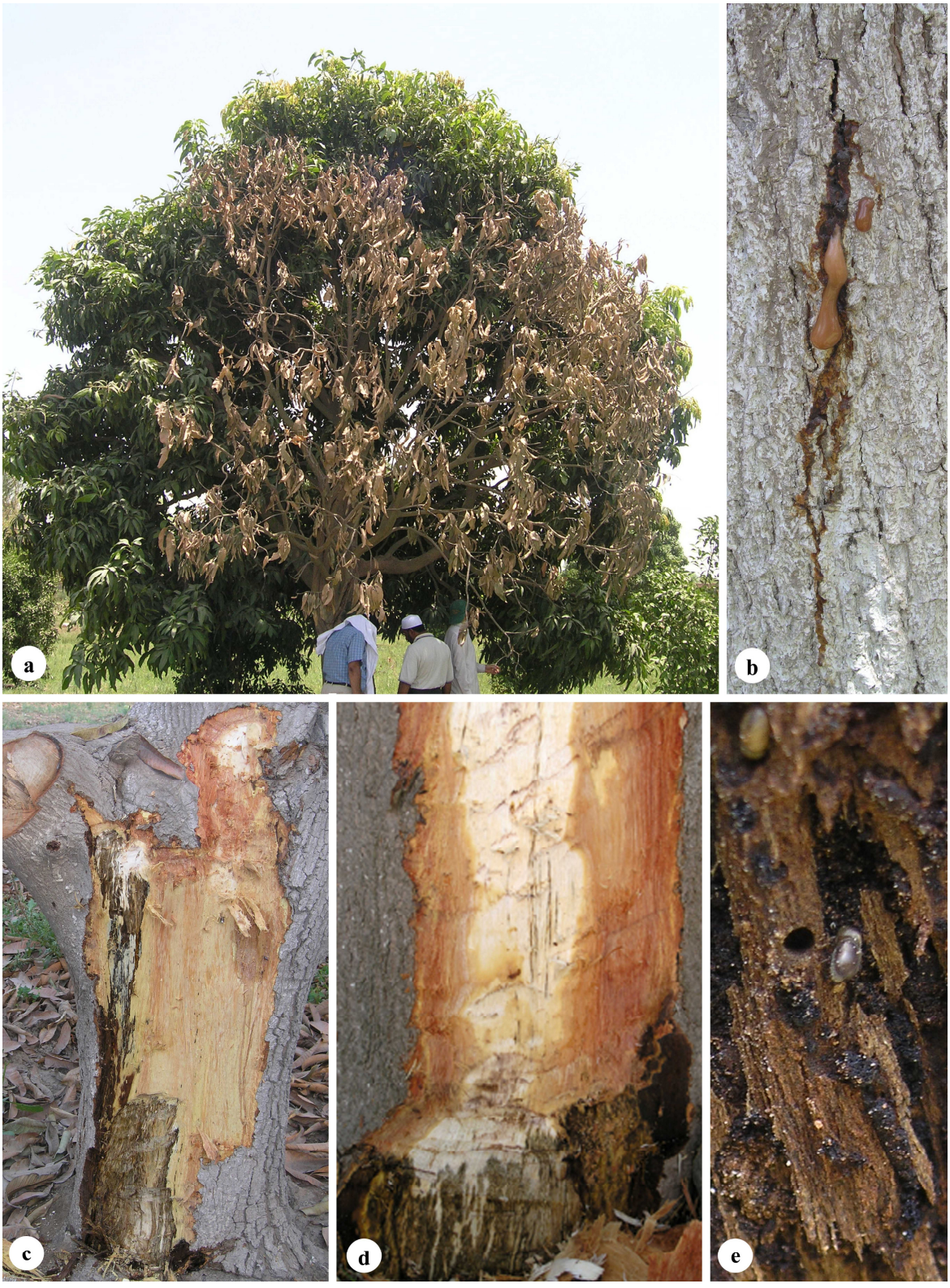


Figure 2. Phylogenetic tree based on the combined regions of the ITS, β-tubulin and EF1-α for *C. manginecans* and other species in the *C. fimbriata s.l.* species complex. Bootstrap values are indicated above the branches.

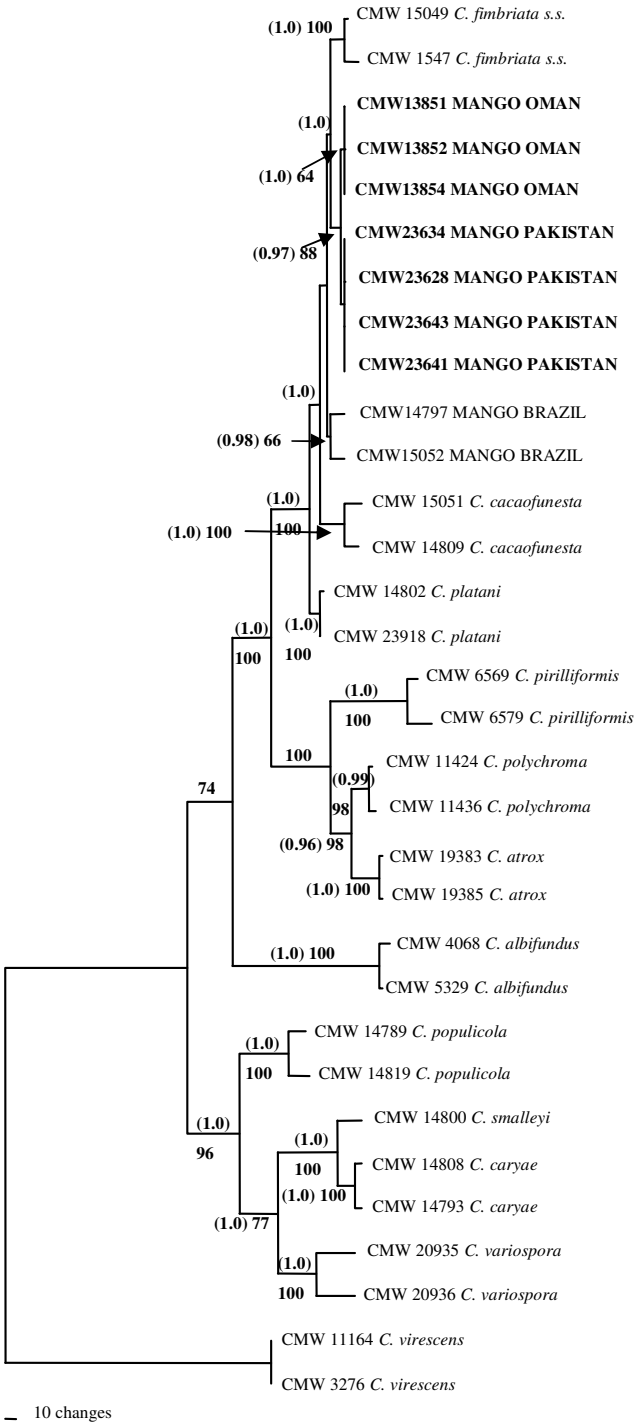
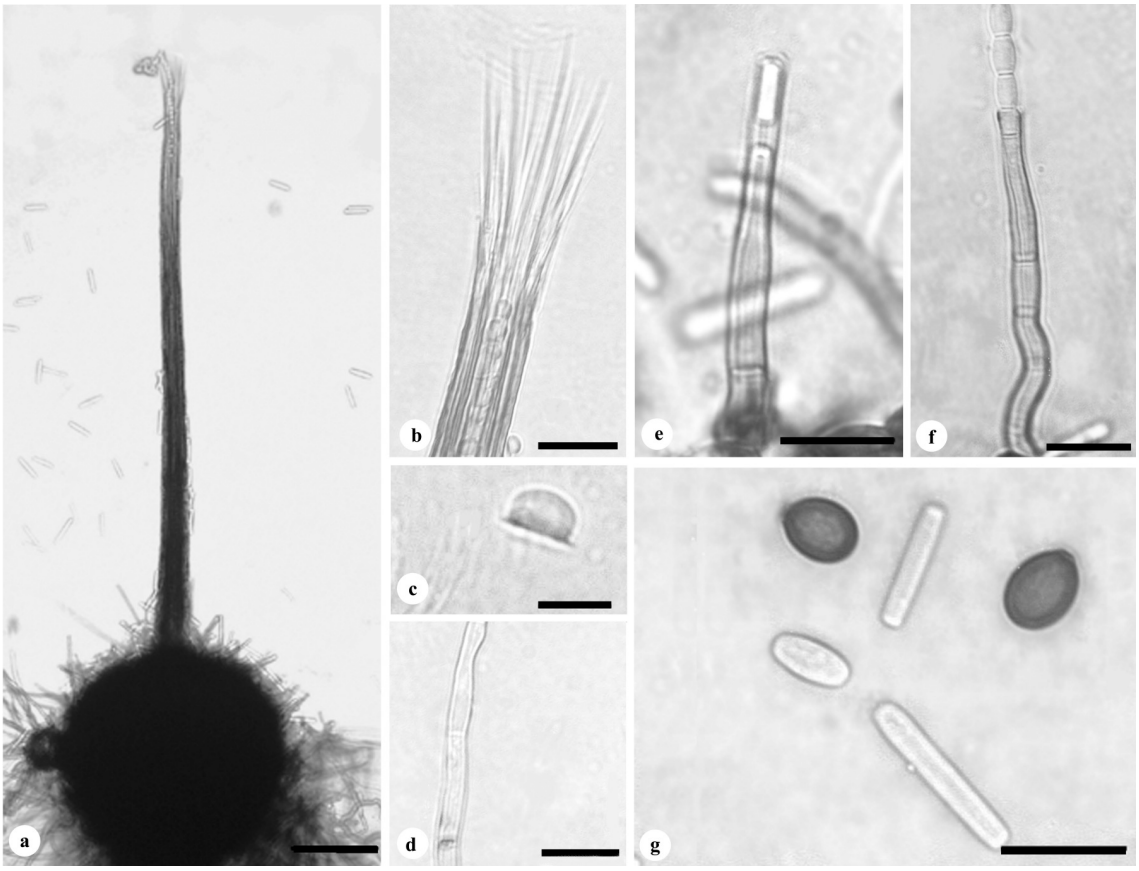


Figure 3. Morphological characteristics of *Ceratocystis manginecans* (from holotype): A. Globose ascomata. B. Divergent ostiolar hyphae. C. Hat-shaped ascospores. D. Segmented hyphae. E. Primary phialidic conidium with emerging cylindrical conida. F. Secondary conidium with emerging chain of barrel-shaped conidia. G. Dark coloured chlamydospores, cylindrical- and barrel shaped conidia. Scale bars: A = 100 µm, B = 20 µm, C = 5 µm, D = 20 µm, E = 20 µm, F = 20 µm, G = 20 µm.



CHAPTER 5

Ceratocystis atrox sp. nov associated with *Phoracantha acanthocera* infestations on *Eucalyptus grandis* in Australia

Van Wyk M., Pegg G., Lawson' S. & Wingfield M.J. (2007) *Ceratocystis atrox* sp. nov associated with *Phoracantha acanthocera* infestations on *Eucalyptus grandis* in Australia. *Australasian Plant Pathology* **36**: 407-414.

ABSTACT

Ceratocystis spp. include important pathogens of trees as well as apparently saprophytic species. Four species have been recorded on *Eucalyptus grandis* in Australia of which only one, *C. pirilliformis* Barnes and M.J. Wingf. is known to be pathogenic. A recent survey of pests and diseases of *Eucalyptus* trees in northern Queensland revealed a species of *Ceratocystis* associated with the tunnels made by the aggressive wood-boring insect, *Phoracantha acanthocera* (Macleay) (Cerambycidae: Coleoptera). The aim of this study was to identify the fungus based on morphological characteristics and comparisons of DNA sequence data for three gene regions. The fungus peripherally resembles *C. fimbriata* Ell. and Halst. but differs from this species most obviously having much darker mycelium, longer ascomatal necks, segmented hyphae and the absence of chlamydospores. Comparisons of combined sequence data confirmed that the *Ceratocystis* sp. from *P. acanthocera* represents an undescribed taxon, which is provided with the name *Ceratocystis atrox* sp. nov. *Ceratocystis atrox* appears to have a close relationship with *P. acanthocera*, although its role in the biology of the insect is unknown and its pathogenicity has not been considered.

INTRODUCTION

Species of *Ceratocystis* include some of the most important pathogens of trees in the world (Kile *et al.* 1993, Redfern *et al*. 1987, Christiansen & Solheim 1990). They also include wound-infecting saprophytes, agents of sap stain and species of unknown ecology. The pathogenic species include two discrete groups. These include species that are either vectored by bark beetles (Coleoptera: Scolytidae) in a specific mutualistic relationship (Redfern *et al*. 1987, Christiansen & Solheim 1990, Marin *et al*. 2003) or those that infect wounds and are important wilt pathogens broadly treated as species of the *Ceratocystis fimbriata sensu lato* (*s.l.*) complex (Webster & Butler 1967, Kile *et al*. 1993).

Recent studies have shown that species in the *Ceratocystis fimbriata s.l.* complex represent a relatively large number of cryptic taxa (Wingfield *et al.* 1996, Barnes *et al*. 2003a, Van Wyk *et al*. 2004b, Baker-Engelbrecht & Harrington 2005, Johnson *et al*. 2005). Convincing evidence for the existence of these species has largely arisen from the application of the phylogenetic species concept and DNA sequence comparisons. There is also some evidence for host-specific taxa in this group (Baker-Engelbrecht & Harrington 2005) although overlapping of host ranges are also found (Marin *et al*. 2003).

A number of *Ceratocystis* spp. have been recorded from *Eucalyptus* spp. in various parts of the world. The most important of these is *C. fimbriata s.l.* that causes a serious vascular wilt disease of *Eucalyptus* spp. in Uruguay (Barnes *et al*. 2003b), Congo (Roux *et al*. 1999), Uganda (Roux *et al.* 2001) and is known in South Africa in the absence of an associated disease (Roux *et al*. 2004). In Australia, where most *Eucalyptus* spp. are native, four species of *Ceratocystis* have been found on these trees. They include *C. moniliformis* Hedgc., *C. moniliformopsis* Yuan and Mohammed (Yuan & Mohammed 2002), *C. eucalypti* Yuan and Kile (Kile *et al.* 1996) and *C. pirilliformis* (Barnes *et al*. 2003a). Of these, *C. pirilliformis* has been shown to be pathogenic and this has only been on greenhouse grown trees in South Africa, where the fungus also occurs on *Eucalyptus* (Roux *et al*. 2004).

During a recent survey of *Eucalyptus* pests and diseases in northern Queensland, a *Ceratocystis* sp. was found sporulating in the tunnels of the aggressive wood boring insect *Phoracantha acanthocera* (Cerambycidae: Coleoptera; syn *Tryphocaria acanthocera*) (Wang *et al*. 1999), commonly known as the bulls-eye borer. This insect is native to Australia and can cause serious damage to *Eucalyptus* spp. grown in plantations (Phillips 1993a, b) and in

re-growth forests (Abbott *et al*. 1991, Farr *et al*. 2000). The aim of the present study was to identify the unknown *Ceratocystis* sp. that occurs in association with *P. acanthocera*.

METHODS

Isolates

Tunnels of *P. acanthocera* in 7 year old *Eucalyptus grandis* trees growing in a plantation west of Cairns were examined (Figure 1). Wood associated with the tunnels had very distinct vascular staining (Figure 1) and fungi were commonly found sporulating on the surface of the discoloured wood. The most common of these fungi had ascomata with globose bases and long necks, resembling species of *Ceratocystis* and *Ophiostoma*.

Samples representing the inner surface of tunnels of *P. acanthocera* were collected from five trees and transferred to the laboratory for further study. Spore droplets from the apices of perithecia were transferred to 2% (w/v) malt extract agar (MEA) (Biolab, Midrand, South Africa) supplemented with streptomycin sulphate (0.001 g vol^{-1}, SIGMA, Steinheim, Germany) and incubated at 25 ºC. In addition a selective carrot baiting technique (Moller & De Vay 1968) was used to obtain isolates of *Ceratocystis.*

All isolates from the tunnels of *P. acanthocera* were purified on 2% MEA. They were subsequently stored in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and representative isolates have been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Representative cultures were dried on glycerol and deposited with the Nation Herbarium of South Africa (PREM).

Morphology

Morphological characters were described from two-week-old cultures grown on 2% MEA. Fungal structures were mounted on glass slides in lactophenol and examined under a Zeiss Axio Vision microscope. Fifty measurements were made for each taxonomically relevant structure. Ranges, averages and standard deviations (st. dev) were determined for each of these structures. The measurements are presented as: (minimum -) mean minus st. dev. - mean plus st. dev. (- maximum). Colors of cultures were defined based on the mycological colour charts of Rayner (1970). Growth studies were performed, on the type of the species as well as a paratype, by placing a 5mm plug from an actively growing culture (2-weeks-old) in the

center of a 90mm 2% MEA Petri dish. The plates were incubated at five different temperatures ranging from 5-35°C (with 5°C intervals). Measurements were made after 1 week, this study was repeated.

DNA isolation, PCR reactions and sequence analysis

DNA of four isolates was extracted as described by Van Wyk *et al*. (2006). PCR reactions for the Internal Transcribed Spacer regions (ITS) 1 and 2 including the 5.8S rDNA region, the Beta-tubulin (*β*-tubulin) region and the Transcription Elongation Factor 1α (EF-1α) region were prepared. The primers used to amplify the DNA for these three regions were those of White *et al*. (1990), Glass and Donaldson (1995) and Jacobs *et al*. (2004), respectively.

PCR reaction mixtures, for all three gene regions, consisted of 1 x Expand HF Buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme), 200 µM of each dNTP, FastStart *Taq* enzyme (2 U) (Roche Diagnostics, Mannheim, Germany), 200 ηM of the forward and reverse primers, and 2-10 ng DNA. Reaction volumes were adjusted to 25 µL with sterile water. The PCR programme was set for 4 min at 95 °C for initial denaturation of the double stranded DNA. This was followed by 10 cycles consisting of a denaturation step at 95 °C for 40 s, an annealing step for 40 s at 55 °C and an elongation step for 45 s at 70 °C. Subsequently, 30 cycles consisting of 94 ºC for 20 s, 55 ºC for 40 s with a 5 s extension step, after each cycle and 70 ºC for 45 s were performed. A final step of 10 min at 72 ºC completed the programme. Amplification of the DNA for the three gene regions 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 6 % Sephadex G-50 columns (Steinheim, Germany).

PCR amplicons were sequenced in both directions using the ABI PRISM^{TM} Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California), with the same primers as those used for DNA amplication. Sequencing reactions were run on an ABI PRISM^{TM} 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 with known species of *Ceratocystis* obtained from GenBank and analysed using PAUP version 4.0b10* (Swofford 2002). To determine whether the sequences for the three gene regions could be combined in one dataset, a partition homogeneity test (Swofford 2002) was conducted. Gaps were treated as a fifth character and trees were obtained via stepwise addition of 1 000

replicates with the Mulpar option in effect. The heuristic search option based on parsimony with stepwise addition was used to obtain the phylogram. Confidence intervals using 1 000 bootstrap replicates were calculated. *Ceratocystis virescens* (Davidson) Moreau was designated as the out-group taxon. All sequences derived from this study were deposited in GenBank (Table 1).

RESULTS

Isolates

Isolations from ascomata in the tunnels of *P. acanthocera* yielded cultures of a fungus that had a very distinct dark green colour. The perithecia exuded hat-shaped ascospores in sticky spore drops, typical of many species of *Ceratocystis* and *Ophiostoma*. Cultures of the fungus had distinct *Thielaviopsis* anamorph that is specific to Ophiostomatoid fungi residing in *Ceratocystis*.

Four isolates of a *Ceratocystis* sp. (CMW 19383/ CBS 120517, CMW 19385/ CBS12051, CMW 19387/ CBS 120519, CMW 19389/ CBS 120225) were collected from four of the five *E. grandis* trees sampled. Based on their very dark green colour, the isolates were distinct from all known species of this genus. Chlamydospores were also absent in this fungus. Two types of conidiophores were found. The more common of these had long conidiogenous cells and others were shorter with wider apices. Both cylindrical and barrel-shaped conidia were present. The optimum growth range for these isolates was 20 – 25 °C. No growth was observed at 5, 10 and 35 °C. At 15 °C the isolates grew ~12 mm in seven days. At 20, 25 and 30 °C the isolates grew ~26mm, 33 mm and 17 mm, respectively.

DNA isolation, PCR reactions & sequence analysis

DNA sequencing yielded amplicons of ~500bp for both the ITS and β-tubulin gene regions and amplicons of ~800bp was obtained for the EF1-α. Partition homogeneity tests showed that the data could be combined (P=0.05). Two most parsimonious trees were obtained, one of which was selected for representation (Figure 2). This tree had a length of 1472 base pairs, the total amount of characters were 1913, with 1150 of these characters being constant, 301 characters being parsimony-uninformative and 462 characters being parsimony-informative, with CI = 0.7554, HI = 0.2446, RI = 0.8216 and RC = 0.6207.

In the phylogenetic tree, *C. fimbriata sensu stricto* (*s.s.*), *C. platani* Engelbrecht and Harrington, *C. cacaofunesta* (Walter) Engelbrecht and Harrington, *C. pirilliformis, C. polychroma* M. van Wyk and M.J. Wingfield, *C. albifundus* M.J. Wingf., De Beer and M.J. Morris, *C. caryae* J.A. Johnson and Harrington, *C. smalleyi* J.A. Johnson and Harrington, *C. variospora* (Davids.) C. Moreau and *C. populicola* J.A. Johnson and Harrington all formed distinct clades, supported by high bootstrap values. The four isolates of the morphologically distinct *Ceratocystis* sp. from the tunnels of *P. acanthocera* on *E. grandis* in Australia formed a separate and distinct clade (Figure 2).

TAXONOMY

Based on morphological characteristics and DNA sequence comparisons for three gene regions, the *Ceratocystis* sp. considered in this study clearly represents a unique taxon. It is thus described as follows:

Ceratocystis atrox M. van Wyk and M.J. Wingf. **sp. nov.** **(Figure 4-5)**
MB511138

Etymology: Name refers to the dark-colored cultures of the fungus from the Latin word *atrocis* meaning dark, fierce, fearsome.

Coloniae atro-olivaceae, hyphae laeves segmentatae, 3–4 µm latae. Bases ascomatum atrobrunneae vel nigrae, globosae, (120-)140–180(-222) µm diametro. Colla ascomatum basin versus atrobrunnea, (21-)26–34(-40) µm lata, apicem versus pallescentia, (13-)14–16(-19) µm lata, (277-)313–401(-451) µm longa. Hyphae ostiolares divergentes, hyalinae, (18-)20–26(-28) µm longae. Asci non visi. Ascosporae in massa rotundata alba vel luteo-bubalina in apicibus collorum ascomatum crescunt, lateraliter visa cucullatae vel pileatae, non septatae, hyalinae, vaginatae, 3–4 µm longae, 4–6 µm latae. *Anamorpha Thielaviopsis*: conidiophorae biformes, in mycelio singuli, conidiophorum primarium hyalinum, (78-)87–151(-218) µm longum, basi 5–7(-13) µm, apice 4–8(-9) µm latum, conidiophorum secondarium hyalinum, phialido primario brevius, (39-)43–57(-66) µm longum, basi 5–7(-9) µm, apice 4–6(-7) µm latum. Evolutio conidiorum phialidica per formatione parietum annularium, conidia biformia, singula vel concatenata, primaria (9-)11–15(-17) µm longa, 3–5 µm lata, secondaria (7-)8–12(-14) µm longa, (5-)6–8(-9) µm lata. Chlamydosporae desunt.

Colonies on 2% MEA dark-olive (21"m) in colour. *Hyphae* smooth and segmented, 3 – 4 µm wide. *Ascomatal bases* dark brown to black, globose, (120-)140–180(-222) µm in diameter. *Ascomatal necks* dark brown at base becoming lighter towards apex, (21-)26–34(-40) µm wide at base of neck, (13-)14–16(-19) µm wide at tip of neck, (277-)313–401(-451) µm in length. *Ostiolar hyphae* divergent, hyaline, (18-)20–26(-28) µm in length. *Asci* not observed. *Ascospores* accumulate in a round, white to yellow (yellow-buff 19d) mass at the apices of the ascomatal necks, cucullate (hat-shaped) in side view, aseptate, hyaline, invested in sheath, 3–4 µm in length by 4–6 µm in width. *Thielaviopsis anamorph*: conidiophores of two types occurring singly on mycelium, primary conidiophores hyaline, long, (78-)87–151(-218) µm in length, 5–7(-13) µm wide at base, 4–8(-9) µm wide at tip, secondary conidiophores hyaline, shorter than primary phialide, (39-)43–57(-66) µm in length, 5–7(-9) µm wide at base, 4–6(-7) µm wide at tip. Phialidic *conidium* development through ring wall building, *conidia* of two types formed singly or in chains, primary conidia (9-)11–15(-17) µm in length, 3–5 µm wide, secondary conidia (7-)8–12(-14) µm in length, (5-)6–8(-9) µm wide. *Chlamydospores* not present. Optimum growth range is between 20 – 25 °C.

Habitat: tunnels of *Phoracantha acanthocera* in *Eucalyptus grandis* trees
Known distribution: Australia.
Material examined: **Australia,** Queensland, isolated from tunnels of *Phoracantha acanthocera* in *Eucalyptus grandis* trees, M.J. Wingfield, holotype Herb. PREM59012, CMW19385, CBS120518 (Aug. 2005). **Australia**, Queensland, isolated from tunnels of *Phoracantha acanthocera* in *Eucalyptus grandis* trees, M.J. Wingfield, paratype PREM59013, CMW19383, CBS120517 (Aug. 2005).

DISCUSSION

Results of this study have led to the discovery of a new species of *Ceratocystis* from Australia. This fungus, which has been given the name *C. atrox,* together with *C. pirilliformis*, *C. eucalypti*, *C. moniliformis* and *C. moniliformopsis*, is the fifth species to have been described from *Eucalyptus* spp. in the country. *Ceratocystis atrox* is also the first *Ceratocystis* sp. to have been found associated with the tunnels of a wood-boring insect in Australia.

Ceratocystis atrox has very obvious hat shaped ascospores and a *Thielaviopsis* anamorph, which places it in either the *C. moniliformis s.l.* or *C. fimbriata s.l.* complex (Baker-Engelbrecht & Harrington 2005, Van Wyk *et al*. 2005). The absence of spines on the bases of

the perithecia and the absence of a disk-like shape at the base of the perithecial necks makes it typical of species residing in the latter group. *Ceratocystis atrox* can easily be distinguished from all other species in the *C. fimbriata* species complex based on various morphological characteristics. One of the most obvious of these is its very dark green colour in culture. It is also unique amongst species of *Ceratocystis* in the *C. fimbriata s.l.* species complex based on its segmented hyphae, short ascomatal necks and the fact that no chlamydospores are found in this species.

Based on cultural characteristics, *C. atrox* differs from all other species of the genus found in Australia. *Ceratocystis moniliformis* has white to grey colored cultures, *C. moniliformopsis* cultures are cream to brown colored (Van Wyk *et al*. 2004a) and both species produce a very fruity aroma. *Ceratocystis pirilliformis* has an olivacious green colour (Barnes *et al*. 2003a) and bears some similarity to *C. atrox*, although the cultures of the latter species are much darker. *Ceratocystis eucalypti* has a dark greenish grey to black colour (Kile *et al*. 1996). When *C. atrox* is compared to the other *Ceratocystis* spp. residing in the same phylogenetic clade, it is also clearly distinct. In this regard, *C fimbriata s.s.*, *C. polychroma*, *C. cacaofunesta* and *C. platani* all have relatively dark-colored cultures but *C. atrox* is a considerable darker green colour than any of them.

All five of these species of *Ceratocystis* occurring on *Eucalyptus* in Australia share common morphological characteristics. These include the formation of hat-shaped ascospores and divergent ostiolar hyphae. They can, however, be distinguished based on the shapes of the ascomatal bases. The ascomatal bases of *C. pirilliformis* are pear-shaped base (Barnes *et al*. 2003a) compared to the globose bases of the remaining four species. *Ceratocystis moniliformis* and *C. moniliformopsis* both have conical spines on their bases. *Ceratocystis atrox* has segmented hyphae that are not seen in any of the other four species as they all have non-segmented hyphae.

DNA sequence comparisons for three gene regions have shown that *C. atrox* resides in the *C. fimbriata* species complex, yet in a discrete clade separate from all other taxa in this group. Comparisons with *C. fimbriata sensu stricto*, *C. cacaofunesta*, *C. platani*, *C. pirilliformis*, *C. polychroma*, *C. caryae*, *C. populicola*, *C. smalleyii, C. variospora* and *C. albifundus* and using *C. virescens* as the monophyletic sister out-group indicate that the closest relative of *C. atrox* is *C. polychroma*. This is a species, thought to be native to Sulawesi (Indonesia), which is associated with a severe die-back disease on clove trees (*Syzygium aromaticum*) (Van Wyk

et al. 2004b). It is interesting that *C. polychroma* and *C. atrox* share similar ecological habitats associated with cerambycid beetles.

Nothing is known regarding the ecology of the association between *C. atrox* and *P. acanthocera*. The fungus was consistently found in tunnels of the insect on all trees examined and this implies a close relationship between the two organisms. The very clear discolouration of the wood associated with the tunnels and the fungus growing in them, implies that the fungus is able to penetrate the wood deeply and that it is more than a surface inhabitant in the tunnels. It is possible that it plays a role in excluding other fungi such as moulds from this niche, as has been suggested for the ophiostomatoid fungi occurring in the infructesences of *Protea* spp. in South Africa (Marais *et al*. 1998). *Ceratocystis atrox* may also be a mild pathogen, contributing to the development of its insect associate, but it is unlikely to be highly pathogenic, as trees infested with *P. acanthocera* were never found to be dying.

REFERENCES

Abbott I., Smith R., Williams M. & Voutier R. (1991) Infestation of regenerated stands of karri (*Eucalyptus diversicolor*) by bullseye borer (*Tryhocaria acanthocera*, Cerambycidae) in Western Australia. *Australian Forestry* **54**: 66–74.

Baker-Engelbrecht C.J. & Harrington T.C. (2005) Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. *Mycologia* **97**: 57–69.

Barnes I., Roux J., Wingfield B.D., Dudzinski M.J., Old K.M. & Wingfield M.J. (2003a) *Ceratocystis pirilliformis*, a new species from *Eucalyptus nitens* in Australia. *Mycologia* **95**: 865–871.

Barnes I., Roux J., Wingfield B.D., O'Neil M. & Wingfield M.J. (2003b) *Ceratocystis fimbriata* infecting *Eucalyptus grandis* in Uruguay. *Australasian Plant Pathology* **32**: 361–366.

Christiansen E. & Solheim H. (1990) The bark beetle-associated blue-stain fungus *Ophiostoma polonicum* can kill various spruces and Douglas fir. *European Journal of Forest Pathology* **20**: 436–446.

Farr J.D., Dick S.G., Williams M.R. & Wheeler I.B. (2000) Incidence of bullseye borer (*Phoracantha acanthocera* (Macleay) Cerambicidae) in 20–35 year old regrowth karri in the south west of Western Australia. *Australian Forestry* **63**: 107–123.

Glass N.L. & 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.

Jacobs K., Bergdahl D.R., Wingfield M.J., Halik S., Seifert K.A., Bright D.E. & Wingfield B.D. (2004) *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* **108**: 411–418**.**

Johnson J.A., Harrington T.C. & Engelbrecht C.J.B. (2005) Phylogeny and taxonomy of the North American clade of the *Ceratocystis fimbriata* complex. *Mycologia* **97**: 1067–1092.

Kile G.A., Harrington T.C., Yaun Z.Q., Dudzinski M.J. & Old K.M. (1996) *Ceratocystis eucalypti* sp. nov., a vascular stain fungus from eucalypts in Australia. *Mycological Research* **100**: 571–579.

Marais G.J., Wingfield M.J., Viljoen C.D. & Wingfield B.D. (1998) A new ophiostomatoid genus from *Protea* infructescences. *Mycologia* **90**: 136–141.

Marin M., Castro B., Gaitan A., Preisig O., Wingfield B.D. & Wingfield M.J. (2003) Relationships of *Ceratocystis fimbriata* isolates from Colombian coffee-growing regions based on molecular data and pathogenicity. *Journal of Phytopathology* **151**: 395–405.

Moller W. & DeVay J (1968) Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* **58**: 1499–1508.

Phillips C.L. (1993a) Forest Insects. Longicorn beetles 2. Bulls-eye borer – *Tryphocaria acanthocera* Macleay. Forest Health Fact sheet 18. (http://www.forestry.sa.gov.au).

Phillips C.L. (1993b) Insect pest problems of eucalypt plantations in Australia. 5. South Australia. *Australian Forestry* **56**: 378–380.

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

Redfern D.B., Stoakley J.T., Steele H. (1987) Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae. Plant Pathology* **36**: 467–480.

Roux J., Coutinho T.A., Munjuni Byabashaija D., Wingfield M.J. (2001) Diseases of plantation *Eucalyptus* in Uganda. *South African Journal of Science* **97**: 16–18.

Roux J., Van Wyk M., Hatting H., Wingfield M.J. (2004) *Ceratocystis* species infecting stem wounds on *Eucalyptus grandis* in South Africa. *Plant Pathology* **53**: 414–421.

Roux J., Wingfield M.J., Wingfield B.D., Bouillett J.P. & Alfenas A.C. (1999) A serious new disease of *Eucalyptus* caused by *Ceratocystis fimbriata* in Central Africa. *Forest Pathology* **30**: 175–184.

Swofford D.L. (2002) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Van Wyk M., Roux J., Barnes I., Wingfield B.D., Chhetri D.B., Kirisits T. & 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. & Wingfield M.J. (2004b) *Ceratocystis polychroma* sp. nov., a new species from *Syzygium aromaticum* in Sulawesi. *Studies in Mycology* **50**: 273–282.

Van Wyk M., Roux J., Barnes I., Wingfield B.D. & Wingfield M.J. (2005) Molecular phylogeny of the *Ceratocystis moniliformis* complex and description of *C. tribiliformis*. *Fungal Diversity* **21**: 181–201.

Van Wyk M., Van der Merwe N.A., Roux J., Wingfield B.D., Kamgan G.N. & Wingfield M.J. (2006) Population biology of *Ceratocystis fimbriata* from *Eucalyptus* trees in South Africa. *South African Journal of Science* **102**: 259–263.

Wang Q., Thornton I.W.B., New T.R. & Wang Q. (1999) A cladistic analysis of the phoracanthine genus *Phoracantha* Newman (Coleoptera: Cerambycidae: Cerambycinae), with discussion of biogeographic distribution and pest status. *Annals of the Entomological Society of America* **92**: 631–638.

Webster R.K. & Butler E.E. (1967) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45**: 1457–1467.

White T.J., Bruns T., Lee S. & 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. Innis MA, Gelfand DH, Sninsky JJ, White TJ) 315-322. Academic Press, San Diego.

Wingfield M.J., De Beer C., Visser C. & Wingfield B.D. (1996) A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematic and Applied Microbiology* **19**: 191–202.

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

Table 1. *Ceratocystis* isolates used in this study.

Species	Isolate no.	GenBank accession no.	Host	Geographical origin	Collector(s)
C. albifundus	CMW4068	DQ520638 EF070429 EF070400	*Acacia mearnsii*	RSA	J. Roux
C. albifundus	CMW5329	AF388947 DQ371649 EF070401	*Acacia mearnsii*	Uganda	J. Roux
C. cacaofunesta	CMW15051 CBS152.62	DQ520636 EF070427 EF070398	*Theobroma cacao*	Costa Rica	A.J. Hansen
C. cacaofunesta	CMW14809 CBS115169	DQ520637 EF070428 EF070399	*Theobroma cacao*	Ecuador	C. Suarez
C. fimbriata	CMW15049 CBS141.37	DQ520629 EF070442 EF070394	*Ipomaea batatas*	USA	C.F. Andrus
C. fimbriata	CMW1547	AF264904 EF070443 EF070395	*Ipomaea batatas*	Papua New Guinea	E.C.H. McKenzie
C. pirilliformis	CMW6569	AF427104 DQ371652 AY528982	*Eucalyptus nitens*	Australia	M.J. Wingfield
C. pirilliformis	CMW6579	AF427105 DQ371653 AY528983	*Eucalyptus nitens*	Australia	M.J. Wingfield
C. platani	CMW14802 CBS115162	DQ520630 EF070425 EF070396	*Platanus occidentalis*	USA	T.C. Harrington
C. polychroma	CMW11424 CBS115778	AY528970 AY528966 AY528978	*Syzygium aromaticum*	Indonesia	M.J. Wingfield
C. polychroma	CMW11436 CBS115777	AY528971 AY528967 AY528979	*Syzygium aromaticum*	Indonesia	M.J. Wingfield
C. atrox	CMW19383 CBS120517	EF070414 EF070430 EF070402	*Eucalyptus grandis*	Australia	M.J. Wingfield

Species	Isolate no.	GenBank accession no.	Host	Geographical origin	Collector(s)
C. atrox	CMW19385 CBS120518	EF070415 EF070431 EF070403	*Eucalyptus grandis*	Australia	M.J. Wingfield
C. atrox	CMW19387 CBS120519	EF070416 EF070432 EF070404	*Eucalyptus grandis*	Australia	M.J. Wingfield
C. atrox	CMW19389 CBS120225	EF070417 EF070433 EF070405	*Eucalyptus grandis*	Australia	M.J. Wingfield
C. populicola	CMW14789 CBS119.78	EF070418 EF070434 EF070406	*Populus* sp.	Poland	J. Gremmen
C. populicola	CMW14819 CBS114725	EF070419 EF070435 EF070407	*Populus* sp.	USA	T. Hinds
C. caryae	CMW14793 CBS114716	EF070424 EF070439 EF070412	*Carya cordiformis*	USA	J. Johnson
C. caryae	CMW14808 CBS115168	EF070423 EF070440 EF070411	*Carya ovata*	USA	J. Johnson
C. smalleyii	CMW14800 CBS114724	EF070420 EF070436 EF070408	*Carya cordiformis*	USA	G. Smalley
C. variospora	CMW20935 CBS114715	EF070421 EF070437 EF070409	*Quercus alba*	USA	J. Johnson
C. variospora	CMW20936 CBS114714	EF070422 EF070438 EF070410	*Quercus robur*	USA	J. Johnson
C. virescens	CMW11164	DQ520639 EF070441 EF070413	*Fagus americanum*	USA	D. Houston

Figure 1. Disease symptoms and damage caused by *P. acanthocera* on *Eucalyptus* trees in Australia. (A) Cracking bark, (B) Damage caused by larvae, (C-D) Fungal staining associated with insect tunnels and *C. atrox*.

Figure 2. Phylogenetic tree based on the combined regions of the ITS, β-tubulin and Ef1-α for *Ceratocystis atrox* and other species in the *C. fimbriata species* complex. The phylogram was obtained using the heuristic search option based on parsimony. Bootstrap values are indicated above the branches. *C. virescens* is used as the out-group taxon.

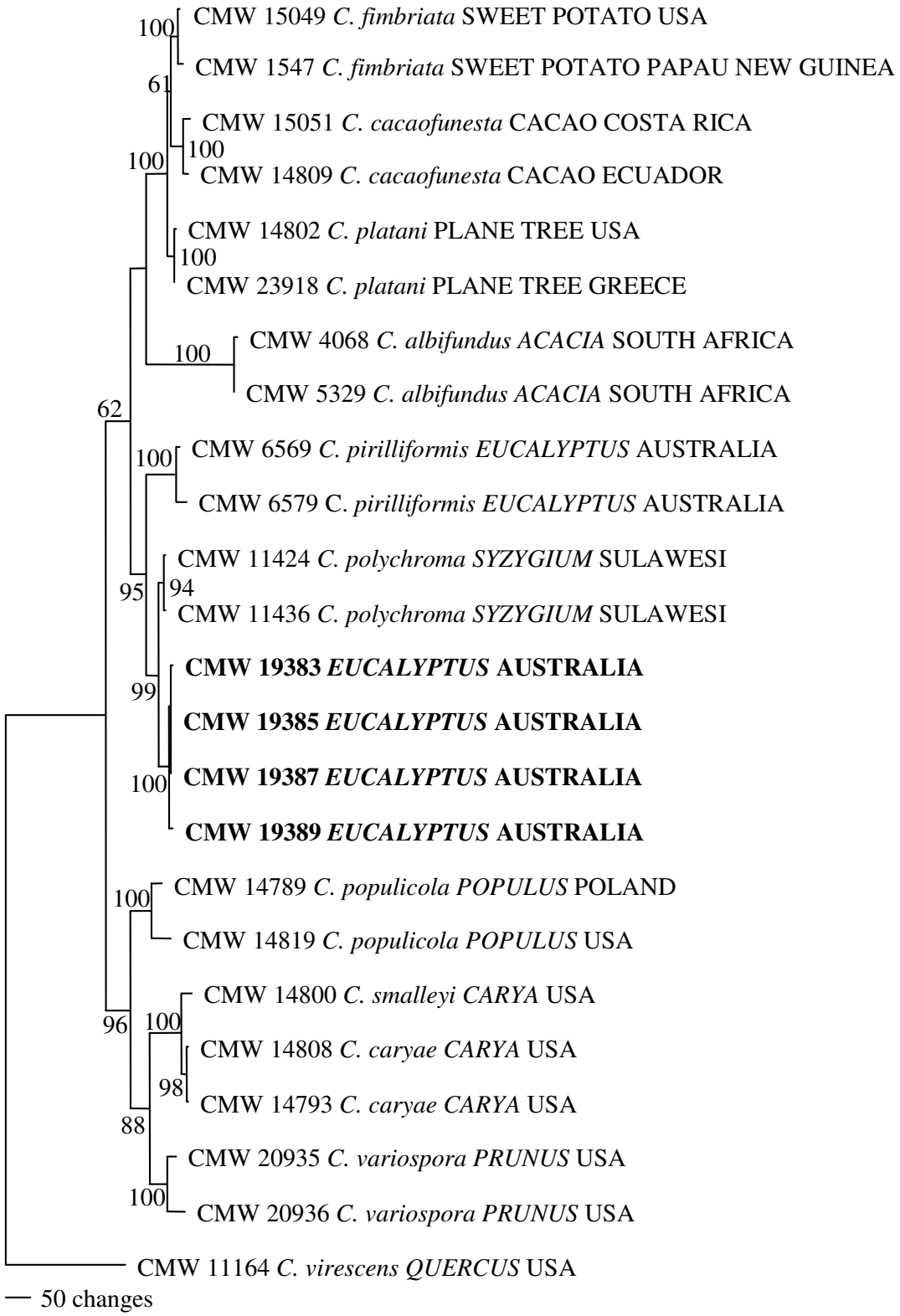
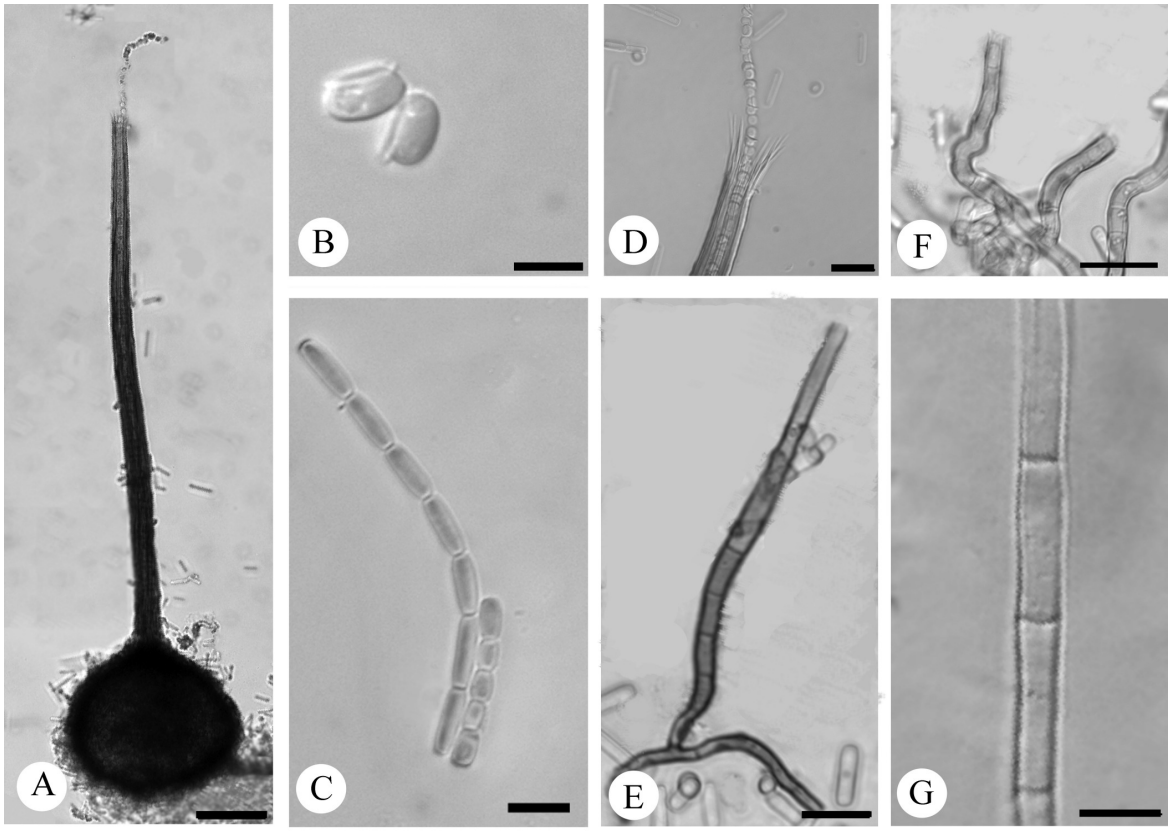


Figure 3. Morphological characteristics of *Ceratocystis atrox*: A. Globose ascomata. B. Hat-shaped ascospores. C. Cylindrical (bottom) and barrel (top) conidia in chains. D. Divergent ostiolar hyphae with emerging hat-shaped ascospores. E. Primary phialidic conidium. F. Secondary conidium. G. Segmented hyphae. Scale bars: A = 20, B = 20, C = 10, D = 5, E = 10, F = 5 µm, G = 5 µm.



CHAPTER 6

Ceratocystis fimbriatomima a new species in the *C. fimbriata sensu lato* complex from *Eucalyptus* trees in Venezuela

Van Wyk M, Wingfield B.D., Mohali S. & Wingfield M.J. (2009). *Ceratocystis fimbriatomima* a new species in the *C. fimbriata sensu lato* complex from *Eucalyptus* trees in Venezuela. *Fungal Diversity* **34:** 173-183.

ABSTRACT

Species of *Ceratocystis* represent a group of important plant pathogens as well as saprobes that occur, primarily on woody substrates. The number of species in *Ceratocystis* has increased substantially in recent years, particularly as DNA-based methods have allowed for the recognition of cryptic taxa. The aim of this study was to identify isolates of a *Ceratocystis* sp. collected from freshly cut stumps of *Eucalyptus* trees in Venezuela. This was carried out using morphological comparisons with similar fungi as well as DNA sequence comparisons for the Internal Transcribed Spacer regions 1 and 2 including the 5.8S rDNA operon, part of the beta-tubulin gene and part of the Transcription Elongation Factor 1-alpha gene region. Characteristics of the fungus in culture and its morphology resembled most species in the *C. fimbriata sensu lato* species complex. Microscopically, the fungus was most similar to *C. fimbriata sensu stricto*. Based on phylogenetic analyses, it was distinct from other species of *Ceratocystis sensu lato* having *C. manginecans* as its closest relative. The *Ceratocystis* sp. from *Eucalyptus* in Venezuela clearly represents a distinct taxon for which the name *C. fimbriatomima* sp. nov. is provided.

INTRODUCTION

Ceratocystis spp. in the *C. fimbriata sensu lato* (*s.l.*) species complex are mostly pathogens causing diseases of a large number of woody and some herbaceous plants (Kile 1993). *Ceratocystis fimbriata* Ell. & Halst., the causal agent of black rot on sweet potato (*Ipomoea batatas* L.) was the first species to be described and it typifies the genus (Halsted 1890). Subsequent to its first discovery, fungi identified as representing this species were isolated from a wide variety of hosts in many different parts of the world (Alexopoulos 1962, Kile 1993, Seifert *et al.* 1993).

Ceratocystis fimbriata has long been recognised to represent a complex of cryptic species (Webster & Butler 1967a,b, Kile 1993, Harrington 2000). Studies based on DNA sequence data have confirmed this view and a recent trend has been to describe species that represent monophyletic lineages that occur in particular niches (Wingfield *et al.* 1996, Barnes *et al*. 2003, Marin *et al*. 2003, Engelbrecht & Harrington 2005, Johnson *et al*. 2005, Van Wyk *et al*. 2004, 2007a,b). The first of these to be described was *C. albifundus* M.J. Wingf., De Beer & Morris which emerged as a pathogen of plantation-grown non-native *Acacia* spp. in South Africa in the early 1990's (Morris *et al.* 1993, Wingfield *et al.* 1996). Subsequently, many new species have been described in the *C. fimbriata s.l.* species complex including *C. pirilliformis* Barnes and M.J. Wingf. (Barnes *et al*. 2003), *C. polychroma* M. van Wyk and M.J. Wingf. (Van Wyk *et al*. 2004), *C. cacaofunesta* Engelbrecht and Harrington (Engelbrecht and Harrington, 2005), *C. platani* Engelbrecht and Harrington (Engelbrecht & Harrington 2005) and *C. atrox* M. van Wyk & M.J. Wingf. (Van Wyk *et al*. 2007b). *Ceratocystis fimbriata* is restricted to isolates from sweet potato and is appropriately referred to as *C. fimbriata sensu stricto* (*s.s.*) (Engelbrecht & Harrington 2005).

Most *Ceratocystis* species in the *C. fimbriata s.l.* species complex cause or are associated with plant diseases (Kile 1993). Symptoms associated with these fungi include root rot in tubular plants, vascular staining, cankers and vascular wilts in woody hosts. Some species threaten the propagation of woody crop plants such as coffee (Marin *et al*. 2003), cacao (Engelbrecht & Harrington 2005), mango (Al Adawi *et al*. 2006, Van Wyk *et al*. 2007a) and timber crops such as *Eucalyptus* (Roux *et al*. 2004, Rodas *et al*. 2007) and *Acacia* (Morris *et al*. 1993, Wingfield *et al*. 1996, Roux *et al*. 2007). On *Eucalyptus*, *C. fimbriata s.l.* has been associated with serious canker and vascular wilt diseases in African and South American countries (Laia *et al*. 1999, Roux *et al.* 2000, Rodas *et al*. 2007).

Many recent studies have recorded species of *Ceratocystis* in the *C. fimbriata s.l.* complex from countries in South America (Baker *et al*. 2003, Marin *et al*. 2003, Rodas *et al.* 2007). Other than reports of *C. fimbriata s.l.* from cacao and coffee in Venezuela in the 1950's (Pontis 1951, Malaguti 1952a,b, De Reyes 1988), very little is known regarding these fungi in Venezuela. During the course of a recent survey of *Eucalyptus* diseases in Venezuela, a *Ceratocystis* sp. resembling *C. fimbriata s.l.* was commonly encountered on the freshly cut stumps of *Eucalyptus* trees.

MATERIALS AND METHODS

Isolates

Samples bearing ascomata typical of *Ceratocystis* spp. were collected from stumps of recently (three-week-old) felled *Eucalyptus grandis* x *E. urophylla* hybrid trees near Acarigua, Portuguesa State in Venezuela. The samples were wrapped in newspaper and placed in separate plastic bags and transported to the laboratory. Ascomata on the wood were inspected one week after collection and masses of ascospores were transferred to 2% Malt Extract Agar (MEA: 20% w/v; Biolab, Midrand, South Africa) supplemented with 100mg/L streptomycin sulphate (SIGMA). Pure cultures were obtained and these have been deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), The University of Pretoria, South Africa. Representative isolates were also lodged with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

PCR and sequencing reactions

Six isolates (Table 1) were grown for two weeks on 2% MEA, after which the mycelium was scrapped from the surface of cultures. DNA was extracted as described by Van Wyk *et al*. (2006) and PCR reactions were run for three gene regions as described by Van Wyk *et al*. (2006). The gene regions selected for sequencing were the Internal Transcribed Spacer region (ITS) one and four including the 5.8S rDNA operon, part of the beta-tubulin (β-tubulin) gene and part of the Transcription Elongation Factor 1-alpha (EF1-α) gene region. The primers selected for the PCR and sequencing reactions were ITS1 and ITS4 developed by White *et al*. (1990), βt1a and βt1b developed by Glass and Donaldson (1995) and EF1F and EF1R developed by Jacobs *et al*. (2004).

For sequencing, two separate reactions were used for the forward and reverse primers, respectively. The reactions were run using the ABI PRISM^{TM} Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California) on an ABI PRISM^{TM} 3100 Autosequencer (Applied BioSystems, Foster City, California, USA). The resultant sequences were

analyzed using the software programme Sequence Navigator (version 1.0.1) (Applied BioSystems, Foster City, California). These sequences, together with sequences for other *Ceratocystis* spp. from GenBank (Table 1) were aligned using the software programme MAFFT (http://timpani.genome.ad.jp/%7emafft/server/) (Katoh *et al*. 2002).

A partition homogeneity test (PHT) was conducted in PAUP version 4.0b10* to determine whether the data sets could be combined (Swofford 2002). In PAUP, all characters had equal weight, and gaps were treated as "fifth base". The heuristic search option based on parsimony was selected to search for optimal trees using heuristic algorithms (Swofford 2002). The starting trees were obtained via stepwise addition, the sequences were randomly added and this was repeated 1000 times. To generate trees, the branch-swapping algorithm was set to tree-bisection-reconnection with the steepest decent not enforced. Polytomies were created by collapsing branches, if the maximum branch length was zero. The "Multrees" option was selected and topological constraints were not enforced. The tree was rooted with two isolates of *C. virescens* (Davidson) Moreau representing the outgroup taxon. Confidence intervals were obtained by calculating 1000 bootstrap replicates. All sequences derived from this study have been deposited in GenBank (Table 1).

The software program MrBayes (version 3.1.1) with the Markov Chain Monte Carlo (MCMC) algorithm was used to produce phylogenetic trees based on Bayesian probabilities (Ronquist & Huelsenbeck 2003). A model of nucleotide substitution was determined for each gene region, using Mrmodeltest2 (Nylander 2004). The nucleotide substitutions obtained were included for each gene partition in MrBayes. One million random trees were generated using the MCMC procedure with four chains, including hot and cold chains, and sampled every 100th generation. Tree likelihood scores were assessed to determine the number of trees that were formed before the stabilization, to prevent including trees that were formed before convergence. Trees outside the point of convergence were discarded by means of the burn-in procedure (Ronquist & Huelsenbeck 2003).

Culture characteristics and morphology

Isolates (CMW24174, CMW24176, CMW24376 and CMW24377) morphologically resembling a species of *Ceratocystis* in the *C. fimbriata s.l.* species complex were grown for two weeks on 2% MEA. Subsequently, 4 mm plugs were transferred to the centres of five 90mm Petri dishes, containing 2% MEA, for seven different temperatures to be tested for growth for each of the isolates. These plates were incubated at 5°C to 35°C at 5°C intervals. Growth was assessed by taking two diameter measurements at right angles to each other for all plates after seven days of incubation. Averages of the ten diameter measurements for each isolate at each temperature were

computed and the entire experiment was repeated once. The colour charts of Rayner (1970) were used to standardise the descriptions of colony colour.

For microscopic measurements, fungal structures, taken from 10 d-old cultures on 2% MEA were mounted in lactic acid. Fifty measurements were made of each taxonomically relevant structure from the culture CMW24174 and 10 measurements for these structures were made for isolates CMW24176, CMW24376 and CMW24377. The minimum, maximum, average and standard deviation (stdv) was calculated for the measurements of each structure. The measurements are thus presented as (minimum-) stdv minus the mean – stdv plus the mean (-maximum). A Carl Zeiss microscope with a Zeiss Axio Vision camera system was used to asses the measurements and to capture photographic images of all relevant taxonomic structures.

RESULTS

Isolates

Fresh fungal structures were commonly found on the specimens collected from *Eucalyptus* stumps in Venezuela. The structures were characteristic of *Ceratocystis* spp. having a *Thielaviopsis* anamorph. Seventeen isolates of the *Ceratocystis* sp. were made from the samples taken from five *Eucalyptus* trees. One isolate (CMW24174) was chosen to represent the fungus and three additional isolates (CMW24176, CMW24376 and CMW24377) were chosen as additional specimens for description. These cultures, grown on 2% MEA, were dried down and have been deposited with the National Collection of Fungi (PREM), Pretoria, South Africa (Table 1.)

PCR and sequencing reactions

Amplicons of ~500 bp (ITS and β-tubulin) and ~800 bp (EF1-α) were obtained from the six isolates chosen for DNA sequence analysis (Table 1). The PHT resulted in a low P-value (P=0.01), possibly attributed to the minimal variation in the β-tubulin gene region. Although the P-value was low, studies (Sullivan, 1996; Cunningham, 1997) suggest that the data could still be combined. The combined dataset for the three gene regions had a total of 1944 characters. Of these 1944 characters, 1164 were constant, 45 were parsimony-uninformative and 735 were parsimony informative. Thirty-six most parsimonious trees were obtained, one of which was selected for presentation (Figure 7). This tree had a length of 1501 steps and is described as follows: Consistency Index = 0.7382, Retention Index = 0.8805 and Rescaled Consistency Index = 0.6500.

Based on the phylogenetic analysis, the *Ceratocystis* sp. from *Eucalyptus* in Venezuela grouped separately from all the described *Ceratocystis* spp. in the *C. fimbriata s.l.* species complex. The closest phylogenetic relative of this fungus was *C. manginecans* (Figure 7). The posterior probabilities for the tree emerging from the phylogenetic analysis were high with the *Ceratocystis* sp. from Venezuela supported 100%. All other species used in this study for comparison resided in groups with high bootstrap support and represented distinct phylogenetic taxa (Figure 7).

For both the ITS and the β-tubulin gene regions, MrModeltest2 selected the GTR+G model to support the datasets best. The HKY+G model was selected for the EF1-α gene region. These model settings were included in the Bayesian analysis and 3000 trees were discarded due to the fact that they were outside of the point of convergence (burn-in) when analysing the Bayesian inference. The posterior probability of the branch nodes of the combined tree obtained with the Bayesian inference supported the bootstrap values obtained with PAUP (Figure 7).

Culture characteristics and morphology

The cultures of the *Ceratocystis* sp. from *Eucalyptus* in Venezuela had a greenish olivaceous (33'"f) colour (Rayner 1970). No growth was observed at 5°C, 10°C and 35°C, and limited growth was observed after seven days at 15°C (26mm) and 20°C (39mm). At 25°C and 30°C the cultures grew rapidly, reaching 50mm and 45mm, respectively in seven days. The cultures had a banana odour similar to that of many *Ceratocystis* spp.

TAXONOMY

The *Ceratocystis* sp. from Venezuela isolated from freshly cut *Eucalyptus* stumps is phylogenetically distinct from all other *Ceratocystis* spp. residing in the *C. fimbriata s.l.* clade. It is also morphologically different to all of these species and is, therefore, described as a new species as follows:

Ceratocystis fimbriatomima M. van Wyk & M.J. Wingf. **sp. nov.** (Figure 1-6)
MycoBank: 511432

Etymology: The name of this fungus is derived from the Latin word *fimbriato* = *fimbriata* and *mima* = mimicking, chosen to describe its morphological similarity to *C. fimbriata s.s.*

Ascosporae lateraliter visae cucullato-pileiformes, non septatae, hyalinae, in vagina inclusa; vagina exclusa 2-4 x 4-6 µm. *Conidiophora secondaria* (phialide infundibuliformi) et *conidia secondaria* (doliiformia) desunt. *Chlamydosporae* umbrinae (6-) 10-14 (-15) µm longae, (6-) 7-11 (-12) µm latae.

Ascomatal bases dark, globose, un-ornamented (142-) 173-215 (-234) µm in diam. *Ascomatal necks* dark at bases becoming lighter towards the apices, (446-) 660-890 (-1070) µm long, apices (16-) 18-24 (-28) µm wide, bases (28-) 32-42 (-47) µm wide. *Ostiolar hyphae* divergent, (40-) 49-61 (-68) µm long. *Ascospores* hyaline, hat-shaped in side view, invested in sheath, 2-4 µm long, 4-6 µm wide, accumulating in buff-yellow masses at tips of ascomatal necks. *Anamorph*: *Thielaviopsis*, *Primary conidiophores* phialidic, flask-shaped, (49-) 60-94 (-122) µm long, 3-5 µm wide at the apices, 5-9 µm wide at broadest points and 4-7 µm wide at bases. *Secondary conidiophores* flaring or wide mouthed absent. *Primary conidia* cylindrical in shape (14-) 20-28 (-31) µm long, 3-5 µm wide. *Secondary conidia*, barrel-shaped conidia, absent. *Chlamydospores* hair brown (17""i), subglobose (6-) 10-14 (-15) µm long, (6-) 7-11 (-12) µm wide.

Habitat: On cut stumps of recently (three-week-old) felled *Eucalyptus grandis* x *E. urophylla* hybrids.
Known distribution: Venezuela.
Material examined: **Venezuela,** Acarigua, Portuguesa State, isolated from bases of felled *Eucalyptus* trees, M.J. Wingfield, PREM59439, CMW24174, CBS121786 (July 2006). **Venezuela,** Acarigua, Portuguesa State, isolated from *Eucalyptus* trees, M.J. Wingfield, PREM59437, CMW24176, CBS121787 (July 2006). **Venezuela,** Acarigua, Portuguesa State, isolated from *Eucalyptus* trees, M.J. Wingfield, PREM59615, CMW24376, CBS121788 (July 2006). **Venezuela,** Acarigua, Portuguesa State, isolated from *Eucalyptus* trees, M.J. Wingfield, CMW24177 (July 2006).

DISCUSSION

A new species of *Ceratocystis* from the stumps of freshly-cut *Eucalyptus* trees in Venezuela has emerged from this study. Primary recognition of this fungus as distinct from other species in the genus is based on phylogenetic analyses of sequence data for the ITS, β-tubulin and EF1-α gene regions. In this respect, the fungus clearly resides in the *C. fimbriata s.l.* species complex. Its morphology, with hat-shaped ascospores produced from ascomata without spines on their bases, which would reside in the *C. moniliformis s.l.* group, is also consistent with this taxonomic placement.

Phylogenetic data for the three gene regions combined, produced a high level of confidence that *C. fimbriatomima* from Venezuela is distinct from all described species. Phylogenetically, the species closest to *C. fimbriatomima* is *C. manginecans* but *C. fimbriata s.s.* is also relatively closely related to it. All other species included in this study for comparative purposes were confirmed as distinct from each other and from *C. fimbriatomima* with high levels of confidences. Other species in the *C. fimbriata s.l.* species complex that have been isolated from *Eucalyptus* are *C. atrox* (Van Wyk *et al*. 2007b), *C. pirilliformis* (Barnes *et al.* 2003) and *C. neglecta* (Rodas *et al*. 2007) are clearly different to *C. fimbriatomima.*

Morphological characteristics of *C. fimbriatomima* are most similar to those of *C. fimbriata s.s*., and its name has been chosen to reflect this fact. Both these fungi lack flaring secondary phialides as well as the barrel-shaped conidia that are produced from such phialides. These structures are found in all other species in the *C. fimbriata s.l.* complex. *Ceratocystis fimbriatomima* can be distinguished from its closest relative *C. fimbriata s.s*. based on the ostiolar hyphae and primary conidiophores that are both shorter in *C. fimbriata s.s.* than in *C. fimbriatomima*. Furthermore, the ascospores of *C. fimbriata s.s.* are much longer than those of *C. fimbriatomima*.

Various *Ceratocystis* spp. have been found on *Eucalyptus* spp. but only those in the *C. fimbriata s.l.* species complex might be confused with *C. fimbriatomima*. *Ceratocystis atrox* is known only from Australia and it has a very distinct association with the wood boring insect *Phoracanthta acanthocera* (Macleay) (Cerambicydae: Coleptera) (Van Wyk *et al*. 2007b). *Ceratocystis pirilliformis* was first found on *Eucalyptus* in Australia (Barnes *et al*. 2003) but it is also known from South Africa, where it is thought to be introduced (Roux *et al*. 2004). *Ceratocystis fimbriatomima* is very different to *C. pirilliformis* in having globose as opposed to pear-shared ascomatal bases (Barnes *et al*. 2003, Roux *et al.* 2004). *Ceratocystis neglecta*, recently found on *Eucalyptus* in Colombia (Rodas *et al*. 2007) differs from *C. fimbriatomima* in that it has secondary conidiophores and secondary, barrel-shaped, conidia. The ascomatal necks of *C. fimbriatomima* are also longer and the primary conidiophores shorter than those of *C. neglecta*. It is thus unlikely that *C. fimbriatomima* could be confused with other *Ceratocystis* spp. in the *C. fimbriata s.l.* species complex that occurs on *Eucalyptus* spp.

Nothing is known regarding the pathogenicity of *C. fimbriatomima* or whether it might cause a disease on *Eucalyptus* in Venezuela. It was found on freshly cut stumps where infections were

typically on green tissue. This ecological niche might indicate that the fungus is a pathogen although inoculation tests will be required to resolve this question.

REFERENCES

Al Adawi A.O., Deadman M.L., Al Rawahi A.K., Al Maqbali Y.M., Al Jahwari A.A., Al Saadi B.A., Al Amri I.S. & Wingfield M.J. (2006) Aetiology and causal agents of mango sudden decline disease in the Sultanate of Oman. *European Journal of Plant Pathology* **116**: 247–254.

Alexopoulos C.J. (1962) *Introductory Mycology*. 2nd edn. John Wiley & Sons, Inc., United States of America.

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

Barnes I., Roux J., Wingfield M.J., Old K.M. & Dudzinski M. (2003) *Ceratocystis pirilliformis*, a new species from *Eucalyptus nitens* in Australia. *Mycologia* **95:** 865–871.

Cunningham C.W. (1997) Can three incongruence tests predict when data should be combined? *Molecular Biology and Evolution* **14**: 733–740.

De Reyes L.C. (1988) Necrosis del tronco y ramas del cacao en Venezuela. 10a Conferencia Internacional de Investigacion en Cacao, Venezuela, 485–489.

Engelbrecht C.J. & Harrington T.C. (2005) Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. *Mycologia* **97**: 57–69.

Glass N.L. & 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.

Halsted B.D. (1890) Some fungous disease of the sweet potato. *Agricultural College Experiment Station Bulletin* **76**: 1–32.

Harrington T.C. (2000) Host specialization and speciation in the American wilt pathogen *Ceratocystis fimbriata*. *Fitopatologia Brasileira* **25**: 262–263.

Jacobs K., Bergdahl D.R., Wingfield M.J., Halik S., Seifert K.A., Bright D.E. & Wingfield B.D. (2004) *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* **108**: 411–418**.**

Johnson J.A., Harrington T.C. & Engelbrecht C.J.B. (2005) Phylogeny and taxonomy of the North American clade of the *Ceratocystis fimbriata* complex. *Mycologia* **97**: 1067–1092.

Katoh K., Misawa K., Kuma K. & Miyata T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**: 3059–3066.

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.A. Webber). APS Press, St. Paul, Minnesota, USA: 173–183.

Laia M.L., Alfenas A.C. & Harrington T.C. (1999) Isolation, detection in soil, and inocultion of *Ceratocystis fimbriata*, causal agent of wilting, dieback and canker in *Eucalyptus*. In : *Proceedings of the 12th Biennial Conference of the Australasian Plant Pathology Society, Canberra, Australia, 27-30 September*. 77. (ed. L. Morin).

Malaguti G. (1952a) *Ceratostomella fimbriata* en el cacao de Venezuela. Acta Cientifica deVenezuela **3**: 94–97.

Malaguti G. (1952b) Una podredumbre del tallo de *Crotalaria juncea*, causada por *Ceratostomella fimbriata*. *Agronomica Tropical, Maracay* **4**: 287–292.

Marin M., Castro B., Gaitan A., Preisig O., Wingfield B.D. & Wingfield M.J. (2003) Relationships of *Ceratocystis fimbriata* isolates from Colombian coffee-growing regions based on molecular data and pathogenicity. *Journal of Phytopathology* **151**: 395–405.

Morris M.J., Wingfield M.J. & De Beer C. (1993) Gummosis and wilt of *Acacia mearnsii* in South Africa caused *Ceratocystis fimbriata*. *Plant Pathology* **42**: 814–817.

Nylander J.A.A. (2004) MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Pontis R.E. (1951) A canker disease of the coffee tree in Colombia and Venezuela. *Phytopathology* **41**: 178–184.

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

Rodas C., Roux J., Van Wyk M., Wingfield B.D. & Wingfield M.J. (2007) *Ceratocystis neglecta* sp. nov., infecting *Eucalyptus* trees in Colombia. *Fungal Diversity* **28**: 73–84.

Ronquist F. & Huelsenbeck J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Roux J., Van Wyk M., Hatting H. & Wingfield M.J. (2004) *Ceratocystis* species infecting stem wounds on *Eucalyptus grandis* in South Africa. *Plant Pathology* **53**: 414–421.

Roux J., Wingfield M.J., Wingfield B.D., Bouillett J.P. & Alfenas A.C. (2000) A serious new disease of *Eucalyptus* caused by *Ceratocystis fimbriata* in Central Africa. *Forest Pathology* **30**: 175–184.

Roux J., Heath R.N., Labuschagne L., Nkuekam G.K. & Wingfield M.J. (2007) Occurrence of the wattle wilt pathogen, *Ceratocystis albifundus* on native South African trees. *Forest Pathology* **37**: 1–11.

Seifert K.A., Wingfield M.J. & Kendrick W.B. (1993) A nomenclature for described species of *Ceratocystis*, *Ophiostoma*, *Ceratocystiopsis*, *Ceratostomella* and *Sphaeronaemella*. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology, and Pathogenicity* (eds. M.J. Wingfield, K.A. Seifert and J.A. Webber). APS Press, St. Paul, Minnesota, USA: 269–287.

Sullivan J. (1996) Combining data with different distributions of among-site variation. *Systematic Biology* **45**: 375–380.

Swofford D.L. (2002) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Van Wyk M., Al-Adawi A.O., Khan I.A., Deadman M.L., Al-Jahwari A., Wingfield B.D., Ploetz R. & Wingfield M.J. (2007a) *Ceratocystis manginecans* sp. nov., causal agent of a destructive mango wilt disease in Oman and Pakistan. *Fungal Diversity* **27**: 213–230.

Van Wyk M., Pegg G., Lawson S. & Wingfield M.J. (2007b) *Ceratocystis atrox* sp. nov associated with *Phoracanthta acanthocera* infestations on *Eucalyptus* in Australia. *Australian Journal of Plant Pathology* **36**: 407–414.

Van Wyk M., Roux J., Barnes I., Wingfield B.D., Liew E.C.Y., Assa B., Summerell B.A. & Wingfield M.J. (2004) *Ceratocystis polychroma* sp. nov., a new species from *Syzygium aromaticum* in Sulawesi. *Studies in Mycology* **50**: 273–282.

Van Wyk M., Van der Merwe N.A., Roux J., Wingfield B.D., Kamgan G.N. & Wingfield M.J. (2006) Population genetic analyses suggest that the *Eucalyptus* fungal pathogen *Ceratocystis fimbriata* has been introduced into South Africa. *South African Journal of Science* **102**: 259–263.

Webster R.K. & Butler E.E. (1967a) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45**: 1457–1468.

Webster R.K. & Butler E.E. (1967b) The origin of self-sterile, cross-fertile strains and culture sterility in *Ceratocystis fimbriata*. *Mycologia* **59**: 212–221.

Wingfield M.J., De Beer C., Visser C. & Wingfield B.D. (1996) A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematic and Applied Microbiology* **19**: 191–202.

White T.J., Bruns T., Lee S. & 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, USA: 315–322.

Table 1. Isolates of *Ceratocystis* spp used in this study.

Species	Isolate no.	GenBank accession no.	Host	Geographical origin	References (Sequence data)
C. albifundus	CMW4068	DQ520638 EF070429 EF070400	*Acacia mearnsii*	RSA	Van Wyk *et al.* (2007b)
C. albifundus	CMW5329	AF388947 DQ371649 EF070401	*Acacia mearnsii*	Uganda	Van Wyk *et al.* (2007b)
C. atrox	CMW19383 CBS120517	EF070414 EF070430 EF070402	*Eucalyptus grandis*	Australia	Van Wyk *et al.* (2007b)
C. atrox	CMW19385 CBS120518	EF070415 EF070431 EF070403	*Eucalyptus grandis*	Australia	Van Wyk *et al.* (2007b)
C. cacaofunesta	CMW15051 CBS152.62	DQ520636 EF070427 EF070398	*Theobroma cacao*	Costa Rica	Van Wyk *et al.* (2007b)
C. cacaofunesta	CMW14809 CBS115169	DQ520637 EF070428 EF070399	*Theobroma cacao*	Ecuador	Van Wyk *et al.* (2007b)
C. caryae	CMW14793 CBS114716	EF070424 EF070439 EF070412	*Carya cordiformis*	USA	Van Wyk *et al.* (2007b)
C. caryae	CMW14808 CBS115168	EF070423 EF070440 EF070411	*Carya ovata*	USA	Van Wyk *et al.* (2007b)
C. fimbriata s.s	CMW15049 CBS141.37	DQ520629 EF070442 EF070394	*Ipomaea batatas*	USA	Van Wyk *et al.* (2006, 2007b)
C. fimbriata s.s.	CMW1547	AF264904 EF070443 EF070395	*Ipomaea batatas*	Papua New Guinea	Roux *et al.* (2000) Van Wyk *et al.* (2007b)
C. fimbriata s.l.	CMW8857	AY233868 AY233878 EU241483	*Annona muricata*	Colombia	Marin *et al.* (2003) Present study
C. fimbriata s.l.$^{+}$	CMW8856 CBS121793	AY233867 AY233874 EU241484	*Citrus lemon*	Colombia	Marin *et al.* (2003) Present study
C. fimbriata s.l.`	CMW10844	AY177238 AY177229 EU241481	*Coffea arabica*	Colombia	Marin *et al.* (2003) Present study
C. fimbriata s.l.	CMW9565 CBS 121790	AY233864 AY233870 EU241487	*Soil*	Colombia	Marin *et al.* (2003) Present study
C. fimbriata s.l.	CMW5751 CBS121792	AY177233 AY177225 EU241493	*Coffea arabica*	Colombia	Marin *et al.* (2003) Present study
C. fimbriata s.l.	CMW9572	AY233863 AY233871 EU241488	*Mandarin*	Colombia	Marin *et al.* (2003) Present study
C. fimbriata s.l.	CMW14797 CBS114721	AY953382 EF433307 EF433316	*Mangifera indica*	Brazil	Van Wyk *et al.* (2007a)
C. fimbriata s.l.	CMW15052 CBS600.70	EF433298 EF433306 EF433315	*Mangifera indica*	Brazil	Van Wyk *et al.* (2007a)
C. fimbriatomima	**CMW24174 CBS121786**	**EF190963 EF190951 EF190957**	***Eucalyptus* sp.**	**Venezuela**	**Present study**
C. fimbriatomima	**CMW24176**	**EF190964**	***Eucalyptus* sp.**	**Venezuela**	**Present study**

	CBS121787	**EF190952 EF190958**			
C. fimbriatomima	**CMW24376 CBS121788**	**EF190965 EF190953 EF190959**	***Eucalyptus* sp.**	**Venezuela**	**Present study**
C. fimbriatomima	**CMW24377**	**EF190966 EF190954 EF190960**	***Eucalyptus* sp.**	**Venezuela**	**Present study**
C. fimbriatomima	**CMW24378**	**EF190967 EF190955 EF190961**	***Eucalyptus* sp.**	**Venezuela**	**Present study**
C. fimbriatomima	**CMW24379**	**EF190968 EF190956 EF190962**	***Eucalyptus* sp.**	**Venezuela**	**Present study**
C. manginecans	CMW13851 CBS121659	AY953383 EF433308 EF433317	*Mangifera indica*	Oman	Van Wyk *et al.* (2007a)
C. manginecans	CMW13852 CBS121660	AY953384 EF433309 EF433318	*Hypocryphalus mangifera*	Oman	Van Wyk *et al.* (2007a)
C. pirilliformis	CMW6569	AF427104 DQ371652 AY528982	*Eucalyptus nitens*	Australia	Barnes *et al.* (2003) Van Wyk *et al.* (2007b)
C. pirilliformis	CMW6579 CBS118128	AF427105 DQ371653 AY528983	*Eucalyptus nitens*	Australia	Barnes *et al.* (2003) Van Wyk *et al.* (2007b)
C. platani	CMW14802 CBS115162	DQ520630 EF070425 EF070396	*Platanus occidentalis*	USA	Van Wyk *et al.* (2007b)
C. platani	CMW23918	EF070426 EF070397 EU426554	*Platanus* sp.	Greece	Van Wyk *et al.* (2007b)
C. polychroma	CMW11424 CBS115778	AY528970 AY528966 AY528978	*Syzygium aromaticum*	Indonesia	Van Wyk *et al.* (2004)
C. polychroma	CMW11436 CBS115777	AY528971 AY528967 AY528979	*Syzygium aromaticum*	Indonesia	Van Wyk *et al.* (2004)
C. populicola	CMW14789 CBS119.78	EF070418 EF070434 EF070406	*Populus* sp.	Poland	Van Wyk *et al.* (2007b)
C. populicola	CMW14819 CBS114725	EF070419 EF070435 EF070407	*Populus* sp.	USA	Van Wyk *et al.* (2007b)
C. smalleyi	CMW14800 CBS114724	EF070420 EF070436 EF070408	*Carya cordiformis*	USA	Van Wyk *et al.* (2007b)
C. smalleyi	CMW26383 CBS114724	EU426553 EU426555 EU426556	*Carya cordiformis*	USA	Van Wyk *et al.* (2007b) This study
C. variospora	CMW20935 CBS114715	EF070421 EF070437 EF070409	*Quercus alba*	USA	Van Wyk *et al.* (2007b)
C. variospora	CMW20936 CBS114714	EF070422 EF070438 EF070410	*Quercus robur*	USA	Van Wyk *et al.* (2007b)
C. virescens	CMW11164	DQ520639 EF070441 EF070413	*Fagus americanum*	USA	Van Wyk *et al.* (2007b)
C. virescens	CMW3276	AY528984 AY528990 AY529011	*Quercus robur*	USA	Van Wyk *et al.* (2004)

Figure 1-6. Morphological characteristics of *Ceratocystis fimbriatomima.* **1.** Ascomata with globose base and long neck. **2.** Divergent ostiolar hyphae. **3.** Primary conidiophore, flask-shaped phialides producing cylindrical conidia. **4.** Dark, sub-globose chlamydospore and cylindrical conidia. **5.** Hat-shaped ascospores. **6.** Chain of cylindrical conidia. Bars; **1.** = 100 µm, **2.** = 10 µm, **3.** = 20 µm, **4.** = 10 µm, **5.** = 10 µm, **6.** = 10 µm.

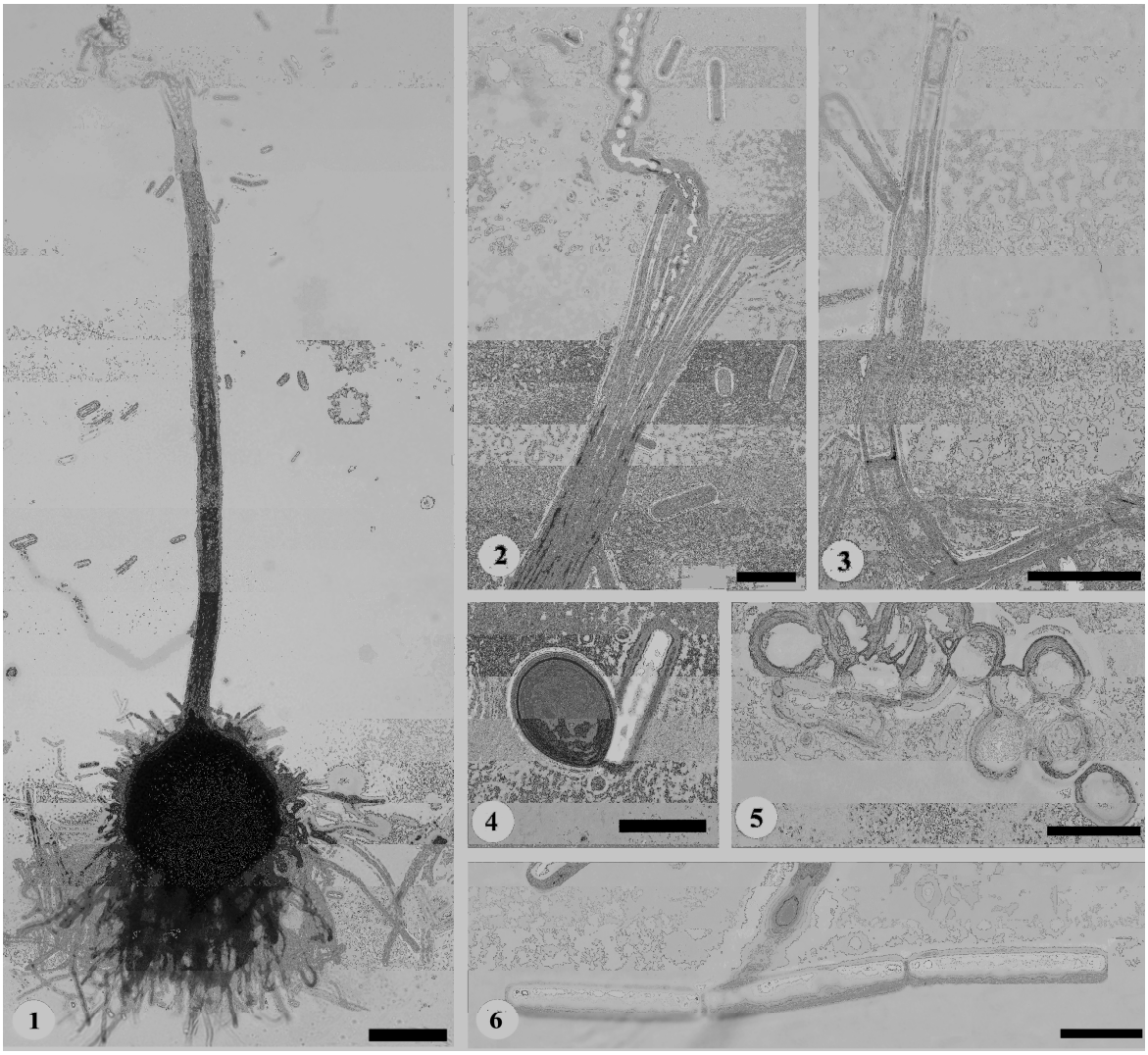
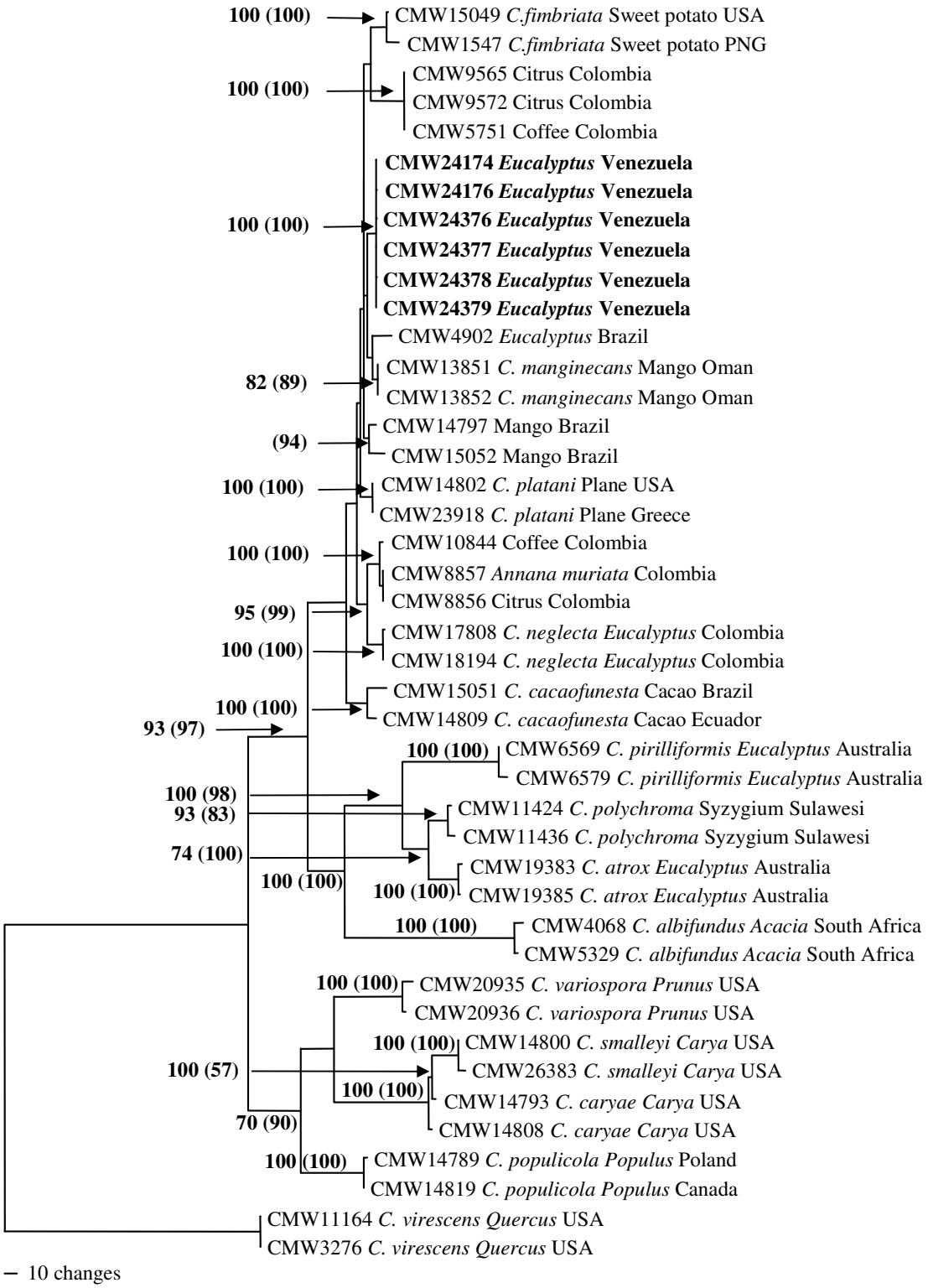


Figure 7. Phylogenetic tree based on the combined regions of the ITS, β-tubulin and Ef1-α for *C. fimbriatomima* and other species in the *C. fimbriata s.l. species* complex. *Ceratocystis virescens* represents the out-group taxon. Bootstrap values are indicated at the branch nodes while Bayesian values are indicated in brackets.



CHAPTER 7

Ceratocystis larium sp. nov., a new species from *Styrax benzoin* wounds associated with incense harvesting in Indonesia

Van Wyk M., Wingfield B.D., Clegg P.A. & Wingfield M.J. (2009). *Ceratocystis larium* sp. nov., a new species from *Styrax benzoin* wounds associated with incense harvesting in Indonesia. *Persoonia* **22**: 75-82.

ABSTRACT

Styrax benzoin trees, native to the island Sumatra, Indonesia are wounded to produce resin that is collected and burned as incense. These wounds on trees commonly develop into expanding cankers that lead to tree death. The aim of this study was to consider whether Ophiostomatoid fungi, typically associated with wounds on trees might be associated with resin harvesting on *S. benzoin*. Samples were collected from the edges of artificially induced wounds, and particularly where cankers and staining of the vascular tissue was evident. Tissue samples were incubated in moist chambers and carrot baiting was also used to detect the presence of *Ceratocystis* spp. Fruiting structures with morphology typical of species in the *C. fimbriata* sensu lato species complex and species in the anamorph genus *Thielaviopsis* were found, both on the incubated wood and on the carrot baits. DNA sequences were generated for the Internal Transcribed Spacer regions one and two including the 5.8S rRNA gene, part of the *β*-tubulin and the Transcription Elongation Factor 1-α gene regions. These data were compared with those of other species in the *C. fimbriata* sensu lato species complex and *Thielaviopsis* using phylogenetic analysis. Morphology of the isolates in culture as well as phylogenetic inference showed that the *Thielaviopsis* sp. present on the wounds was *T. basicola.* The *Ceratocystis* sp. from *S. benzoin* represents a new taxon in the *C. fimbriata sensu lato* complex described here as *C. larium* sp. nov.

INTRODUCTION

Trees in the genus *Styrax* L. are native to the Northern hemisphere including eastern and south eastern Asia and South America, where they occur in warm temperate areas (Burkill 1935, Fernandez 2004). There are about 150 species of *Styrax* and many are used to produce resin that is aromatic when burned. *Styrax benzoin* Dryabd trees in Indonesia, specifically Sumatra, commonly referred to as Sumatra Benzoin are tapped for resin, which is collected and dried. The dried resin produces fragrant aromas when burned and is thus a valuable source of incense, which is believed to have magical properties (Wheatley 1959). More than 18 000 families in Northern Sumatra alone are dependant on benzoin production (Wollenberg *et al*. 2004).

Wounds on *S. benzoin* trees often develop into cankers that can eventually girdle and kill them. Such wounds are commonly associated with vascular staining, typical of that resulting from infection by ophiostomatoid fungi (Wingfield *et al.* 1993). These fungi and particularly species of *Ceratocystis sensu lato* (*s.l.*) have the capacity to infect wounds and kill trees (Bretz 1952, Norris 1953, De Vay *et al.* 1963, Kile 1993).

Ceratocystis s. l. represents a diverse species complex with distinct groups of taxa separated by clear phylogenetic, morphological and ecological boundaries. These groups are in the process of being assigned generic status. Many of these fungi infect wounds on trees but some are also symbionts of conifer infesting bark beetles. Various *Ceratocystis* spp. have been found infecting wounds on trees made during agronomic practices or bark harvesting, often resulting in serious disease problems (De Vay *et al*. 1963, Kile 1993, Marin *et al*. 2003)

The aim of this study was to consider whether wounds made on *S. benzoin* trees in the resin harvesting process might be infected with *Ceratocystis* spp. and to identify these fungi based on morphology and phylogenetic analyses.

MATERIALS AND METHODS

Isolates

Wounds made on *S. benzoin* trees (Figure 1) were inspected and samples were taken where vascular staining and gummosis was evident. Samples were wrapped in newspaper and transported to the laboratory. Wood samples were incubated in a moist environment and inspected directly for fungal growth (Figure 1). Spores produced by fungal structures on the wood surface were transferred onto 2

% Malt Extract Agar (MEA: 20 % w/v; Biolab, Midrand, South Africa) supplemented with 100mg/L streptomycin sulphate (SIGMA). Pieces of wood were also placed between two slices of 10mm carrot pieces that were initially treated with streptomycin sulphate to bait for species of *Ceratocystis* (Moller & De Vay 1968a). Pure cultures were obtained (Figure 1) and these were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), The University of Pretoria, South Africa. Representative isolates were also lodged with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Representative cultures were dried and deposited with the Nation Herbarium of South Africa (PREM).

Phylogenetic analyses

DNA was extracted, as described by Van Wyk *et al*. (2006) for six selected isolates representing two morphological groups. PCR reactions for the Internal Transcribed Spacer regions (ITS) 1 and 2 including the 5.8S rDNA region, the Beta-tubulin (*β*-tubulin) region and the Transcription Elongation Factor 1α (EF-1α) region were prepared as described by Van Wyk *et al*. (2006). The conditions for the PCR's were as described by Van Wyk *et al*. (2006) with the annealing temperature at 55 ºC for all three gene regions. The primers used to amplify the DNA for these three regions were those of White *et al*. (1990), Glass & Donaldson (1995) and Jacobs *et al*. (2004), respectively.

An ABI PRISM^{TM} Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California) was used to prepare the PCR amplicons for sequencing. An ABI PRISM^{TM} 3100 Autosequencer (Applied BioSystems, Foster City, California, USA) was used to run the sequencing reactions. Sequences were analyzed with Chromas Lite 2.01 (http://www.technelysium.com.au). The obtained sequences were subjected to Blast analysis in the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) to confirm the identity of the genera present. This showed the presence of isolates representing the *C. fimbriata s.l.* complex and others of a *Thielaviopsis* species.

The sequences obtained, together with other closely related species, obtained from GenBank (Table 1) were aligned using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/software/source.html) (Katoh *et al.* 2002) for each dataset. The alignments were manually inspected and corrected where necessary. Sequences were analyzed using Phylogenetic Analysis Using Parsimony (PAUP) version 4.0b10* (Swofford 2002). A partition homogeneity test (Swofford 2002) was run to determine whether sequence data for three gene regions could be combined. In PAUP, gaps were treated as a fifth character and trees were obtained via stepwise addition of 1 000 replicates, the Mulpar option

was in effect and the heuristic search option based on parsimony with stepwise addition was selected. Confidence intervals using 1 000 bootstrap replicates were calculated. *C. virescens* (Davidson) Moreau was the designated outgroup for the dataset containing the *C. fimbriata s.l.* species. *Ceratocystis fimbriata sensu stricto* (*s.s.*) was designated as the out-group for the *Thielaviopsis* dataset. All sequences derived from this study were deposited in GenBank (Table 1 and 2).

Morphology

Cultures were grown on 2 % MEA for two weeks prior to assessment of morphological characters of the unknown *Ceratocystis* sp. Fungal structures were mounted on glass slides in lactic acid and these were examined using a Zeiss Axio Vision microscope. Fifty measurements were made for each taxonomically relevant structure. Ranges, averages and standard deviations (st. dev) were determined for each of these characters. Colours of structures and cultures were assessed using the mycological colour charts of Rayner (1970).

To determine the optimum temperature for growth of isolates, growth studies were performed on three isolates representing the unknown *Ceratocystis* sp. A 5 mm plug from the margin of an actively growing culture (2-weeks-old) was placed at the centers of 90 mm 2 % MEA Petri dishes. There were five replicates for each isolate at each temperature and growth was assessed between 5–35 °C at 5 °C intervals after seven days. The entire study was repeated once.

RESULTS

Isolates

Fresh fungal structures were found on the wood surface of the samples collected from wounded *S. benzoin* trees in Indonesia. The fungal structures were characteristic of two different fungi, one with perithecia similar to those of *Ceratocystis* spp. in the *C. fimbriata s.l.* species complex and the other, a *Thielaviopsis* sp. with septate chlamydospores. Sixteen isolates were collected of which six represented a *Thielaviopsis* sp. and the remaining cultures were of a *Ceratocystis* sp.

Phylogenetic analyses

For the *C. fimbriata s.l.* isolates, amplicons of ~500 bp (ITS and β-tubulin) and ~800 bp (EF1-α) were obtained. A P-value of 0.01 was obtained for the PHT showing that the three data sets could be combined (Sullivan 1996, Cunningham 1997). This combined dataset consisted of 1988 characters, of which 1102 were constant, 46 were parsimony uninformative and 840 were parsimony informative. Seven most parsimonious trees were obtained, one of which was selected for

presentation (Figure 6). The tree is described as follows; Tree length (TL) = 2030 steps, Consistency Index (CI) = 0.7, Retention Index (RI) = 0.9 and Rescaled Consistency Index (RC) = 0.6.

The isolates representing *C. fimbriata s.l.* grouped phylogenetically separate from all other described species in this species complex with 100 % support. The species phylogenetically closest to the isolates from *S. benzoin* was *C. albifundus* (Figure 2). All posterior probabilities were high, supporting the separate species within the *C. fimbriata s.l.* species complex.

MrModeltest2 selected the HKY+I+G model for the ITS gene region as the most suited. For the β-tubulin gene region, the GTR+G model was selected while the HKY+I+G model were selected for the EF1-α gene region. The selected models were incorporated into the Bayesian analysis. Two thousand trees were discarded to exclude any trees that were drawn outside of the point of convergence. All posterior probabilities that were obtained with parsimony were confirmed with the Bayesian analyses (Figure 2).

In the case of the *Thielaviopsis* isolates, amplicons of ~500 bp (ITS and β-tubulin) and ~800 bp (EF1-α) were obtained. A *P*-value of 0.01 was obtained for the PHT which suggested combinability of the datasets (Sullivan, 1996; Cunningham, 1997). The *Thielaviopsis* dataset consisted of 1956 characters, of which 1206 were constant, 54 were parsimony uninformative and 696 were parsimony informative. One most parsimonious tree was obtained and presented (Figure 3). The tree is described as follows: TL = 1730 steps, CI = 0.7, RI = 0.9 and RC = 0.6. The *Thielaviopsis* sp. grouped phylogenetically close to *Thielaviopsis basicola* (Berk. et Br.) Ferr. with a high bootstrap support (100 %).

The models obtained from MrModeltest2 for the ITS, β-tubulin gene region and the EF1-α gene region were the GTR+G, GTR+I+G and GTR+I+G, respectively. Two thousand trees were discarded. All posterior probabilities that were obtained with parsimony were confirmed with the Bayesian analyses (Figure 3).

Morphology

Thielaviopsis basicola is a very well known fungus with characteristic and distinct segmented chlamydospores. An isolate (CMW25438) was selected randomly to confirm that morphologically these isolates are representative of *T. basicola* .Dark clumps of conidiophores were evident in cultures. The very distinct chlamydospores of *T. basicola* were also present.

For the *C. fimbriata s.l.* isolates; one isolate (CMW25434) was chosen to represent the fungus and three additional isolates (CMW25435, CMW25436 and CMW25437) were chosen as additional specimens for description purposes. The cultures of *C. fimbriata s.l.* isolates had a light greyish olive (21’’’’’b) colour (Rayner 1970). These isolates were slow growing. No growth was observed at 4 ºC and 35 ºC. Limited growth was observed at 10 ºC (5mm), 15 ºC (10mm) and 30 ºC (6.5mm). Intermediate growth was observed at 20 ºC (12.4mm) with optimal growth at 25 ºC (13.5mm) in seven days. The cultures had a strong banana odour similar to that of many *Ceratocystis* spp. Micro-morphological characteristics distinct for the isolates from Indonesia included the pirilliform ascomatal bases and both the cylindrical and barrel-shaped conidia were of variable size. Similarly variable sizes were observed for the chlamydospores.

The *Ceratocystis* isolates from wounds on *S. benzoin* trees are phylogenetically and morphologically distinct from all other *Ceratocystis* spp. residing in the *C. fimbriata s.l.* clade. These isolates are therefore, described as representing a new species as follows:

Ceratocystis larium M. van Wyk & M.J. Wingf. sp. nov. (Figure 4)
Mycobank 512564

Etymology - The name refers to the guardian spirits of a home or town and reflects the spiritual properties the incense obtained from *S. benzoin* trees.

Bases ascomatum fuscae pirilliformes inornatae (101–) 120–184 (–243) µm latae (113–) 139–201 (–254) µm longae. *Conidia primaria* cylindrica vel oblonga apicibus truncatis (8–) 11–21 (–28) µm longa (2–) 3–5 (–6) µm lata. Conidia secondaria doliiformia vel obtusa, (6–) 7–9 (–13) µm longa 4–6 (–7) µm lata. Chlamydosporae badiae, prolate sphaeroideae vel perprolatae (8–) 9–13 (–16) µm longae (7–) 8–10 (–11) µm latae.

Ascomatal bases dark, pirilliform, un-ornamented (101–) 120–184 (–243) µm wide, (113–) 139–201 (–254) µm long. *Ascomatal necks* dark at bases becoming hyaline at the apices, (222–) 347–573 (–808) µm long, apices (10–) 13–19 (–25) µm wide, bases (19–) 24–36 (–44) µm wide. *Ostiolar hyphae* hyaline, divergent, (18–) 22–30 (–35) µm long. *Ascospores* hyaline, hat-shaped in side view, invested in sheath, 2–4 µm long, 3–5 µm wide excluding sheath, 4–7 µm wide including sheath, accumulating in buff-yellow masses at tips of ascomatal necks. *Primary conidiophores* phialidic, flask-shaped, (52–) 64–98 (–141) µm long, (2–) 3–5 µm wide at the apices, 4–6 (–7) µm wide at broadest points and (3–) 4–6 (–7) µm wide at bases. *Secondary conidiophores* phialidic, apices wide,

(44–) 50–86 (–99) µm long, 4–6 µm wide at the apices, 3–5 (–6) µm wide at bases. *Primary conidia* cylindrical to oblong with truncated apices in shape (8–) 11–21 (–28) µm long, (2–) 3–5 (–6) µm wide. *Secondary conidia*, barrel-shaped to obtuse, (6–) 7–9 (–13) µm long, 4–6 (–7) µm wide. *Chlamydospores* hair brown (17""""i), prolate spheroidal to perprolate (8–) 9–13 (–16) µm long, (7–) 8–10 (–11) µm wide.

Habitat: Wounds on *S. benzoin* trees.

Known distribution: Northern Sumatra, Indonesia

Specimens examined: **Indonesia:** Tele, isolated from the wounds created when tapping resin from *S. benzoin* trees, M.J. Wingfield, PREM60193, CMW25434, CBS122512 (March 2007). Same as above, PREM60194, CMW25435, CBS122606. Same as above, PREM60195, CMW25436, CBS122607. Same as above, PREM60196, CMW25437.

DISCUSSION

Two species of *Ceratocystis s.l.* were isolated from wounds on *S. benzoin* trees in this study. These fungi were identified based on morphology and phylogenetic inference and included *Thielaviopsis basicola* and an undescribed species of *Ceratocystis* residing in the *C. fimbriata s.l.* species complex and which has been given the name *C. larium*. Both fungi were commonly found on the surface of wounds on *S. benzoin* trees and *C. larium* was also easily collected from stained tissue using carrot baiting.

Thielaviopsis basicola is a well known soil borne pathogen of many root crops (Nag Raj & Kendrick 1975, Geldenhuis *et al.* 2006) and its presence on the surface of wounds on trees might seem unusual. However, it has been identified as associated with insects that vector the conidia and/or chlamydospores (Labuschagne & Kotze 1991, Stanghellini *et al.* 1999). It is thus possible that insects, for example ants that live in the soil are attracted by the aromatic gum that accumulates at the wound sites of the trees, thereby carrying the soil borne fungus to the sites at which it was isolated in this study. Because it is also a carrot pathogen (Geldenhuis *et al*. 2006), it can be found on carrot baits used to isolate *Ceratocystis* spp. but in the case of this study, it was found sporulating on the surface of wounds and had no association with carrots.

The presence of a *Ceratocystis* sp. associated with wounds on *S. benzoin* trees is not surprising as these fungi are commonly found on wounds on trees (Kile 1993). Indeed, various species of

Ceratocystis have been trapped from the environment by artificially wounding trees (Barnes *et al*. 2003). In this case, wounds are visited by sap-feeding insects that are also attracted to the fruity aromas produced by many *Ceratocystis* spp. (Moller & De Vay 1968b). We hence assume that *C. larium* was carried to wounds on *S. benzoin* by such insects.

Ceratocystis larium represents a discrete taxon. Based on phylogenetic inference for the ITS, β-tubulin and the EF1-α gene regions, *C. larium* is most closely related to *C. albifundus*. *Ceratocystis albifundus* is most distinct from all the other species within the *C. fimbriata s.l.* species complex with no species phylogenetically closely related to it. *Ceratocystis larium*, residing in a phylogenetically sister group to *C. albifundus,* is thus also clearly distinct from all other species in the *C. fimbriata s.l.* species complex.

Morphologically, *C. larium* is similar to other species in the *C. fimbriata s.l.* species. In this regard, it has as grey to green colony colour and a fruity odor. Similar to *C. pirilliformis* (Barnes *et al*. 2003) and *C. obpyriformis* (Heath *et al*. 2009), it has pirilliform ascomatal bases. However, the cylindrical conidia in *C. larium* differ substantially in size and shape from each other and this distinct variation is also true for the barrel-shaped conidia. Although variation is expected within a species, there is no other species in the *C. fimbriata s.l.* species complex that displays this remarkable variability in size and shape of the conidia. Chlamydospores in *C. larium* are also variable in shape, ranging from prolate spheroidal to perprolate and these structures are also abundant in this species.

Ceratocysis larium is clearly an opportunistic fungus that infects wounds made to tap the gum of *S. benzoin* trees. Nothing is known regarding the pathogenicity of this fungus or *T. basicola* on these trees. However, many wounds made to the trees develop into significant cankers that appear to eventually lead to tree death. Pathogenicity of these fungi should thus be tested and if they are contributing to the death of tees, efforts should be made to restrict their presence.

REFERENCES

Barnes I., Roux J., Wingfield B.D., Dudzinski M.J., Old K.M. & Wingfield M.J. (2003) *Ceratocystis pirilliformis*, a new species from *Eucalyprus nitens* in Australia. *Mycologia* **95**: 865–871.

Bretz T.W. (1952) The ascigerious stage of the oak wilt fungus. *Phytopathology* **42**: 435–437.

Burkill I.H. (1935) Styrax. In: A Dictionary of the Economic Products of the Malay Peninsula. The Government of the Straits Settlements and Federated Malay States. Vol. II 2101–2108. The Crown Agents for the Colonies, London. Vol. II 2101–2108.

Cunningham C.W. (1997) Can three incongruence tests predict when data should be combined? *Molecular Biology and Evolution* **14**: 733–740.

De Vay J.E., Lukezic F.L., English W.H. & Trujillo E.E. (1963) *Ceratocystis* canker of stone fruit trees. *Phytopathology* **53**: 873.

Geldenhuis M.M., Roux J., Cilliers A.J., Wingfield B.D. & Wingfield M.J. (2006) Clonality in South African isolates and evidence for a European origin of the root pathogen *Thielaviopsis basicola*. *Mycological Research* **110**: 306–311.

Glass N.L. & 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.

Heath R.N., Wingfield B.D., Wingfield M.J., Meke G., Mbaga A. & Roux J. (2009) *Ceratocystis* spp. on *Acacia mearnsii* and *Eucalyptus* spp. in eastern and southern Africa including six new species. *Fungal Diversity* **34**: 41–68.

Jacobs K., Bergdahl D.R., Wingfield M.J., Halik S., Seifert K.A., Bright D.E. & Wingfield B.D. (2004) *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* **108**: 411–418**.**

Katoh K., Misawa K., Kuma K. & Miyata T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**: 3059–3066.

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

Labuschagne N. & Kotze J.M. (1991) Incidence of *Chalara elegans* in groundnut seed samples and seed transmission of blackhull. *Plant Pathology* **40**: 639–642.

Marin M., Castro B., Gaitan A., Preisig O., Wingfield B.D. & Wingfield M.J. (2003) Relationships of *Ceratocystis fimbriata* isolates from Colombian coffee-growing regions based on molecular data and pathogenicity. *Journal of Phytopathology* **151**: 395–405.

Moller W.J. & De Vay J.E. (1968a) Carrot as a species-selective isolation medium for *Ceratocysits fimbriata*. *Phytopathological notes* **58**: 123–126.

Moller W.J. & De Vay J.E. (1968b) Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* **58**: 1499–1508.

Nag Raj T.R. & Kendrick B. (1975) A Monograph of Chalara and Allied Genera. Wilfrid Laurier University Press, Waterloo.

Norris D.N. (1953) Insect transmission of oak wilt in Iowa. *Plant Disease Report* **37**: 417–418.

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

Stanghellini M.E., Rasmussen S.L. & Kim D.H. (1999) Aerial transmission of *Thielaviopsis basicola*, a pathogen of corn-salad, by adult shore flies. *Phytopathology* **89**: 476–479.

Sullivan J. (1996) Combining data with different distributions of among-site variation. *Systematic Biology* **45**: 375–380.

Swofford D.L. (2002) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Van Wyk M., Roux J., Barnes I., Wingfield B.D. & Wingfield M.J. (2006). Molecular phylogeny of the *Ceratocystis moniliformis* complex and description of *C. tribiliformis* sp. nov. *Fungal Diversity* **21**: 181–201.

Wheatley P. (1959) Geographical Notes on some Commodities involved in Sung Maritime Trade. Journal of the Malayan Branch of the Royal Asiatic Society. Singapore: Tien Wah Press Ltd.

White T.J., Bruns T., Lee S. & Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds), PCR Protocols: A sequencing guide to methods and applications: 315–322. Academic Press, San Diego, USA.

Wingfield M.J., Seifert K.A. & Webber J.F. (1993) *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology, and Pathogenicity. APS Press, St. Paul, Minnesota.

Wollenberg E., Belcher B., Sheil D., Dewi S. & Moeliono M. (2004) Governace brief 2004. Why are forest areas relevant to reducing poverty in Indonesia? **4**: 1–6.

Zhou X.D., Jacobs K., Kirisits T., Chhetri D.B. & Wingfield M.J. (2008) *Leptographium bhutanense* sp. nov., associated with the root collar weevil *hylobitelus chenkupdorjii* on *Pinus wallichiana* in Bhutan. *Persoonia* **21**: 1–8.

Table 1. Isolates of *Ceratocystis fimbriata* s.l. spp used in this study.

Species	Isolate no.	GenBank accession no.	Host	Geographical origin
C. albifundus	CMW4068	DQ520638 EF070429 EF070400	*Acacia mearnsii*	RSA
C. albifundus	CMW5329	AF388947 DQ371649 EF070401	*Acacia mearnsii*	Uganda
C. atrox	CMW19383 CBS120517	EF070414 EF070430 EF070402	*Eucalyptus grandis*	Australia
C. atrox	CMW19385 CBS120518	EF070415 EF070431 EF070403	*Eucalyptus grandis*	Australia
C. cacaofunesta	CMW15051 CBS152.62	DQ520636 EF070427 EF070398	*Theobroma cacao*	Costa Rica
C. cacaofunesta	CMW14809 CBS115169	DQ520637 EF070428 EF070399	*Theobroma cacao*	Ecuador
C. caryae	CMW14793 CBS114716	EF070424 EF070439 EF070412	*Carya cordiformis*	USA
C. caryae	CMW14808 CBS115168	EF070423 EF070440 EF070411	*Carya ovata*	USA
C. colombiana	CMW9565 CBS121790	AY233864 AY233870 EU241487	*Soil*	Colombia
C. colombiana	CMW5751 CBS121792	AY177233 AY177225 EU241493	*Coffea arabica*	Colombia
C. colombiana	CMW9572	AY233863 AY233871 EU241488	*Mandarin*	Colombia
C. fimbriata s.s	CMW15049 CBS141.37	DQ520629 EF070442 EF070394	*Ipomaea batatas*	USA
C. fimbriata s.s.	CMW1547	AF264904 EF070443 EF070395	*Ipomaea batatas*	Papua New Guinea
C. fimbriatomima	CMW24174 CBS121786	EF190963 EF190951 EF190957	*Eucalyptus* sp.	Venezuela
C. fimbriatomima	CMW24176 CBS121787	EF190964 EF190952 EF190958	*Eucalyptus* sp.	Venezuela
C. laurium	**CMW25434 CBS122512**	**EU881906 EU881894 EU881900**	***Styrax benzoin***	**Indonesia**
C. laurium	**CMW25435 CBS122606**	**EU881907 EU881895 EU881901**	***Styrax benzoin***	**Indonesia**
C. laurium	**CMW25436 CBS122607**	**EU881908 EU881896 EU881902**	***Styrax benzoin***	**Indonesia**
C. laurium	**CMW25437**	**EU881909 EU881897**	***Styrax benzoin***	**Indonesia**

		EU881903		
C. manginecans	CMW13851	AY953383	*Mangifera indica*	Oman
	CBS121659	EF433308		
		EF433317		
C. manginecans	CMW13852	AY953384	*Hypocryphalus mangifera*	Oman
	CBS121660	EF433309		
		EF433318		
C. neglecta	CMW17808	EF127990	*Eucalyptus* sp.	Colombia
	CBS121789	EU881898		
		EU881904		
C. neglecta	CMW18194	EF127991	*Eucalyptus* sp.	Colombia
	CBS121017	EU881899		
		EU881905		
C. obpyriformis	CMW23807	EU245004	*Acacia mearnsii*	South Africa
	CBS122608	EU244976		
		EU244936		
C. obpyriformis	CMW23808	EU245003	*Acacia mearnsii*	South Africa
	CBS122511	EU244975		
		EU244935		
C. papilata	CMW8857	AY233868	*Annona muricata*	Colombia
		AY233878		
		EU241483		
C. papilata	CMW8856	AY233867	*Citrus lemon*	Colombia
	CBS121793	AY233874		
		EU241484		
C. papilata	CMW10844	AY177238	*Coffea arabica*	Colombia
		AY177229		
		EU241481		
C. pirilliformis	CMW6569	AF427104	*Eucalyptus nitens*	Australia
		DQ371652		
		AY528982		
C. pirilliformis	CMW6579	AF427105	*Eucalyptus nitens*	Australia
	CBS118128	DQ371653		
		AY528983		
C. platani	CMW14802	DQ520630	*Platanus occidentalis*	USA
	CBS115162	EF070425		
		EF070396		
C. platani	CMW23918	EF070426	*Platanus* sp.	Greece
		EF070397		
		EU426554		
C. polychroma	CMW11424	AY528970	*Syzygium aromaticum*	Indonesia
	CBS115778	AY528966		
		AY528978		
C. polychroma	CMW11436	AY528971	*Syzygium aromaticum*	Indonesia
	CBS115777	AY528967		
		AY528979		
C. polyconidia	CMW23809	EU245006	*Acacia mearnsii*	South Africa
	CBS122289	EU244978		
		EU244938		
C. polyconidia	CMW23818	EU245007	*Acacia mearnsii*	South Africa
	CBS122290	EU244979		
		EU244939		
C. populicola	CMW14789	EF070418	*Populus* sp.	Poland
	CBS119.78	EF070434		
		EF070406		
C. populicola	CMW14819	EF070419	*Populus* sp.	USA
	CBS114725	EF070435		
		EF070407		
C. smalleyi	CMW14800	EF070420	*Carya cordiformis*	USA
	CBS114724	EF070436		
		EF070408		
C. smalleyi	CMW26383	EU426553	*Carya cordiformis*	USA

	CBS114724	EU426555 EU426556		
C. tanganyicensis	CMW15991 CBS122295	EU244997 EU244969 EU244929	*Acacia mearnsii*	Tanzania
C. tanganyicensis	CMW15999 CBS122294	EU244998, EU244970, EU244939	*Acacia mearnsii*	Tanzania
C. tsitsikammensis	CMW14276 CBS121018	EF408555 EF408569 EF408576	*Rapanea melanophloeos*	South Africa
C. tsitsikammensis	CMW14278 CBS121019	EF408556 EF408570 EF408577	*Rapanea melanophloeos*	South Africa
C. variospora	CMW20935 CBS114715	EF070421 EF070437 EF070409	*Quercus alba*	USA
C. variospora	CMW20936 CBS114714	EF070422 EF070438 EF070410	*Quercus robur*	USA
C. virescens	CMW11164	DQ520639 EF070441 EF070413	*Fagus americanum*	USA
C. virescens	CMW3276	AY528984 AY528990 AY529011	*Quercus robur*	USA
C. zombamontana	CMW15235	EU245002 EU244974 EU244934	*Eucalyptus* sp.	Malawi
C. zombamontana	CMW15236	EU245000 EU244972 EU244932	*Eucalyptus* sp.	Malawi

Table 2. Isolates of *Thielaviopsis* spp. and associated *Ceratocystis* spp. used in this study.

Species	Isolate no.	GenBank accession no.	Host	Geographical origin
Thielaviopsis australis / Ceratocystis australis	CMW2333	FJ411325 FJ411351 FJ411299	*Nothofagus cunninghamii*	Australia
T. australis / C. australis	CMW2653	FJ411326 FJ411352 FJ411300	*Nothofagus cunninghamii*	Australia
T. eucalypti / C. eucalypti	CMW3254	FJ411327 FJ411353 FJ411301	*Eucalyptus sieberi*	Australia
T. eucalypti / C. eucalypti	CMW4453	FJ411328 FJ411354 FJ411302	*Eucalyptus sieberi*	Australia
T. basicola	CMW6714	FJ411331 FJ411357 FJ411305	Carrots	Australia
T. basicola	CMW7625 CBS117828	FJ411332 FJ411358 FJ411306	Chicory	South Africa
T. basicola	CMW25438	FJ411333 FJ411359 FJ411307	***Styrax benzoin***	**Indonesia**
T. basicola	CMW25439	FJ411334 FJ411360 FJ411308	***Styrax benzoin***	**Indonesia**
T. basicola	CMW25440	FJ411335 FJ411361 FJ411309	***Styrax benzoin***	**Indonesia**
T. neocaledoniae	CMW3270	FJ411329 FJ411355 FJ411303	Unknown	USA
T. neocaledoniae	CMW26392 CBS149.83	FJ411330 FJ411356 FJ411304	*Coffea robusta*	USA
T. ovoidea	CMW22733 CBS354.76	FJ411343 FJ411369 FJ411317	Fire wood	Netherlands
T. paradoxa / C. paradoxa	CMW8779	FJ411324 FJ411349 FJ411298	Coconut	Indonesia
T. paradoxa / C. paradoxa	CMW8790	FJ411323 FJ411350 FJ411297	Coconut	Indonesia
T. populi	CMW26387 CBS484.71	FJ411336 FJ411362 FJ411310	*Populus robusta.*	Belgium
T. populi	CMW26388 CBS486.71	FJ411337 FJ411363 FJ411311	*Populus gelrica*	Belgium
T. punctulata / C. radicicola	CMW26389 CBS167.67	FJ411338 FJ411368 FJ411316	*Lawsonia inermis*	Europe
T. punctulata / C. radicicola	CMW1032 CBS114.47	FJ411339 FJ411364 FJ411312	*Phoenix dactylifera*	USA
T. punctulata / C. radicicola	CMW6728	FJ411340 FJ411365 FJ411313	*Daucus carrota*	Australia
T. quercina / C. fagacearum	CMW2039	FJ411344	*Quercus* sp.	USA

		FJ411370		
		FJ411318		
T. quercina / C. fagacearum	CMW2658	FJ411345	*Quercus* sp.	USA
		FJ411371		
		FJ411319		
T. thielavioides	CMW22736	FJ411342	*Lupinus albus*	Italy
	CBS148.37	FJ411367		
		FJ411315		
T. thielavioides	CMW22737	FJ411341	*Populus* sp.	Belgium
	CBS180.75	FJ411366		
		FJ411314		
T. ungeri / C. coerulescens	CMW26364	FJ411321	*Picea* sp.	USA
		FJ411347		
		FJ411295		
T. ungeri / C. coerulescens	CMW26365	FJ411322	*Picea abies*	Germany
	CBS140.37	FJ411348		
		FJ411296		
T. ungeri / C. coerulescens	CMW26366	FJ411320	*Picea abies*	Finland
	CBS489.80	FJ411346		
		FJ411294		
C. fimbriata s.s	CMW15049	DQ520629	*Ipomaea batatas*	USA
	CBS141.37	EF070442		
		EF070394		
C. fimbriata s.s.	CMW1547	AF264904	*Ipomaea batatas*	Papua New Guinea
		EF070443		
		EF070395		

Figure. 1. Isolation of species of fungi from *S. benzoin* in Indonesia. a. Triangular wounds created with a knife for gum exudation; b. exposed wound from *S. benzoin* trees illustrating gummosis and wood discolouration**;** c. fungal growth on collected pieces of wood that were sampled; d. Pure culture of a *Ceratocystis fimbriata s.l.* species; e. pure culture of a *Thielaviopsis* species.

Figure. 2. One of seven most parsimonious phylogenetic trees, based on the combined regions of the ITS, β-tubulin and Ef1-α for *Ceratocystis larium* and other species in the *C. fimbriata s.l.* species complex. *Ceratocystis virescens* represents the out-group taxon. Bootstrap values are indicated at the branch nodes and Bayesian values in parentheses.

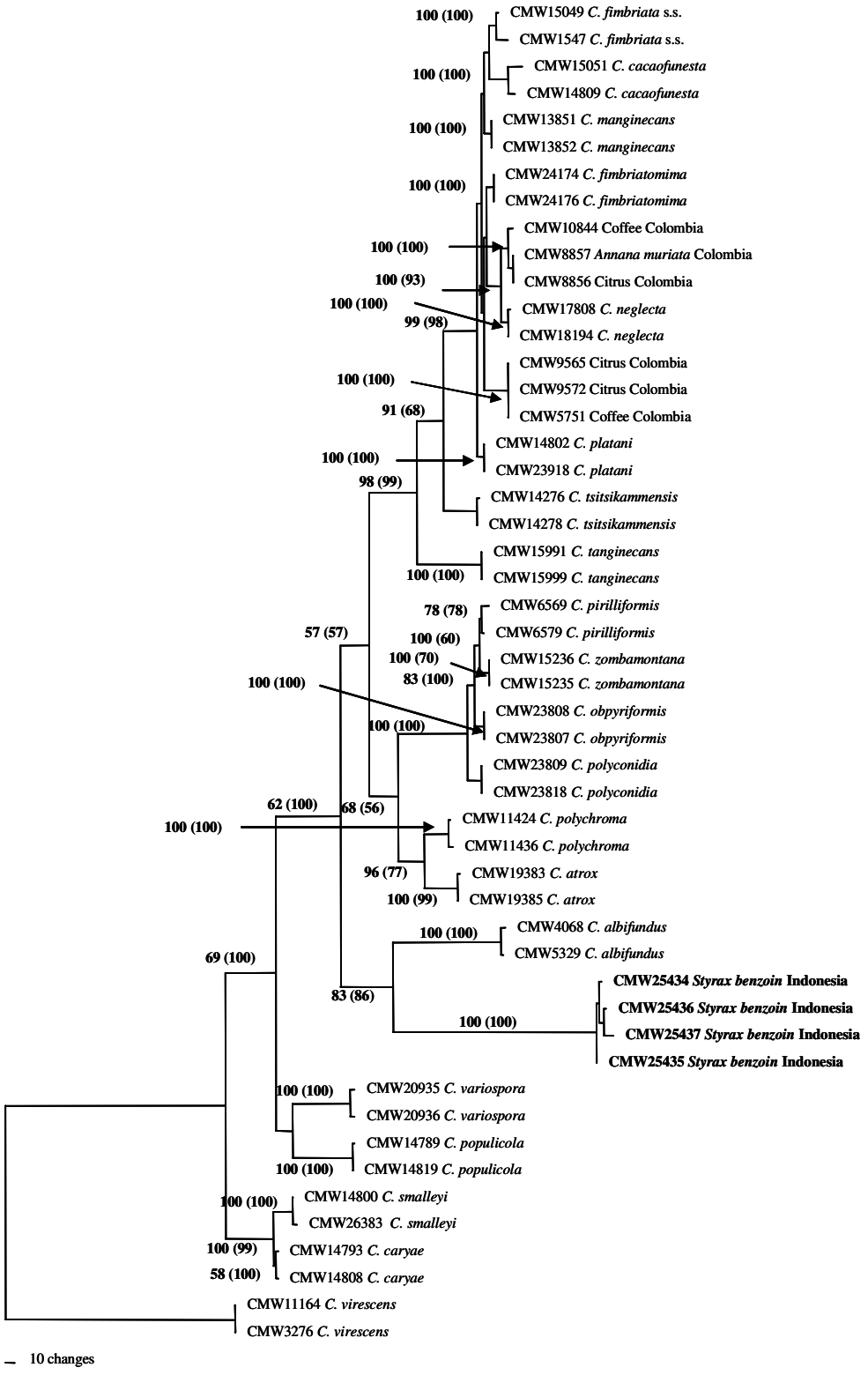


Figure 3. Most parsimonious tree based on the combined regions of the ITS, β-tubulin and Ef1-α for *T. basicola* and other species in the *Thielaviopsis* genus. *Ceratocystis fimbriata s.s.* represents the out-group taxon. Bootstrap values are indicated at the branch nodes and Bayesian values in parenthesis.

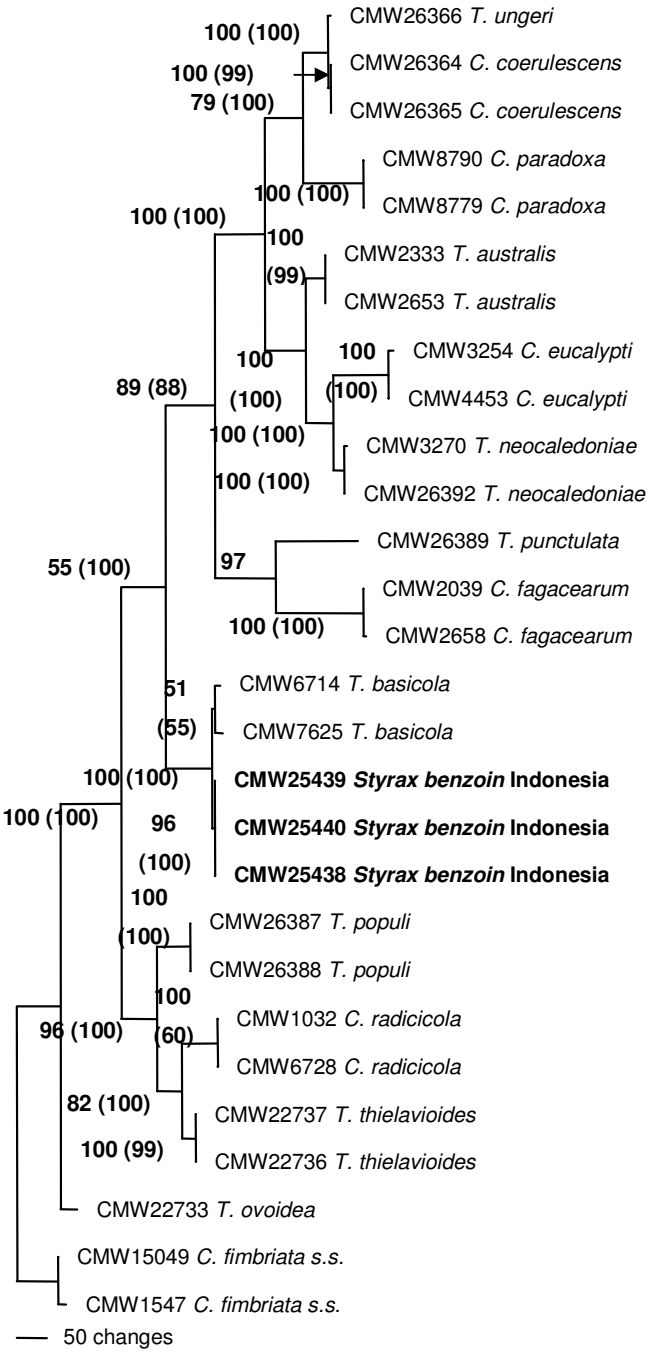
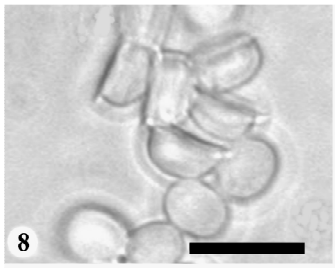
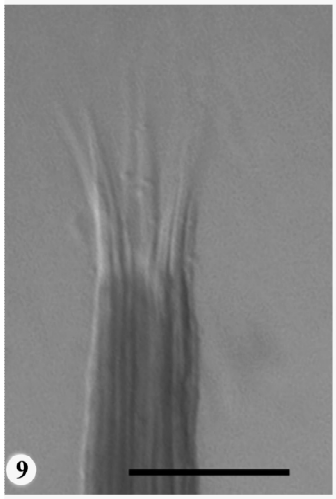
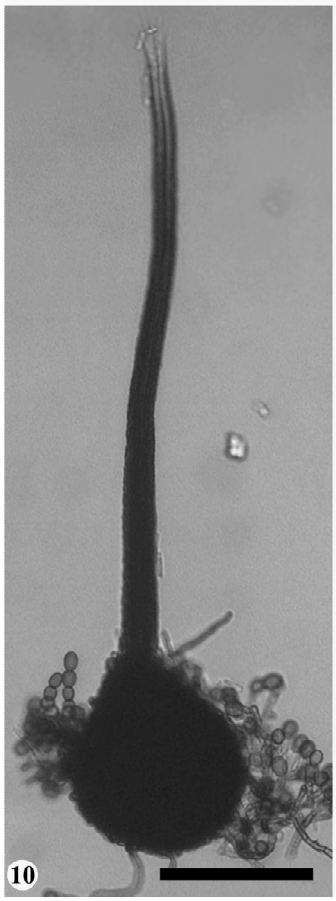
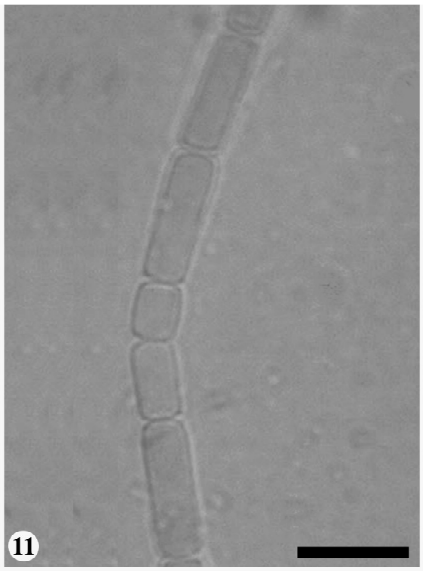


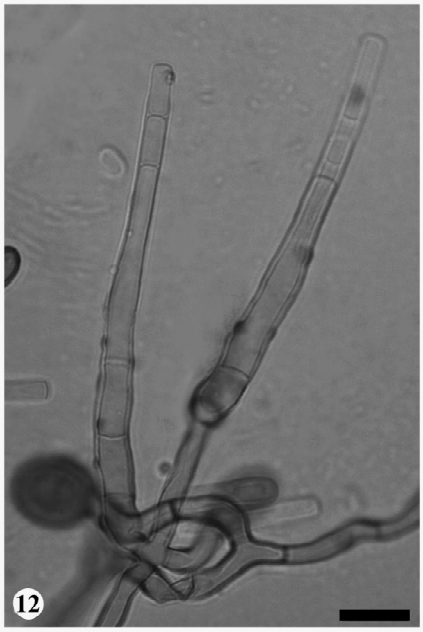
Figure 4. Morphological characteristics of *C. larium*, scalebars indicated in brackets. a. Hat-shaped ascospores (5µm); b. various shapes of the primary conidia, mainly cylindrical in shape (10µm); c. secondary conidia, barrel-shaped to obtuse (10µm); d. divergent ostiolar hyphae (20µm); e. flask-shaped primary conidiophores (10µm); f. secondary conidiophores with emerging barrel-shaped conidia (10µm); g. ascomata with pirilliform base (100µm); h. chlamydospores of various shapes (10µm); i. numerous chlamydospores visible in culture (10µm)

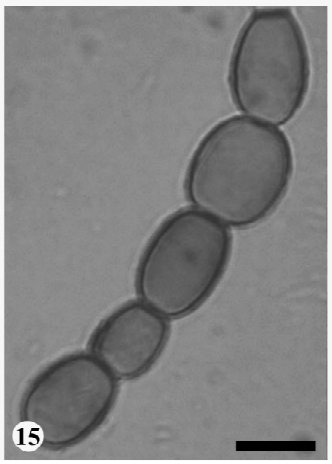


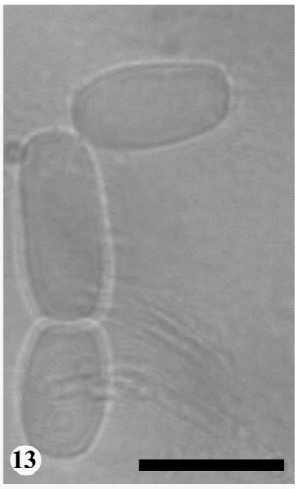


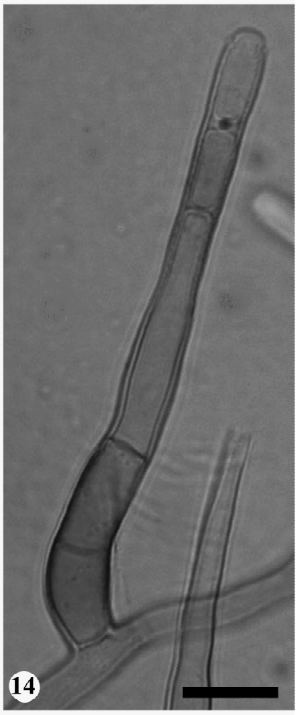


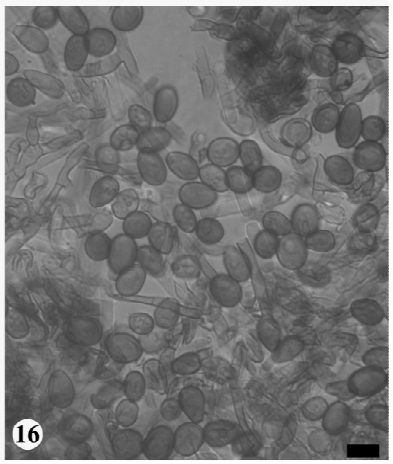












CHAPTER 8

New *Ceratocystis* species infecting coffee, cacao, citrus and native trees in Colombia

Van Wyk M., Wingfield B.D., Marin M. & Wingfield M.J. (2010). New *Ceratocystis* species infecting coffee, cacao, citrus and native trees in Colombia. *Fungal Diversity* **40**: 103-117.

ABSTRACT

Ceratocystis fimbriata sensu lato includes a large number of plant and especially tree pathogens. In Colombia, isolates of this fungus cause a serious cankerstain disease on coffee as well as other fruit trees. Large collections of these isolates have been shown to occur in two distinct phylogenetic lineages based on ITS sequence comparisons. The aim of this study was to compare representatives of these two groups of isolates from coffee, citrus, cacao and native trees in Colombia, based on morphology and DNA-sequences for three gene regions. Host-specificity of the fungus was also considered. Representatives of the two groups of isolates were morphologically distinct and could be distinguished based on DNA sequence comparisons. They are also distinct from other species in the *C. fimbriata sensu lato* species complex and the sweet potato pathogen *C. fimbriata sensu stricto* and are provided with the names *C. colombiana* sp. nov and *C. papillata* sp. nov. There was no evidence for host-specificity amongst isolates of these two fungi that collectively represent a serious threat to coffee production in Colombia.

INTRODUCTION

Colombia is the world's largest producer of coffee (*Coffea arabica* L.) and depends on this product for foreign exchange. It is consequently worrying that this crop is seriously affected by a canker-stain disease caused by *Ceratocystis fimbriata* Ellis & Halst. *sensu lato* (s.l.). The first record of coffee canker disease caused by this fungus, was from the island of Java (Indonesia) in 1900 (Pontis 1951) and it was first recorded in Colombia in 1932 (Zimmerman 1900, Castaño 1951).

Canker stain disease caused by *C. fimbriata s.l.* in Colombia is found on a wide range of hosts other than coffee. These include citrus (*Citrus reticulata* Blanco, *Citrus sinensis* (L.) Osbeck and *Citrus limon* (L.) Burm. f.) barinas nut (*Caryodendron orinocense* H. Karst.), mango (*Mangifera indica* L.), rubber (*Hevea brasiliensis* (Willd.) Müll. Arg.) cacao (*Theobroma cacao* L.) and several native forest trees including *Schizolobium parahybum* (Vell.) S.F. Blake and *Annona muricata* L. (Castaño 1951, Webster & Butler 1967, Kile 1993, Mourichon 1994, Pardo-Cardona 1995, Marin *et al*. 2003). Coffee is frequently cultivated in close proximity to citrus and native trees and it is likely that inoculum of the pathogen is readily exchanged between these plants.

Ceratocystis fimbriata s.l. requires wounds for infection and these are created during pruning and other cultivation practices (Pontis 1951, Marin *et al*. 2003). In some cases, the source of infection is chlamydospore-infested soil (Rossetto & Ribeiro 1990, Marin *et al*. 2003, Marin 2004). *Ceratocystis fimbriata* was first described based on isolates of the fungus causing black rot of sweet potato in the United States of America (Halsted 1890). Subsequently, the fungus was associated with diseases of a large number of different plant species in many parts of the world. While morphological differences between isolates of *C. fimbriata s.l.* were not clearly evident, Webster and Butler (1967) recognised that the fungus probably represented more than one entity. The availability of molecular techniques and particularly DNA sequence comparisons has dramatically changed the current taxonomic position of *C. fimbriata s.l.*

A contemporary view of *C. fimbriata s.l.* is that it represents a complex of many cryptic species. Thus, the sweet potato black rot pathogen is treated as *C. fimbriata sensu stricto* (*s.s.*). Numerous cryptic species that would previously have been treated as C. fimbriata have been described during the course of the last decade (Engelbrecht & Harrington 2005, Johnson *et al*. 2005, Van Wyk *et al*. 2004, 2006, 2007a, b). It also is likely that many other discrete and cryptic taxa reside amongst isolates of this fungus that have not yet been studied in detail.

Barnes *et al*. (2001) undertook the first DNA-based study on isolates in the *C. fimbriata s.l.* complex including isolates of *C. fimbriata s.l.* from coffee in Colombia. DNA sequence comparisons and analyses using microsatellite markers showed that the isolates from Colombia resided in two very distinct phylogenetic lineages (Barnes *et al*. 2001). Marin *et al*. (2003) compared 50 isolates of *C. fimbriata* s.l. from 11 different provinces in Colombia using DNA sequence data, RAPDs, RFLPs and pathogenicity tests. Their results also showed conclusively that isolates from Colombia represented two distinct entities that were highly variable.

The aim of this study was to compare isolates representing the two groups (Marin *et al*. 2003) encompassing *C. fimbriata s.l.* from Colombia. Comparisons of isolates were based on morphology and on sequences of three gene regions. Because isolates representing the two phylogenetic assemblages originated from different hosts, their ability to infect these trees was also considered using pathogenicity tests.

MATERIALS AND METHODS

Isolates

Isolates used in this study were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1). These were also all used in the previous study of Marin *et al.* (2003). The isolates were selected based on host, geographic occurrence and groupings emerging from the studies of Barnes *et al.* (2001) and Marin *et al*. (2003).

PCR and sequencing reactions

DNA was extracted from isolates as described by Van Wyk *et al.* (2006). Three gene regions were chosen for comparisons. These were the ITS 1 and 4 regions including the 5.8S gene of the rDNA operon, part of the β-tubulin gene and a portion of the Transcription Elongation Factor 1-α gene. The primers used for these reactions were those developed by White *et al.* (1990), Glass and Donaldson (1995) and Jacobs *et al.* (2004), respectively.

The PCR and sequencing conditions and reactions were as described by Van Wyk *et al.* (2006). Sequences for other closely related Ceratocystis species were obtained from previous studies (Van Wyk *et al*. 2004, 2007a, b, 2009) and analysed using PAUP version 4.0b10* (Swofford 2002). Sequences were aligned with the programme MAFFT (Katoh *et al*. 2002) and manually confirmed. A partition homogeneity test (PHT) (Swofford 2002) was used to determine whether the three datasets could be combined. Gaps were treated as a fifth character and the trees were obtained by

the stepwise addition of 1,000 replicates with the Mulpar option in effect. The phylogram was obtained by selecting the heuristic search option with stepwise addition. Confidence intervals using 1,000 bootstrap replicates were calculated. Two isolates of *C. virescens* (R.W. Davidson) C. Moreau were designated to represent the outgroup and all sequences derived from this study were deposited in GenBank (Table 1).

The Markov Chain Monte Carlo (MCMC) algorithm was used to create a phylogenetic tree based on Bayesian probabilities using the software program MrBayes version 3.1.1 (Ronquist and Huelsenbeck 2003). For each of the three genes, a model of nucleotide substitution was determined using MrModeltest 2.2 (Nylander 2004). The nucleotide substitutions obtained were included for each gene partition in MrBayes. One million random trees were generated using the MCMC procedure with four chains and sampled every 100th generation. To avoid including trees that had been sampled before convergence, tree likelihood scores were assessed to determine the number of trees that were formed before the stabilization. Trees outside the point of convergence were discarded by means of the burn-in procedure in MrBayes (Ronquist & Huelsenbeck 2003).

The two groups of isolates from Colombia as well as C. neglecta, previously described from Eucalyptus in Colombia (Rodas *et al*. 2007), were analyzed separately for each of the three gene regions mentioned above. The PAUP settings were the same as those for the combined dataset, but without an outgroup. Molecular Evolutionary Genetics Analysis (MEGA) 4 (Tamura *et al*. 2007) was utilized to determine the level of variation between the isolates from Colombia including C. neglecta. Sequences for each gene region were examined to determine the number of fixed alleles between them. The software package TCS (Clement *et al*. 2000) was used to construct an allele network to observe the shared alleles between isolates representing the two groups from Colombia and the Colombian species *C. neglecta*.

Culture characteristics and morphology

Three isolates representing each of the two groups identified by Marin *et al*. (2003) (CMW5751, CMW5761, CMW10871 and CMW8856, CMW8857, CMW8850) were selected for morphological studies. Colony colour was determined using the colour tables of Rayner (1970). A growth study was conducted to determine the temperatures for optimal growth of the two groups of isolates. Cultures were grown on 2% (w/v) malt extract agar (MEA) (Biolab, Midrand, South Africa) supplemented with Streptomycin Sulphate (0.001 g vol−1, SIGMA, Steinheim, Germany) for 2 weeks. A 4 mm plug was taken from the edges of actively growing cultures and placed at the centres of 90 mm Petri dishes containing 2% MEA. Five replicate plates for each isolate were incubated at temperatures ranging form 5°C to 35°C at 5°C intervals. Growth was assessed by

measuring colony diameters after 7 days. The average growth and standard deviations were then calculated. The entire experiment was repeated once.

The same six isolates, representing the two groups, used in the growth studies were used for morphological examinations. Fungal structures were mounted in lactic acid on glass slides and examined using a Carl Zeiss microscope and the photographic images were captured with a Zeiss Axio Vision camera system. Fifty measurements were taken for each taxonomically relevant structure in isolates CMW5751 and CMW8856, which were chosen to represent the two groups respectively. In addition, ten measurements were made of the relevant structures for the four remaining isolates representing the two groups. Ranges, averages, and standard deviations of all measurements were calculated and measurements are presented as (minimum-) mean minus standard deviation minus—mean plus standard deviation (-maximum).

Reciprocal inoculation tests

In order to test differences in pathogenicity amongst isolates obtained from coffee, cacao and citrus (Valencia orange), a greenhouse inoculation experiment was conducted in Chinchiná, Colombia. Four randomly chosen isolates from coffee, two from each group (Marin *et al*. 2003), two isolates from cacao and two isolates from citrus, were used. These eight strains were inoculated into the stems of nine-month-old plants of coffee variety Caturra, eight-month-old plants of cacao variety IMC-67 and 12-month-old plants of Valencia orange.

A cambial disc was removed from the main stems of 40 plants using a 5-mm-diameter cork borer. A similar sized plug taken from the actively growing margins of cultures of each isolate growing on 2% MEA plates were placed in the wounds and covered with Parafilm to reduce desiccation and contamination. A set of 40 plants of each host was inoculated in a similar way, but using sterile MEA discs as a control treatment. After 21 weeks, the lengths of necrotic lesions in the phloem (coffee) or the vertical extension of discoloration in the xylem (cacao and citrus) was determined. Data were analysed using one-way analysis of variance (ANOVA) for each host. Tukey's HSD multiple comparisons were performed to test for statistically significant differences amongst isolates (SYSTAT, SPSS Inc., Chicago).

RESULTS

PCR and sequence analyses

Amplicons of ~500 bp were obtained for the ITS and β-tubulin regions and a ~800 bp fragment was obtained for the Transcription Elongation Factor 1-α region. The PHT gave a value of P=0.01,

which allowed for the datasets being combined (Cunningham 1997). The dataset consisted of 1,971 characters, 1,104 were constant characters, 46 were parsimony uninformative and 821 were parsimony informative. Four most parsimonious trees were obtained from this dataset, one of which one was selected for presentation (Figure 1). This tree had a length of 2205, a consistency index of 0.7, a retention index of 0.9 and a rescaled consistency index of 0.6.

For the dataset representing the ITS region, the model obtained with MrModeltest2 that was used in the Bayesian analysis was the GTR+I+G model. For the partial β-tubulin gene dataset, the GTR+G model was selected while for the EF1-α gene dataset, the HKY+I+G model was selected. Two thousand trees were discarded due to the fact that they were outside the point of convergence (burn-in). Bayesian inference used to obtain the posterior probability of the branch nodes of the combined tree supported the bootstrap values obtained with PAUP (100% and 100%, respectively) (Figure 1). In the phylogenetic tree (Figure 1), two groups of isolates from Colombia were phylogenetically distinct from each other. The groups had a high bootstrap support of 99% and 80%, respectively. Isolates in both groups showed a high level of variation in sequences and both groups included isolates from native trees, citrus, cacao and coffee. The species most closely related to those representing the two groups from Colombia were *C. fimbriata s.s.*, *C. cacaofunesta* Engelbr. & T.C. Harr., *C. platani* Englbr. & T.C. Harr., *C. fimbriatomima* M. van Wyk & M.J. Wingf, *C. neglecta* M. van Wyk, Jol. Roux & Rodas and *C. manginecans* M. van Wyk, Al Adawi, M.J. Wingf.

Phylogenetic trees derived from sequences for the single gene regions (ITS, βT and EF1-α, respectively), separated the two groups of isolates from each other and from *C. neglecta* and had structures (Figure 2) very similar to that for the tree (Figure 1) representing the combined data set for the three gene regions. However, the suport for the branches separating the groups was not as high as that found in the combined dataset.

Fixed polymorphisms between isolates representing the two groups from Colombia and *C. neglecta* were observed for all three gene regions. For the ITS region, there were 16 differences between the two groups of isolates from Colombia. Likewise, there were 19 fixed polymorphisms separating *C. neglecta* and Group 1 isolates and six differences between *C. neglecta* and Group 2 isolates in the ITS region. For the βT region there was one fixed allele separating the two groups of isolates form Colombia. Likewise, there were three fixed alleles separating *C. neglecta* and Group 1 isolates and two fixed alleles separating *C. neglecta* and Group 2 isolates. In the EF1-α region there were no fixed alleles separating the two groups of isolates from Colombia but there were two fixed alleles separating both *C. neglecta* and Group 1 and the same was true for *C. neglecta* and Group 2 isolates. The allele networks (Figure 3) drawn to show genetic linkage between the two groups of

isolates from Colombia or between those groups and *C. neglecta* showed no linkage between these three groups.

Culture characteristics and morphology

Cultures representing isolates occurring in the two groups from Colombian *C. fimbriata s.l.* clades were morphologically different. Isolates (CMW5751, CMW5761, CMW10871) representing Group 1 had a stellate appearance with ascomata abundant in the cultures that were a light brownish olive (19″k) colour. Group 2 isolates (CMW8856, CMW8857, CMW8850) displayed a deep greyish olive colour (21′′′′i).

Growth comparisons in culture showed that isolates (CMW8856, CMW 8857, CMW8850) representing Group 2 grew faster than isolates (CMW5751, CMW5761, CMW10871) in Group 1 at 15°C, 20°C, 25°C and 30°C. Neither group of isolates showed growth at 5°C, 10°C and 35°C. The optimum temperature for growth of isolates in Group 1 was 25°C with the cultures having an average diameter of 32 mm in 7 days. Group 2 isolates also grew optimally at 25°C attaining an average diameter of 31 mm (20°C), 36 mm (25°C) and 23 mm (30°C) respectively in 7 days.

Reciprocal inoculation tests

After 21 weeks, all isolates, irrespective of the host plant of origin, produced lesions on the stems of inoculated coffee plants. Seven of the isolates gave rise to necrotic lesions in the phloem ranging on an average from 50 to 106 mm long (Figure 4a). These plants also developed chlorotic foliage and epicormic shoots below the inoculation points. Isolate CMW9561 from cacao caused lesions that were on average only 21 mm long, while the other isolate from cacao gave rise to lesions with an average length similar to those associated with the coffee isolates. Statistical analyses indicated significant differences in the lengths of lesions in the phloem caused by the isolates (Figure 4a). Isolate CMW8850 from Valencia orange was the most pathogenic to coffee plants and gave rise to an average lesion length of 106 mm. Isolates residing in the two groups from coffee did not produce significantly different lesion lengths on that host.

Extensive xylem discoloration was produced by all isolates inoculated onto cacao. The average lengths of internal discoloration ranged from 173 mm (CMW5746 from coffee) to 264 mm (CMW9561 from cacao) (Figure 4b). Isolate CMW9561 from cacao produced the longest average lesion lengths, but no significant differences were detected between this isolate and other isolates. The high level of susceptibility of this host originating from isolates obtained from different hosts was evident from the very long individual lesions on some plants, for example 650 mm (CMW5761 from coffee), 620 mm (CMW8860 from cacao) and 470 mm (CMW10844 from coffee) (Figure 4b).

Xylem discoloration on Valencia orange plants ranged from 53 mm (CMW9561 from cacao) to 193 mm (CMW5761 from coffee). Five isolates (CMW10844 from coffee, CMW5746 from coffee, CMW8850 from citrus, CMW8860 from cacao and CMW5751 from coffee) recovered from different hosts produced statistically indistinguishable lengths of discoloration (Figure 4c). Citrus isolates were moderately pathogenic, while isolate CMW5761 from coffee was most pathogenic on this host (Figure 4c). Control inoculations did not give rise to lesions on any of the hosts tested (Figure 4a–c).

TAXONOMY

The *C. fimbriata s.l.* isolates from Colombia considered in this study clearly represent two unique taxa. These differ from each other based on morphology and DNA sequence comparisons for three gene regions. They also differ from all described species of *Ceratocystis* based on morphological characteristics and they are thus described as follows:

Ceratocystis colombiana M. van Wyk & M.J. Wingf., sp. nov. (Figure 5a–h)
MB511243.
Etymology—Name refers to the Colombian origin of this fungus.

Ascomata typica generis, atrobrunnea vel nigra, collo apicem versus pallescenti. Bases peritheciorum globosae. Colla ascomatum, apice cum hyphis ostiolaribus divergentibus. Ascosporae pileiformes. Conidiophorae biformes; phialides primariae ampulliformes apicem versus contractae. Conidia primaria cylindrica. Conidiophorae secondariae apice subinfundibuliformes. Conidia secondaria doliiformia Chlamydosporae coffeinae vel umbrinae.

Culture morphology stellate, ascomata abundant, light brownish olive (19″k). Ascomata dark-brown to black with the necks becoming lighter towards the apices. Ascomatal bases globose (140–)177–237(–294) µm diam. Ascomatal necks (375–)448–560(–676) µm long, (24–)27–35(–43) µm wide at base, (12–)14–18(–19) µm wide at apices. Ascomatal necks terminating in divergent ostiolar hyphae, (28–)38–46(–52) µm long. Ascospores hat-shaped 3–4 µm long, (3–)4–6(–7) µm wide excluding sheaths, 6–8(–11) µm including sheaths. Conidiophores of two types. Primary conidiophores flask-shaped phialides tapering towards the apices. Primary phialides (58–)65–83(–106) µm long, 4–6(–8) µm wide at base, (3–)6–8(–9) µm wide at the broadest point and 3–5(–6) µm wide at apices. Primary conidia, cylindrical, (12–)16–24(–29) µm long and 4–6 µm wide. Secondary conidiophores flaring at apices. Secondary phialides (42–)49–71(–85) µm long, (4–)5–7

µm wide at the base, (5–)6–8 µm wide at apices. Secondary conidia, barrel-shaped, 9–14 µm long, 6–8(–11) µm wide. Chlamydospores hair-brown, globose, 11–14 µm long, 11–15(–17) µm wide.

Habitat: *Coffea arabica*, soil, citrus trees, *Schizolobium parahybum*.
Known distribution: Colombia, South America.
Material examined: **Colombia**, Valle del Cauca, from diseased *Coffea arabica* trees, M. Marin, PREM59434, CMW5751, CBS121792 (2000). **Colombia**, *Coffea arabica* trees, M. Marin, PREM59435, CMW5761, CBS121791 (2000). **Colombia**, Caldas, from soil in citrus orchard, B. Castro PREM59619, CMW9565, CBS121790 (2002). **Colombia**, Caldas, from soil in citrus (Tangelo) orchard, B. Castro PREM59619, CMW9565 (2002). **Colombia**, Caldas, Mandarin, B. Castro PREM60177, CMW9572 (2002). **Colombia**, Caldas, *C. arabica*, M. Marin, PREM60175, CMW5768 (2000). **Colombia**, Santander, *Schizolobium parahybum*, unknown, PREM60176, CMW11280 (2001). **Colombia**, Caldas, *Citrus sinensis* (Valencia orange), M. Marin, PREM60178, CMW10871 (2001).

Notes: Ceratocystis colombiana can be distinguished from its closest phylogenetic relative by the presence of secondary conidiophores and the secondary barrel-shaped conidia.

Ceratocystis papillata M. van Wyk & M.J. Wingf., sp. nov., (Figure 6a–h)
MB511244.

Etymology—Name refers to the papilla like form of the apices of the ascomatal bases.

Ascomata atrobrunnea vel nigra, globosa collis elongatis apicem versus pallescentibus; bases globosae. Supra cum structura torulosa; colla. Hyphae ostiolares divergentes. Ascosporae pileiformes. Conidiophora primaria ampulliformis, apicem versus contracta. Conidia primaria cylindrica. Phialides secondariae tubiformes. Conidia secondaria ellipsoideo-doliiformia. Chlamydosporae subglobosae vel globosae, coffeinae vel umbrinae.

Culture morphology deep greyish olive in colour (21′′′′i). Ascomata dark-brown to black in colour, globose, necks long becoming lighter towards the apices. Ascomatal bases globose, with papillate apex, (160–)177–233(–258) µm diam. Ascomatal necks (472–)614–724(–753) µm long, (30–)33–47(–58) µm wide at base, (13–)17–21(–25) µm wide at apices. Ostiolar hyphae divergent, (44–)55–71(–78) µm long. Ascospores hat-shaped, 3–4 µm long, (3–)4–6 µm in width without sheath, 5–7(–8) µm wide including the sheath. Primary conidiophores flask shaped, tapered towards the apices, (106–)116–164(–184) µm long, the bases are (3–)4–6(–7) µm wide, at the broadest point (6–)7–9

µm wide and at the apices 3–5(–6) µm wide. Primary conidia cylindrical, (17–)19–25(–29) µm long, (3–)4–6(–7) µm wide. Secondary phialides tubular, (46–) 48–66 (–76) µm long, bases 3–5 µm wide, apices 3–6 µm wide. Secondary conidia barrel-shaped, (6–)9–11 µm long, 5–7(–8) µm wide. Chlamydospores subglobose to globose, hair-brown, (10–)12–14(–16) µm long, (8–)10–12(–14) µm wide.

Habitat: *Coffea arabica*, soil, *Theobroma cacao*, *Schizolobium parahybum*, Citrus x tangelo, *Citrus limon*, *Annona muricata*.
Known distribution: Colombia, South America.
Material examined: **Colombia**, Caldas, Citrus x tangelo B. Castro, PREM59438, CMW8856, CBS121793 (2001). **Colombia**, Quindio, *Citrus limon*, B. Castro, PREM59620, CMW8850, CBS121794 (2001). **Colombia**, Quindio, *Schizolobium parahybum*, B. Castro, PREM59621, CMW8858, CBS121795 (2001). **Colombia**, Risaralda, *Annona muricata*, B. Castro, PREM60173, CMW8857 (2001). **Colombia**, Caldas, *C. arabica*, M.J. Wingfield, PREM60171, CMW5746 (2000). **Colombia**, Antioquia, *C. arabica*, M. Marin, PREM60172, CMW10844 (1998). **Colombia**, Caldas, *Theobroma cacao*, B. Castro, PREM60174, CMW8860 (2001). **Colombia**, Caldas, *Theobroma cacao*, B. Castro, CMW9561 (2001).

Notes: *Ceratocystis papillata* can be distinguished from most species in the *C. fimbriata s.l.* species complex by the presence of the cap-like morphology of the apices of the ascomatal bases. The only other species that have a similar ascomatal base morphology are *C. caryae*, *C. smalleyi*, *C. variospora* and *C. populicola* J.A. Johnson & T.C. Harr., but there are clear differences between *C. papillata* and these species. These differences include absence of primary conidiophores in *C. smalleyi,* the smaller sizes of both conidiophores of *C. papillata* compared to those of *C. caryae*, the shorter necks of *C. populicola* and the larger ascomatal bases of *C. variospora*.

DISCUSSION

Mutligene phylogeny has proved useful in distinguishing species complexes in several genera of fungi including *Ceratocystis* (Van Wyk *et al*. 2004, 2007a, b, 2009), *Colletotrichum* (Yang *et al*. 2009, Prihastuti *et al*. 2009), *Diplodia* and *Lasiodiplodia* (Lazzizera *et al*. 2008, Alves *et al*. 2008), *Fusarium* (Kvas *et al*. 2009), *Mycosphaerella* (Crous 2009) and *Phomopsis* (Santos & Phillips 2009) and has also revealed two cryptic species in this study. Previous studies have shown that isolates of *C. fimbriata s.l.* from coffee in Colombia reside in two distinct phylogenetic lineages (Barnes *et al*. 2001, Marin *et al*. 2003). Results of the present investigation confirm those results and they have shown that the species in the two groups differ phylogenetically based on sequences

for three gene regions, from all described species in the *C. fimbriata s.l.* species complex. Fixed polymorphisms were found in two of the three gene regions between representatives of the two groups of isolates and in three gene regions between these fungi and *C. neglecta*, which is a closely related species also from Colombia. Allele networks based on sequence data for three gene regions also showed robust separation between isolates representing the two groups of isolates from Colombia. Furthermore, isolates residing in the two phylogenetic lineages from Colombia were morphologically distinct from each other. These results support the conclusion that isolates representing the two phylogenetic groups of isolates from Colombia represent novel taxa for which the names *C. papillata* and *C. colombiana* have been provided.

Isolates of *C. papillata* and *C. colombiana* reside in robust phylogenetic clades distinct from all other species in the *C. fimbriata s.l.* species complex with high bootstrap and Bayesian support. There was considerable variation in the sequences of the individual isolates considered. These are clearly species with high genetic variability and are most likely native to the area in which they were collected. Sequence variation might also suggest that these fungi could represent species complexes that cannot be resolved at the present time. Single gene trees gave the same phylogenetic separation of isolates as that derived for the combined dataset, although a few isolates did not have strong support for their final grouping. The species phylogenetically most closely related to *C. papillata* and *C. colombiana* are *C. fimbriata s.s.*, *C. platani*, *C. manginecans*, *C. neglecta*, *C. fimbriatomima* and *C. cacaofunesta*.

Ceratocystis papillata can be distinguished from *C. colombiana* based on differences in culture morphology on 2% MEA. The stellate appearance of *C. colombiana* cultures is very distinct and makes it possible to easily distinguish between the two species. They also differ in their growth optima in culture and in various morphological characteristics. The most obvious morphological difference between the two species is found in the fact that isolates of *C. papillata* have ascomatal bases that have papillate apices. These are very distinct from the globose bases of *C. colombiana*. *Ceratocystis papillata* also has much longer ostiolar hyphae and the ascospore sheaths are much shorter than those of *C. colombiana*. Furthermore, the primary phialides of *C. papillata*, which are most commonly encountered, are double the length of those of *C. colombiana*. The barrel-shaped conidia in *C. papillata* were also substantially smaller than those of *C. colombiana*.

Ceratocystis papillata is morphologically similar to other species in the *C. fimbriata s.l.* species complex. The most distinct difference between this and other species is the characteristic “cap-like” morphology of the apices of the ascomatal bases in *C. papillata*. Furthermore, *C. papillata* can be distinguished from *C. fimbriata s.s.* by the absence of secondary conidiophores and secondary

conidia, both of which are present in *C. papillata*. Other differences are the longer ascomatal necks and ostiolar hyphae of *C. fimbriata s.s.* when compared with *C. papillata*. The two species can also be separated by the shorter primary conidiophores of *C. fimbriata s.s.*, compared with those of *C. papillata*.

The cap-like apices of the ascomatal bases of *C. papillata* have been observed in other species in the *C. fimbriata s.l.* species complex, e.g. *C. caryae*, *C. smalleyi*, *C. variospora* and *C. populicola* (Johnson *et al*. 2005). However, this morphological feature is always present in *C. papillata* while it is only occasionally found in *C. caryae* (Johnson *et al*. 2005). This morphology in *C. caryae* was described as a part of the ascomatal neck (Johnson *et al*. 2005) while in *C. papillata* it is more clearly part of the ascomatal base. Furthermore, the ascomatal necks, the primary and secondary conidiophores, the cylindrical conidia and the chlamydospores of *C. papillata* are smaller than those reported for *C. caryae* (Johnson *et al*. 2005). *Ceratocystis smalleyi* has only one conidiophore type while *C. papillata* has both primary and secondary conidiophores. Further, *C. smalleyi* lacks the strong sweet banana aroma which is very evident in *C. papillata*. *Ceratocystis variospora* possess much larger ascomatal bases and longer ascomatal necks than *C. papillata*. *Ceratocystis populicola* is different from *C. papillata* in that both types of conidiophores are longer in *C. populicola* and the ascomatal necks are shorter for isolates in *C. populicola*.

Ceratocystis colombiana is closely related to *C. papillata* and morphologically similar to those species in the socalled “Latin-American” clade of *C. fimbriata s.l.* (Johnson *et al*. 2005). Unlike *C. papillata*, *C colombiana* does not have a single characteristic that distinguishes it from the other species in this group. In this case, reliance on DNA sequencing and phylogenetic placement is required to identify it with confidence.

Ceratocystis colombiana can be distinguished from *C. fimbriata s.s*. by the presence of the secondary conidiophores and secondary conidia that are absent in *C. fimbriata s.s*. The ascomatal bases of *C. colombiana* are smaller than those of *C. fimbriata s.s*. Other differences between these species are the shorter ascomatal necks, ostiolar hyphae and primary conidiophores of *C. colombiana* when compared with those of *C. fimbriata s.s.*

Ceratocystis papillata and *C. colombiana* have been isolated from a wide range of hosts including three cultivated tree crops as well as native trees in Colombia. Isolates from all three crop plants (coffee, cacao and citrus) were able to infect their hosts of origin, but also the other plants considered. There was thus no indication of host specificity of these two pathogens. Within *C. fimbriata s.l.*, some species are known to be highly host specific, infecting only a single host

species, e.g. *C. platani* only infects plane trees (Johnson *et al*. 2005). However, there are pathogenic species in the *C. fimbriata s.l.* complex that are not host specific, e.g. *C. albifundus* M.J. Wingf., De Beer & M.J. Morris which has been isolated from nine different host genera (Roux *et al*. 2007). The relatively large number of species residing in the *C. fimbriata s.l.* species complex that are found in Latin American, suggests that this may be a centre of diversity for the group (Harrington 2000, Johnson *et al*. 2005). The natural hosts of *C. papillata* and *C. colombiana* could be native tree species and their ability to infect non-native crop plants might indicate that they are species that have wide host ranges in their native environment.

Ceratocystis papillata and *C. colombiana* are both virulent pathogens of coffee in Colombia, where this tree is one of the county's most important sources of income and employment. Recognising these fungi as distinct taxa could have significant implications for managing the diseases that they cause. Although they seem not to be host specific, selection of resistant cultivars will need to consider differences between these fungal species. Furthermore, Marin (2004) has shown that isolates of the two species tend to be limited predominately to a single orchard and to different areas of the country. Every effort should be made to reduce the chances of local spread of these species.

REFERENCES

Alves A., Crous P.W., Correia A. & Phillips A.J.L. (2008) Morphological and molecular data reveal cryptic speciation in *Lasiodiplodia theobromae*. *Fungal Diversity* **28**:1–13

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

Castaño J. (1951) Interpretatión de los síntomas y los signos de la enfermedad de la macana en café para el establecimiento de la diagnosis. *Cenicafé* **2**:7–32

Clement M., Posada D. & Crandall K. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**:1657–1660

Crous P.W. (2009) Taxonomy and phylogeny of the genus *Mycosphaerella* and its anamorphs. *Fungal Diversity* **38**:1–24

Cunningham C.W. (1997) Can three incongruence tests predict when data should be combined? *Molecular Biology and Evolution* **14**:733–740

Engelbrecht C.J. & Harrington T.C. (2005) Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. *Mycologia* **97**:57–69

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

Halsted B.D. (1890) Some fungous disease of the sweet potato. *Agricultural College Experiment Station Bulletin* **76**:1–32

Harrington T.C. (2000) Host specialization and speciation in the American wilt pathogen *Ceratocystis fimbriata*. *Fitopatologia Brasiliensis* **25**:262–263

Jacobs K., Bergdahl D.R., Wingfield M.J., Halik S., Seifert K.A., Bright D.E. & Wingfield B.D. (2004) *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* **108**:411–418

Johnson J.A., Harrington T.C. & Engelbrecht C.J.B. (2005) Phylogeny and taxonomy of the North American clade of the *Ceratocystis fimbriata* complex. *Mycologia* **97**:1067–1092

Katoh K., Misawa K., Kuma K. & Miyata T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**:3059–3066

Kile G.A. (1993) In: Wingfield MJ, Seifert KA, Webber JA (eds) *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. APS, St. Paul, pp 173–183

Kvas M., Marasas W.F.O., Wingfield B.D., Wingfield M.J. & Steenkamp E.T. (2009) Diversity and evolution of *Fusarium* species in the *Gibberella fujikuroi* complex. *Fungal Diversity* **34**:1–21

Lazzizera C., Frisullo S., Alves A., Lopes J. & Phillips A.J.L. (2008) Phylogeny and morphology of *Diplodia* species on olives in southern Italy and description of *Diplodia olivarum* sp. nov. *Fungal Diversity* **31**:63–71

Marin M. (2004) Phylogenetic and molecular population biology studies on *Ceratocystis* spp. associated with conifer and coffee diseases. PhD thesis, Department of Plant pathology and Microbiology, University of Pretoria, South Africa

Marin M., Castro B., Gaitan A., Preisig O., Wingfield B.D. & Wingfield M.J. (2003) Relationships of *Ceratocystis fimbriata* isolates from Colombian coffee-growing regions based on molecular data and pathogenicity. *Journal of Phytopathology* **151**:395–405

Mourichon X. (1994) Serious Citrus dieback in Colombia caused by *Ceratocystis fimbriata*. Fruits **49**:415–416

Nylander J.A.A. (2004) MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University Pardo-Cardona VM (1995) Hongos fitopatógenos de Colombia. Medellín, Colombia. Dissertation, Universidad Nacional de Colombia

Pontis R.E. (1951) A canker disease of the coffee tree in Colombia and Venezuela. *Phytopathology* **41**:179–184

Prihastuti H., Cai L., Chen H. & Hyde K.D. (2009) Characterization of *Colletotrichum* species associated with coffee berries in Chiang Mai, Thailand. *Fungal Diversity* **39**:89–109

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

Rodas C., Roux J., Van Wyk M., Wingfield B.D. & Wingfield MJ (2007) *Ceratocystis neglecta* sp. nov., infecting Eucalyptus trees in Colombia. *Fungal Diversity* **28**:73–84

Ronquist F. & Huelsenbeck J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**:1572–1574

Rossetto C.J. & Ribeiro I.J.A. (1990) Mango wilt. XII. Recommendations for control. *Revista de Agricultura Piracicaba* **65**:173–180

Roux J., Heath R.N., Labuschagne L., Nkuekam G.K. & Wingfield M.J. (2007) Occurrence of the wattle wilt pathogen, *Ceratocystis albifundus* on native South African trees. *Forest Pathology* **37**:1–11

Santos J.M. & Phillips A.J.L. (2009) Resolving the complex of *Diaporthe* (*Phomopsis*) species occurring on *Foeniculum vulgare* in Portugal. *Fungal Diversity* **34**:111–125

Swofford D.L. (2002) PAUP*: phylogenetic analysis using parsimony (* and other methods). Version: 4. Sinauer, Sunderland

Tamura K., Dudley J., Nei M. & Kumar S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**:1596–1599

Van Wyk M., Roux J., Barnes I., Wingfield B.D., Liew E.C.Y., Assa B., Summerell B.A. & Wingfield M.J. (2004) *Ceratocystis polychroma* sp. nov., a new species from *Syzygium aromaticum* in Sulawesi. *Studies in Mycology* **50**:273–282

Van Wyk M., Van der Merwe N.A., Roux J., Wingfield B.D., Kamgan G.N. & Wingfield M.J. (2006) Population genetic analyses suggest that the Eucalyptus fungal pathogen *Ceratocystis fimbriata* has been introduced into South Africa. *South African Journal of Science* **102**:259–263

Van Wyk M., Al Adawi A.O., Khan I.A., Deadman M.L., Al Jahwari A., Wingfield B.D., Ploetz R.C. & Wingfield M.J. (2007a) *Ceratocystis manginecans* sp. nov., causal disease of a destructive mango wilt disease in Oman and Pakistan. *Fungal Diversity* **27**:213–230

Van Wyk M., Pegg G., Lawson S. & Wingfield M.J. (2007b) *Ceratocystis atrox* sp. nov associated with *Phoracantha acanthocera* infestations on *Eucalyptus* in Australia. *Australasian Plant Pathology* **36**:407–414

Van Wyk M., Wingfield B.D., Mohali S. & Wingfield M.J. (2009) *Ceratocystis fimbriatomima,* a new species in the *C. fimbriata sensu lato* complex isolated from *Eucalyptus* trees in Venezuela. *Fungal Diversity* **34**:173–183

Webster R. & Butler E. (1967) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45**:1457–1468

White T.J., Bruns T., Lee S. & Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a sequencing guide to methods and applications. Academic, San Diego, pp 315–322

Yang Y.L., Liu Z.Y., Cai L., Hyde K.D., Yu Z.N. & Mckenzie E.H.C. (2009) *Collectrotrichum anthracnose* of *Amaryllidaceae*. *Fungal Diversity* **39**:123–146

Zimmerman A. (1900) Ueber den Krebs von *Coffea arabica*, verursacht *Rostrella coffeae* gen. et sp.n. *Bulletin of the Institute of Botanical Gardens Buitenzorg* **4**:19–22

Table 1. Isolates of *Ceratocystis* used in this study.

Species	Isolate no.	GenBank accession no.	Host	Geographic origin
C. albifundus	CMW4068	DQ520638 EF070429 EF070400	*Acacia mearnsii*	RSA
C. albifundus	CMW5329	AF388947 DQ371649 EF070401	*Acacia mearnsii*	Uganda
C. atrox	CMW19383 CBS120517	EF070414 EF070430 EF070402	*Eucalyptus grandis*	Australia
C. atrox	CMW19385 CBS120518	EF070415 EF070431 EF070403	*Eucalyptus grandis*	Australia
C. cacaofunesta	CMW15051 CBS152.62	DQ520636 EF070427 EF070398	*Theobroma cacao*	Costa Rica
C. cacaofunesta	CMW14809 CBS115169	DQ520637 EF070428 EF070399	*Theobroma cacao*	Ecuador
C. caryae	CMW14793 CBS114716	EF070424 EF070439 EF070412	*Carya cordiformis*	USA
C. caryae	CMW14808 CBS115168	EF070423 EF070440 EF070411	*Carya ovata*	USA
C. colombiana	**CMW9565 CBS121790**	**AY233864 AY233870 EU241487**	**Soil in coffee platation**	**Colombia**
C. colombiana	**CMW9572**	**AY233863 AY233871 EU241488**	***Mandarin***	**Colombia**
C. colombiana	**CMW5768**	**AY177235 AY177222 EU241491**	***Coffea Arabica***	**Colombia**
C. colombiana	**CMW5761 CBS121791**	**AY177234 AY177224 EU241492**	***Coffea aabica***	**Colombia**
C. colombiana	**CMW5751 CBS121792**	**AY177233 AY177225 EU241493**	***Coffea aabica***	**Colombia**
C. colombiana	**CMW11280**	**AY233861 AY233873 EU241490**	***Schizolobium parahybum***	**Colombia**
C. colombiana	**CMW10871**	**AY233862 AY233872 EU241489**	***Valencia orange***	**Colombia**
C. fimbriata s.s.	CMW15049 CBS141.37	DQ520629 EF070442 EF070394	*Ipomaea batatas*	USA
C. fimbriata s.s.	CMW1547	AF264904 EF070443 EF070395	*Ipomaea batatas*	Papua New Guinea
C. fimbriatomima	CMW24174 CBS121786	EF190963 EF190951 EF190957	*Eucalyptus* sp.	Venezuela
C. fimbriatomima	CMW24176 CBS121787	EF190964 EF190952 EF190958	*Eucalyptus* sp.	Venezuela
C. manginecans	CMW13851	AY953383	*Mangifera indica*	Oman

	CBS121659	EF433308 EF433317		
C. manginecans	CMW13852 CBS121660	AY953384 EF433309 EF433318	*Mangifera indica*	Oman
C. manginecans	CMW13854	AY953385 EF433310 EF433319	*Mangifera indica*	Oman
C. neglecta	CMW17808 CBS121789	EF127990 EU881898 EU881904	*Eucalyptus*	Colombia
C. neglecta	CMW18194 CBS121017	EF127991 EU881899 EU881905	*Eucalyptus*	Colombia
C. papillata	**CMW10844**	**AY177238 AY177229 EU241481**	***Coffea Arabica***	**Colombia**
C. papillata	**CMW5746**	**EU241479 EU241480 EU241482**	***Coffea Arabica***	**Colombia**
C. papillata	**CMW8857**	**AY233868 AY233878 EU241483**	***Annona muricata***	**Colombia**
C. papillata	**CMW8856 CBS121793**	**AY233867 AY233874 EU241484**	***Citrus lemon***	**Colombia**
C. papillata	**CMW8850 CBS121794**	**AY233866 AY233875 EU241485**	***Tangelo mineola***	**Colombia**
C. papillata	**CMW8858 CBS121795**	**AY233865 AY233877 EU241486**	***Schizolobium parahybum***	**Colombia**
C. papillata	**CMW8860**	**GQ478239 GQ478241 GQ478237**	***Theobromae cacao***	**Colombia**
C. papillata	**CMW9561**	**GQ478240 GQ478242 GQ478238**	***Theobromae cacao***	**Colombia**
C. pirilliformis	CMW6569	AF427104 DQ371652 AY528982	*Eucalyptus nitens*	Australia
C. pirilliformis	CMW6579 CBS118128	AF427105 DQ371653 AY528983	*Eucalyptus nitens*	Australia
C. platani	CMW14802 CBS115162	DQ520630 EF070425 EF070396	*Platanus occidentalis*	USA
C. platani	CMW23918	EF070426 EF070397	*Platanus occidentalis*	Greece
C. polychroma	CMW11424 CBS115778	AY528970 AY528966 AY528978	*Syzygium aromaticum*	Indonesia
C. polychroma	CMW11436 CBS115777	AY528971 AY528967 AY528979	*Syzygium aromaticum*	Indonesia
C. populicola	CMW14789 CBS119.78	EF070418 EF070434 EF070406	*Populus* sp.	Poland
C. populicola	CMW14819 CBS114725	EF070419 EF070435 EF070407	*Populus* sp.	USA
C. smalleyi	CMW26383 CBS114724	EU426553 EU426555 EU426556	*Carya cordiformis*	USA

C. smalleyi	CMW14800 CBS114724	EF070420 EF070436 EF070408	*Carya cordiformis*	USA
C. tanganyicensis	CMW15991 CBS122295	EU244997 EU244969 EU244929	*Acacia mearnsii*	Tanzania
C. tanganyicensis	CMW15999 CBS122294	EU244998, EU244970, EU244939	*Acacia mearnsii*	Tanzania
C. tsitsikammensis	CMW14276 CBS121018	EF408555 EF408569 EF408576	*Rapanea melanophloeos*	South Africa
C. tsitsikammensis	CMW14278 CBS121019	EF408556 EF408570 EF408577	*Rapanea melanophloeos*	South Africa
C. variospora	CMW20935 CBS114715	EF070421 EF070437 EF070409	*Quercus alba*	USA
C. variospora	CMW20936 CBS114714	EF070422 EF070438 EF070410	*Quercus robur*	USA
C. virescens	CMW11164	DQ520639 EF070441 EF070413	*Fagus americanum*	USA
C. virescens	CMW3276	AY528984 AY528990 AY529011	*Quercus robur*	USA

Bold face indicate the cultures used in this study

Figure 1. Most parsimonious phylogenetic tree representing isolates in the *C. fimbriata s.l.* species complex and based on combined sequence data for the ITS, βT and EF1-α gene regions. The two distinct groups representing isolates from Colombia (Group 1 and Group 2) represent the new species *C. papillata* and *C. colombiana*. The dataset consisted of 1971 characters, 46 parsimony uninformative characters, 821 parsimony informative characters. Tree length was 2205, consistency index = 0.7, retention index = 0.9 and rescaled consistency index = 0.6.

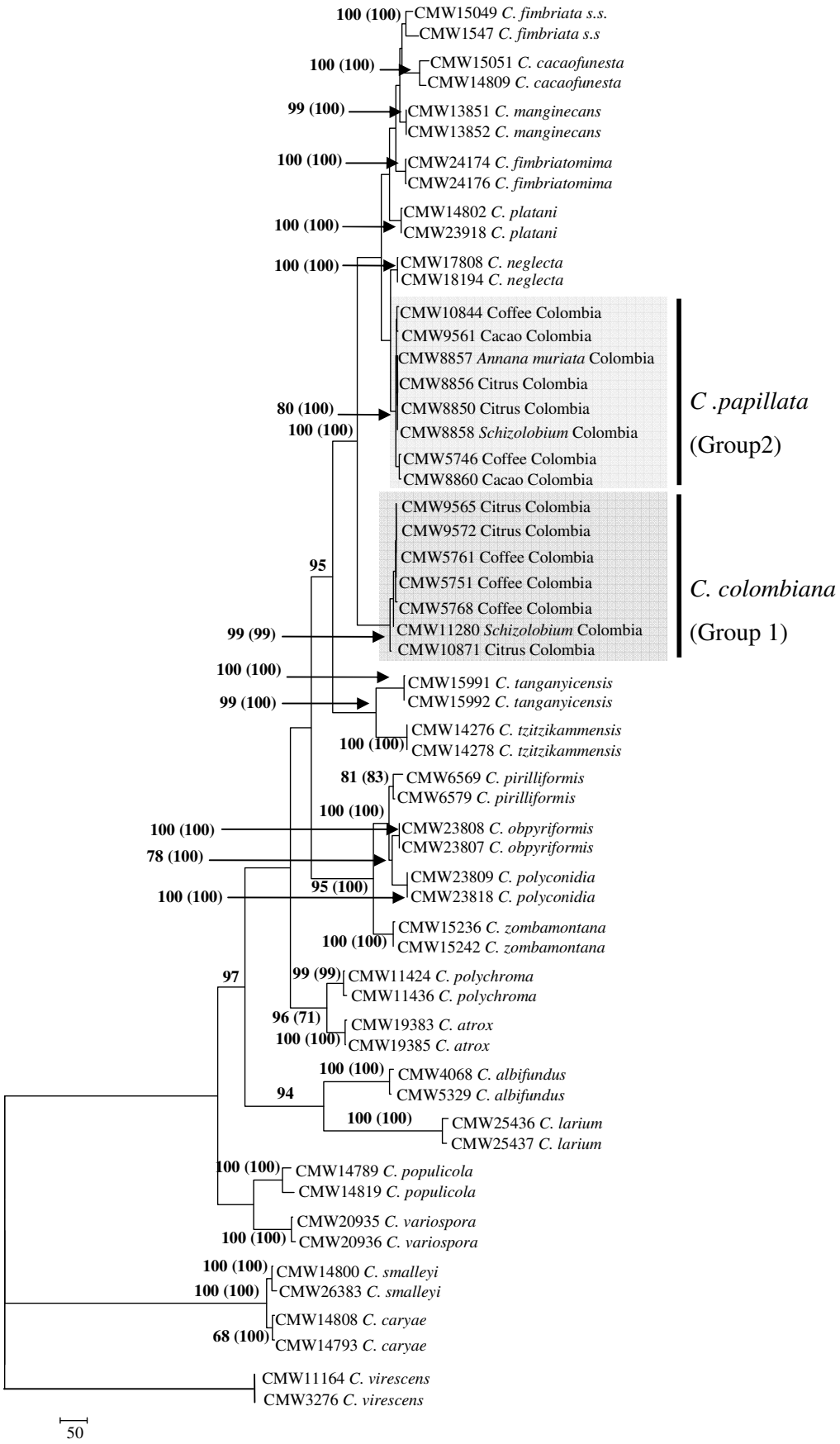
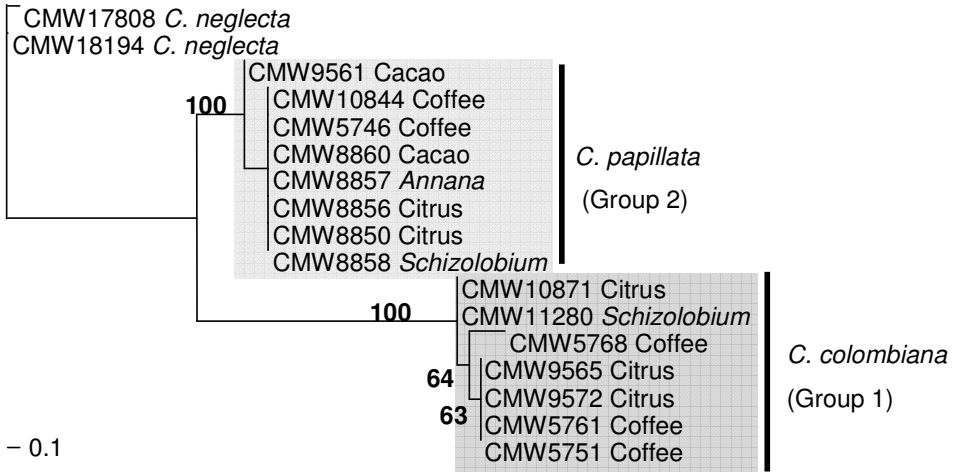
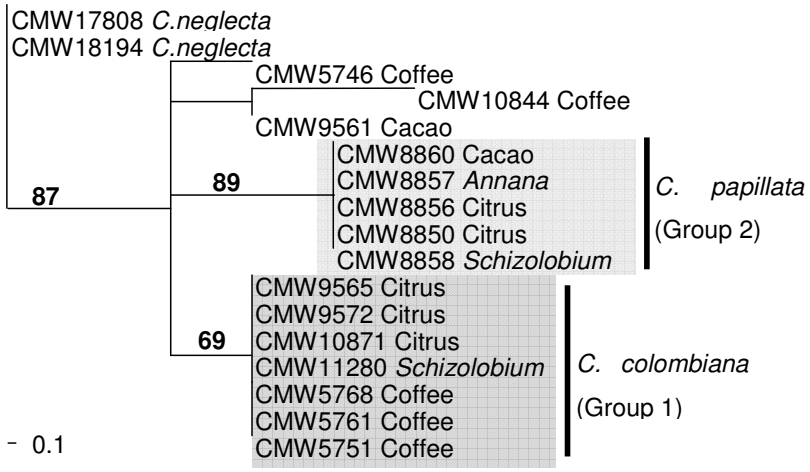


Figure 2. Three separate unrooted phylogenetic trees representing the a. ITS, b. the βT and the c. EF1-α gene regions for isolates representing the two groups (Group 1 = *C. colombiana*; Group 2 = *C. papillata*) from Colombia and *C. neglecta*. The two groups of isolates from Colombia were separated from each other and from *C. neglecta* and had structures very similar to that for combined dataset. For the ITS tree, the bootstrap support was high for both groups of isolates. For the βT tree the bootstrap support was high for *C. papillata* and lower for *C. colombiana* and there were some outlying isolates, however, the structure remained the same as the combined tree. For the EF1-α tree there was support for separation of *C. colombiana* and *C. papillata* but there were some outlying isolates.

a.



b.



c.

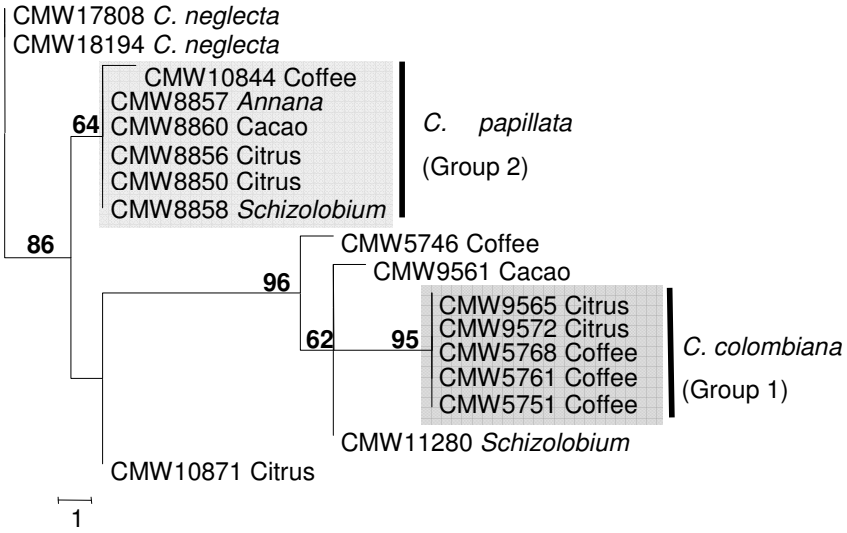


Figure 3. Three allele networks derived from a single dataset of all three gene regions representing *C. colombiana*, *C. papillata* and *C. neglecta.* All numbers in allele network represent CMW numbers that are listed in Table 1. Within the *C. colombiana* network, there was high variation and up to 6 differences between some isolates. Within the *C. papillata* network there were also high levels of variation with up to 11 differences between some isolates. In both *C. colombiana* and *C. papillata* there was a single genotype that was dominant.

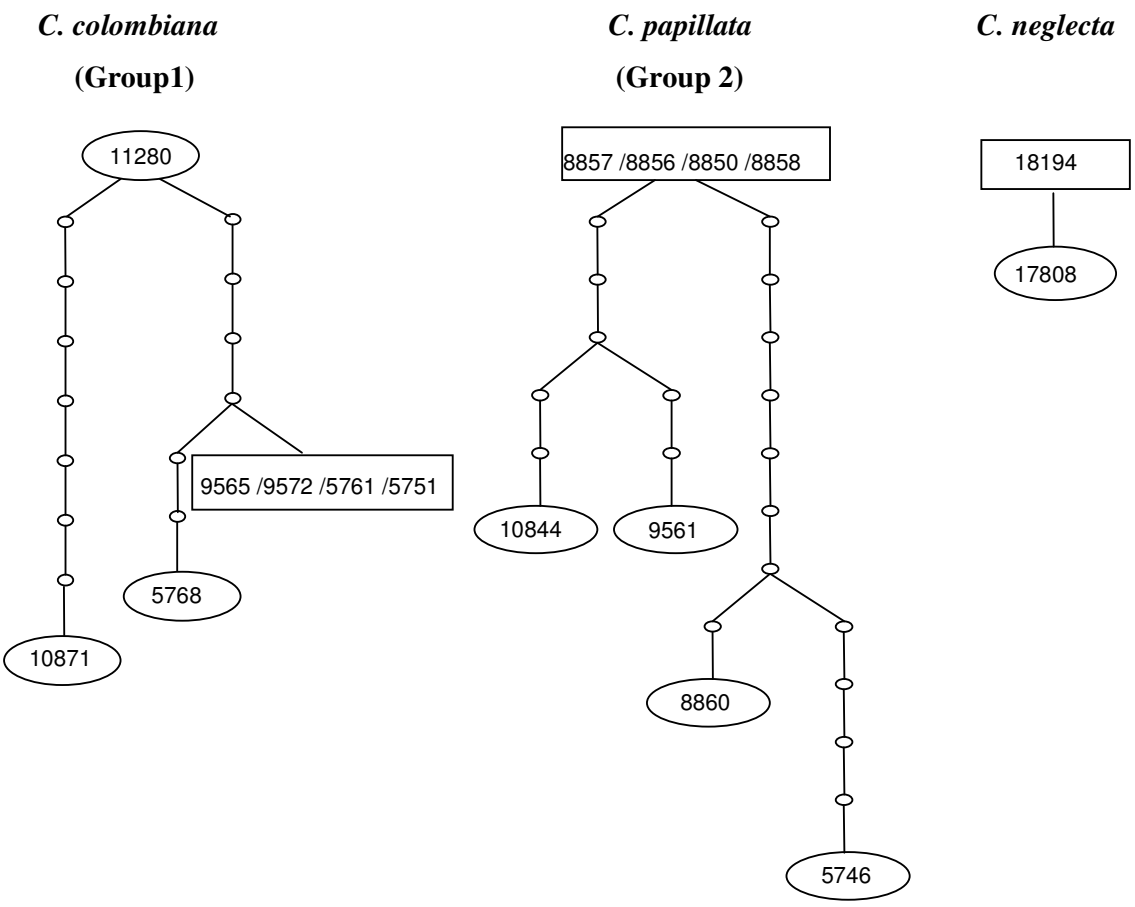


Figure 4. Mean lesion lengths (±SEM) in the phloem after inoculation with eight isolates representing *C. colombiana* and *C. papillata* obtained from coffee (CMW5761, CMW5751; CMW10844, CMW5746), cacao (CMW8860, CMW9561) and citrus (CMW8851, CMW8852). **a.** Inoculations on nine-month-old coffee plants (variety Caturra). **b.** Inoculation on eight-month-old cacao plants (variety IMC-67). **c.** Inoculation on twelve-month-old Valecia orange plants. Bars in the histogram annotated with different letters differ significantly from each other Isolates followed by different letters differ significantly from each other in terms of lesion lengths ($P < 0.05$).

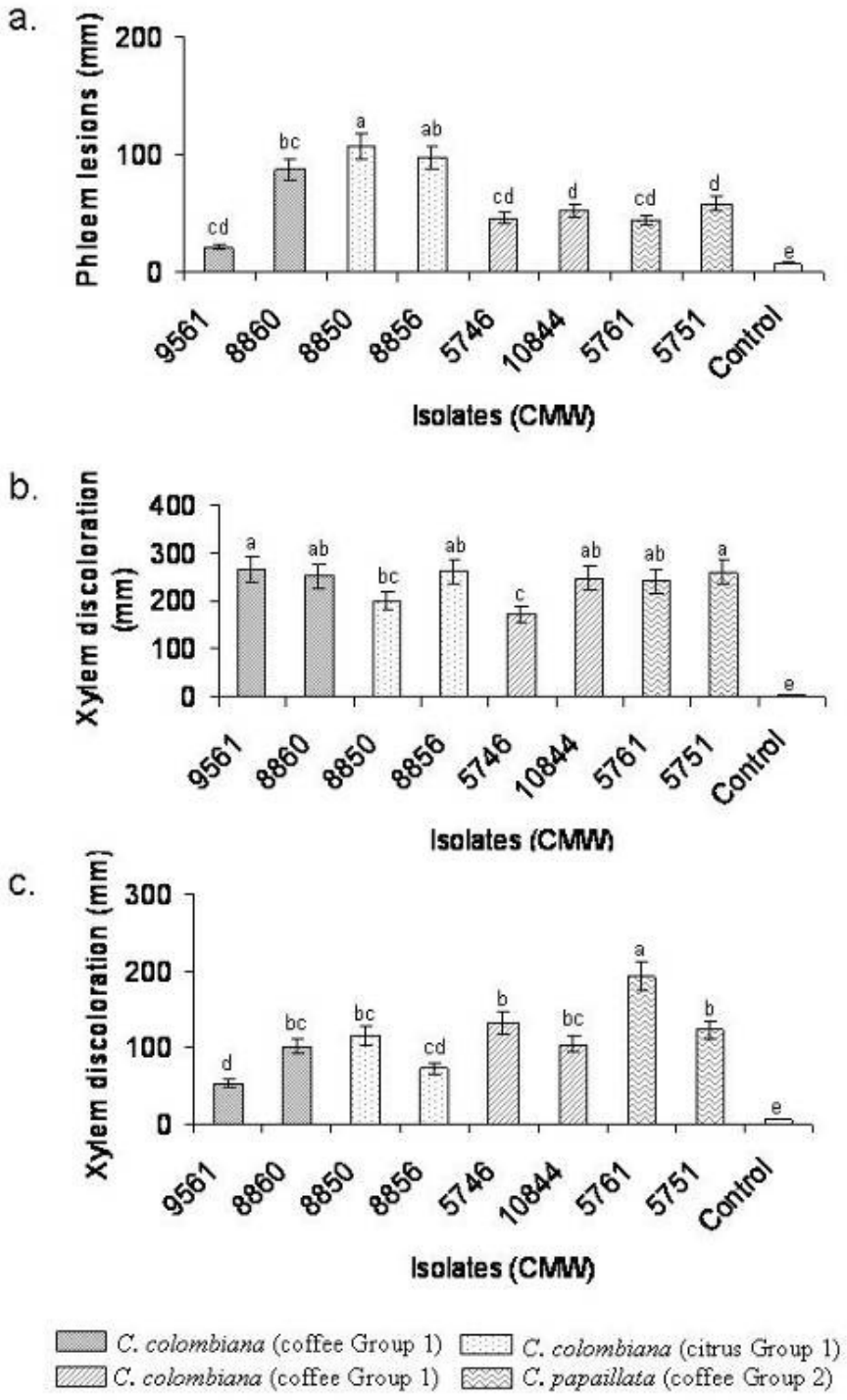


Figure 5. Morphological characteristics of isolate (CMW 5751) representing *C. colombiana*. A. Ascoma with globose base and elongated neck terminating in divergent ostiolar hyphae. B. Divergent ostiolar hyphae. C. Hat-shaped ascospores. D. Chlamydospores. E. Cylindrical conidia. F. Chain of barrel-shaped conidia. G. Primary flask-shaped phialides. H. Secondary phialides with flared apex. (Scale bars: A = 100µm, B = 20µm, C = 5µm, D, F-H = 10µm, E = 20µm).

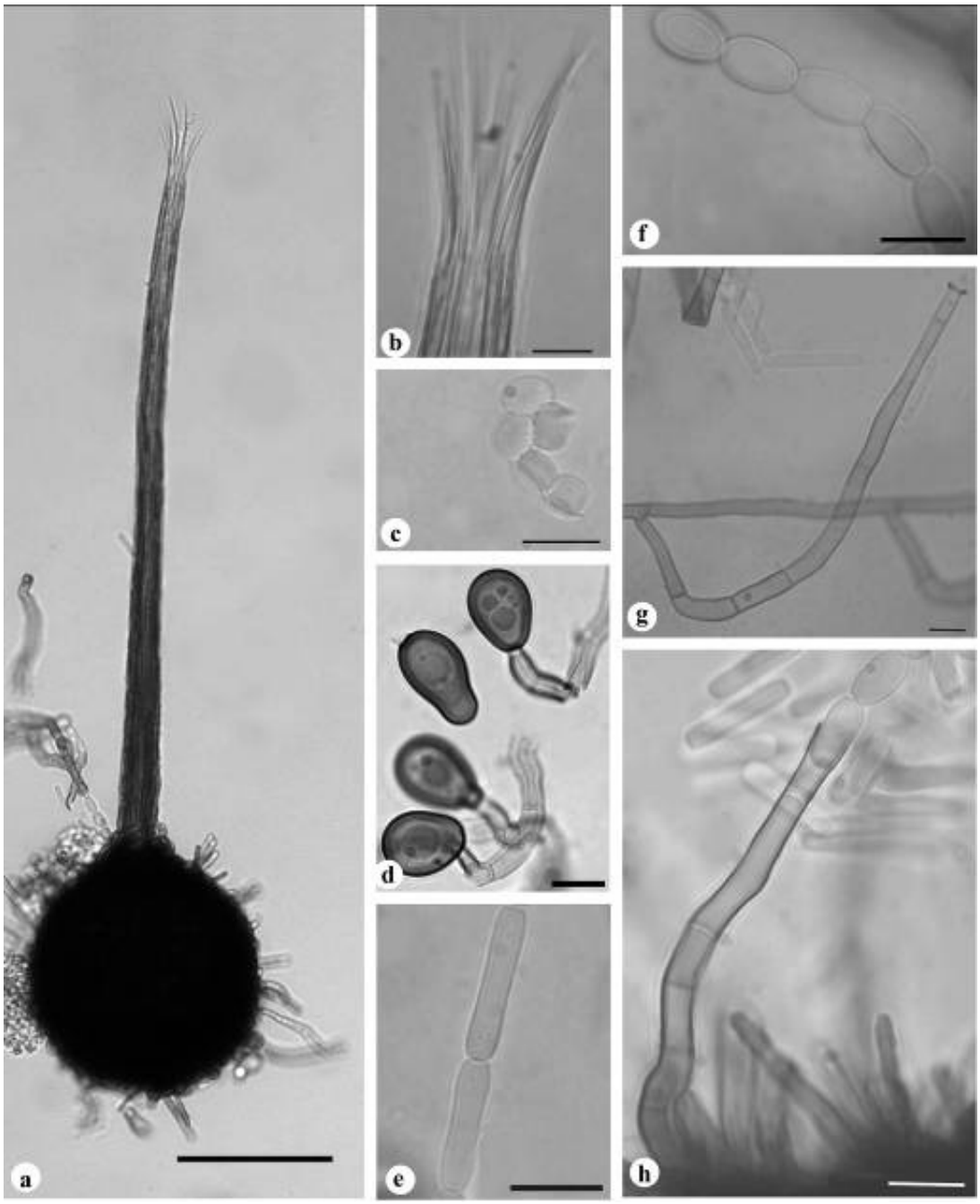
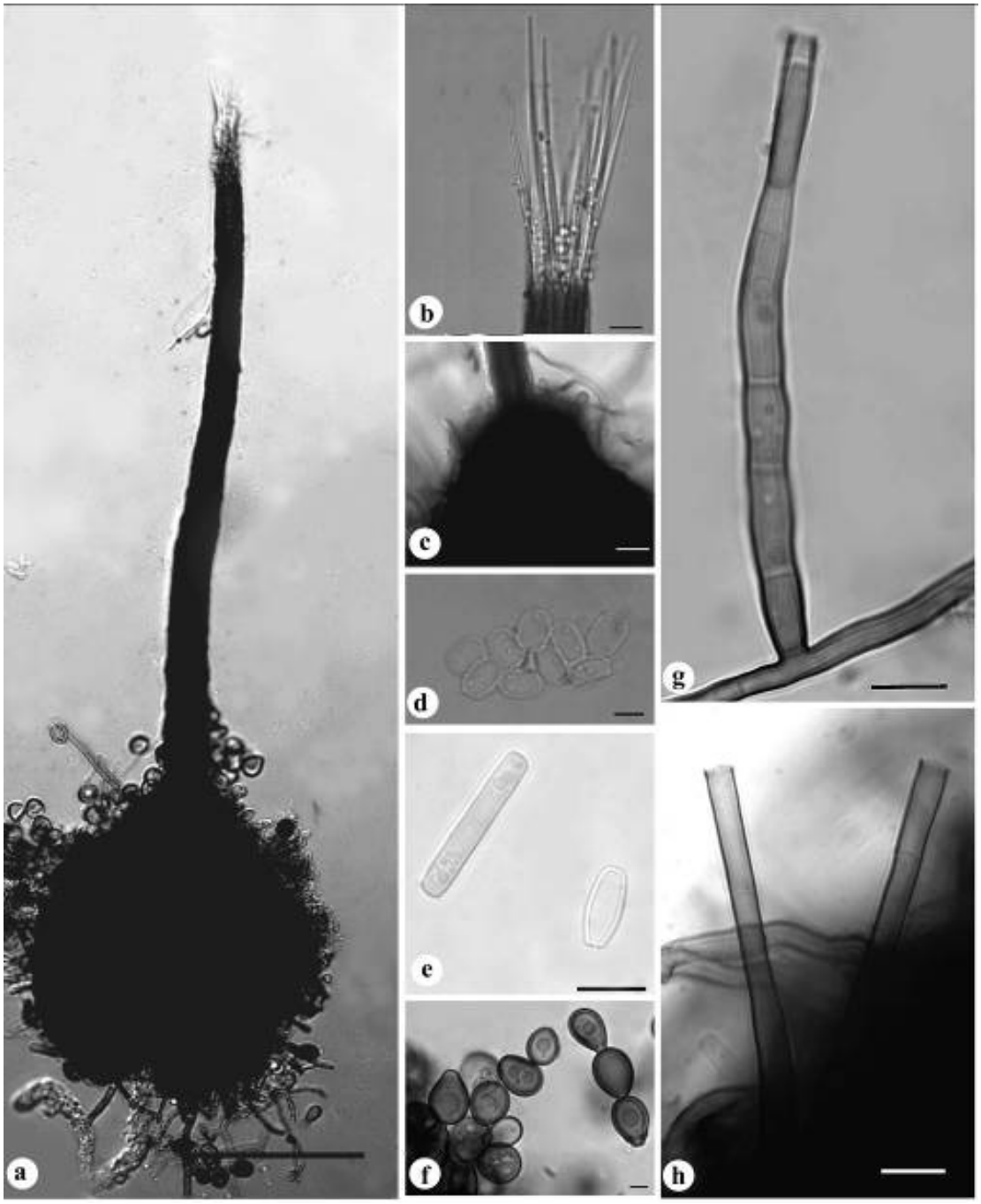


Figure 6. Morphological characteristics of *C. papillata* (CMW 8856) from Colombia isolated from coffee trees. A. Globose ascomata. B. Divergent ostiolar hyphae. C. Ascomatal base with papillate apex. D. Hat-shaped ascospores. E. Cylindrical and barrel-shaped conidia. F. Chlamydospores. G. Primary flasked-shaped phialide. H. Secondary flaring phialide. (Scale A = 100µm, B = 20µm, C = 100µm, D = 5µm, E- H = 10µm).



CHAPTER 9

Four new *Ceratocystis* spp. infecting wounds on *Eucalyptus*, *Schizolobium* and *Terminalia* trees in Ecuador

Van Wyk M., Wingfield B.D. & Wingfield M.J. (2011) Four new *ceratocystis* spp. infecting wounds on *Eucalyptus, Schizolobium* and *Terminalia* trees in Ecuador. *Fungal Diversity* **46**: 111-131.

ABSTRACT

Species of *Ceratocystis* commonly infect wounds on trees. In this study, artificially induced wounds were made on the stems of *Eucalyptus*, *Schizolobium* and *Terminalia* trees in Ecuadorian lowland forests, in an effort to determine the presence of *Ceratocystis* spp. in that environment. Species belonging to the *C. fimbriata sensu lato* (*s.l.*) complex and others in the *C. moniliformis s.l.* complex were collected. Phylogenetic analyses for both major groups in *Ceratocystis* using three gene regions (ITS, *β*-tubulin and EF1-*α*) revealed three distinct clades in the *C. fimbriata s.l.* complex and two in the *C. moniliformis s.l.* complex. Isolates in the three clades representing the *C. fimbriata s.l.* complex represent morphologically distinct species that are described here as *C. curvata* sp. nov., *C. ecuadoriana* sp. nov. and *C. diversiconidia* sp. nov. Isolates in one of the two clades in the *C. moniliformis* species complex represented *C. moniliformis sensu stricto* (*s.s.*) and the other was of a species with a distinct morphology that is described here as *C. sublaevis* sp. nov.

INTRODUCTION

Ceratocystis spp. are perithcial Ascomycetes that commonly infect wounds or the cut surfaces of recently felled trees (Kile 1993, Barnes *et al*. 2003, Van Wyk *et al*. 2009). They are transferred to these infection sites by sap-feeding insects such as nitidulid beetles (Juzwik 1999). In addition, a small number of *Ceratocystis* species are associates of conifer-infesting bark beetles (Wingfield *et al*. 1997).

Some species of *Ceratocystis* are important pathogens of fruit and forest trees or root crops (Kile 1993). In addition, many species are weakly pathogenic or saprophytes causing sap stain on timber. Most species that are weakly pathogenic or saprophytic reside in the *C. moniliformis* (Hedgc.) C. Moreau species complex (Hedgcock 1906, Van Wyk *et al*. 2006a) and some of the species related to *C. coerulescens* (Münch) B.K. Bakshi also cause sap stain particularly in conifers (Wingfield *et al*. 1997, Witthuhn *et al*. 1998). Species in the *C. fimbriata* Ellis & Halst. *s.l.* complex include important pathogens of trees causing vascular wilt and canker stain diseases (Roux *et al*. 1999, Engelbrecht *et al*. 2007, Roux & Wingfield 2009).

Early studies treated *Ceratocystis moniliformis* and *C. fimbriata* as single species although it was recognised that *C. fimbriata* included substantial variability (Webster & Butler 1967). In recent years, numerous cryptic species that would previously have been accommodated in *C. moniliformis* have been described (Yuan & Mohammed 2002, Van Wyk *et al*. 2004, 2006a). Likewise, *C. fimbriata s.l.* accommodates a relatively large number of cryptic taxa (Van Wyk *et al*. 2004, Engelbrecht & Harrington 2005, Johnson *et al.* 2005) with *C. fimbriata s.s.* defined by isolates phylogenetically related to those from sweet potato in the USA (Engelbrecht & Harrington 2005). It is likely that DNA sequence phylogenies and other emerging species concepts will reveal many other cryptic species in this group.

In Ecuador, plantation forestry is based largely on *Eucalyptus* spp. (~50%) and *Pinus* spp. (~40%) but various other trees are being tested for their potential as plantation species. These include the non-native *Terminalia ivorensis* A. Chev. and the native *Schizolobium parahyba* (Vell.) S.F. Blake. One of the key considerations in evaluating species for plantation development in Ecuador concerns their health and in this regard, various disease problems have been encountered (Geldenhuis *et al*. 2004, Lombard *et al*. 2008). In a previous study, *C. fimbriata s.l.* and *C. moniliformis* were isolated from machete wounds on diseased *S. parahyba* in Ecuador (Geldenhuis *et al*. 2004). Given the importance of members of the *C.*

fimbriata s.l. complex as tree pathogens (Kile 1993, Roux *et al*. 2004, Heath *et al*. 2009, Roux & Wingfield 2009), there has been concern that these fungi could present constraints to forestry in Ecuador.

The tropical environment in areas of Ecuador where hardwood species are grown, results in a lush vegetation and considerable competition due to rapidly growing understory plants. These are typically cleared manually using machetes and physical wounds are common at the bases of trees. These wounds present ideal sites for infection by species of *Ceratocystis s.l.* Therefore, this study was conducted to determine which of these fungi might be present in the local environment. This was done by artificially wounding tree stems and collecting *Ceratocystis* spp. for identification.

MATERIALS AND METHODS

Isolates

Wounds were made on the stems of *Eucalyptus deglupta* Blume, *Terminalia ivorensis* and *Schizolobium parahyba* trees in Ecuador at three different sites and times. A first wounding trial was conducted on trees growing on the farms Rio Silanche and La Celica near Salinas in March 2004, where wounds were made on *E. deglupta* trees. A second trial was conducted at Rio Pitzara near Salinas during November 2005 and in this case, wounds were made on *E. deglupta* and *T. ivorensis* trees. A third trial at Rio Pitzara was conducted during February 2006 where wounds were induced on *S. parahyba* trees. Wounds were made on the stems of trees using a machete and were similar to those described by Barnes *et al*. (2003).

Pieces of wood were collected from the treated trees approximately four weeks after wounding and fungi were isolated directly from structures on the wood surface or through carrot baiting (Moller & De Vay 1968). Spore droplets were removed from ascomatal necks, transferred to 2% (w/v) malt extract agar (MEA) (Biolab, Midrand, South Africa) supplemented with Streptomycin Sulphate (0.001 g vol^{-1}, SIGMA, Steinheim, Germany) and incubated at 25ºC until cultures sporulated. All isolates collected in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and representative isolates have been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Material consisting of dried cultures on 2% MEA of representative isolates were lodged with the National Fungal Herbarium (PREM), Pretoria, South Africa.

DNA sequence comparisons

DNA was extracted from selected isolates as described by Van Wyk *et al*. (2006b). PCR conditions and reactions for three gene regions; the Internal Transcribed Spacer (ITS) regions 1 and 2 including the 5.8S rRNA operon, part of the Beta tubulin (βt) gene and part of the Transcription Elongation Factor 1-alpha (EF1-α), were as described by Van Wyk *et al*. (2006b). The primers utilized in these PCR reactions were ITS1 & ITS4 (White *et al*. 1990) for the ITS region, βt1a & βt1b (Glass & Donaldson 1995) for the βt gene region and EF1F & EF1R (Jacobs *et al*. 2004) for the EF1-α gene region. Purification of these amplified products was achieved with 6% Sephadex G-50 columns (Steinheim, Germany). The amplicons were sequenced in both directions using the ABI PRISM$_{TM}$ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California) as described in previous studies (Van Wyk *et al*. 2006b).

Sequence and phylogenetic analyses

The chromatographs were analysed using the freeware Cromas Lite 2.01 (www.technelysium.com.au). All isolates were subjected to ITS PCR and sequences were submitted to BLAST on NCBI (http://www.ncbi.nlm.nih.gov) for initial identification before they were selected for further sequencing. The sequences were subsequently divided into two groups based on the results of these analyses. They either grouped in the *C. fimbriata s.l.* complex or in the *C. moniliformis s.l.* complex. Sequence data for closely related species were taken from previous studies (Van Wyk *et al*. 2006a, 2007a,b, 2009) on these two groups of fungi. The sequences for the three gene regions for each of the two datasets (*C. moniliformis s.l.* and *C. fimbriata s.l.*) were aligned using MAFFT version 6.606 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) (Katoh *et al*. 2002), and the alignments were confirmed manually in MEGA4 (Tamura *et al*. 2007).

Phylogenetic analyses based on parsimony were done using PAUP version 4.0b10* (Swofford 2002). A Partition Homogeneity Test (PHT) was run to determine whether the sequences of three gene regions for each of the two datasets could be combined (Swofford 2002). Phylograms were generated using a heuristic tree search algorithm. Sequences were randomised (reps = 1000), Mulpar was effective and a TBR branch swapping algorithm was used in the search. Gaps were treated as a fifth character. Confidence intervals of branch nodes using 1000 bootstrap replicates with the full heuristic search option were calculated. *Ceratocystis virescens* (R.W. Davidson) C. Moreau was used as the out-group taxon for both the datasets. All sequences derived from this study were deposited in GenBank (Table 1).

MrModeltest2 (Nylander 2004) was used to determine the most appropriate model for the analyses of each gene region. The suggested partition-specific models were then included in the Bayesian analyses. Phylogenetic trees based on Bayesian inference were generated using MrBayes version 3.1.1. (Ronquist & Huelsenbeck 2003). One million generations were run during which random trees were generated using the MCMC procedure. Four chains were applied and sampled every 100th generation. To avoid including trees that had been sampled before convergence, tree likelihood scores were assessed to determine the number of trees that were formed before stabilization. Trees outside the point of convergence were discarded by means of the burn-in procedure in MrBayes.

The three gene regions of the five groups of isolates from Ecuador (three in the *C. fimbriata s.l.* group and two in the *C. moniliformis s.l.* group) were analyzed separately for each gene region (ITS, βt and EF1-α). The PAUP settings were the same as above for the combined dataset excluding an outgroup. To determine the amount of variation between the isolates from Ecuador, Molecular Evolutionary Genetics Analysis (MEGA) 4 (Tamura *et al.* 2007) was used. Fixed alleles were sought in each gene region of the respective groups. Allele networks were drawn using the software TCS (Clement *et al*. 2000) in order to observe the shared alleles between the three groups in the *C. fimbriata s.l.* dataset and the two groups in the *C. moniliformis s.l.* dataset.

Culture characteristics and morphology

Culture morphology was assessed after 3 or 7 days of growth on 2% MEA (w/v) supplemented with Streptomycin Sulphate (100mg per litre). Colony colour was assigned using the colour charts of Rayner (1970). Two distinctly different colony morphologies and colony colours were observed in the isolates from Ecuador when grown on 2% MEA. The two colony morphologies were representative of species in the *C. fimbriata s.l.* and *C. moniliformis s.l*. complexes.

It was not possible to distinguish between isolates representing the *C. fimbriata s.l.* complex based on colony morphology. Three clades in this group were, however, identified based on phylogenetic inference. Three isolates representing each of these phylogenetic clades in the *C. fimbriata s.l.* group were selected for further morphological comparisons.

The isolates representing species in the *C. moniliformis s.l.* complex had indistinguishable colony morphology. Two clades were identified in this group based on phylogenetic inference. One of these represented *C. moniliformis s.s.* and the other an undescribed taxon.

Three isolates representing the latter group were thus selected for morphological characterisation.

Optimum growth temperatures were determined by placing 4mm plugs taken from the margins of actively growing cultures, mycelium side down, at the centres of 90mm 2% MEA plates. Five replicates were made for each culture at seven different temperatures between 5ºC and 35ºC at five ºC intervals. The growth was assessed seven days after incubation for the isolates representative of species in the *C. fimbriata s.l.* complex. For isolates representing species in the *C. moniliformis s.l.* complex, growth was measured three days after incubation.

For morphological characterisation, fungal structures, taken from 10 d-old cultures grown on 2% MEA were mounted in lactic acid. Photographic images were captured for all taxonomically important structures with a Carl Zeiss microscope and a Zeiss Axio Vision camera system. Three isolates were selected for each of the discrete phylogenetic clades in the *C. fimbriata s.l.* and *C. moniliformis s.l.* complexes. Fifty measurements were taken for all taxonomically useful characteristics for one isolate in each group and an additional 10 measurements were made for the remaining two isolates. Minimum, maximum, average and standard deviation (stdv) were calculated for each structure and these are presented as (minimum-) stdv minus the mean – stdv plus the mean (-maximum).

RESULTS

Isolates

A total of 57 isolates representing *Ceratocystis* spp. were collected from the artificially induced wounds on trees. Of these, 42 were collected from *E. deglupta*, six were from *T. ivorensis* and nine were from wounds made on *S. parahyba*. Based on morphology, these isolates represented two discrete groups, one typical of species in the *C. moniliformis s.l.* complex and one in the *C. fimbriata s.l.* complex. Of these, nine in the *C. moniliformis* complex were from *S. parahyba* trees and two were from *T. ivorensis* trees. Forty two isolates in the *C. fimbriata s.l.* complex were from *E. deglupta* trees while four were from *T. ivorensis* trees.

Phylogeny

Two separate phylogenetic datasets were constructed. One of these was for isolates in the *C. fimbriata s.l.* complex and the other for isolates in the *C. moniliformis s.l.* complex.

***C. fimbriata s.l.* species complex**

A *P*-value of 0.01 was obtained from the PHT for the dataset that included the three gene regions considered. Although this value was low, it indicated that the datasets could be combined (Sullivan 1996, Cunningham 1997). A total of 1996 characters represented this dataset of which 1087 were constant, 44 were parsimony uninformative and 865 characters were parsimony informative. Four most parsimonious trees were obtained after a heuristic search, one of which was selected for presentation (Figure 1). The tree length and goodness of character fit indices were as follows: tree length = 1956 steps, consistency index (CI) = 0.7, retention index (RI) = 0.9 and a rescaled consistency index (RC) of 0.6.

The HKY+G model was determined to be appropriate for the ITS gene region with the software MrModeltest2. The GTR+G model was best suited for the βt gene region, while the HKY+G model was found to be best suited for the EF1-α gene region. These model settings were included in the Bayesian analyses with 4000 trees discarded because they were outside the point of convergence. The posterior probabilities of the branch nodes supported the bootstrap values of the selected tree obtained in PAUP (Figure 1).

Parsimony analysis placed isolates from Ecuador in three separate clades with 100, 96 and 100% support respectively (Figure 1). The clades were designated as CF1, CF2, and CF3 on the phylogram and are, henceforth, referred to using this notation. All isolates in Groups CF1 and CF2 were from *E. deglupta* with isolates CMW22432, CMW22433, CMW22435, CMW22442 residing in CF1 and isolates CMW22092, CMW22093, CMW22097, CMW22405 residing in Group CF2 (Figure 1). Isolates in Group CF1 were phylogenetically distinct from all species in the *C. fimbriata s.l.* species complex with 100% bootstrap and Bayesian support. The isolates in Group CF1 were sister to *C. colombiana* M. van Wyk & MJ. Wingf., a pathogen of various tree crops in Colombia (Van Wyk *et al*. 2010a) (Figure 1). Isolates residing in Group CF2 were phylogenetically distinct (Bootstrap 96%, Bayesian 98%) from all other species in the *C. fimbriata s.l.* species complex and formed a sister group to *C. neglecta* M. van Wyk, Jol. Roux & Rodas, a pathogen of *Eucalyptus* trees in Colombia (Rodas *et al*. 2008). Isolates in Group CF3 included those that were obtained from *T. ivorensis* trees (Figure 1). This group was phylogenetically distinct from all species in this complex with 100% bootstrap and Bayesian support, with no sister group present.

The tree topologies for each of the datasets of the seperate gene regions appeared similar when compared to the tree topology of the dataset for the combined gene regions (Figure 2). The three seperate groups could still be identified, however, support for the branches was not

as high as in the combined dataset. All three gene regions had fixed polymorphisms for all three groups within the *C. fimbriata s.l.* dataset. In the ITS region, there were 33 differences between CF and CF2, 52 differences between CF2 and CF3 and 64 differences between CF1 and CF3. In the BT region there were four differences between CF1 and CF2, nine differences between CF2 and CF3 and seven differences between CF1 and CF3. In the EF region, there were six differences between CF1 and CF2, 9 differences between CF2 and CF3 and nine differences between CF1 and CF3. The allele networks obtained showed that the three groups observed in the phylogenetic trees were also seen in the allele network as the single dataset of th three gene regions produced three separate allele networks (Figure 3).

***C. moniliformis s.l.* species complex**

The PHT for the isolates in the *C. moniliformis* complex gave a *P*-value of 0.01, and the datasets were thus combined (Sullivan 1996, Cunningham 1997). This dataset consisted of 1824 characters of which 1389 were constant, 11 parsimony-uninformative and 424 characters were parsimony informative. One of six trees obtained was selected for presentation (Figure 4) and it had a length of 610 steps, a CI of 0.8, a RI of 0.9 and a RC of 0.8.

With MrModeltest2, the HKY+I model was selected for the ITS dataset while the GTR+G model was selected for the βt gene region and the SYM+G model was best suited for the EF1-α gene region. These models were incorporated into the Bayesian analyses in MrBayes. Three thousand trees were discarded (burn-in) as they were outside the point of convergence. The posterior probabilities for the nodes were supportive of the bootstrap values obtained in PAUP (Figure 4).

Two well resolved clades emerged from the phylogenetic analyses. In one of these, referred to as CM1 (CMW22458, CMW22459, CMW22462, CMW22460) isolates from Ecuador grouped with *C. moniliformis s.s.* (61% bootstrap and 100% Bayesian) and they were all from *S. parahyba* (Figure 4). Isolates (CMW22415, CMW22422, CMW22444, CMW22449) in the second clade (CM2) were phylogenetically distinct from all other isolates (97% bootstrap, 100% Bayesian) in the *C. moniliformis s.l.* species complex (Figure 4). The latter isolates originated from both *T. ivorensis* and *E. deglupta* trees.

Analyses of the datasetes for the two groups in the *C. moniliformis s.l.* complex, based on single gene regions, gave results diferent to those emerging from the combination of the three gene regions (Figure 5). For the ITS gene tree, there was no support for either of the two

groups. For the *β*t gene region, all the isolates from Ecuador grouped in one clade. In the EF1-α gene tree, the two groups were well-defined with high bootsrap support and the tree had a topology similar to that for the combined gene sequences.

Fixed polymorphisms were observed in two of the three gene regions considered. The βt gene region had one base pair difference while the EF1-α gene region had 14 differences between *C. moniliformis* and the isolates representing an apparently different species from Ecuador. There were no differences between isolates based on sequence comparisions for the ITS gene region (Van Wyk *et al*. 2006a, 2010b). The allele network obtained for the combined genes for the isolates in the *C. moniliformis s.l.* complex from Ecuador together with the *C. moniliformis s.s.* isolates is presented in Figure 6. All isolates representing *C. moniliformis s.s.* grouped together in one cluster while the isolates representing the CM2 clade grouped in seperately from them..

Culture characteristics and morphology

The isolates from Ecuador could be separated into two groups based on their culture morphology. The first group included isolates that grew slowly and had a greenish colour. The second group grew faster and were white to light brown with abundant aerial mycelium or little to no mycelium but abundant ascomata that covered the plates. The first group of isolates were morphologically most similar to species in the *C. fimbriata s.l.* complex and the remaining group was morphologically most similar to species in the *C. moniliformis s.l.* species complex. These two morphological groups were also consistent with the two groups emerging from DNA sequence comparisons.

***C. fimbriata s.l.* species complex**

Based on phylogenetic analyses, three distinct groups (CF1, CF2, CF3) were identified in the *C. fimbriata s.l.* complex. However, only one group could be identified based on culture morphology. All isolates that represented *C. fimbriata s.l.* had greenish to brown colonies and a banana odour. They, however, differed from each other with regard to growth in culture. After seven days of incubation, isolates representing Group CF1 (CMW22432, CMW22435 and CMW22442) had an optimum growth at 25℃ (36mm). No growth was observed at 5℃, 10℃ or 35℃, limited growth (14mm) occurred at 15℃ and intermediate growth was observed at 20℃ (28mm) and 30℃ (32mm). Isolates representing Group CF2 (CMW22092, CMW22093 and CMW22097) had an optimum growth at 25℃ (47mm). No growth was observed at 5℃, 10℃ and 35℃, limited growth was observed at 15℃ (20mm) and 30℃

(24mm) and intermediate growth was observed at 20℃ (36mm). Isolates representing Group CF3 (CMW22445, CMW22446 and CMW22447) had an optimum growth at 25℃ (21mm). No growth was observed at 5℃, 10℃ or 35℃, limited growth was observed at 15℃ (8mm) and intermediate growth was observed at 20℃ (16mm) and 30℃ (14mm).

***C. moniliformis s.l.* species complex**

Based on phylogenetic analyses, two groups (CM1, CM2) of isolates were identified in the *C. moniliformis s.l.* species complex. However, no clear distinction could be made between them based on colony morphology. Both groups displayed very rapid growth, had white to light brown colonies with abundant aerial mycelium and a banana odour. Isolates representing Group CM1 (CMW22451, CMW22456, CMW22458, CMW22459, CMW22460, CMW22462) were identified as *C. moniliformis s.s.* based on the phylogenetic analysis and no further morphological characterisation was made for them. Isolates representing Group CM2 (CMW22444, CMW22449, CMW22410 and CMW22415) had an optimum growth after three days at 25℃ (56mm). No growth was observed at 5℃, 10℃ or 35℃, limited growth was observed at 15℃ (19mm) and intermediate growth was observed at 20℃ (41mm) and 30℃ (40mm).

TAXONOMY

The *Ceratocystis* isolates from Ecuador considered in this study clearly represent four previously unknown taxa, three in the *C. fimbriata s.l.* species complex and one in the *C. moniliformis s.l.* species complex. These differ from each other based on minor morphological characteristics and they are primarily distinguished based on DNA sequence comparisons for three gene regions. They are thus described as follows:

C. curvata M. van Wyk & M.J. Wingf. **sp. nov.** (Figure 7a-i)
MB512846

Etymology: The name refers to the curved ascomatal necks in this species.

Bases ascomatum nigrae globosae vel obpyriformes (162-) 185-239 (-280) µm longae, (170-) 192-240 (-280) µm latae. *Colla ascomatum* atrobrunnea vel nigra, apicibus undulates. *Hyphae ostiolares* divergentes, (34-) 39-49 (-59) µm longae. Conidiophora biformia in mycelio singuli disposita, *primaria* phialidica hyalina basi tumescentia, apicem versus contracta (44-) 55-95 (-139) µm longa; *secondaria* tubularia, expansa hyalina, (34-) 42-66 (-90) µm longa.

Chlamydosporae atro-brunneae, parietibus crassis, globosae vel subglobosae 11-17 x 9-12 µm.

Colony brownish olive (19"m) on malt extract agar. *Mycelium* submerged and aerial. *Optimal temperature* for growth 25ºC, no growth below 15ºC and above 35ºC. *Hyphae* smooth, not constricted at septa. *Ascomatal bases* black, globose to obpyriform, no ornamentation, bases (162-) 185-239 (-280) µm long, (170-) 192-240 (-280) µm wide, (166-) 188-240 (-280) µm in diam. *Ascomatal necks* dark brown to black, undulating at apices, (419-) 498-644 (-714) µm long,(12-) 15-21 (-25) µm wide at apices, (19-) 23-35 (-56) µm wide at bases. *Ostiolar hyphae* divergent, hyaline, (34-) 39-49 (-59) µm long. *Asci* not observed. *Ascospores* hat-shaped in side view, aseptate, hyaline, invested in a sheath, 3-5 × 4-6 µm without sheath, 3-5 × 5-8 µm with sheath. Ascospores accumulating in buff-yellow (19d) mucilaginous masses at the apices of ascomatal necks. *Thielaviopsis anamorph*: *Conidiophores* of two types occurring singly on mycelium, *primary conidiophores*, phialidic, hyaline, swollen at the base, tapering towards the apices, (44-) 55-95 (-139) µm long, 4-6 µm wide at bases, 4-7 µm wide at widest point in middle, 3-4 µm wide at apices. *Secondary conidiophore*, phialidic, flaring, hyaline, (34-) 42-66 (-80) µm long, 3-6 µm wide at bases, 4-6 µm wide at apices. *Conidia* of two types: *Primary conidia*, hyaline, aseptate, cylindrical, (10-) 13-21 (-31) × 3-6 µm. *Secondary conidia*, hyaline, aseptate, barrel-shaped 7-11 × 4-8 µm. *Chlamydospores* dark brown, thick walled, globose to perprolate, 11-17 × (7-) 9-11 (-12) µm.

Habitat: Freshly wounded *Eucalyptus deglupta* wood.

Known distribution: Ecuador.

Material examined: **Ecuador**, near Salinas isolated from wood of *E. deglupta*, M.J. Wingfield, PREM60151, CMW22442, CBS122603 (2004). Same data, PREM60154, CMW22432. Same data PREM60153, CMW22433. Same data PREM60152, CMW22435, CBS122604.

Notes: Ceratocystis curvata is distinct from all other species within the *C. fimbriata s.l.* complex in that it generally has ascomatal necks that are curved at the apices. Several other structures differ in size when compared to other species in the *C. fimbriata s.l.* complex (Table 3).

C. ecuadoriana M. van Wyk & M.J. Wingf. **sp. nov.** (Figure 8a-h)
MB512847

Etymology: Name reflects the country where the fungus was first collected.

Bases ascomatum nigrae globosae, (180-) 215-265 (-290) µm longae, (184-) 208-250 (-279) µm latae. *Colla ascomatum* atrobrunnea vel nigra, (515-) 615-851 (-1021) µm longa. Conidiophora biformia in mycelio singuli disposita, *primaria* phialidica hyalina basi tumescentia, apicem versus contracta (60-) 72-94 (-98) µm longa; *secondaria* phialidica expansa hyalina, (52-) 61-85 (-98) µm longa. *Conidia* biformia: *primaria* hyalina non septata cylindrica (9-) 14-20 (-24).

Colony brownish olive (19"m) on malt extract agar. *Mycelium* submerged and aerial. *Optimal temperature* for growth 25ºC, no growth below 15ºC and above 35ºC. *Hyphae* smooth, not constricted at septa. *Ascomatal bases* black, globose, no ornamentation, bases (180-) 215-265 (-290) µm long, (184-) 208-250 (-279) µm wide, (182-) 212-258 (-285) µm in diam. *Ascomatal necks* dark brown to black, (515-) 615-851 (-1021) µm long, (14-) 17-23 (-28) µm wide at apices, (24-) 28-38 (-46) µm wide at bases. *Ostiolar hyphae* divergent, hyaline, (29-) 32-42 (-50) µm long. *Asci* not observed. *Ascospores* hat-shaped in side view, aseptate, hyaline, invested in sheath, 3-4 × 3-6 µm without sheath, 3-4 × 5-8 µm with sheath. Ascospores accumulating in buff-yellow (19d) mucilaginous masses on the apices of ascomatal necks. *Thielaviopsis anamorph*: *Conidiophores* of two types occurring singly on mycelium, *primary conidiophore*, phialidic, hyaline, swollen at the base, tapering towards the apices, (60-) 72-94 (-98) µm long, 4-6 µm wide at bases, 5-7 µm wide at widest point in middle, 3-5 µm wide at apices. *Secondary conidiophore*, tubular, flaring, hyaline, (52-) 61-85 (-98) µm long, 3-6 µm wide at bases, 4-6 µm wide at apices. *Conidia* of two types: *Primary conidia*, hyaline, aseptate, cylindrical, (9-) 14-20 (-24) × 3-5 µm. *Secondary conidia*, hyaline, aseptate, barrel-shaped 7-9 (-11) × 4-7 µm. *Chlamydospores* dark brown, thick walled, globose to sub-globose, (9-) 11-15 (-16) × (7-) 9-11 (-12) µm.

Habitat: Freshly wounded wood of *Eucalyptus deglupta*.

Known distribution: Ecuador.

Material examined: **Ecuador**, near Salinas isolated from wood of *E. deglupta*, M.J. Wingfield, PREM60155, CMW22092, CBS124020 (2004). Same data: PREM60156, CMW22093, CBS124021. Same data: PREM60158, CMW22097, CBS124022. Same data PREM60157, CMW22405.

Notes: There is no single morphological characteristic that distinguishes this species from other species within the *C. fimbriata s.l.* complex. It does, however, have very long ascomatal necks when compared to other species. Differences in the sizes and shapes of various other structures are also seen when compared to the other species within the *C. fimbriata s.l.* complex (Table 3).

C. diversiconidia M. van Wyk & M.J. Wingf. **sp. nov.** (Figure 9a-i)
MB 512848

Etymology: Name reflects the wide range of variation in size of both the cylindrical and barrel shaped conidia.

Bases ascomatum nigrae globosae, (97-) 115-155 (-196) µm longae, (97-) 122-170 (-223) µm latae. *Colla ascomatum* atrobrunnea vel nigra, (245-) 368-520 (-599) µm longa, basi (19-) 23-29 (-34) µm lata. *Hyphae ostiolares* divergentes, hyalinae, (40-) 45-61 (-72) µm longae. Conidiophora biformia in mycelio singuli disposita, *primaria* phialidica hyalina basi tumescentia, apicem versus contracta (58-) 82-132 (-162) µm longa; *secondaria* phialidica expansa hyalina, (40-) 49-65 (-68) µm longa.

Colony brownish olive (19"m) on malt extract agar. *Mycelium* submerged and aerial. *Optimal temperature* for growth 25ºC, no growth below 15ºC and above 35ºC. *Hyphae* smooth, not constricted at septa. *Ascomatal bases* black, globose no ornamentation, bases (97-) 115-155 (-196) µm long, (97-) 122-170 (-223) µm wide, (97-) 119-163 (-210) µm in diam. *Ascomatal necks* dark brown to black, (245-) 368-520 (-599) µm long, (12-) 15-21 (-25) µm wide at apices, (19-) 23-29 (-34) µm wide at bases. *Ostiolar hyphae* divergent, hyaline, (40-) 45-61 (-72) µm long. *Asci* not observed. *Ascospores* hat-shaped in side view, aseptate, hyaline, invested in sheath, 3-5 × 4-7 µm without sheath, 3-5 × 6-8 µm with sheath. Ascospores accumulating in buff-yellow (19d) mucilaginous masses on the apices of ascomatal necks.
Thielaviopsis anamorph: *Conidiophores* of two types occurring singly on mycelium, *primary conidiophores*, phialidic, hyaline, swollen at the base, tapering towards the apices, (58-) 82-132 (-162) µm long, 4-7 µm wide at bases, 4-9 µm wide at widest point in middle, 3-6 µm wide at apices. *Secondary conidiophores*, phialidic, flaring, hyaline, (40-) 49-65 (-68) µm long, 4-6 µm wide at bases, 5-7 µm wide at apices. *Conidia* of two types: *Primary conidia*, hyaline, aseptate, cylindrical to oblong, apices truncate, (10-) 13-21 (-30) × 4-6 µm. *Secondary conidia*, hyaline, aseptate, barrel-shaped (5-) 8-10 (-11) × 4-8 µm.

Chlamydospores dark brown, thick walled, pyriform to obpyriform, (12-) 14-18 (-19) × (9-) 10-12 (-15) µm.

Habitat: Freshly wounded wood of *Terminalia ivorensis*.

Known distribution: Ecuador.

Material examined: **Ecuador**, near Salinas, isolated from wood of *T. ivorensis*, M.J. Wingfield, PREM60160, CMW22445, CBS123013 (2004). Same data: PREM60162, CMW22448, CBS122605. Same data: PREM60161, CMW22446. Same data: PREM60159, CMW22447, CBS122818.

Notes: Ceratocystis diversiconidia is distinct from most species in the *C. fimbriata s.l.* complex due to its diverse conidial and chlamydospore sizes. There are also differences in sizes of various other structures when compared to other species in the *C. fimbriata s.l.* complex (Table 3).

C. sublaevis M. van Wyk & M.J. Wingf., **sp. nov.** (Figure 10a-h)
MB512849.

Etymology: The name refers to the limited number of conical spines on the ascomatal bases that are somewhat smooth in comparison to other species in this group.

Colonia in MEA alba vel laete brunnea. *Mycelium* abundans, plerumque aerium. *Bases ascomatum* nigrae globosae sparse spinis nigris et hyphis ornatae, spinae (2-) 4-10 (-13) µm, bases (98-) 131-173 (-187) µm longae, (102-) 144-192 (-231) µm latae. *Colla ascomatum* atrobrunnea vel nigra (100-) 137-183 (-209) µm longa, discoidea vel papillata. *Hyphae ostiolares* divergentes, hyalinae, (15-) 18-24 (-25) µm longae. Conidiophora biformia in mycelio singuli disposita, *primaria* phialidica hyalina basi tumescentia, apicem versus contracta (15-) 23-37 (-50) µm longa; *secondaria* tubularia apicem versus non contracta, hyalina rara (19-) 25-34 (-36) µm longa. *Conidia* biformia: *primaria* hyalina non septata cylindrica 5-8 × 1-3 µm; *secondaria* hyalina non septata doliiformia rara 3-6 × 2-3 µm.

Colony white to light brown (19"f) on malt extract agar. *Mycelium* abundant mostly aerial. *Optimal temperature* range for growth 20-30ºC, no growth below 15ºC and above 35ºC. *Hyphae* smooth, not constricted at septa. *Ascomatal bases* black, globose, ornamented sparsely with spines and hyphae, spines black, (2-) 4-10 (-13) µm long, bases (98-) 131-173 (-

187) µm long, (102-) 144-192 (-231) µm wide, (100-) 137-183 (-209) µm in diam. *Ascomatal necks* dark brown to black, undulating, (522-) 598-802 (-990) µm long, (9-) 12-16 (-19) µm wide at apices, (24-) 31-45 (-55) µm wide at bases, with a disc to papillate base. *Ostiolar hyphae* divergent, hyaline, (15-) 18-24 (-25) µm long. *Asci* not observed. *Ascospores* hat-shaped in side view, aseptate, hyaline, invested in sheath, 2-4 × 3-5 µm without sheath, 2-4 × 4-6 µm with sheath. Ascospores accumulating in buff-yellow (19d) mucilaginous masses on the apices of ascomatal necks. *Thielaviopsis anamorph*: *Conidiophores* of two types occurring singly on mycelium, *primary conidiophores*, phialidic, hyaline, swollen at the base, tapering towards the apices, (15-) 23-37 (-50) µm long, 2-5 µm wide at bases, 2-5 µm wide at widest point in middle, 1-3 µm wide at apices. *Secondary conidiophores*, tubular, not-tapering towards apices, hyaline, scarce, (19-) 25-34 (-36) µm long, 2-4 µm wide at bases, 2-4 µm wide at apices. *Conidia* of two types: *Primary conidia*, hyaline, aseptate, cylindrical, 5-8 × 1-3 µm. *Secondary conidia*, hyaline, aseptate, scarce, barrel-shaped 3-6 × 2-3 µm.

Habitat: Freshly wounded wood of *Eucalyptus deglupta* and *Terminalia ivorensis*.

Known distribution: Ecuador.

Material examined: **Ecuador**, Salinas area, isolated from freshly wounded wood of *T. ivorensis*, M.J. Wingfield, PREM60164, CMW22444, CBS122518 (2004). **Ecuador**, isolated from wood of *T. ivorensis*, M.J. Wingfield, PREM60163, CMW22449, CBS122517 (2004). **Ecuador**, isolated from wood of *E. deglupta*, M.J. Wingfield, PREM60165, CMW22415 (2004). **Ecuador**, isolated from wood of *E. deglupta*, M.J. Wingfield, PREM60166, CMW22422, CBS122603 (2004).

Notes: Ceratocystis sublaevis is morphologically distinct from all other species in the *C. moniliformis s.l.* complex. The most obvious distinguishing characteristic is found in its reatively small number of conical spines on the ascomatal bases. The very distinct disk-shaped structures at the bases of the ascomatal necks, typical of this group of fungi, is also very small in comparison to those seen in other species (Table 4).

DISCUSSION

Five *Ceratocystis* spp. were isolated from freshly made wounds on the stems of plantation-grown trees in the lowland forests of Ecuador. Two of these species, *C. sublaevis* and *C. moniliformis s.s.* reside in the *C. moniliformis s.l.* species complex. The three remaining species reside in the *C. fimbriata s.l.* complex and they have been provided with the names *C.*

curvata, C. ecuadoriana and *C. diversiconidia*. Both *C. ecuadoriana* and *C. curvata* strains were isolated from *Eucalyptus* trees, while *C. diversiconidia* was isolated from *Terminalia* trees. The *C. moniliformis s.s.* isolates were isolated from *Schizolobium* while *C. sublaevis* was found on both *Eucalyptus* and *Terminalia* trees.

The three new Ecuadorian *Ceratocystis* spp. in the *C. fimbriata s.l.* complex reside in three distinct phylogenetic groups. *Ceratocystis curvata* in clade CF1 is most closely related to *C. colombiana*. The latter species is a pathogen of various tree crops (Van Wyk *et al*. 2010a) in Colombia that is geographically close to Ecuador where *C. curvata* was found. *Ceratocystis ecuadoriana* in clade CF2 was sister to *C. neglecta* that is a pathogen of *Eucalyptus* trees also in Colombia (Rodas *et al*. 2008). In contrast, *C. diversiconidia* in clade CF3 was distant from other species in the *C. fimbriata s.l.* complex without clearly defined phylogenetic neighbours. Based on phylogenetic inference, it should not be difficult to recognise these three new species from other taxa in the *C. fimbriata s.l.* complex that have been defined in previous studies (Johnson *et al*. 2005, Van Wyk *et al*. 2004, 2007b).

It was not unusual to isolate *C. moniliformis* from wounds on trees in Ecuador as this fungus has previously been found in a similar habitat in the country (Geldenhuis *et al*. 2004). The new species, *C. sublaevis,* residing in the *C. moniliformis s.l.* complex was phylogenetically distinct from all species in this group. It was, however, closest related to *C. moniliformis s.s.* No differences were observed in the sequences of the ITS gene region when comparing isolates representing *C. moniliformis s.s.* and the isolates representing *C. sublaevis*. This was expected as it is known that, in contrast to isolates in the *C. fimbriata s.l.* complex, isolates in the *C. moniliformis s.l.* complex have no differentiation in the ITS gene region, while the EF1-α gene region has the highest degree of resolution (Van Wyk *et al*. 2006a, 2010b).

The morphological characteristics of species in the *C. fimbriata s.l.* complex are very similar and new species can be recognised with confidence only through the application of phylogenetic inference. Yet, *C. curvata* has very distinct undulating ascomatal necks, *C. diversiconidia* has conidia and chlamydospores that differ significantly in size from other species in the group and *C. ecuadoriana* has unusually long ascomatal necks, which should assist in their identification.

Ceratocystis sublaevis can be distinguished from other members of the *C. moniliformis s.l.* complex morphologically. The most distinct differences observed are the limited occurrences

of conical spines on the ascomatal bases as well as its unusually small basal plateau giving rise to the ascomatal necks.

Many *Ceratocystis* species are important plant pathogens (Kile 1993, Roux & Wingfield 2009). In this regard, nothing is known regarding the pathogenicity of the new *Ceratocystis* spp. described in this study. Given that there were no disease symptoms found on the wounded trees, it is possible that they all represent wound-infecting saprophytes. It would be worthwhile to consider their pathogenicity on the hosts from which they were isolated. In contrast, species in the *C. moniliformis s.l.* complex are typically non-pathogenic colonists of freshly wounded wood (Van Wyk *et al*. 2006a, Heath *et al*. 2009) and this is probably true for *C. moniliformis s.s*. and *C. sublaevis* found in this study.

The discovery of a relatively large number of new taxa in a well-known group of fungi, reflects strongly the fact that these fungi have been poorly studied in Ecuador. It is likely that similar studies in that country will yield additional new species in this group and some of these are potentially important pathogens. Their discovery will enhance the understanding of fungal diversity in Ecuador and their description would augment a growing understanding of an intriguing and ecologically important group.

REFERENCES

Barnes I., Roux J., Wingfield B.D., Dudzinski M.J., Old K.M. & Wingfield M.J. (2003) *Ceratocystis pirilliformis*, a new species from *Eucalyptus nitens* in Australia. *Mycologia* **95**: 865-871.

Clement M., Posada D. & Crandall K. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**: 1657–1660.

Cunningham C.W. (1997) Can three incongruence tests predict when data should be combined? *Molecular Biology and Evolution* **14**: 733–740.

Engelbrecht C.J.B & Harrington T.C. (2005) Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. *Mycologia* **97**: 57–69.

Engelbrecht C.J.B., Harrington T.C., Alfenas A.C. & Suarez C. (2007) Genetic variation in populations of the cacao wilt pathogen, *Ceratocystis cacaofunesta*. *Plant Pathology* **56**: 923–933.

Geldenhuis M.M., Roux J., Wingfield M.J. & Wingfield B.D. (2004) Development of polymorphic markers for the root pathogen *Thielaviopsis basicola* using ISSR-PCR. *Molecular Ecology Notes*. **4**: 547–550.

Glass N.L. & 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.

Heath R.N., Wingfield B.D., Wingfield M.J., Meke G., Mbaga A. & Roux J. (2009) *Ceratocystis* species on *Acacia mearnsii* and *Eucalyptus* spp. in eastern and southern Africa including six new species. *Fungal Diversity* **34**: 41–67.

Hedgcock G.G. (1906) Studies upon some chromogenic fungi which discolor wood. *Missouri Botanical Garden Annual Report* **17**: 59–111.

Jacobs K., Bergdahl D.R., Wingfield M.J., Halik S., Seifert K.A., Bright D.E. & Wingfield B.D. (2004) *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* **108**: 411–418.

Johnson J.A., Harrington T.C. & Engelbrecht C.J.B. (2005) Phylogeny and taxonomy of the North American clade of the *Ceratocystis fimbriata* complex. *Mycologia* **97**: 1067–1092.

Juzwik J. (1999) Overland transmission of *Ceratocystis fagacearum*: Extending our understanding. In: Shade tree wilt diseases: Proceedings from wilt diseases of shade trees: A national conference. (Cynthia L. ed.). St. Paul, MN: APS Press 83–92.

Katoh K., Misawa K., Kuma K. & Miyata T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**: 3059–3066.

Kile G.A. (1993) Plant diseases caused by species of *Ceratocystis* sensu stricto and *Chalara*. In: Wingfield MJ, Seifert KA, Webber JA (eds), *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology and Pathogenicity. APS Press. St. Paul, Minnesota, USA: 173–183.

Lombard L., Bogale M., Montenegro F., Wingfield B.D. & Wingfield M.J. (2008) A new bark canker disease of the tropical hardwood tree *Cedrelinga cateniformis* in Ecuador. *Fungal Diversity* **31**: 73–81.

Moller W. & De Vay J. (1968) Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* **58**: 1499–1508.

Nylander J.A.A. (2004) MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

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

Rodas C., Roux J., Van Wyk M., Wingfield B.D. & Wingfield M.J. (2008) *Ceratocystis neglecta* sp. nov., infecting *Eucalyptus* trees in Colombia. *Fungal Diversity* **28**: 73–84.

Ronquist F. & Huelsenbeck J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Roux J., Dunlop R. & Wingfield M.J. (1999) Susceptibility of elite *Acacia mearnsii* families to Ceratocystis wilt in South Africa. *Journal of Forestry Research* **4**: 187–190.

Roux J., Van Wyk M., Hatting H. & Wingfield M.J. (2004) *Ceratocystis* species infecting stem wounds on *Eucalyptus grandis* in South Africa. *Plant Pathology* **53**: 414–421.

Roux J. & Wingfield M.J. (2009) *Ceratocystis* species: emerging pathogens of non-native plantation *Eucalyptus* and *Acacia* species. *Southern Forests* **71**: 115–120.

Sullivan J. (1996) Combining data with different distributions of among-site variation. *Systematic Biology* **45**: 375–380.

Tamura K., Dudley J., Nei M. & Kumar S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596–1599.

Swofford D.L. (2002) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Van Wyk M., Al-Adawi A.O., Khan I.A., Deadman M.L., Al-Jahwari A., Wingfield B.D., Ploetz R. & Wingfield M.J. (2007a) *Ceratocystis manginecans* sp. nov., causal agent of a destructive mango wilt disease in Oman and Pakistan. *Fungal Diversity* **27**: 213–230.

Van Wyk M., Pegg G., Lawson S. & Wingfield M.J. (2007b) *Ceratocystis atrox* sp. nov associated with *Phoracanthta acanthocera* infestations on *Eucalyptus* in Australia. *Australian Journal of Plant Pathology* **36**: 407–414

Van Wyk M., Roux J., Barnes I., Wingfield B.D., Liew E.C.Y., Assa B., Summerell B.A. & Wingfield M.J. (2004) *Ceratocystis polychroma* sp. nov., a new species from *Syzygium aromaticum* in Sulawesi. *Studies in Mycology* **50**: 273–282

Van Wyk M., Roux J., Barnes I., Wingfield B.D. & Wingfield M.J. (2006a) Molecular phylogeny of the *Ceratocystis moniliformis* complex and description of *C. tribilliformis* sp. nov. *Fungal Diversity* **21**: 181–201

Van Wyk M., Van der Merwe N.A., Roux J., Wingfield B.D., Kamgan G.N. & Wingfield M.J. (2006b) Population genetic analyses suggest that the *Eucalyptus* fungal pathogen *Ceratocystis fimbriata* has been introduced into South Africa. *South African Journal of Science* **102**: 259–263

Van Wyk M., Wingfield B.D., Marin M. & Wingfield M.J. (2010a) Two new *Ceratocystis* species infecting coffee, cacao, citrus and native trees in Colombia. *Fungal Diversity* **40**: 103–117

Van Wyk M., Wingfield B.D., Mohali S. & Wingfield M.J. (2009) *Ceratocystis fimbriatomima,* a new species in the *C. fimbriata sensu lato* complex isolated from *Eucalyptus* trees in Venezuela. *Fungal Diversity* **34**: 173–183

Van Wyk M., Wingfield B.D. & Wingfield M.J. (2010b) *Ceratocystis* species in the *Ceratocystis fimbriata* complex. In: *Ceratophiostoma*. North Stadbroke Island, Brisbane, Australia, Aug 2006 (Abstract)

Webster R.K. & Butler E.E. (1967) A morphological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45**: 1457–1467

White T.J., Bruns T., Lee S. & 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. MA Innis, DH Gelfand, JJ Sninsky and TJWhite). Academic Press, San Diego, USA: 315–322

Witthuhn R.C., Wingfield B.D., Wingfield M.J. & Wolfaart M. (1998) Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. *Mycologia* **90**: 96–101

Wingfield M.J., Harrington T.C. & Solheim H. (1997) Two species in the *Ceratocystis coerulescens* complex from conifers in western North America. *Canadian Journal of Botany* **75**: 827–834

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

Table 1. Isolates and species within the *C. fimbriata s.l.* species complex used in this study.

Species	Isolate no.	GenBank accession no.	Host	Geographical origin
C. albifundus	CMW4068	DQ520638 EF070429 EF070400	*Acacia mearnsii*	RSA
C. albifundus	CMW5329 CBS119681	AF388947 DQ371649 EF070401	*Acacia mearnsii*	Uganda
C. atrox	CMW19383 CBS120517	EF070414 EF070430 EF070402	*Eucalyptus grandis*	Australia
C. atrox	CMW19385 CBS120518	EF070415 EF070431 EF070403	*Eucalyptus grandis*	Australia
C. cacaofunesta	CMW15051 CBS152.62	DQ520636 EF070427 EF070398	*Theobroma cacao*	Costa Rica
C. cacaofunesta	CMW14809 CBS115169	DQ520637 EF070428 EF070399	*Theobroma cacao*	Ecuador
C. caryae	CMW14793 CBS114716	EF070424 EF070439 EF070412	*Carya cordiformis*	USA
C. caryae	CMW14808 CBS115168	EF070423 EF070440 EF070411	*Carya ovata*	USA
C. colombiana	CMW9565 CBS121790	AY233864 AY233870 EU241487	Soil	Colombia
C. colombiana	CMW5751 CBS121792	AY177233 AY177225 EU241493	*Coffea arabica*	Colombia
C. colombiana	CMW9572	AY233863 AY233871 EU241488	Mandarin	Colombia
***C. curvata* (CF1)**	**CMW22432**	**FJ151439 FJ151451 FJ151473**	***Eucalyptus deglupta***	**Colombia**
***C. curvata* (CF1)**	**CMW22433 CBS122513**	**FJ151438 FJ151450 FJ151472**	***Eucalyptus deglupta***	**Colombia**
***C. curvata* (CF1)**	**CMW22435 CBS122604**	**FJ151437 FJ151449 FJ151471**	***Eucalyptus deglupta***	**Colombia**
***C. curvata* (CF1)**	**CMW22442 CBS122603**	**FJ151436 FJ151448 FJ151470**	***Eucalyptus deglupta***	**Colombia**
***C. diversiconidia* (CF3)**	**CMW22445 CBS123013**	**FJ151440 FJ151452 FJ151474**	***Terminalia ivorensis***	**Colombia**
***C. diversiconidia* (CF3)**	**CMW22446**	**FJ151443 FJ151455 FJ151477**	***Terminalia ivorensis***	**Colombia**
***C. diversiconidia* (CF3)**	**CMW22447 CBS122818**	**FJ151442 FJ151454 FJ151476**	***Terminalia ivorensis***	**Colombia**
***C. diversiconidia* (CF3)**	**CMW22448 CBS122605**	**FJ151441 FJ151453 FJ151475**	***Terminalia ivorensis***	**Colombia**
***C. ecuadoriana* (CF2)**	**CMW22092 CBS124020**	**FJ151432 FJ151444**	***Eucalyptus deglupta***	**Colombia**

		FJ151466		
***C. ecuadoriana* (CF2)**	**CMW22093 CBS124021**	**FJ151433 FJ151445 FJ151467**	***Eucalyptus deglupta***	**Colombia**
***C. ecuadoriana* (CF2)**	**CMW22097 CBS124022**	**FJ151434 FJ151446 FJ151468**	***Eucalyptus deglupta***	**Colombia**
***C. ecuadoriana* (CF2)**	**CMW22405**	**FJ151435 FJ151447 FJ151469**	***Eucalyptus deglupta***	**Colombia**
C. fimbriata s.s	CMW15049 CBS141.37	DQ520629 EF070442 EF070394	*Ipomoea batatas*	USA
C. fimbriata s.s.	CMW1547 CBS123010	AF264904 EF070443 EF070395	*Ipomoea batatas*	Papua New Guinea
C. fimbriatomima	CMW24174 CBS121786	EF190963 EF190951 EF190957	*Eucalyptus* sp.	Venezuela
C. fimbriatomima	CMW24176 CBS121787	EF190964 EF190952 EF190958	*Eucalyptus* sp.	Venezuela
C. larium	CMW25434 CBS122512	EU881906 EU881894 EU881900	*Styrax benzoin*	Indonesia
C. larium	CMW25435 CBS122606	EU881907 EU881895 EU881901	*Styrax benzoin*	Indonesia
C. manginecans	CMW13851 CBS121659	AY953383 EF433308 EF433317	*Mangifera indica*	Oman
C. manginecans	CMW13852 CBS121660	AY953384 EF433309 EF433318	*Hypocryphalus mangifera*	Oman
C. neglecta	CMW17808 CBS121789	EF127990 EU881898 EU881904	*Eucalyptus* sp.	Colombia
C. neglecta	CMW18194 CBS121017	EF127991 EU881899 EU881905	*Eucalyptus* sp.	Colombia
C. obpyriformis	CMW23807 CBS122608	EU245004 EU244976 EU244936	*Acacia mearnsii*	South Africa
C. obpyriformis	CMW23808 CBS122511	EU245003 EU244975 EU244935	*Acacia mearnsii*	South Africa
C. papillata	CMW8857	AY233868 AY233878 EU241483	*Annona muricata*	Colombia
C. papillata	CMW8856 CBS121793	AY233867 AY233874 EU241484	*Citrus limon*	Colombia
C. papillata	CMW10844	AY177238 AY177229 EU241481	*Coffea arabica*	Colombia
C. pirilliformis	CMW6569	AF427104 DQ371652 AY528982	*Eucalyptus nitens*	Australia
C. pirilliformis	CMW6579 CBS118128	AF427105 DQ371653 AY528983	*Eucalyptus nitens*	Australia
C. platani	CMW14802 CBS115162	DQ520630 EF070425 EF070396	*Platanus occidentalis*	USA

C. platani	CMW23918	EF070426 EF070397 EU426554	*Platanus* sp.	Greece
C. polychroma	CMW11424 CBS115778	AY528970 AY528966 AY528978	*Syzygium aromaticum*	Indonesia
C. polychroma	CMW11436 CBS115777	AY528971 AY528967 AY528979	*Syzygium aromaticum*	Indonesia
C. polyconidia	CMW23809 CBS122289	EU245006 EU244978 EU244938	*Acacia mearnsii*	South Africa
C. polyconidia	CMW23818 CBS122290	EU245007 EU244979 EU244939	*Acacia mearnsii*	South Africa
C. populicola	CMW14789 CBS119.78	EF070418 EF070434 EF070406	*Populus* sp.	Poland
C. populicola	CMW14819 CBS114725	EF070419 EF070435 EF070407	*Populus* sp.	USA
C. smalleyi	CMW14800 CBS114724	EF070420 EF070436 EF070408	*Carya cordiformis*	USA
C. smalleyi	CMW26383 CBS114724	EU426553 EU426555 EU426556	*Carya cordiformis*	USA
C. tanganyicensis	CMW15991 CBS122295	EU244997 EU244969 EU244929	*Acacia mearnsii*	Tanzania
C. tanganyicensis	CMW15999 CBS122294	EU244998, EU244970, EU244939	*Acacia mearnsii*	Tanzania
C. tzitzikammensis	CMW14276 CBS121018	EF408555 EF408569 EF408576	*Rapanea melanophloeos*	South Africa
C. tzitzikammensis	CMW14278 CBS121019	EF408556 EF408570 EF408577	*Rapanea melanophloeos*	South Africa
C. variospora	CMW20935 CBS114715	EF070421 EF070437 EF070409	*Quercus alba*	USA
C. variospora	CMW20936 CBS114714	EF070422 EF070438 EF070410	*Quercus robur*	USA
C. virescens	CMW11164 CBS123166	DQ520639 EF070441 EF070413	*Fagus americanum*	USA
C. virescens	CMW3276 CBS123216	AY528984 AY528990 AY529011	*Quercus robur*	USA
C. zombamontana	CMW15235 CBS122297	EU245002 EU244974 EU244934	*Eucalyptus* sp.	Malawi
C. zombamontana	CMW15236 CBS122296	EU245000 EU244972 EU244932	*Eucalyptus* sp.	Malawi

* Isolates in bold are the ones obtained with this study

Table 2. Isolates and species in the *C. moniliformis s.l.* species complex used in this study.

Species	Isolate no.	GenBank accession no.	Host	Geographical origin
C. bhutanensis	CMW8217 CBS114289	AY528957 AY528962 AY528952	*Picea spinulosa*	Bhutan
C. bhutanensis	CMW8242 CBS112907	AY528956 AY528961 AY528951	*Picea spinulosa*	Bhutan
C. moniliformis	CMW4114 CBS118151	AY528997 AY528986 AY529007	*Shizolobium parahybum*	Ecuador
C. moniliformis	CMW8379	AY529005 AY528995 AY529016	*Cassia fistula*	Bhutan
C. moniliformis	CMW9590 CBS116452	AY431101 AY528985 AY529006	*Eucalyptus grandis*	South Africa
C. moniliformis	CMW9990 CBS155.62	FJ151423 FJ151457 FJ151479	*Theobroma cacao*	Costa Rica
C. moniliformis	CMW10134 CBS118127	FJ151422 FJ151456 FJ151478	*Eucalyptus grandis*	South Africa
C. moniliformis	**CMW22458**	**FJ151424 FJ151458 FJ151480**	***Shizolobium parahybum***	**Ecuador**
C. moniliformis	**CMW22459**	**FJ151425 FJ151459 FJ151481**	***Shizolobium parahybum***	**Ecuador**
C. moniliformis	**CMW22460**	**FJ151427 FJ151461 FJ151482**	***Shizolobium parahybum***	**Ecuador**
C. moniliformis	**CMW22462**	**FJ151426 FJ151460 FJ151483**	***Shizolobium parahybum***	**Ecuador**
C. moniliformopsis	CMW9986 CBS109441	AY528998 AY528987 AY529008	*Eucalyptus oblique*	Australia
C. moniliformopsis	CMW10214 CBS115792	AY528999 AY528988 AY529009	*Eucalyptus sieberi*	Australia
C. oblonga	CMW23802 CBS122820	EU245020 EU244992 EU244952	*Acacia mearnsii*	South Africa
C. oblonga	CMW23803 CBS122291	EU245019 EU244991 EU244951	*Acacia mearnsii*	South Africa
C. omanensis	CMW3800 CBS117839	DQ074743 DQ074733 DQ074738	*Mangifera indica*	Oman
C. omanensis	CMW11048 CBS115787	DQ074742 DQ074732 DQ074737	*Mangifera indica*	Oman
C. savannae	CMW17278 CBS121019	EF408553 EF408567 EF408574	*Eucalyptus macarthurrii*	South Africa
C. savannae	CMW17300 CBS121151	EF408551 EF408565 EF408572	*Acacia nigrescens*	South Africa
C. tribiliformis	CMW13011 CBS115867	AY528991 AY529001	*Pinus merkusii*	Indonesia

		AY529012		
C. sublaevis	**CMW22415**	**FJ151428**	***Eucalyptus deglupta***	**Ecuador**
		FJ151462		
		FJ151484		
C. sublaevis	**CMW22422**	**FJ151429**	***Eucalyptus deglupta***	**Ecuador**
	CBS122516	**FJ151463**		
		FJ151485		
C. sublaevis	**CMW22444**	**FJ151430**	***Terminalia ivorensis***	**Ecuador**
	CBS122518	**FJ151464**		
		FJ151486		
C. sublaevis	**CMW22449**	**FJ151431**	***Terminalia ivorensis***	**Ecuador**
	CBS122517	**FJ151465**		
		FJ151487		
C. tribiliformis	CMW13012	AY528992	*Pinus merkusii*	Indonesia
	CBS118242	AY529002		
		AY529013		
C. virescens	CMW11164	DQ520639	*Fagus americanum*	USA
	CBS123166	EF070441		
		EF070413		
C. virescens	CMW3276	AY528984	*Quercus robur*	USA
	CBS123216	AY528990		
		AY529011		
Thielaviopsis ceramica	CMW15245	EU245022	*Eucalyptus grandis*	Malawi
	CBS122299	EU244994		
		EU244926		
T. ceramic	CMW15248	EU245024	*Eucalyptus grandis*	Malawi
	CBS122300	EU244996		
		EU244928		

* Isolates in bold are the ones obtained with this study

Table 3. Morphological features of *C. curvata*, *C. ecuadoriana* and *C. diversiconidia* compared with each other and related species in the *C. fimbriata sensu lato* species complex. All measurements are in µm. (Measurements not available are denoted as N.A.)

Species	*C. curvata*	*C. cacaofunesta*	*C. ecuadoriana*	*C. neglecta*	*C. diversiconidia*	*C. fimbriata s.s.*
Characteristics						
Ascomatal bases						
Shape	Globose to obpyriform	Globose	Globose	Globose	Globose	Globose
Length	(162-)185-239(-280)	100-275	(180-)215-265(-290)	(173-)202-244(-281)	(97-)115-155(-196)	110-250
Width	(170-)192-240(-280)	95-305	(184-)208-250(-279)	(153-)178-228(-250)	(97-)122-170(-223)	120-250
Ascomatal necks	Undulating at apices	Straight	Straight	Straight	Straight	Straight
Length	(419-)498-644(-714)	310-1010	(515-)615-851(-1021)	(691-)745-840(-889)	(245-)368-520(-599)	440-770
Width (bases)	(19-)23-35(-56)	20-45	(24-)28-38(-46)	(27-)31-39(-46)	(19-)23-29(-34)	28-40
Width (apices)	(12-)15-21(-25)	12-25	(14-)17-23(-28)	(14-)16-20(-22)	(12-)15-21(-25)	16-24
Ostiolar hyphae						
Shape	Divergent	Divergent	Divergent	Divergent	Divergent	Divergent
Length	(34-)39-49(-59)	30-125	(29-)32-42(-50)	(35-)41-49(-54)	(40-)45-61(-72)	20-120
Ascospores						
Length	3-5	3-4	3-4	3-6	3-5	3-4.5
Width (excluding sheath)	4-6	4.5-6.5	3-6	4-7	4-7	3.5-5
Width (including sheath)	5-8	3.5-5.5	5-8	5-8	6-8	5-7.5
Primary phialides						
Length	(44-)55-95(-139)	12-85	(60-)72-94(-98)	(75-)80-114(-152)	(58-)82-132(-162)	27-60
Width (bases)	4-6	N.A.	4-6	(4-)5-7(-8)	4-7	N.A.
Width (broadest point)	4-7	2-9	5-7	5-9	4-9	4-8.5
Width (apices)	3-4	2-6.5	3-5	(3-)4-6(-7)	3-6	3-6
Secondary phialides						
Length	(34-)42-66(-80)	Present (sizes N.A.)	(52-)61-85(-98)	(38-)48-76(-89)	(40-)49-65(-68)	Absent
Width (bases)	3-6	Present (sizes N.A.)	3-6	(3-)5-7(-8)	4-6	Absent
Width (apices)	4-6	Present (sizes N.A.)	4-6	(3-)5-7(-8)	5-7	Absent
Primary conidia						
Length	(10-)13-21(-31)	8-40	(9-)14-20(-24)	(11-)15-27(-30)	(10-)13-21(-30)	9-33
Width	3-6	2.5-5	3-5	(3-)5-6	4-6	3.5-5
Secondary conidia						
Length	7-11	Present (sizes N.A.)	7-9(-11)	(6-)10-11	(5-)8-10(-11)	Absent
Width	4-8	Present (sizes N.A.)	4-7	(4-)5-7(-9)	4-8	Absent

Species	*C. curvata*	*C. cacaofunesta*	*C. ecuadoriana*	*C. neglecta*	*C. diversiconidia*	*C. fimbriata s.s.*
Chlamydospores						
Shape	Globose to perprolate	Globose to pyriform	Globose to subglobose	Globose	Pyriform to obpyriform	Globose to pyriform
Length	(7-)9-11(-12)	10-20(-37)	(9-)11-16(-16)	(8-)10-12(-13)	(12-)14-18(-19)	11-16
Width	11-17	3.5-11.5	(7-)9-11(-12)	(9-)10-14(-16)	(9-)10-12(-15)	6.5-12
Reference	This study	Baker & Harrington 2005	This study	Rodas *et al.* 2008	This study	Baker & Harrington 2005

Table 4. Comparison of morphological characteristics for *C. moniliformis s.s.*, *C. subleavis* and *C. tribiliformis*. All measurements are in µm. (Measuring data not available denoted as N.A.).

Species	*C. moniliformis s.s.*	*C. subleavis*	*C. tribiliformis*
Characteristics			
Ascomatal bases			
Shape	Globose	Globose	Globose to obpyriform
Length	N.A.	(98-)131-173(-187)	N.A.
Width	N.A.	(102-)144-192(-231)	N.A
Diam	90-180	(100-)137-183(-209)	(196-)203-249(-264)
Conical spines - length	12-16	(2-)4-10(-13)	(4-)6-10(-12)
Ascomatal necks	Straight	Undulating	Straight
Length	N.A	(522-)598-802(-990)	(741-)615-851(-1047)
Width (bases)	N.A	(24-)31-45(-55)	(43-)44-50(-53)
Width (apices)	N.A.	(9-)12-16(-19)	(13-)14-18(-20)
Ostiolar hyphae			
Shape	Divergent	Divergent	Divergent
Length	12-18	(15-)18-24(-25)	(22-)25-31(-32)
Ascospores			
Length	3-4	2-4	2-3
Width (excluding sheath)	4-5	3-4	4-5
Width (including sheath)	N.A.	4-6	5-6
Primary phialides			
Length	N.A.	(15-)23-37(-50)	(21-)72-94(-46)
Width (bases)		2-5	3-4
Width (broadest point)		2-5	N.A.
Width (apices)		1-3	1-3
Secondary phialides	Absent		
Length		(19-)25-34(-36)	N.A.
Width (bases)		2-4	N.A.
Width (apices)		2-4	N.A.
Primary conidia			
Length	6-8	5-8	7-9
Width	1.8-2.2	1-3	2
Secondary conidia	Absent		
Length		3-6	7-9
Width		2-3	3-4
Chlamydospores	Absent	Absent	Absent
Shape			
Length			
Width			
Reference	Hedgcock 1906	This study	Van Wyk *et al.* 2006a

Figure 1. Isolates of Ecuador obtained from *E. deglupta* and *T. ivorensis* trees in a phylogenetic tree based on the combined regions of the ITS, β-tubulin and Ef1-α for species in the *C. fimbriata s.l.* species complex. *Ceratocystis virescens* represents the out-group taxon. Bootstrap values and Bayesian posterior probabilities (in brackets) are indicated at the branch nodes.

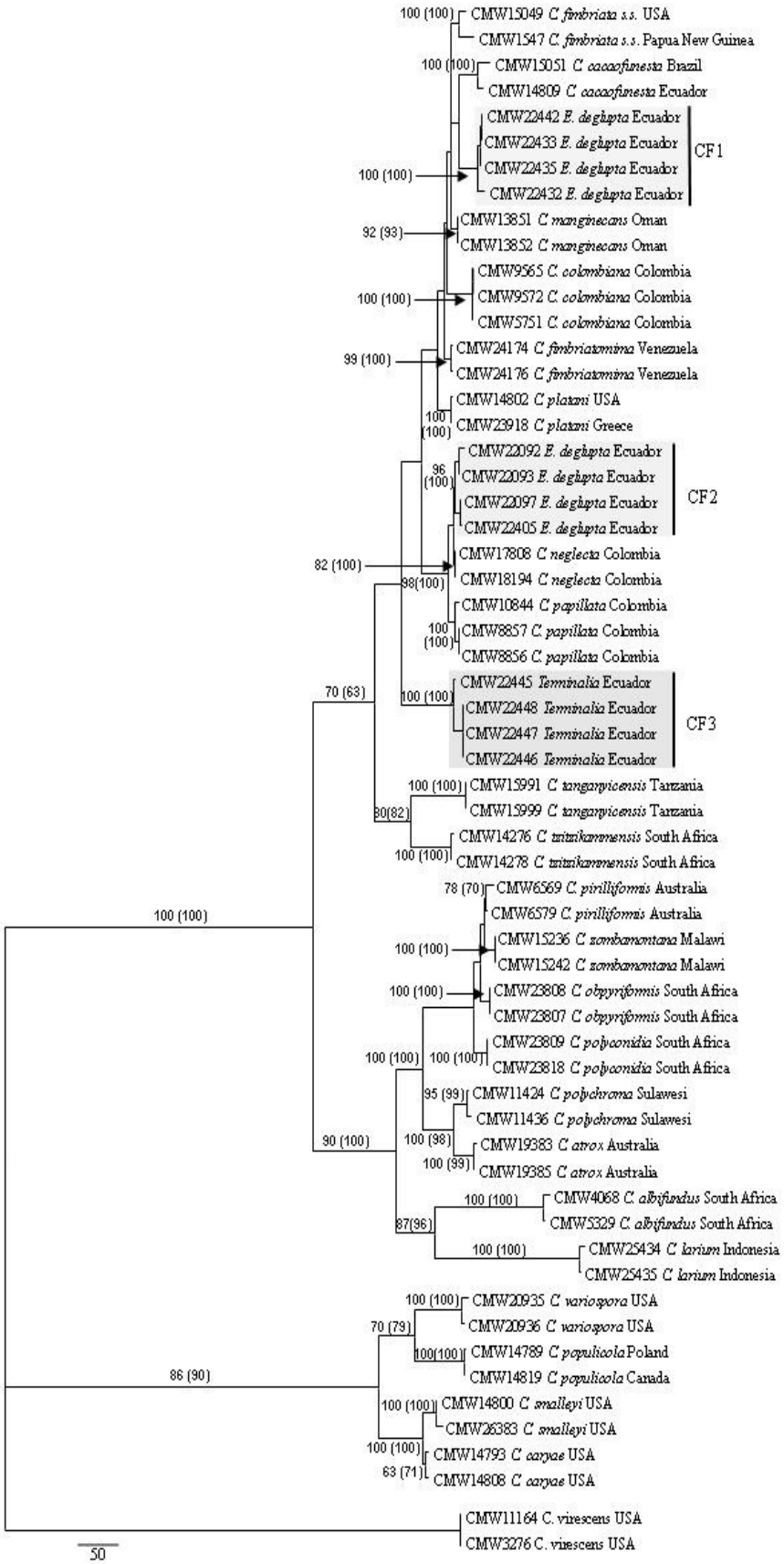
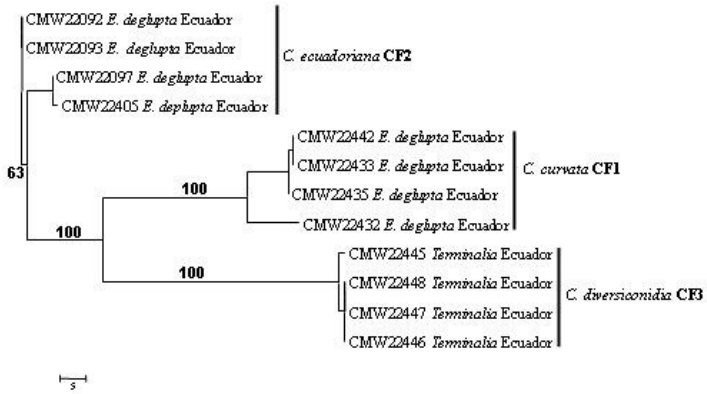
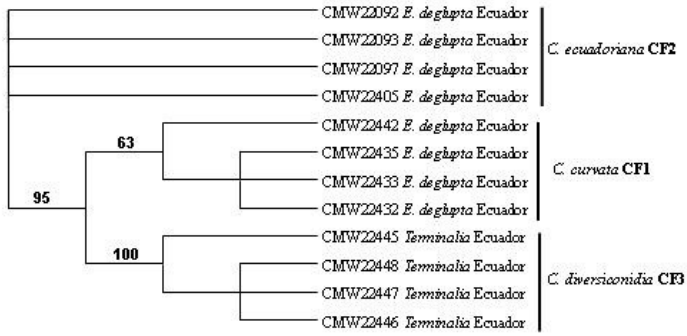


Figure 2. Separate unrooted phylogenetic trees of the three groups in the *C. fimbriata s.l.* dataset representing the three gene regions. a. ITS. b. β-tubulin. c. EF1-α. Bootstrap values are indicated on the branches.

a.



b.



c.

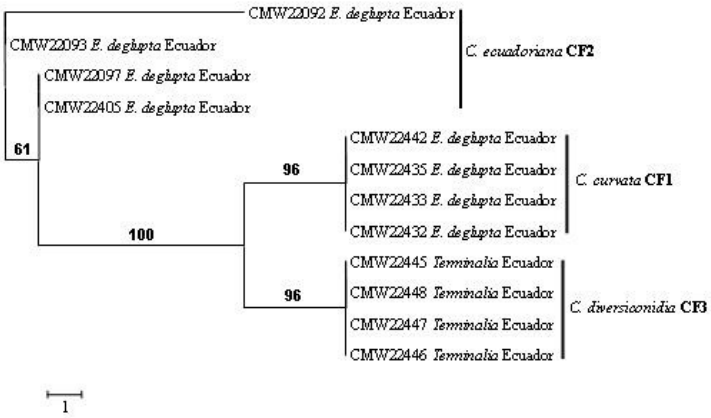


Figure 3. Three separate allele networks obtained from one dataset, within the *C. fimbriata s.l.* complex, representing three gene regions (ITS, β-tubulin and EF1-α) of the isolates obtained from Ecuador on *E. deglupta* and *Terminalia* trees. The numbers represent CMW numbers as listed in Table 1.

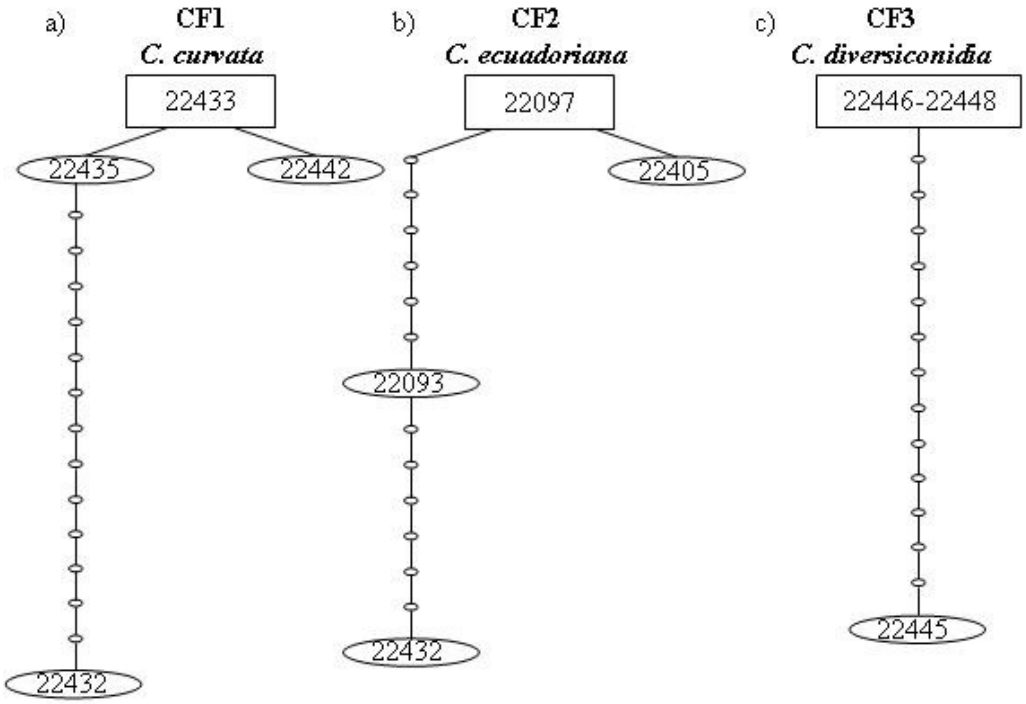


Figure 4. Isolates of Ecuador obtained from *S. parahybum*, *E. deglupta* and *T. ivorensis* trees in a phylogenetic tree based on the combined regions of the ITS, β-tubulin and Ef1-α for species in the *C. moniliformis s.l.* species complex. *Ceratocystis virescens* represents the out-group taxon. Bootstrap values and Bayesian posterior probabilities (in brackets) are indicated at the branch nodes.

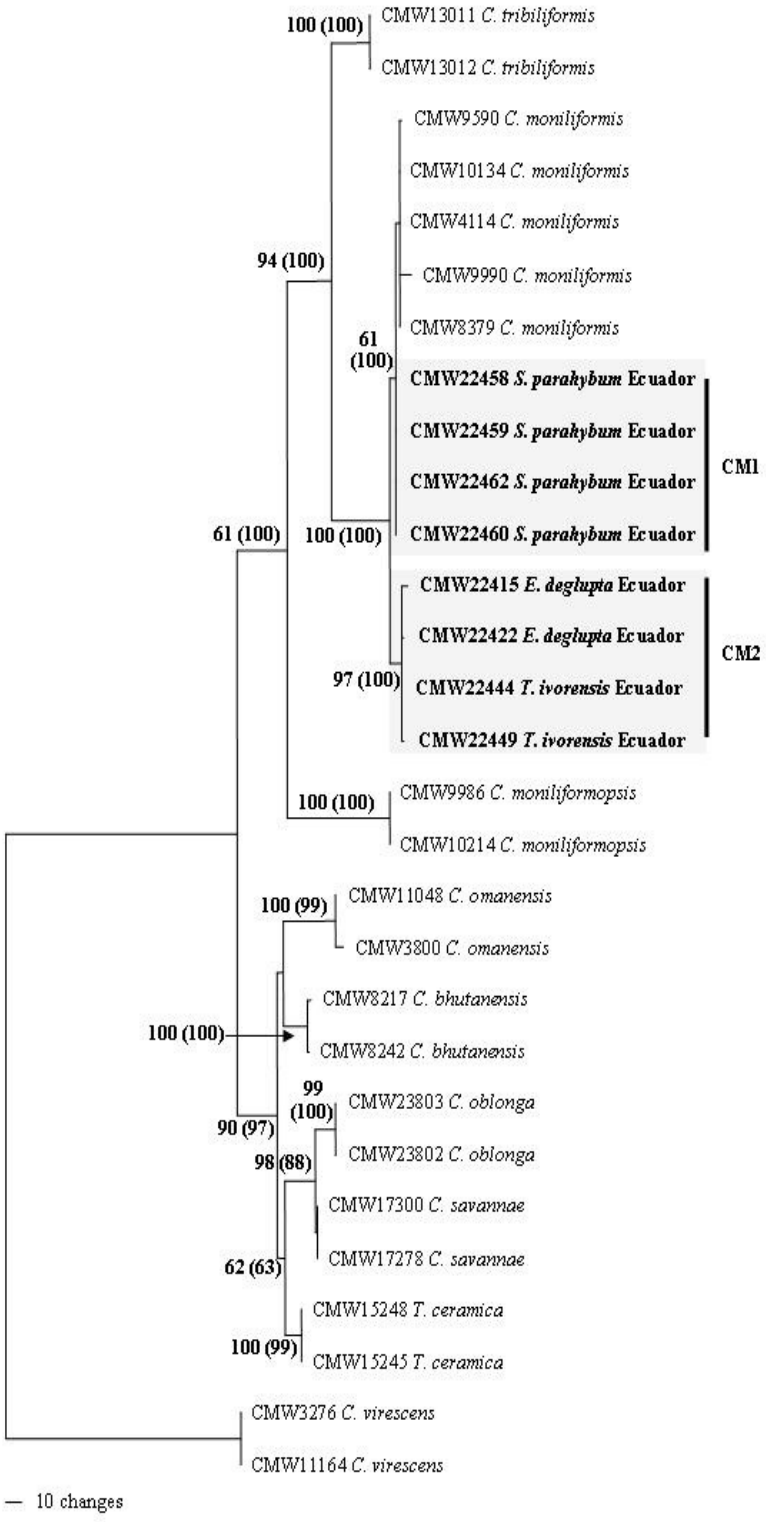
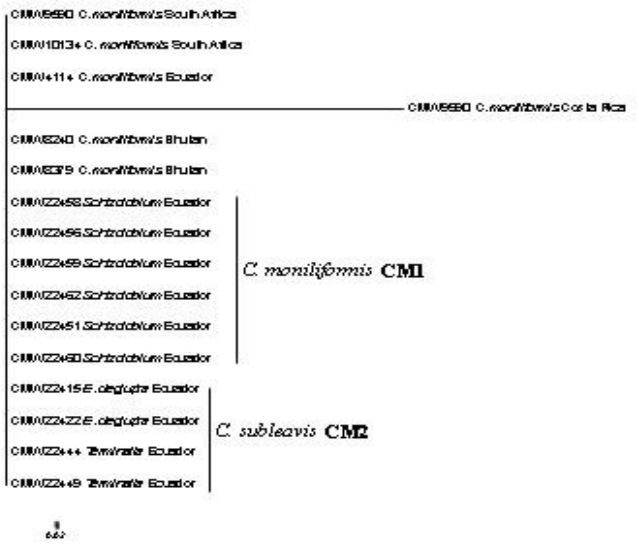
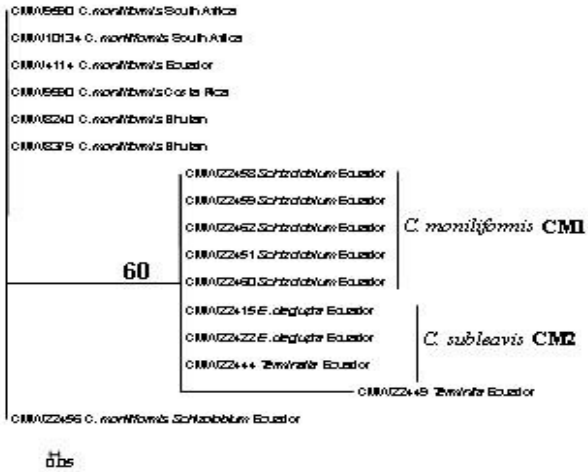


Figure 5. Three separate unrooted phylogenetic trees of the two groups in the *C. moniliformis s.l.* dataset representing three gene regions. a. ITS. b. β-tubulin. c. EF1-α. Bootstrap values are indicated on the branches. Where there are no bootstrap values this indicates that the bootstrap was less than 60%.

a.



b.



c.

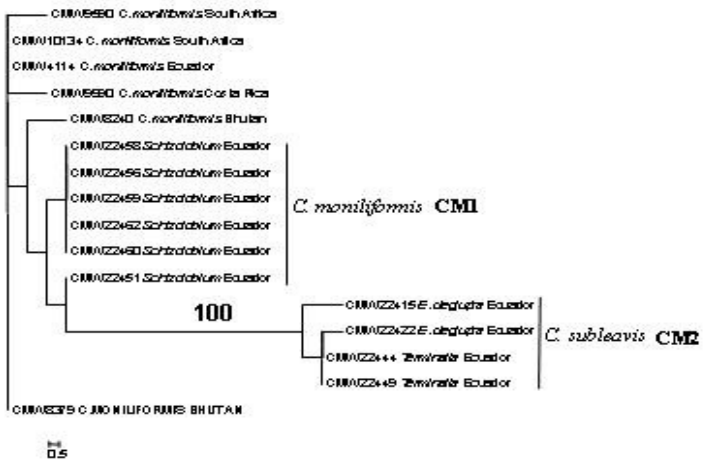
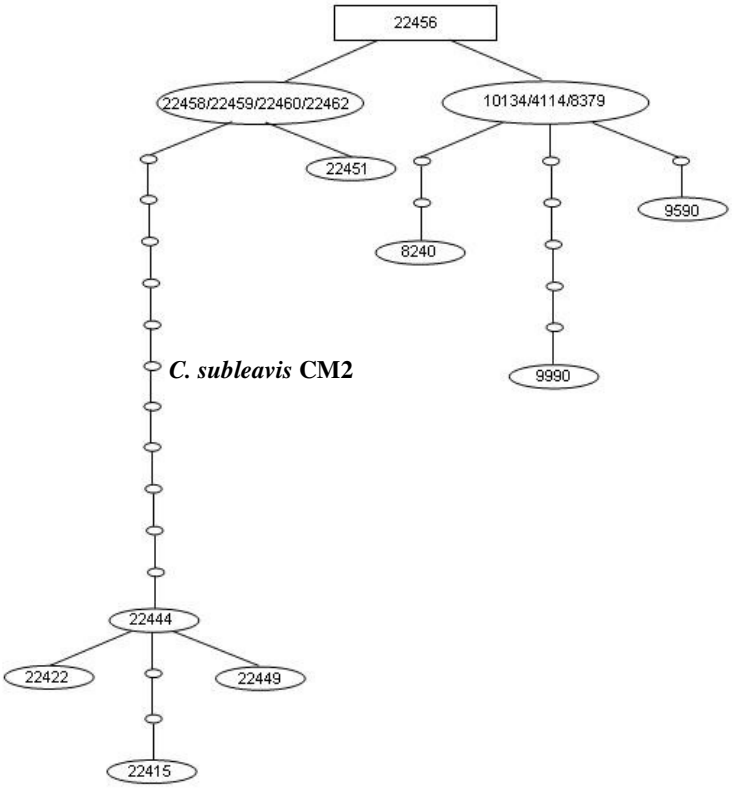


Figure 6. Allele network for representative isolates in the *C. moniliformis s.l.* complex. Isolates representing *C. moniliformis s.s.* and isolates obtained in this study from Ecuador were selected for this dataset. The ITS, βt and EF1-α gene regions were combined and a single allele network was obtained. The numbers in the network represent isolates in the CMW collection (Table 1).

***C. moniliformis s.s.* CM1**



***C. subleavis* CM2**

Figure 7. Morphological characteristics of *Ceratocystis curvata.* **a.** Ascoma with globose to obpyriform base. **b.** Ascomatal neck undulating at apex. **c**. Divergent ostiolar hyphae. **d**. Hat-shaped ascospores. **e.** Primary conidiophore, flask-shaped phialides. **f**. Secondary conidiophore, flask-shaped but flaring at apex. **g**. Chain of cylindrical conidia. **h**. Chain of barrel-shaped conidia. **i**. Dark, globose to perprolate chlamydospores Bars; **a.** = 100 µm. **b, d, h** = 5µm. **c, e, f, g, i** = 10µm.

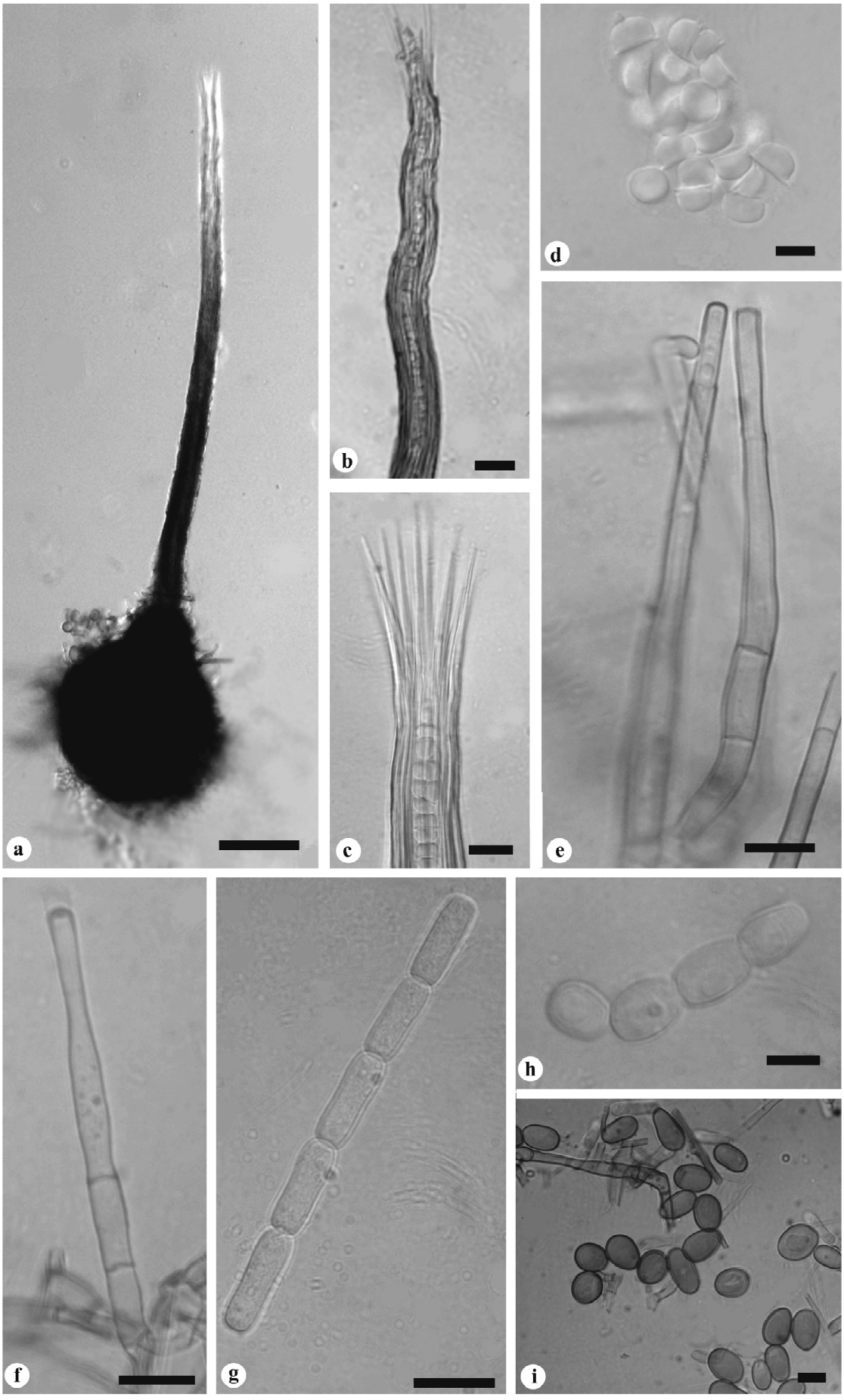


Figure 8. Morphological characteristics of *Ceratocystis ecuadoriana.* **a.** Ascoma with globose base. **b.** Divergent ostiolar hyphae. **c**. Hat-shaped ascospores. **d.** Primary conidiophore, flask-shaped phialides. **e**. Secondary conidiophore, tubular flaring at apex. **f**. Cylindrical conidia. **g**. Chain of barrel-shaped conidia. **h**. Dark, globose to sub-globose chlamydospores Bars; **a.** = 100 µm. **c, f, h** = 5µm. **b, d, e, g** = 10µm.

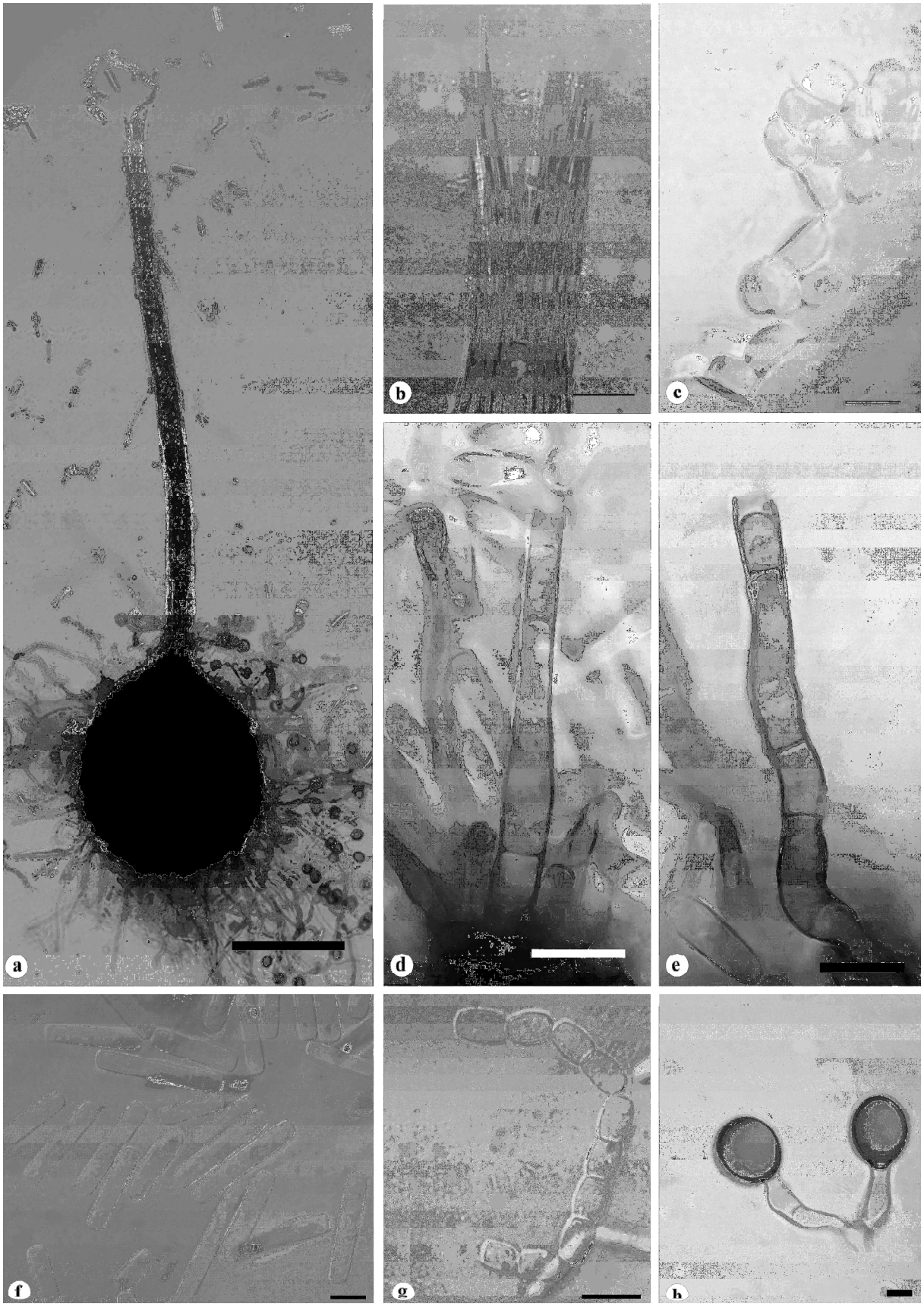


Figure 9. Morphological characteristics of *Ceratocystis diversiconidia.* **a.** Ascoma with globose base. **b.** Divergent ostiolar hyphae. **c**. Hat-shaped ascospore. **d.** Primary conidiophore, flask-shaped phialides. **e**. Secondary conidiophore, flask-shaped but flaring at apex. **f**. Dark, pyriform to obpyriform chlamydospores. **g**. Chain of barrel-shaped conidia with different sizes. **h**. Primary conidia, cylindrical to oblong with truncated ends. **i**. Primary and secondary conidia. Bars; **a.** = 100µm. **b =** 20µm. **c =** 5µm. **d-i** = 10µm.

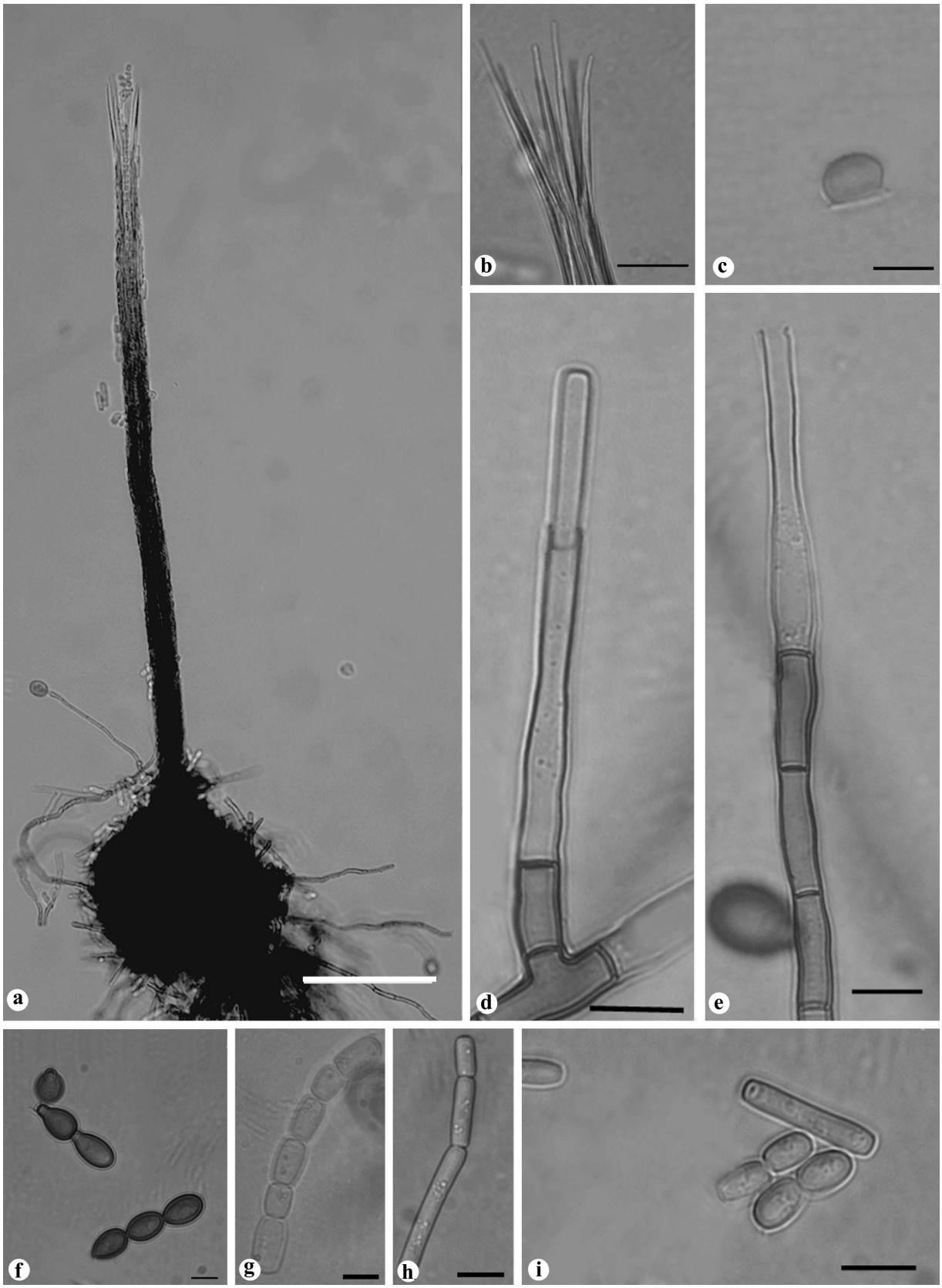
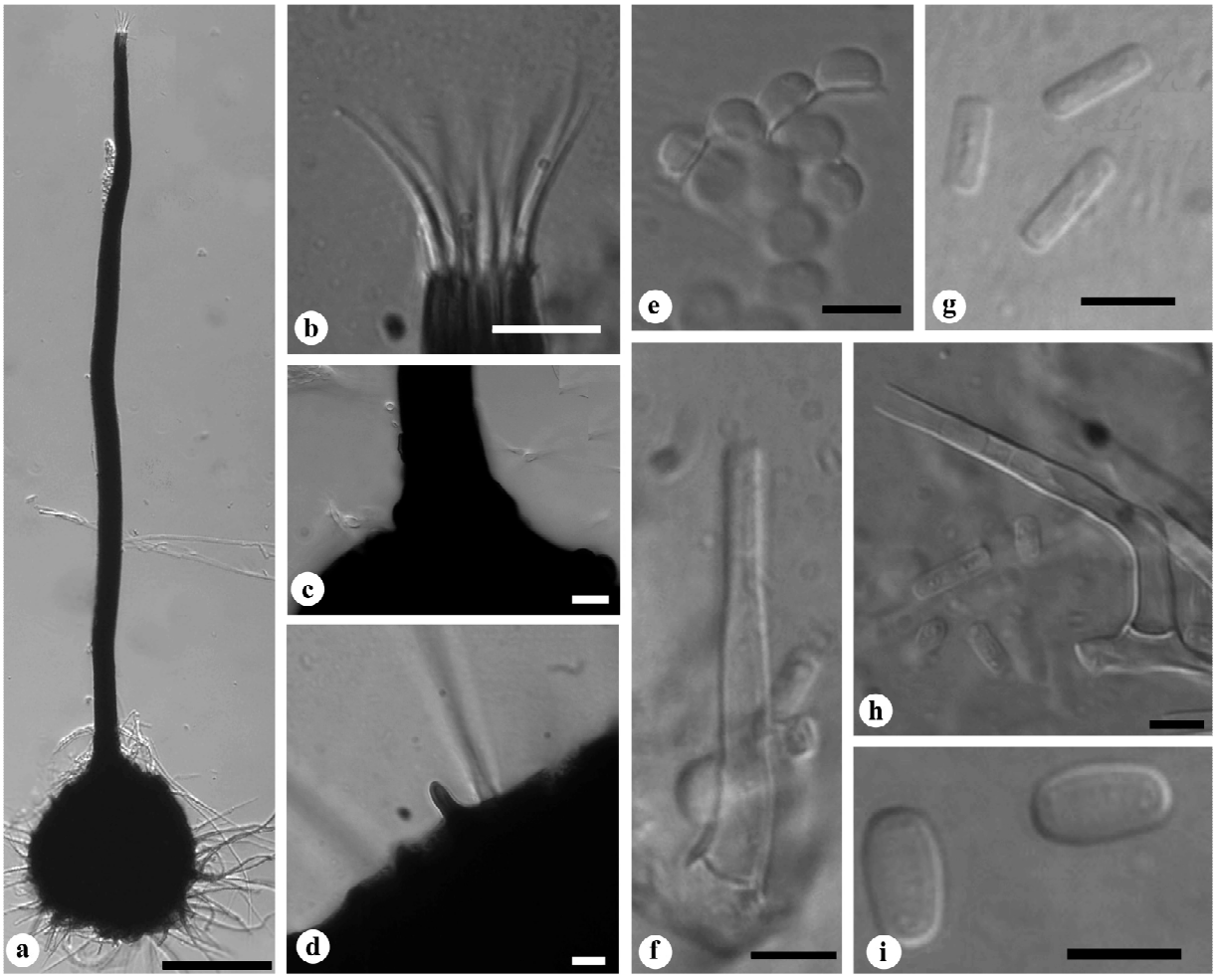


Figure 10. Morphological characteristics of *Ceratocystis subleavis.* **a.** Ascoma with globose base with limited amount of conical spines. **b.** Divergent ostiolar hyphae. **c**. Ascomatal neck disk-shaped at base. **d**. One of a limited number of conical spines on ascomatal base **e.** Hat-shaped ascospores. **f.** Primary conidiophore, flask-shaped phialides. **g**. Primary conidia, cylindrical with truncated ends. **h**. Secondary conidiophore, tubular flaring at apex. **i**. Barrel-shaped conidia. Bars; **a.** = 100µm. **b =** 10µm. **c-i** = 5µm.



CHAPTER 10

Two *Ceratocystis* species associated with mango disease in Brazil

Van Wyk M., Wingfield B.D., Al Adawi A.O., Rossetto C., Ito M.F. & Wingfield M.J. (2009). Two new *Ceratocystis* species associated with mango disease in Brazil. *Mycotaxon* **117**: 381–404.

ABSTRACT

The disease of *Mangifera indica* known as mango blight, murcha or seca da mangueira in Brazil is caused by the canker wilt pathogen *Ceratocystis fimbriata sensu lato*. It is also closely associated with infestation by the non-native wood boring beetle *Hypocryphalus mangiferae*. The aim of this study was to characterize *Ceratocystis* isolates obtained from diseased mango trees in Brazil. Identification was based on sequence data for the Internal Transcribed Spacer region 1 and 2 including the 5.8S rRNA operon, part of the Beta-tubulin 1 gene and part of the Transcription Elongation Factor 1-alpha gene. The isolates from Brazil grouped in two well defined and unique clades within *C. fimbriata* s.l. These were also distinct from *C. manginecans* that causes a similar disease associated with *H. mangiferae* in Oman and Pakistan. Based on sequence comparisons and morphological characteristics, isolates representing the two phylogenetic clades are described as *C. mangicola sp. nov.* and *C. mangivora sp. nov.*

INTRODUCTION

A disease typified by wilting of the leaves, flowers and stems of mango trees (*Mangifera indica* L.) was first reported from Brazil in the 1930's (Viégas 1960, Ploetz 2003). The disease, commonly referred to as "mango blight", "seca" or "murcha da mangueira", represents one of the most important constraints to mango production in Brazil (Ploetz 2003). The causal agent of this disease was identified as *Ceratocystis fimbriata* Ellis and Halst. *sensu lato* (s.l.) (Viégas 1960, Piza 1966, De Toledo Piza 1966, Ribeiro 1980).

Ceratocystis fimbriata s.l. was first recognised as possibly encompassing more than one taxon by Webster & Butler (1967a, b) who showed host specificity amongst isolates of the fungus. Isolates of *C. fimbriata* s.l. are morphologically similar but many can be separated from each other based on phylogenetic inference using DNA sequences. Thus, during the course of the past decade, numerous new and cryptic species in the *C. fimbriata* complex have been described. Examples include the African fungus *C. albifundus* MJ Wingf., De Beer and MJ Morris (Wingfield *et al*. 1996, Barnes *et al*. 2005), *C. larium* M. van Wyk and MJ Wingf. (Van Wyk *et al*. 2009a), *C. cacaofunesta* Engelbr. and TC. Harr. (Engelbrecht and Harrington 2005), *C. fimbriatomima* M. van Wyk and MJ Wingf. (Van Wyk *et al*. 2009b) and *C. curvata* M. van Wyk and MJ Wingf., *C. ecuadoriana* M. van Wyk and MJ Wingf. and *C. diversiconidia* M. van Wyk and MJ Wingf. (Van Wyk *et al*. 2011). In the strict sense, *C. fimbriata* is restricted to those isolates related to the sweet potato black rot pathogen, first described by Halsted (1890) from diseased *Ipomoea batatas* (L.) Lam. tubers in the USA (Engelbrecht & Harrington 2005). An alternative view is that phylogenetically different isolates of *C. fimbriata* s.l. from various hosts in Brazil might represent populations of *C. fimbriata* s.s. rather than discrete taxa (Ferreira *et al*. 2010).

Ceratocystis spp. requires wounds to infect trees (De Vay *et al*. 1963, Kile 1993). In Brazil, mango blight is closely associated with the wood-boring beetle *Hypocryphalus mangiferae* Stebbing (Coleoptera: Scolytinae) that is native to southern Asia (Wood 1982, Butani 1993, Atkinson & Peck 1994). It has been hypothesised that this insect aids in the dissemination of the fungus in Brazil (Ribeiro 1980, Yamashiro & Myazaki 1985, Ploetz 2003). Interestingly, the same beetle is associated with *Ceratocystis manginecans* M. van Wyk, A. Adawi and MJ Wingf. that causes a serious disease of Mango trees in Oman and Pakistan (Al Adawi *et al*. 2006, Van Wyk *et al.* 2005, 2007a) and that has the same symptoms as mango blight in Brazil.

At the time of describing *C. manginecans* (Van Wyk *et al*. 2007a), only two isolates of *C. fimbriata* s.l. from diseased mango in Brazil were included. These isolates were phylogenetically different to C. *manginecans* but they were not treated as novel due to the small number of isolates available. Recently, a larger collection of isolates of *C. fimbriata* s.l. associated with mango blight in Brazil has become available for study. The aim of this investigation was to compare these isolates with *C. manginecans* and thus to determine their identity.

MATERIALS AND METHODS

Isolates

A total of 15 isolates from diseased Mango trees obtained in Sao Paulo State in Brazil were included in this study (Table 1). Isolates were transferred to 2% Malt Extract Agar (MEA) (20 g/L) (Biolab, Midrand, South Africa) and maintained at room temperature (~25°C). All cultures 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 Schimmelcultures (CBS), Utrecht, The Netherlands. Cultures of representative isolates bearing fruiting structures of the fungi were dried on 30% glycerol and have been deposited with the National Collection of Fungi (PREM), South Africa.

Phylogenetic comparisons

DNA was extracted from the isolates obtained from mango in Brazil using the techniques described by Van Wyk *et al*. (2006). Three sets of analyses were run on these isolates. The first dataset consisted of only the Internal Transcribed Spacer region 1 and 2 including the 5.8S rRNA operon (ITS) and included sequences for all species in the *C. fimbriata* s.l. complex as well as most sequences for this fungus available in GenBank and from a variety of plants in Brazil. For the second dataset, three gene regions were targeted for PCR including the ITS, part of the Beta-tubulin 1 (βt) gene and part of the Transcription Elongation Factor 1-alpha gene (EF1-α). Data for, these three gene regions were combined. The third dataset consisted of only the isolates from Brazil and isolates from mango and each gene region (ITS, βt and EF1-α) was treated separately.

DNA amplification was achieved with the primer sets ITS1 and ITS4 (White *et al*. 1990), βt1a and βt1b (Glass and Donaldson 1995) and EF1F and EF1R (Jacobs *et al*. 2004), following the protocols

described by Van Wyk *et al*. (2006). Amplification was assessed with the aid of gel electrophoresis in the presence of ethidium bromide. PCR amplicons were purified using 6% Sephadex G-50 columns (Steinheim, Germany) and sequenced in both directions using the ABI PRISM^{TM} Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California), with the same primers as those used for DNA amplification. Sequencing reactions were run on an ABI PRISM^{TM} 3100 Autosequencer (Applied BioSystems, Foster City, California, USA).

Sequence analyses were done using the software programme Chromas Lite 2.01 (http://www.technelysium.com.au). Sequence data obtained in this study for isolates from mango in Brazil were compared with those residing in the *C. fimbriata* s.l. clade for *Ceratocystis* obtained from GenBank (http://ncbi.nlm.nih.gov) or those previously published (Van Wyk *et al*. 2005, 2007a,b, 2009a,b, 2011). These sequences were aligned using MAFFT (http://timpani.genome.ad.jp/%7emafft/server/) (Katoh *et al.* 2002) and confirmed manually. Thereafter, the *C. fimbriata* s.l. dataset was analyzed using PAUP version 4.0b10* (Swofford 2002). Sequences for the three gene regions were analyzed separately and a partition homogeneity test (Swofford 2002) was used to determine whether the three datasets (ITS, βt and EF1-α) could be combined. The combined analyses were run as described in Van Wyk *et al*. (2009b). Sequences derived from this study were deposited in GenBank (Table 1) and the datasets and trees described here were deposited in TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S11057).

A modeltest (MrModeltest2) was run on each gene region to determine the rate of nucleotide substitution (Nylander 2004). These substitution rates were incorporated into Bayesian analyses (MrBayes version 3.1.1), which were used to determine whether nodes obtained with PAUP had statistical support (Ronquist and Huelsenbeck 2003). One million trees were generated using the Markov Chain Monte Carlo (MCMC) procedure. Four chains, two hot and two cold, were utilized to obtain the results. Trees were sampled every 100th generation and printed. Tree likelihood scores were assessed to determine the number of trees that had formed before stabilization, and thus to avoid including trees that had formed before convergence. Trees outside the point of convergence were discarded by means of the burn-in procedure (Ronquist & Huelsenbeck 2003).

Molecular Evolutionary Genetics Analysis (MEGA) 4 (Tamura *et al.* 2007) was used to determine the level of variation between the isolates from a wide range of hosts in Brazil for the ITS region only. In addition, this approach was applied for the ITS, βt and EF1-α for the isolates obtained from

mango trees in Brazil and including *C. manginecans* previously described from Oman and Pakistan. Sequences for each of the three gene regions were inspected to determine the number of fixed alleles between them.

An allele network was drawn using the software TCS (Clement *et al*. 2000). The dataset consisted of the combined gene regions (ITS, *β*t and EF1-*α*) of all the isolates obtained from mango in Brazil and including *C. manginecans* and *C. fimbriatomima* (Figure 4).

Culture characteristics and morphology

Based on the phylogenetic comparisons, two groups (B1 and B2) of isolates emerged. Three representatives from each of the two groups (CMW14797, CMW27306, CMW28907 and CMW15052, CMW27304, CMW27305) were randomly selected for growth studies in culture at different temperatures. The isolates were grown for 14 days on 2% MEA, after which 5mm plugs were transferred to the centres of 90mm Petri plates containing 2% MEA. These plates were then incubated at temperatures between 5°C and 35°C at five degree intervals. Five plates were used for each isolate at each of the test temperatures and the entire experiment was repeated once. The colony colours for isolates were assigned using the colour charts of Rayner (1970).

For microscope studies, the same six isolates, representing the two groups (B1 and B2) that were used to compare culture characteristics were selected. These cultures were grown for 10 days on 2% MEA plates. Fungal structures were selected and mounted in lactic acid on glass slides. Photographic images were captured with a Carl Zeiss compound microscope and using a Zeiss Axio Vision camera system. For isolates CMW14797 and CMW28305, 50 measurements were made for taxonomically relevant morphological characteristics, while 10 measurements were taken for isolates CMW27306, CMW28907, CMW15052 and CMW27304. The averages and standard deviations (stdv) were computed for all the measurements that are presented in the descriptions as (minimum-) stdv minus the mean – stdv plus the mean (-maximum). Where the minimum value was the same as the mean minus the stdv, the value in brackets (minimum-) was not included.

RESULTS

Phylogenetic comparisons

ITS sequences for species in *C. fimbriata* s.l. including unidentified isolates from *Colocasia*, *Mangifera*, *Gmelina* and *Ficus* in Brazil, gave a 614 bp dataset for 83 isolates. This dataset consisted of 234 constant, 11 parsimony uninformative and 369 parsimony informative characters. Five trees were obtained in these analyses, one of which was selected for presentation (Figure 1). The tree had the following characteristics; tree length = 1279, Consistency Index = 0.6, Rescaled Index = 0.5, Retention Index = 0.9.

MrModeltest2 selected the GTR+I+G model for the ITS gene region. These settings were included in the Bayesian analyses and four thousand trees were discarded because they were obtained outside the point of convergence. The Bayesian probabilities obtained in MrBayes were included in the tree (Figure 1) obtained in PAUP. These probabilities obtained in the Bayesian analyses were similar to those obtained in PAUP.

The isolates from Brazil grouped into several polyphyletic clades (Figure 1). These included a well supported clade (Bootstrap 86%) represented by two isolates, one from *Acacia* the other from *Eucalyptus*. A second clade included only isolates from Taro (Bootstrap 85%). Isolates from mango and a *Eucalyptus* and *Gmelina* isolate resided in a discrete clade (Bayesian 91%) and *C. manginecans* isolates were in a clade sister to that clade (Bootstrap 87%). A group of isolates from mango resided in a clade with strong support (Bootstrap 99%, Bayesian 86%) and an isolate from fig was sister to that clade.

Amplicons of ~500 bp (ITS and β-tubulin) and ~800 bp (EF1-α) were obtained for the Brazilian isolates of *C. fimbriata* s.l. from mango (Table 1). The PHT resulted in a low P-value (P=0.01), possibly due to the small amount of variation in the βt gene region. Although the P-value was low, this value remained acceptable (Sullivan 1996, Cunningham 1997) to support combination of the data for the three gene regions. The combined dataset for the three gene regions consisted of a total of 1971 characters, 1066 of which were constant, 57 were parsimony-uninformative and 848 were parsimony informative. Twenty two most parsimonious trees were obtained, one of which (Figure 2) was selected for presentation (Tree length = 2361, Consistency Index = 0.6, Rescaled Index = 0.5, Retention Index = 0.9).

MrModeltest2 selected the GTR+I+G model for the ITS gene region, the GTR+G model for the βt gene region and the HKY+I+G for the EF1-α gene region. These settings were included in the

Bayesian analyses. Two-thousand trees were discarded as they were outside of the point of convergence. The posterior probabilities for the branch nodes were included in the tree obtained with PAUP (Figure 2). The posterior probabilities supported the bootstrap values obtained using PAUP.

The isolates from mango in Brazil grouped in two distinct clades (B1 and B2), with high bootstrap support (100% and 100%, respectively). These two phylogenetic groups were sister to *C. manginecans*, the species most closely related to them. All other species considered in the study, formed well supported and distinct clades, confirming their unique nature.

The single gene tree for the ITS gene region (Figure 3) had a structure similar to the tree based on combined sequences for the three gene regions. The two groups of isolates from mango (B1 and B2) in Brazil grouped apart from *C. fimbriatomima* and *C. manginecans* with high bootstrap support. The single gene trees for the *β*t and EF1-α gene region did not distinguish between the two groups of isolates from mango in Brazil but they did distinguish *C. fimbriatomima* and *C. manginecans* from these two groups.

The number of fixed alleles between the four groups (three from mango and one from *Eucalyptus*), *C. manginecans,* the two groups of isolates obtained from Mango in Brazil (Group B1 and Group B2) and *C. fimbriatomima* varied within and between groups (Table 2). The combined dataset for the three gene regions resulted in two allele trees (Figure 4). *Ceratocystis fimbriatomima* was represented on its own while all three taxa from diseased mango including *C. manginecans* and the two groups identified in this study, resided in a single allele tree.

Culture characteristics and morphology

Isolates representing Group B1 were morphologically similar to other species in *C. fimbriata* s.l. They produced a banana odour, typical of fungi in this group. After 2 weeks on 2% MEA, the colonies had a dark brown (snuff brown, 15"k) colour (Rayner 1970) with large numbers of perithecia visible on the surface of the cultures. At 5ºC and 35ºC, no growth was observed after 7 days. At 10ºC (8mm), 15ºC (22mm), 20ºC (36mm) and 30ºC (20mm) diminished growth was observed after 7 days while the optimum temperature for growth of these isolates was 25ºC (44mm).

Isolates residing in Group B2 were similar to those in Group B1 producing a banana odour and they had a similar morphology. After 2 weeks on 2% MEA, the colonies also had a dark brown (snuff brown, 15''k) colour (Rayner 1970) with many perithecia produced on the culture surface. As with the isolates in Group B1, after seven days, there was no growth at either 5 or 35ºC. Some growth was observed at the other temperatures tested 10ºC (7mm) and 15ºC (22mm), 20ºC (41mm), 30ºC (36mm) and 25ºC (45mm) represented the optimum temperature for growth.

TAXONOMY

Based on DNA sequence comparisons and to a lesser extent morphology, isolates from mango in Brazil could be separated into two distinct groups. These groups represent previously unknown species that are described as follows:

Ceratocystis mangicola M van Wyk and MJ Wingf. **sp. nov.** (Figure 5)

(B1; CMW14797) MB511886

Etymology: The name refers to the fact that the fungus occurs on mango

Coloniae olivaceae in 2%MEA, odore Musae. Hyphae laeves, segmentatae. Bases ascomatum globosae vel subglobosae, atrobrunneae vel nigrae, (125-)139-199(-230) µm latae, (115-)136-192(-236) µm longae. Colla ascomatum brunnea, apicem versus pallescentia vel hyalina, (541-)766-980(-1103) µm longa, basi (21-)26-36(-46) µm, apice (15-)19-27(-33) µm lata. Hyphae ostiolares hyalinae divergentes convergentesque, (47-)57-73(-79) µm longa. Asci fugaces, non visi. Ascosporae hyalinae, pileiformes, 3-4 µm longae, vagina exclusa 3-4 µm, inclusa 5-6 µm lata. Anamorpha Thielaviopsis: Conidiophorae biformes; primariae phialidicae, lageniformes, hyalinae, (59-)71-119(-140) µm longae, basi (3-)4-6(-7) µm, maxime 5-7(-8) µm, apice 3-5(-8) µm latae; secondariae copiosae, tubiformes, apicibus expansis, hyalinae, (53-)72-114(-148) µm longae, basi 4-6(-7) µm, apice 6-8(-9) µm latae. Conidia biformia; primaria hyalina, cylindrica, (15-)18-24(-29) µm longa, (3-)4-6 µm lata; secondaria copiosa, hyalina, doliiformia vel subglobosa, (6-)7-9(-11) µm longa, 6-8 µm lata. Chlamydosporae sparsae, brunneae, pachydermae, globosae vel subglobosae, (12-)14-16(-17) µm longae, (9-)11-13(-14) µm latae.

Colonies brown (15''k) in colour on 2% MEA. Odour banana. Hyphae smooth and segmented. Ascomatal bases globose to sub-globose, dark-brown to black, (125-)139-199(-230) µm wide, (115-)136-192(-236) µm in length. Ascomatal necks brown becoming lighter towards apices (541-)766-980(-1103) µm long, (21-)26-36(-46) µm wide at base, (15-)19-27(-33) µm wide at tip. Ostiolar hyphae of two types; hyaline, divergent and convergent, (47-)57-73(-79) µm long. Asci evanescent, not seen. Ascospores hyaline, hat-shaped, 3-4 µm in length, 3-4 µm wide excluding sheath, 5-6 µm wide including sheath. *Thielaviopsis* anamorph: Conidiophores of two morphological forms. Primary conidiophores phialidic, lageniform, hyaline, (59-)71-119(-140) µm long, (3-)4-6(-7) µm wide at base, 5-7(-8) µm wide at broadest point, 3-5(-8) µm wide at tips. Secondary conidiophores, abundant, tube-like, flaring at apices, hyaline, (53-)72-114(-148) µm long, 4-6(-7) µm wide at bases and 6–8(-9) µm wide at tips. Conidia of two types. Primary conidia, hyaline, cylindrical, (15-)18-24(-29) µm in length, (3-)4-6 µm wide. Secondary conidia, abundant, hyaline, barrel to sub-globose shaped, (6-)7-9(-11) µm in length, 6-8 µm wide. Chlamydospores rare, brown, thick-walled, globose to sub-globose, (12-)14-16(-17) µm in length by (9-)11-13(-14) µm wide.

Known distribution: São Paulo State, Brazil

Habitat: Isolated from *Mangifera indica* trees and associated with the wood-boring scolytine, *Hypocryphalus mangiferae*.

Specimens examined: **Brazil**, São Paulo State, from diseased *Mangifera indica* trees, C.J. Baker, PREM60182, CMW14797, CBS114721 (2000). **Brazil,** São Paulo State, from diseased *Mangifera indica* trees, C. Rossetto, PREM60183, CMW27306 (2007). **Brazil,** São Paulo State, from diseased *Mangifera indica* trees, C. Rossetto, PREM60184, CMW28907 (2008). **Brazil,** São Paulo State, from diseased *Mangifera indica* trees, C. Rossetto, PREM60185, CMW28908 (2008). **Brazil,** São Paulo State, from diseased *Mangifera indica* trees, C. Rossetto, PREM60186, CMW28913 (2008). **Brazil,** São Paulo State, from diseased *Mangifera indica* trees, C. Rossetto, PREM60187, CMW28914 (2008).

Notes: *Ceratocystis mangicola* is distinguished from all other species in the *C. fimbriata* s.l. complex based primarily on phylogenetic inference. However, it also has ostiolar hyphae that are both divergent and convergent as opposed to being only divergent in most species of this genus.

Ceratocystis mangivora M van Wyk and MJ Wingf. **sp. nov.** (Figure 6)

(B2; CMW27305) MB512368

Etymology: Name reflects the fact that this fungus causes a disease on mango.

Coloniae brunneae in 2% MEA, odore arienae. Hyphae laeves segmentatae. Bases ascomatum globosae vel obpyriformes, atrobrunneae vel nigrae, (171-)188-244(-295) µm latae, (174-)192-256(-310) µm longae. Colla ascomatum brunnea, apicem versus pallescentia, apice in duo vel plura ramosa, (394-)437-575(-654) µm longa, basi (21-)26-34(-40) µm, apice (16-)19-29(-35) µm lata. Hyphae ostiolares hyalinae divergentes convergentesque, (60-)75-91(-96) µm longae. Asci evanescentes, non visi. Ascosporae hyalinae, pileiformes, 3-5 µm longae, vagina exclusa 4-6 µm, illa inclusa 5-8 µm lata. Anamorpha Thielaviopsis: Conidiophorae biformes; primariae phialidicae lageniformes hyalinae (70-)78-106(-124) µm longae, basi (3-)5-7 µm, maxime 5-7(-8) µm, apice 3-5 µm latae; secondariae abundantes tubiformes apice expansae hyalinae (42-)62-100(-118) µm longae, basi (3-)4-6 µm, apice (4-)6-8(-9) µm latae. Conidia biformia; primaria hyalina cylindrica (12-)16-24(-31) µm longa, 2-5 µm lata; secondaria abundantia hyalina doliiformia (8-)9-13(-15) µm longa, (5-)6-8(-9) µm lata. Chlamydosporae absunt.

Colonies brown (15"k) in colour on 2% MEA. Odour banana. Hyphae smooth and segmented. Ascomatal bases globose to obpyriform, dark-brown to black, (171-)188-244(-295) µm wide, (174-)192-256(-310) µm in length. Ascomatal necks brown becoming lighter towards apices, branching at apices into two or more necks, (394-)437-575(-654) µm long, (21-)26-34(-40) µm wide at base, (16-)19-29(-35) µm wide at tip. Ostiolar hyphae hyaline, divergent and convergent, (60-)75-91(-96) µm long. Asci evanescent, not seen. Ascospores hyaline, hat-shaped, 3-5 µm in length, 4-6 µm wide excluding sheath, 5-8 µm wide including sheath. *Thielaviopsis* anamorph: Conidiophores of two morphological forms. Primary conidiophores phialidic, lageniform, hyaline, (70-)78-106(-124) µm long, (3-)5-7 µm wide at base, 5–7(-8) µm wide at broadest point, 3-5 µm wide at tips. Secondary conidiophores, abundant, tube-like, flaring at apices, hyaline, (42-)62-100(-118) µm long, (3-)4-6 µm wide at bases and (4-)6-8(-9) µm wide at tips. Conidia of two types. Primary conidia, hyaline, cylindrical, (12-)16-24(-31) µm in length, 2-5 µm wide. Secondary conidia, abundant, hyaline, barrel-shaped, (8-)9-13(-15) µm in length, (5-)6-8(-9) µm wide. Chlamydospores absent.

Known distribution: São Paulo State, Brazil

Habitat: Isolated from *Mangifera indica* trees. Associated with the wood-boring scolytid *Hypocryphalus mangiferae*.

Material examined: **Brazil,** São Paulo State, from diseased *Mangifera indica* trees, C. Rossetto, PREM60570, CMW27305, CBS128340 (2001). **Brazil,** São Paulo State, from diseased *Mangifera indica* trees, C. Rossetto, PREM60188, CMW15052, CBS600.70 (1970). **Brazil,** São Paulo State, from diseased *Mangifera indica* trees, C. Rossetto, PREM60189, CMW27304, CBS127204 (2001). **Brazil,** São Paulo, from diseased *Mangifera indica* trees, C. Rossetto, PREM60190, CMW27307 (2001). **Brazil,** São Paulo State, from diseased *Mangifera indica* trees, C. Rossetto, PREM60191, CMW28909 (2008). **Brazil,** São Paulo State, from diseased *Mangifera indica* trees, C. Rossetto, PREM60192, CMW28910 (2001).

Notes: Isolates of *C mangivora* can have ascomatal necks that branch dichotomously at the apices with ostiolar hyphae being either divergent or convergent. Isolates of this species also did not produce chlamydospores in culture.

DISCUSSION

Results of this study showed that a relatively large collection of isolates of *Ceratocystis* from mango trees suffering from Mango blight in Brazil, reside in two distinct phylogenetic clades. These groups are, furthermore, distinct from *C. manginecans* that causes a similar disease of mango in Oman and Pakistan (Al Adawi *et al*. 2006, Van Wyk *et al.* 2005, 2007a). The isolates residing in these two groups are consequently treated as distinct taxa and the names *C. mangicola* and *C. mangivora* have been provided for them.

The blight disease of mango trees in Brazil has been known for almost a century and was previously ascribed to *C. fimbriata* s.l., a fungus that we now recognise as representing a relatively large number of cryptic taxa. These species are morphologically very similar and although individual species can be distinguished from their closest relatives, recognition based solely on morphological characteristics would be very difficult. This situation is the same as that found for many groups of fungi, for example species of *Fusarium* in the *Gibberella fujikuroi* species complex (Leslie *et al*. 1992, O'Donnel *et al*. 2000). *Ceratocystis mangicola* is phylogenetically most closely related to *C. manginecans* a known pathogen of mango and other crops in Oman and Pakistan. *Ceratocystis mangivora* also described in this study has no sister group but is also closely related to the other two mango pathogens. Isolates of these species were not only distinct from each other but also phylogenetically distinct from the mango pathogen, *C. manginecans*. The species phylogenetically

most closely related to these mango pathogens from Brazil was *C. fimbriatomima*, which was first isolated from *Eucalyptus* trees in Venezuela (Van Wyk *et al.* 2009b).

Phylogenetic analyses of sequences for the ITS gene region gave strong support for the separation of *C. mangicola* and *C. mangivora*. In contrast the *β*t and EF1-*α* gene regions for the single gene trees showed little or no variation between *C. mangicola* and *C. mangivora*. This is not uncommon for species in the *C. fimbriata* complex (Van Wyk *et al*. 2010). Similarly the allele trees for four species; *C. fimbriatomima* and the three mango pathogens, *C. manginecans*, *C. mangicola* and *C. mangivora* showed that the three species from mango were most closely related to each other. This suggests a common ancestor for the three mango pathogens and the fact that they have probably undergone speciation relatively recently.

Ceratocystis mangicola and *C. mangivora* are morphologically very similar, both having cultures that are dark brown in colour and that produce a banana odour that is characteristic of most species of *Ceratocystis.* However, isolates representing the two species could be distinguished from each other based on various micro-morphological characteristics. Thus, *C. mangicola* isolates have ostiolar hyphae that are both divergent and convergent and this is a characteristic has not been described for other species of *C. fimbriata* s.l. other than in *C. mangivora* described in this study. Isolates of *C. mangivora* consistently display branched ascomatal necks that give rise to either convergent or divergent ostiolar hyphae. Furthermore, similar to some species in *C. fimbriata* s.l., *C. mangivora* did not produce chlamydospores in culture while these structures are very obvious in cultures of *C. mangicola. Ceratocystis mangicola* isolates also have globose to sub-globose ascomatal bases compared to the globose to obpyriform bases in *C. mangivora.*

A previous study on *C. fimbriata* s.l. from different hosts including mango in Brazil, treated the isolates as a genetically diverse population representing a single taxon (Fereirra *et al*. 2010). It would have been interesting to include data for isolates from that study in the present investigation and this might also have provided a more robust species delimitation for *C. mangicola* and *C. mangivora*. These isolates were, however, not available for study and sequence data have also not been deposited in GenBank precluding comparisons at that level.

An interesting aspect of the mango blight disease in Brazil and in Oman is the fact that the pathogens are associated with the same wood boring insect *H. mangiferae* in both areas of the world.

Hypocryphalus mangiferae is a monophagous bark beetle found only on species of *Mangifera* (Schedl 1961). Its area of origin is thus likely to be the same as mango trees in tropical Asia (Wood 1982, Butani 1993, Kostermans & Bompard 1993). Both the tree and the beetle have been introduced into Brazil (Wood 1982, Butani 1993, Kostermans & Bompard 1993).

Both *C. mangicola* and *C. mangivora* are suspected to be native to Brazil. As with most species of *Ceratocystis,* a wound is required for *C. mangicola* and *C. mangivora* to infect mango trees (Silva *et al*.1959). Intensive studies of diseased mango trees in Brazil have shown that in the early stages of the disease, *H. mangiferae* is the only insect present. *Xyleborus* spp. typically appear when the disease spreads down towards the larger branches and Cerambycidae and Curlionidae only appear when the disease reaches the trunk regions (Silva *et al*. 1959, Castro 1960, Medeiros & Rossetto 1966, Rossetto *et al*. 1980). Studies have also shown that *H. mangiferae* is the primary vector of *C. mangicola* and *C. mangivora* in Brazil (Ribeiro & Rossetto 1971). A similar vector relationship has also been shown for *C. manginecans* in Oman (Al Adawi *et al*. 2006, Van Wyk *et al.* 2007)

The fact that *H. mangiferae* has become associated with three cryptic species of *Ceratocystis* is not surprising. Species in this group of fungi easily establish relationships with insects (Kile 1993, Roux & Wingfield 2009) and this is probably facilitated by the fruity aromas that they produce. The association between *C. mangicola*, *C. mangivora* and *C. manginecans* with *H. mangiferae* is very similar to emerging new associations between ambrosia beetles and tree pathogens such as those found in Laurel Wilt Disease in the USA (Mayfield *et al*. 2008) and oak decline in Asia (Kamata *et al*. 2002).

Providing names to species reflected by phylogenetic lineages such as those represented by *C. mangicola, C. mangivora* and *C. manginecans* might be considered a matter of opinion regarding the level of relatedness of these fungi. An alternative view would for example be to treat them as components of a population of the single species *C. fimbriata*. Our view of this situation is that having names that define distinctly different phylogenetic groupings provides a mechanism to easily distinguish differences. Such differences are undoubtedly valuable in studying important diseases, including aspects of host pathogen interaction and resistance. Furthermore, providing names for *C. mangicola, C. mangivora* and *C. manginecans* as we have chosen to do in this and a previous study (Van Wyk *et al*. 2007a) will facilitate quarantine procedures and efforts to curb the global movement of tree pathogens (Wingfield *et al*. 2001, Slippers *et al*. 2005).

REFERENCES

Al Adawi A.O., Deadman M.L., Al Rawahi A.K., Al Maqbali Y.M., Al Jahwari A.A., Al Saadi B.A., Al Amri I.S. & Wingfield M.J. (2006) Aetiology and causal agents of mango sudden decline disease in the Sultanate of Oman. *European Journal of Plant Pathology* **116**: 247–254.

Atkinson T.H. & Peck S.B. (1993) Annotated checklist of the bark and ambrosia beetles (Coleoptera: Platypodidae and Scolytidae) of tropical southern Florida. *Florida Entomologist* **77**: 313–329.

Barnes I., Nakabonge G., Roux J., Wingfield B.D. & Wingfield M.J. (2005) Comparisons of populations of the wilt pathogen *Ceratocystis albifundus* in South Africa and Uganda. *Plant Pathology* **54**: 189–195.

Butani D.K. (1993) *Mango: Pest Problems.* Periodical Expert Book. Agency D-42 Vivek Vihar: Delhi (India).

Castro R. da Silva (1960) Contribuição ao estudo de Hypocryphalus mangiferae (Stebbing, 1914) (Coleoptera, Scolytidae). Ciclo biológico e etologia. Recife. Tese para concurso de professor livre-docente da 9ª cadeira- entomologia e parasitologia- da Escola Superior de Agricultura da Universidade Rural de Pernambuco, 54.

Clement M., Posada D. & Crandall K.A. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**: 1657–1659.

Cunningham C.W. (1997) Can three incongruence tests predict when data should be combined? *Molecular Biology and Evolution* **14**: 733–740.

De Toledo Piza C. (ed.) (1966) Anais do Simposio Sobre a Seca da Mangueira. (Abstract: *Review of Applied Mycology* **46**: 378, 1967

DeVay J.E., Lukezic F.L., English W.H. & Trujillo E.E. (1963) Ceratocystis canker of stone fruit trees. *Phytopathology* **53**: 873.

Engelbrecht C.J.B. & Harrington T.C. (2005) Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. *Mycologia* **97**: 57–69.

Ferreira E.M., Harrington T.C., Thorpe D.J. & Alfenas A.C. (2010) Genetic diversity and interfertility among highly differentiated populations of *Ceratocystis frimbriata* in Brazil. *Plant Pathology* **59**: 721–735.

Glass N.L. & 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.

Halsted B.D. (1890) Some fungous diseases of the sweet potato. *New Jersey Agricultural College Experiment Station.* **76**: 1–32.

Jacobs K., Bergdahl D.R., Wingfield M.J., Halik S., Seifert K.A., Bright D.E. & Wingfield B.D. (2004) *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* **108**: 411–418**.**

Kamata N., Esaki K., Kato K., Igeta Y. & Wada K. (2002) Potential impact of global warming on deciduos oak dieback caused by ambrosia fungus *Raffaelea* sp. carried by ambrosia beetle *Platypus quercivorus* (Coleoptera: Platypodidae) in Japan. *Bulletin of Entomological Research* **92**: 119–126.

Katoh K., Misawa K., Kuma K. & Miyata T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**: 3059–3066.

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 pp.

Kostermans A.J.G.H & Bompard J.M. (1993) The mangoes. Their botany, nomenclature, horticulture and utilization. Academic Press, New York. 233 pp.

Leslie J.F., Plattner R.D., Desjardins A.E. & Klittich C.J.R. (1992) Fumonisin B1 production by strains from different mating populations of *Gibberella fujikuroi* (*Fusarium* section *Liseola*). *Phytopathology* **82**: 341–345.

Mayfield A.E., Smith J.A., Hughes M. & Dreaden T.J. (2008) First report of Laurel wilt disease caused by a *Raffaelea* sp. on Avocado in Florida. *Plant Disease* **92**: 976.

Medeiros J.W.A. & Rossetto C.J. (1966) Seca-da-mangueira. I Observações preliminares. O Agronomico, Campinas, SP, Brasil. **18**:1–11.

Nylander J.A.A. (2004) MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

O'Donnell K., Nirenberg H.I., Aoki T. & Cigelnik I. (2000) A multigene phylogeny of the *Giberella fujikuroi* species complex: Detection of additional phylogenetically distinct species. *Mycoscience* **41**: 61–78.

Piza C de T. (ed.) 1966. Anais do Simposio Sobre a Seca da Mangueira. (Abstract: *Review of Applied Mycology* **46**: 378, 1967).

Ploetz R.C. (2003) Diseases of mango. In: *Diseases of tropical fruit crops* (ed. R.C. Ploetz). CABI Publishing: Wallingford Oxford (UK). **15**:1–35 pp.

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

Ribeiro I.J.A. (1980) Seca da mangueira. Agentes causais e studio da molestia. *In: Anais do I Simposio Brasiliero Sobre a Cultura de Mangueira*. Sociedad Brasileira de Fruticultura, Jacoticobal, November 24-28, 1980, 123–130.

Ribeiro I.J.A. & Rossetto C.J. (1971) Seca-da-mangueira. V Isolamento de *Ceratocystis fimbriata* de *Hypocryphalus mangiferae* e freqüência de sintomas iniciais no campo. Anais do I Cogresso Brasileiro de fruticultura. Campinas, 12-16 de julho 1971. Vol 2, pp. 607–616.

Ronquist F. & Huelsenbeck J.P. (2003) MrBayes 3. Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Rossetto C.J., Ribeiro I.J.A. & Igue T. (1980) Seca-da-mangueira. III. Comportamento de variedades de mangueira, espécies de coleobrocas e comportamento de *Hypocryphalus mangiferae*. Circular 106. Instituto Agronômico de campinas. pp 44.

Roux J. & Wingfield M.J. (2009) *Ceratocystis* species: Emerging pathogens of non-native plantation *Eucalyptus* and *Acacia* species. *Southern Forests* **71**: 115–120.

Schedl K.E. (1961) *Hypocraphalus mangifera* Stebbing. In: Scolytidae und Platypodidae Afrikas I. *Revista de Entomologia Mocambique* **4**: 543–544.

Silva J.N., Gayão T.C. & Castro R.S. (1959) A morte das mangueiras do Recife (Resultados preliminares do estudo dessa doença). Pernambuco, Instituto Agronômico do Nordeste, *Boletim Técnico* **7**: 38.

Slippers B., Stenlid J. & Wingfield M.J. (2005) Emerging pathogens: fungal host jumps following anthropogenic introduction. *Trends in Ecology and Evolution* **20**: 420–421.

Sullivan J. (1996) Combining data with different distributions of among-site variation. *Systematic Biology* **45**: 375–380.

Swofford D.L. (2002) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Tamura K., Dudley J., Nei M. & Kumar S. (2007) MEGA4: Molecular evolutionary genetics analyses (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596–1599.

Van Wyk M., Al Adawi A.O., Wingfield B.D., Al Subhi A.M., Deadman M.L. & Wingfield M.J. (2005). DNA based characterization of *Ceratocystis fimbriata* isolates associated with mango decline in Oman. *Australasian Plant Pathology* **34**: 587–590.

Van Wyk M., Al Adawi A.O., Khan A., Deadman M.L., Al Jahwari A.A., Wingfield B.D., Ploetz R.C. & Wingfield M.J. (2007a) *Ceratocystis manginecans* sp. nov., causal agent of a destructive mango wilt disease in Oman and Pakistan. *Fungal Diversity* **27**: 213–230.

Van Wyk M., Pegg G., Lawson S. & Wingfield M.J. (2007b) *Ceratocystis atrox* sp. nov associated with *Phoracanthta acanthocera* infestations on *Eucalyptus* in Australia. *Australian Journal of Plant Pathology* **36**: 407–414.

Van Wyk M., Roux J., Barnes I., Wingfield B.D. & Wingfield M.J. (2006) Molecular phylogeny of the *Ceratocystis moniliformis* complex and description of *C. tribiliformis* sp. nov. *Fungal Diversity* **21**: 181–201.

Van Wyk M., Wingfield B.D., Clegg P.A. & Wingfield M.J. (2009a) *Ceratocystis larium* sp. nov., a new species from *Styrax benzoin* wounds associated with incense harvesting in Indonesia. *Persoonia* **22**: 75–82.

Van Wyk M., Wingfield B.D., Mohali S. & Wingfield M.J. (2009b). *Ceratocystis fimbriatomima*, a new species in the *C. fimbriata sensu lato* complex isolated from *Eucalyptus* trees in Venezuela. *Fungal Diversity* **34**: 173–183.

Van Wyk M., Wingfield B.D. & Wingfield M.J. (2010) *Ceratocystis* species in the *Ceratocystis fimbriata* complex. In: *Ceratophiostoma*. North Stadbroke Island, Brisbane, Australia, August 2006. (In press)

Van Wyk M., Wingfield B.D. & Wingfield M.J. (2011) Four new *Ceratocystis* spp. associated with wounds on *Eucalyptus*, *Schizolobium* and *Terminalia* trees in Ecuador. *Fungal Diversity* **46**: 111–131.

Viegas A.P. (1960) Mango blight. *Bragantia* **19**: 163–182 (abstracted in Review of Applied *Mycology* **42**: 696, 1963)

Webster R.K. & Butler E.E. (1967a) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45**: 1457–1468.

Webster R.K. & Butler E.E. (1967b) The origin of self-sterile, cross-fertile strains and culture sterility in *Ceratocystis fimbriata*. *Mycologia* **59**: 212–221.

White T.J., Bruns T., Lee S. & 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 (USA). 315–322 pp.

Wingfield M.J., De Beer C., Visser C. & Wingfield B.D. (1996) A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematic and Applied Microbiology* **19**: 191–202.

Wingfield M.J., Slippers B., Roux J. & Wingfield B.D. (2001) Worldwide movement of exotic forest fungi, especially in the tropics and the southern hemisphere. *BioScience* **51**: 134–140.

Wood S.L. (1982) The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. *Great Basin Naturalist, Memoirs* **6**: 1–1356.

Yamashiro T. & Myazaki I. (1985) Principal pests and diseases of mango - *Mangifera indica* L. - in the State of Sao Paulo and updated control methods. *Biológico* **51**:41–50.

Table 1. *Ceratocystis* spp. for which isolates or sequences were used in this study.

Species	Isolate no.	GenBank accession no.	Host	Geographical origin
C. acaciivora	CMW22563	EU588656, EU588636, EU588646	*Acacia mangium*	Indonesia
C. acaciivora	CMW22564	EU588657, EU588637, EU588647	*Acacia mangium*	Indonesia
C. albifundus	CMW4068	DQ520638 EF070429 EF070400	*Acacia mearnsii*	RSA
C. albifundus	CMW5329	AF388947 DQ371649 EF070401	*Acacia mearnsii*	Uganda
C. atrox	CMW19383 CBS120517	EF070414 EF070430 EF070402	*Eucalyptus grandis*	Australia
C. atrox	CMW19385 CBS120518	EF070415 EF070431 EF070403	*Eucalyptus grandis*	Australia
C. cacaofunesta	CMW15051 CBS152.62	DQ520636 EF070427 EF070398	*Theobroma cacao*	Costa Rica
C. cacaofunesta	CMW14809 CBS115169	DQ520637 EF070428 EF070399	*Theobroma cacao*	Ecuador
C. colombiana	CMW9565 CBS121790	AY233864 AY233870 EU241487	Soil	Colombia
C. colombiana	CMW5751 CBS121792	AY177233 AY177225 EU241493	*Coffea arabica*	Colombia
C. colombiana	CMW9572	AY233863 AY233871 EU241488	*Mandarin*	Colombia
C. caryae	CMW14793 CBS114716	EF070424 EF070439 EF070412	*Carya cordiformis*	USA
C. caryae	CMW14808 CBS115168	EF070423 EF070440 EF070411	*Carya ovata*	USA
C. curvata	CMW22442 CBS122603	FJ151436 FJ151448 FJ151470	*Eucalyptus deglupta*	Colombia
C. curvata	CMW22435 CBS122604	FJ151437 FJ151449 FJ151471	*Eucalyptus deglupta*	Colombia
C. diversiconidia	CMW22445 CBS123013	FJ151440 FJ151452 FJ151474	*Terminalia ivorensis*	Colombia
C. diversiconidia	CMW22446	FJ151443 FJ151455 FJ151477	*Terminalia ivorensis*	Colombia
C. ecuadoriana	CMW22092 CBS124020	FJ151432 FJ151444 FJ151466	*Eucalyptus deglupta*	Colombia
C. ecuadoriana	CMW22093 CBS124021	FJ151433 FJ151445 FJ151467	*Eucalyptus deglupta*	Colombia
C. fimbriata s.s.	CMW15049	DQ520629	*Ipomoea batatas*	USA

	CBS141.37	EF070442 EF070394		
C. fimbriata s.s.	CMW1547	AF264904 EF070443 EF070395	*Ipomaea batatas*	Papua New Guinea
C. fimbriata s.l.	C1345	AY157966	*Eucalyptus*	Brazil
C. fimbriata s.l.	C1987	AY585344	*Eucalyptus*	Brazil
C. fimbriata s.l.	C2041	AY585345	*Acacia mearnsii*	Brazil
C. fimbriata s.l.	CMW14811 CBS115171 C1905	AY526288	*Colocasia esculenta*	Brazil
C. fimbriata s.l.	CMW14791 CBS114713 C1865	AY526286	*Colocasia esculenta*	Brazil
C. fimbriata s.l.	C1900	AY526287	*Colocasia esculenta*	Brazil
C. fimbriata s.l.	C2032	AY526289	*Colocasia esculenta*	Brazil
C. fimbriata s.l.	C925	AY157967	*Gmelina arborea*	Brazil
C. fimbriata s.l.	CMW14806 CBS115166 C1782	AY526292	*Ficus carica*	Brazil
C. fimbriata s.l.	CMW14796 CBS114720 C1715	AY526307	*Colocasia esculenta*	USA, Hawaii
C. fimbriata s.l.	CMW14804 CBS115164 C1714	AY526306	*Colocasia esculenta*	USA, Hawaii
C. fimbriata s.l.	BPI596162	AY526305	*Colocasia esculenta*	China
C. fimbriata s.l.	C1558	AY157965	*Mangifera indica*	Brazil
C. fimbriatomima	CMW24174 CBS121786	EF190963 EF190951 EF190957	*Eucalyptus* sp.	Venezuela
C. fimbriatomima	CMW24176 CBS121787	EF190964 EF190952 EF190958	*Eucalyptus* sp.	Venezuela
C. larium	CMW25434 CBS122512	EU881906 EU881894 EU881900	*Styrax benzoin*	Indonesia
C. larium	CMW25435 CBS122606	EU881907 EU881895 EU881901	*Styrax benzoin*	Indonesia
C. mangicola	**CMW14797 CBS114721 C1688**	**AY953382 EF433307 EF433316**	***Mangifera indica***	**Brazil**
C. mangicola	**CMW27306**	**FJ200256 FJ200269 FJ200282**	***Mangifera indica***	**Brazil**
C. mangicola	**CMW28907**	**FJ200257 FJ200270 FJ200283**	***Mangifera indica***	**Brazil**
C. mangicola	**CMW28908**	**FJ200258 FJ200271 FJ200284**	***Mangifera indica***	**Brazil**
C. mangicola	**CMW28913**	**FJ200259 FJ200272 FJ200285**	***Mangifera indica***	**Brazil**
C. mangicola	**CMW28914**	**FJ200260 FJ200273 FJ200286**	***Mangifera indica***	**Brazil**
C. manginecans	CMW13851 CBS121659	AY953383 EF433308 EF433317	*Mangifera indica*	Oman

C. manginecans	CMW13852 CBS121660	AY953384 EF433309 EF433318	*Hypocryphalus mangifera*	Oman
C. manginecans	CMW23634	EF433302	*Mangifera indica*	Pakistan
C. manginecans	CMW23628	EF433303	*Mangifera indica*	Pakistan
C. mangivora	**CMW15052** **CBS600.70** **C74**	**EF433298** **EF433306** **EF433315**	***Mangifera indica***	**Brazil**
C. mangivora	**CMW27304**	**FJ200261** **FJ200274** **FJ200287**	***Mangifera indica***	**Brazil**
C. mangivora	**CMW27305**	**FJ200262** **FJ200275** **FJ200288**	***Mangifera indica***	**Brazil**
C. mangivora	**CMW27307**	**FJ200263** **FJ200276** **FJ200289**	***Mangifera indica***	**Brazil**
C. mangivora	**CMW28909**	**FJ200264** **FJ200277** **FJ200290**	***Mangifera indica***	**Brazil**
C. mangivora	**CMW28910**	**FJ200265** **FJ200278** **FJ200291**	***Mangifera indica***	**Brazil**
C. mangivora	**CMW28911**	**FJ200266** **FJ200279** **FJ200292**	***Mangifera indica***	**Brazil**
C. mangivora	**CMW28912**	**FJ200267** **FJ200280** **FJ200293**	***Mangifera indica***	**Brazil**
C. mangivora	**CMW28916**	**FJ200260** **FJ200281** **FJ200294**	***Mangifera indica***	**Brazil**
C. neglecta	CMW17808 CBS121789	EF127990 EU881898 EU881904	*Eucalyptus* sp.	Colombia
C. neglecta	CMW18194 CBS121017	EF127991 EU881899 EU881905	*Eucalyptus* sp.	Colombia
C. obpyriformis	CMW23807 CBS122608	EU245004 EU244976 EU244936	*Acacia mearnsii*	South Africa
C. obpyriformis	CMW23808 CBS122511	EU245003 EU244975 EU244935	*Acacia mearnsii*	South Africa
C. papillata	CMW8857	AY233868 AY233878 EU241483	*Annona muricata*	Colombia
C. papillata	CMW8856 CBS121793	AY233867 AY233874 EU241484	*Citrus lemon*	Colombia
C. papillata	CMW10844	AY177238 AY177229 EU241481	*Coffea arabica*	Colombia
C. pirilliformis	CMW6569	AF427104 DQ371652 AY528982	*Eucalyptus nitens*	Australia
C. pirilliformis	CMW6579 CBS118128	AF427105 DQ371653 AY528983	*Eucalyptus nitens*	Australia
C. platani	CMW14802 CBS115162	DQ520630 EF070425 EF070396	*Platanus occidentalis*	USA
C. platani	CMW23918	EF070426 EF070397	*Platanus* sp.	Greece

		EU426554		
C. polychroma	CMW11424	AY528970	*Syzygium*	Indonesia
	CBS115778	AY528966	*aromaticum*	
		AY528978		
C. polychroma	CMW11436	AY528971	*Syzygium*	Indonesia
	CBS115777	AY528967	*aromaticum*	
		AY528979		
C. polyconidia	CMW23809	EU245006	*Acacia mearnsii*	South Africa
	CBS122289	EU244978		
		EU244938		
C. polyconidia	CMW23818	EU245007	*Acacia mearnsii*	South Africa
	CBS122290	EU244979		
		EU244939		
C. populicola	CMW14789	EF070418	*Populus* sp.	Poland
	CBS119.78	EF070434		
		EF070406		
C. populicola	CMW14819	EF070419	*Populus* sp.	USA
	CBS114725	EF070435		
		EF070407		
C. smalleyi	CMW14800	EF070420	*Carya*	USA
	CBS114724	EF070436	*cordiformis*	
		EF070408		
C. smalleyi	CMW26383	EU426553	*Carya*	USA
	CBS114724	EU426555	*cordiformis*	
		EU426556		
C. tanganyicensis	CMW15991	EU244997	*Acacia mernsii*	Tanzania
	CBS122295	EU244969		
		EU244929		
C. tanganyicensis	CMW15999	EU244998	*Acacia mearnsii*	Tanzania
	CBS122294	EU244970		
		EU244939		
C. tsitsikammensis	CMW14276	EF408555	*Rapanea*	South Africa
	CBS121018	EF408569	*melanophloeos*	
		EF408576		
C. tsitsikammensis	CMW14278	EF408556	*Rapanea*	South Africa
	CBS121019	EF408570	*melanophloeos*	
		EF408577		
C. variospora	CMW20935	EF070421	*Quercus alba*	USA
	CBS114715	EF070437		
		EF070409		
C. variospora	CMW20936	EF070422	*Quercus robur*	USA
	CBS114714	EF070438		
		EF070410		
C. virescens	CMW11164	DQ520639	*Fagus*	USA
		EF070441	*americanum*	
		EF070413		
C. virescens	CMW3276	AY528984	*Quercus robur*	USA
		AY528990		
		AY529011		
C. zombamontana	CMW15235	EU245002	*Eucalyptus* sp.	Malawi
		EU244974		
		EU244934		
C. zombamontana	CMW15236	EU245000	*Eucalyptus* sp.	Malawi
		EU244972		
		EU244932		

CMW numbers are in the Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa

CBS numbers are in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

C numbers are in the T. Harrington collection Iowa State University, USA

BPI numbers are in the US National Fungus collection.

Table 2. Comparison of sequences and the number of fixed alleles differing between *Ceratocystis* spp. obtained from mango as well as the phylogenetically closely related species *C. fimbriatomima*. Shaded cells indicate variations within each species.

ITS	***C. mangicola***	***C. mangivora***	***C. manginecans***	***C. fimbriatomima***
C. mangicola	**4**	16	6	10
C. mangivora	16	**2**	20	14
C. manginecans	6	20	**0**	14
C. fimbriatomima	10	14	14	**1**

βt	***C. mangicola***	***C. mangivora***	***C. manginecans***	***C. fimbriatomima***
C. mangicola	**1**	0	5	8
C. mangivora	0	**3**	4	7
C. manginecans	5	4	**0**	3
C. fimbriatomima	8	7	3	**1**

EF-1α	***C. mangicola***	***C. mangivora***	***C. manginecans***	***C. fimbriatomima***
C. mangicola	**1**	0	1	0
C. mangivora	0	**9**	1	0
C. manginecans	1	1	**0**	1
C. fimbriatomima	0	0	1	**0**

Figure 1. Phylogenetic tree based of the ITS gene region for *Ceratocystis mangicola* (B1), *C. mangivora* (B2) and other species in the *C. fimbriata* s.l. complex including isolates from Brazil obtained from various hosts. Bootstrap values are indicated at the branches, with Bayesian support in brackets.

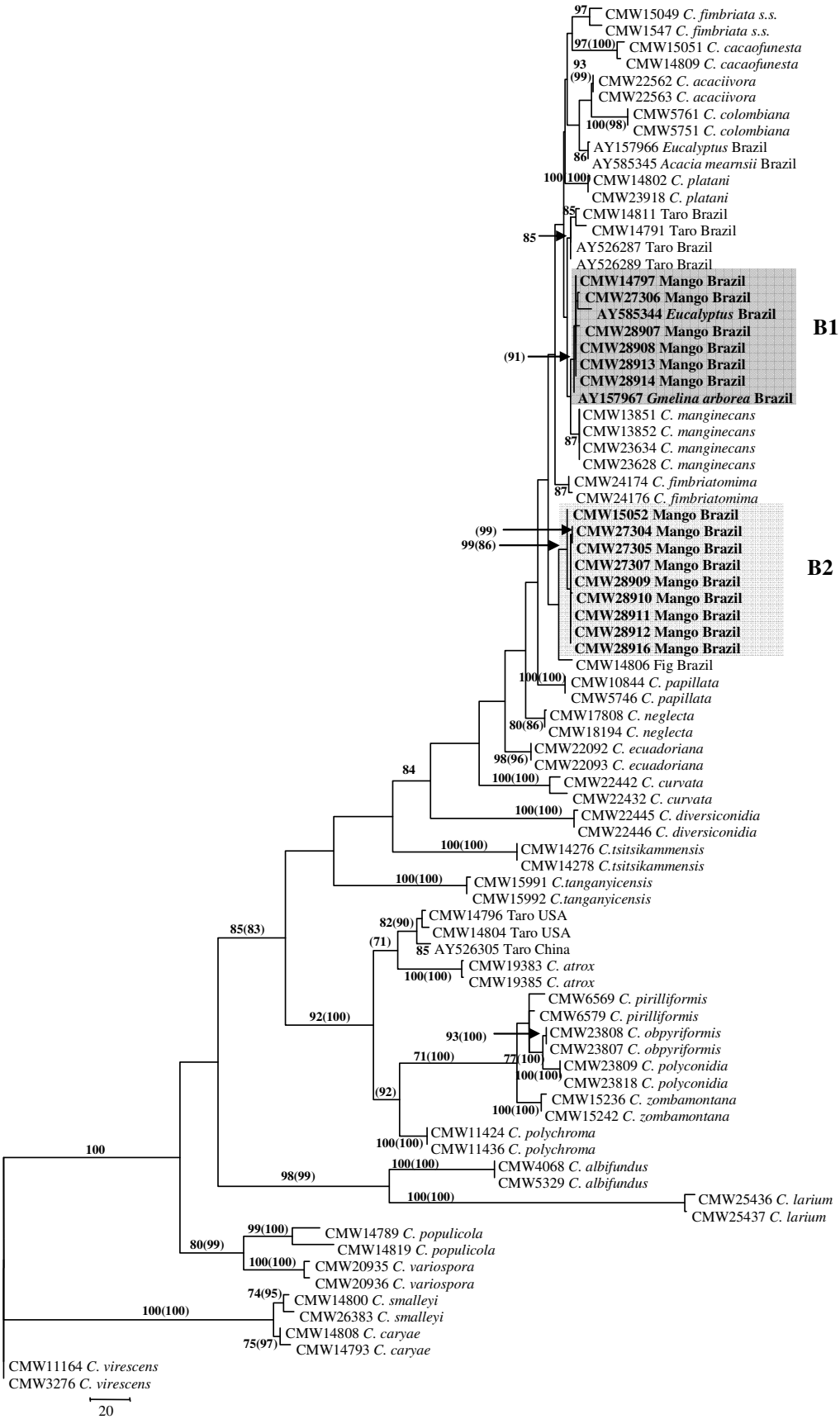


Figure 2. Phylogenetic tree based on the combined regions of the ITS, β-tubulin and EF1-α for *Ceratocystis mangicola* (B1), *C. mangivora* (B2) and other species in the *C. fimbriata* s.l. species complex. Bootstrap values are indicated at the branches, with Bayesian support in brackets.

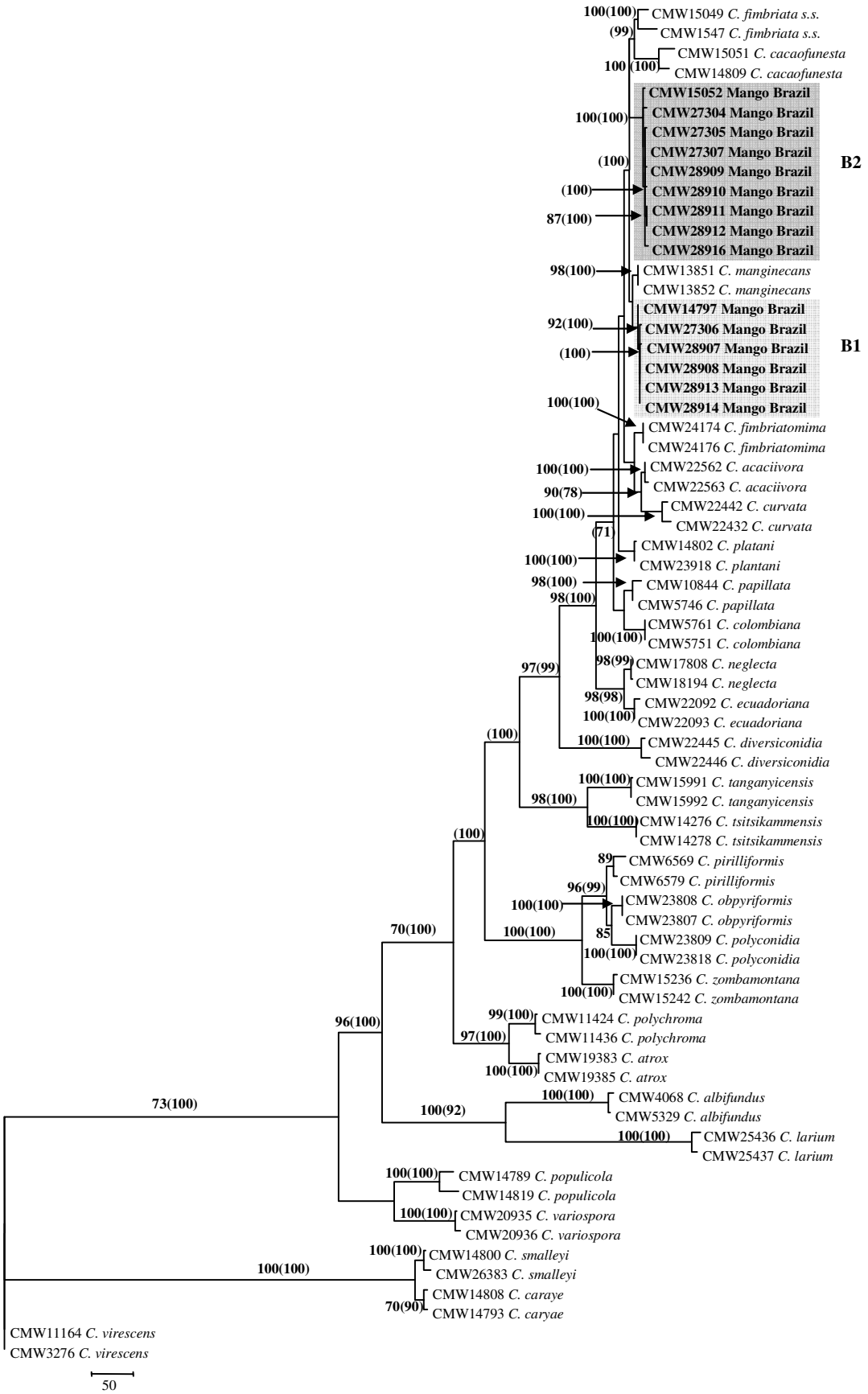
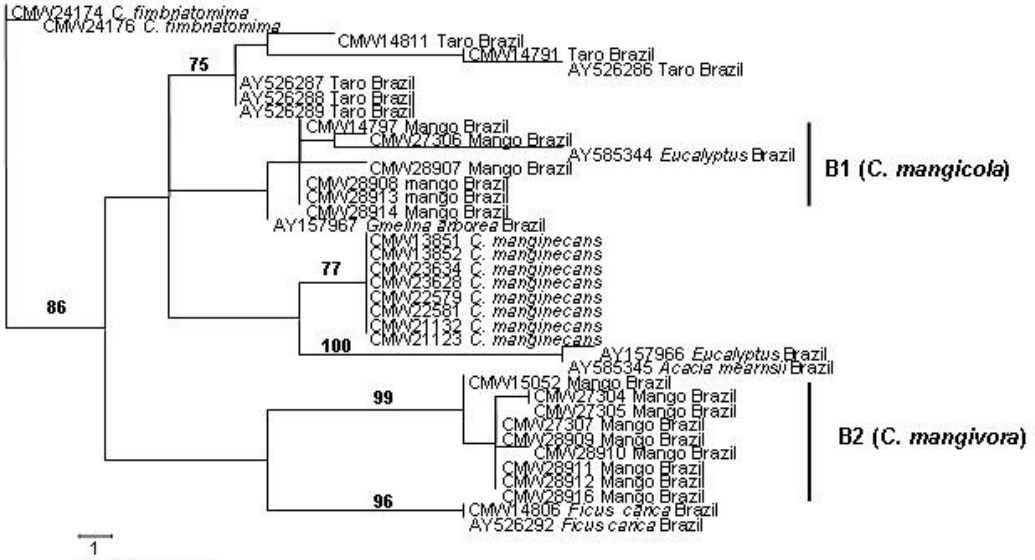
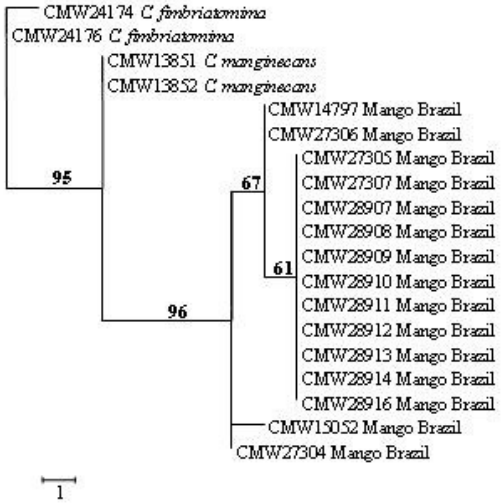


Figure 3. Three separate unrooted phylogenetic trees representing three gene regions. Isolates representing the two groups from Brazil mango as well as *Ceratocystis manginecans* and *C. fimbriatomima* were included. a. ITS. b. β-tubulin. c. EF1-α.

a.



b.



c.

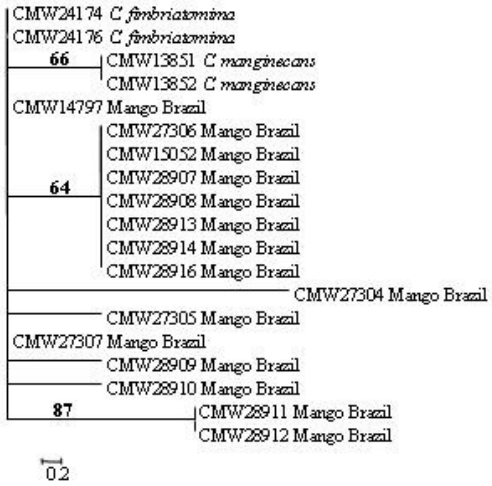


Figure 4. An allele network of the two groups of isolates from Brazil as well as a closely related species *C. manginecans* also isolated from mango trees and *Ceratocystis fimbriatomima*. The numbers represent CMW numbers (Table 1).

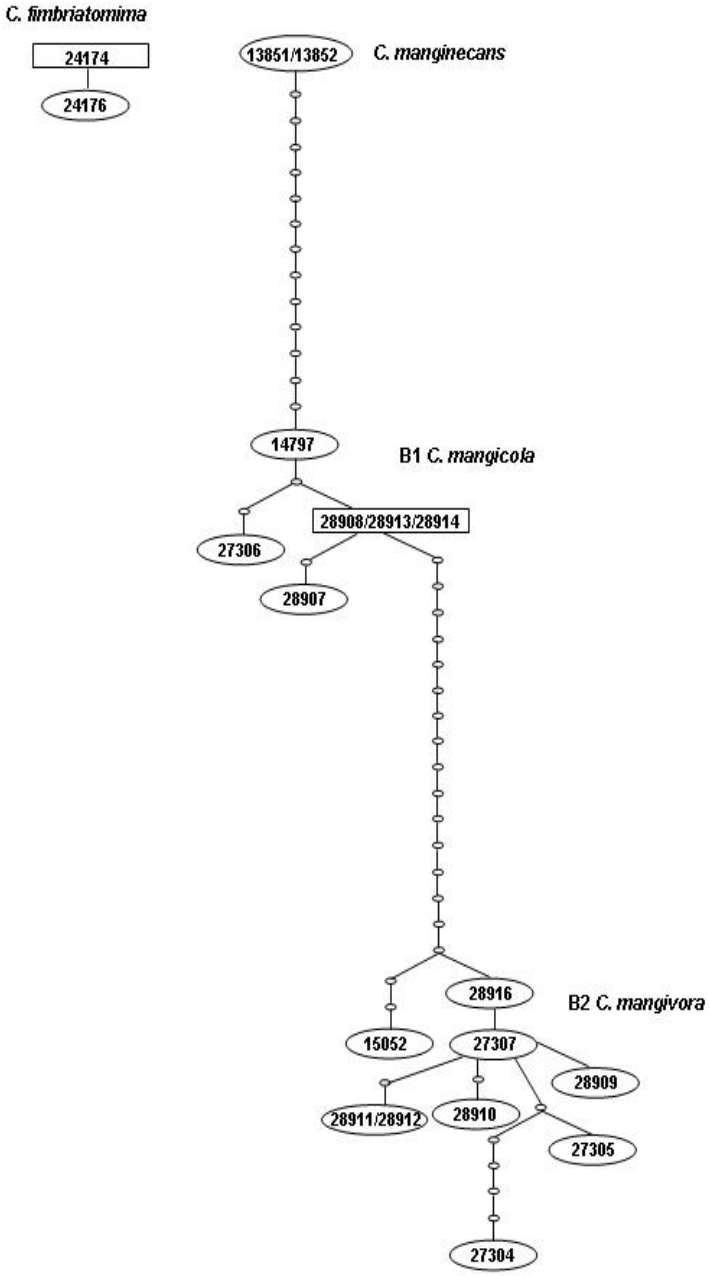


Figure 5. Morphological characteristics of *Ceratocystis mangicola* (from holotype): a. Globose ascomata. b. Divergent ostiolar hyphae. c. Convergent stiolar hyphae. d. Primary phialidic conidiophore. e. Secondary conidiophore with emerging chain of barrel-shaped conidia. f. Dark colored chlamydospores. g. Hat-shaped ascospores. h. Cylindrical conidia. i. Chain of barrel-shaped conidia. Scale bars: a = 100 µm, b, c, e, f, h, i = 10 µm, d = 20 µm, g = 5 µm.

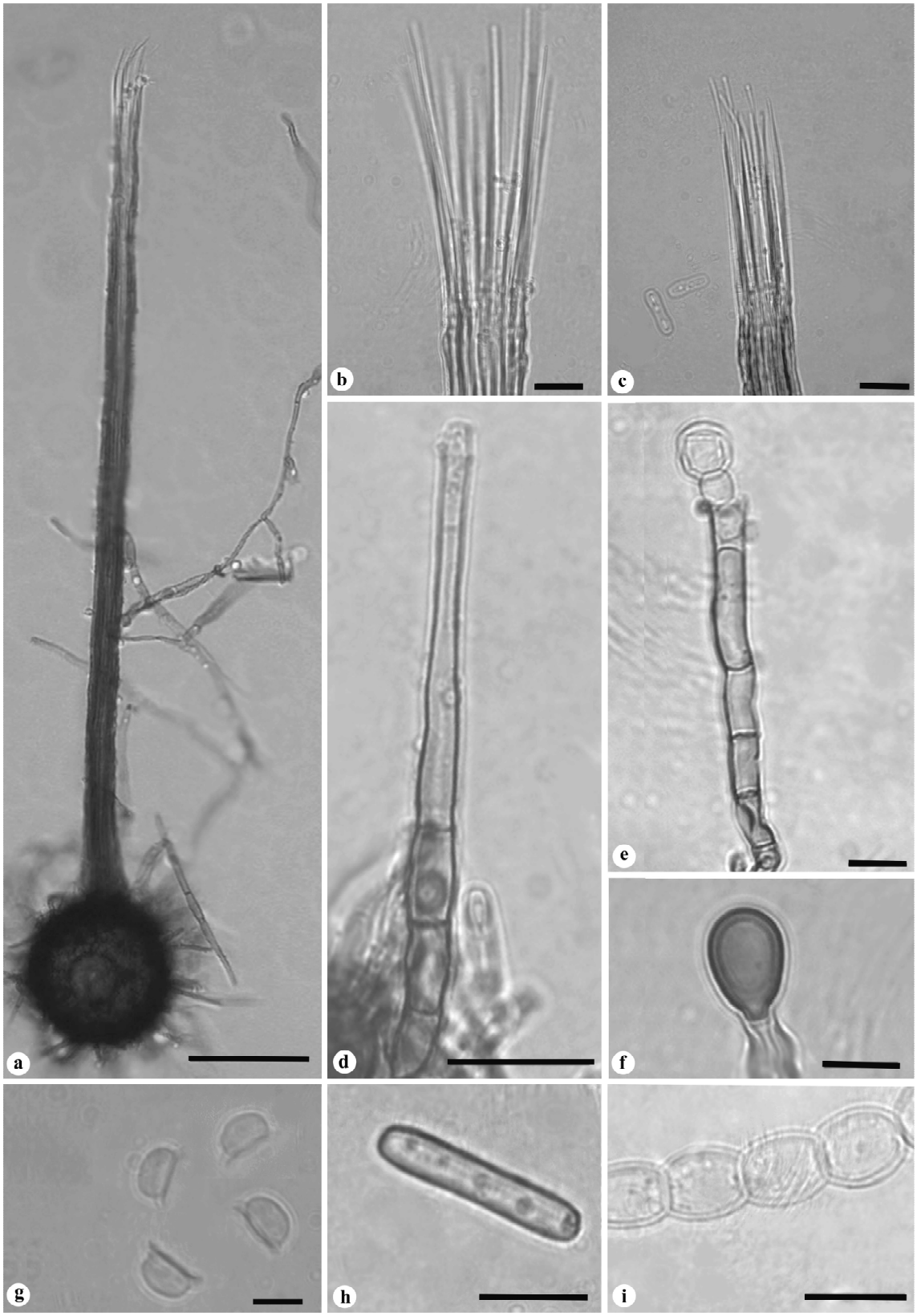
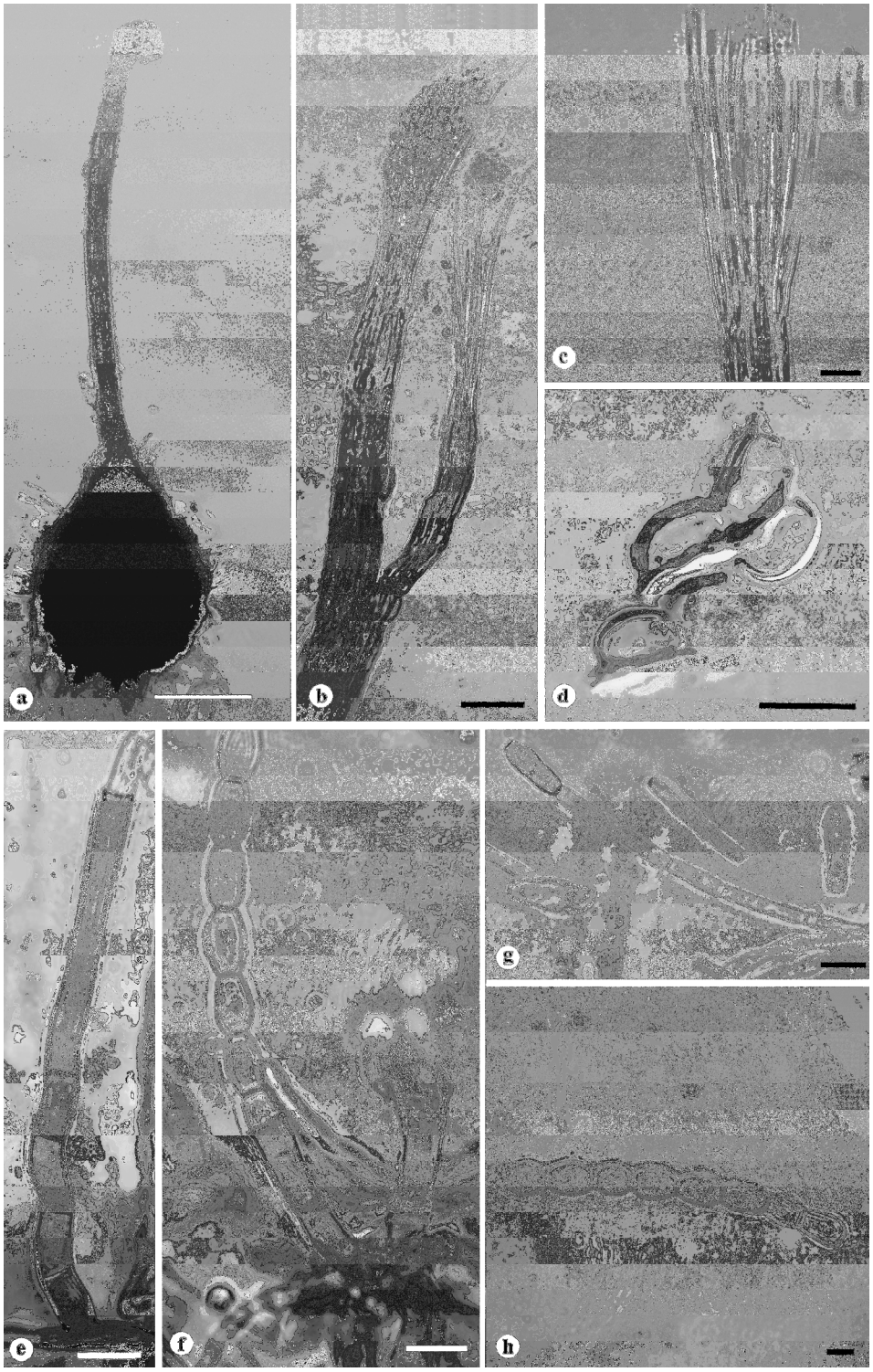


Figure 6. Morphological characteristics of *Ceratocystis mangivora* (from holotype): a. Globose to obpyriform ascomata. b. Ascomatal neck branching into two apices with both convergent and divergent ostiolar hyphae. c. Convergent ostiolar hyphae. d. Hat-shaped ascospores. e. Primary conidiophore. f. Secondary conidiophore with emerging chain of barrel-shaped conidia. g. Chain of cylindrical conidia. h. Chain of barrel-shaped conidia. Scale bars: a = 100 µm, b, c, e, f, g = 10 µm, d, h = 5 µm



CHAPTER 11

Ceratocystis eucalypticola sp. nov., from *Eucalyptus* in South Africa and comparison to global isolates from this tree

Van Wyk M., Roux J., Kamgan Nkuekam G., Wingfield B.D. & Wingfield M.J. (2012) *Ceratocystis eucalypticola* sp. nov. from *Eucalyptus* in South Africa and comparison to global isolates from this tree. *IMA Fungus* **3**: 45-58

ABSTRACT

Eucalyptus trees, mostly native to Australia, are widely planted in the tropics and Southern Hemisphere for the production of wood and pulp. Worldwide surveys of diseases on these trees have yielded a large collection of *Ceratocystis* isolates from dying trees or from wounds on their stems. The aim of this study was to characterise these isolates and to consider their relatedness to each other. Culture appearance, morphological features and a distinctive fruity odour in all cultures were typical of species in the *Ceratocystis fimbriata sensu lato* (*s.l.*) complex. Phylogenetic analyses of sequences for the combined ITS, βt-1 and TEF1-α gene regions revealed a genetically diverse group of isolates residing in a single large clade, that were distinct from all other species in the *C. fimbriata s.l.* complex. Based on morphology and phylogenetic inference, the *Eucalyptus* isolates are recognised as closely related. The South African isolates are described here as a new species, *C. eucalypticola*.

INTRODUCTION

Eucalyptus species are mostly native to Australia, but have been widely planted in the tropics and Southern Hemisphere. This is because they are adapted to a wide range of different environments and are typically fast growing. It has further been suggested that the success of these trees as non–natives is due to the separation from their natural enemies (Wingfield *et al.* 2008, Roux & Wingfield 2009). The potential threat of pests and pathogens to the sustainability of eucalypt plantations in areas where they are not native is consequently great and of substantial concern to forestry industries globally (Old *et al.* 2003, Wingfield *et al.* 2008).

In order to understand and manage the threat of pests and pathogens to *Eucalyptus* species grown as non-natives and in plantations, tree health surveys are undertaken regularly. Amongst the pathogens that have been found on these trees, a *Ceratocystis* sp. in the *C. fimbriata s.l.* complex causes serious disease problems in Brazil, the Republic of Congo, Uganda, and Uruguay (Laia *et al.* 1999, Roux *et al.* 2000, 2001, 2004, Barnes *et al.* 2003a). Various other *Ceratocystis* species in the *C. fimbriata s.l.* complex have also been found on naturally occurring or artificially induced wounds on the stems of trees, in various parts of the world. Some of these have been shown to be cryptic taxa that have been provided with names (van Wyk *et al.* 2007, 2008, 2010a, Rodas *et al.* 2007, Heath *et al.* 2009, Kamgan Nkuekam *et al.* 2012). Several species are thought to be pathogens, while the role of others in tree health is not known.

The genus *Ceratocystis* comprises a diverse group of fungi, including saprophytes causing blue-stain of lumber and serious pathogens that cause mortality (Kile 1993). The genus is typified by *C. fimbriata s.s.* that is a pathogen restricted to root crops, specifically sweet potato (Engelbrecht & Harrington 2005). *Ceratocystis fimbriata s.l.* represents a diverse assemblage of isolates, some of which have been treated as distinct taxa defined based on phylogenetic inference, morphological differences, and mating behaviour (Barnes *et al.* 2001, Engelbrecht & Harrington 2005, Johnson *et al.* 2005, van Wyk *et al.* 2007, 2008, Heath *et al.* 2009). However, Ferreira *et al.* (2010) treated some isolates of the *C. fimbriata sl.* complex from Brazil as representing a particular population of *C. fimbriata s.s.*, rather than as discrete taxa.

Global surveys of the health of *Eucalyptus* species in plantations have yielded a large collection of isolates that can loosely be accommodated in the *C. fimbriata s.l.* complex. The aim of this study was to characterise these isolates and to consider patterns in their distribution on *Eucalyptus* species worldwide.

MATERIALS AND METHODS

Isolates

Isolates used in this study were obtained from: (1) artificially induced wounds on the stems of *Eucalyptus* trees in South Africa, Thailand, and Indonesia (Table 1). The isolates were obtained by directly transferring spore masses from the apices of ascomata produced on the wounded inner bark and wood to agar plates. When sporulating structures were absent, the wood samples were placed in moist chambers to enhance sporulation. Spore masses were transferred to 2 % Malt Extract Agar (MEA) in Petri dishes and incubated at room temperature. Additionally, the carrot baiting technique was used to obtain isolates (Moller & DeVay 1968). (2) cultures were sourced from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa. These isolates had previously been identified as representing the *C. fimbriata s.l.* complex and were from diseased *Eucalyptus* trees in various parts of the world including Brazil, Uganda, Congo, and Uruguay (Table 1).

PCR and sequencing reactions

DNA was extracted from all isolates as described by van Wyk *et al.* (2006a). Three gene regions were selected for PCR amplification, including ITS1 and ITS2, including the 5.8S rDNA operon, part of the beta-tubulin (βt-1) gene, and part of the Transcription Elongation Factor-1 alpha (TEF1-α) gene region. The reactions and programme for amplification were as described by van Wyk *et al.* (2006b). The primers utilized were ITS1 and ITS4 (White *et al.* 1990), βt1a and βt1b (Glass & Donaldson 1995), and EF1F and EF1R (Jacobs *et al.* 2004).

Sequencing reactions were set up and run as described by van Wyk *et al.* (2006a). Sequences of the isolates from *Eucalyptus* were analysed with Chromas Lite 2.01 (http://www.technelysium.com.au). These sequences as well as those for all species in the *C. fimbriata s. lat.* species complex (Table 1) were aligned using MAFFT (http://timpani.genome.ad.jp/%7emafft/server/) (Katoh *et al.* 2002). All sequences derived from this study have been deposited in GenBank (Table 1).

Combined gene tree for all described speciesin the C. fimbriata s.l. complex

Representative isolates of all described species in the *C. fimbriata s.l.* complex were included in this dataset, including those obtained for this study from CMW. The sequences of three gene regions (ITS, βt-1 and TEF1-α) were combined and a partition homogeneity test (PHT) was used to determine if the data from the three regions could be combined, using the software programme PAUP v. 4.0b10 (Swofford 2002). Settings in PAUP were as described in van Wyk *et al.* (2010a). *Ceratocystis virescens* was selected as the outgroup taxon.

MrModeltest2 (Nylander 2004) was used to determine the most appropriate model of nucleotide substitution for each of the three gene regions, respectively. These models were then included in the Bayesian analyses using MrBayes (Ronquist & Huelsenbeck 2003). The Bayesian analyses were run as described in van Wyk *et al.* (2010a).

Combined and separate gene trees of unnamed Ceratocystis fimbriata s.l. isolates obtained from Eucalyptus

This dataset consisted only of *Ceratocystis fimbriata s.l.* isolates from *Eucalyptus* trees and that have not yet been described as separate species. A closely related and previously described species, *C. colombiana*, also obtained from *Eucalyptus,* was included as an outgroup. This was done to determine whether these isolates represent one group with no separate grouping or whether geographical grouping exists, as has been documented in *C. fimbriata s.l.* (Engelbrecht & Harrington 2005, Ferreira *et al.* 2010).

Models were obtained for each of the ITS, βt-1 and TEF1-α gene regions with the use of MrModeltest2 (Nylander 2004). Consistent with both the first datasets, these models were incorporated into MrBayes (Ronquist & Huelsenbeck 2003) in order to run Bayesian analyses.

Utilising the *C. fimbriata s. lat.* isolates from *Eucalyptus* trees obtained from the CMW culture collection, the Molecular Evolutionary Genetics Analysis software (MEGA) 4 (Tamura *et al.* 2007) was used to determine the amount of variation for each gene region. The three gene regions were inspected to determine the number of fixed alleles between them. Allele trees were drawn using the software TCS (Clement *et al.* 2000) from the combined dataset for the *Eucalyptus* isolates, including the closely related species *C. colombiana,* known only from *Eucalyptus*.

Culture characteristics and morphology

Two isolates of *Ceratocystis fimbriata s.l.* from *Eucalyptus* were selected from each country, other than Brazil, for which only one *Eucalyptus* isolate was available. These were used to describe morphological characteristics. Isolates were transferred to each of five 2 % Malt Extract Agar (MEA) plates and incubated in the dark. The isolates were incubated at 30 ºC for 7 d, after which the growth was assessed.

Microscopic examinations were made of isolates from Indonesia, Uruguay, Thailand, and South Africa. Isolates from other countries were excluded because the cultures did not produce ascomata. All taxonomically informative structures were measured from 10 d old cultures on 2 % MEA, mounted in lactic acid. Ten measurements were made for each of the two isolates from Indonesia, Uruguay, Thailand, and South Africa.

A preliminary study of isolates representing the larger collection of *C. fimbriata s. lat.* isolates from *Eucalyptus,* and nested together in the same phylogenetic clade, showed that they are morphologically very similar. Consequently, four isolates (CMW 9998, CMW 15054, CMW 10000 and CMW 11536) from *Eucalyptus* in South Africa were selected for more detailed study. These South African isolates were transferred to five 2 % MEA plates each and incubated at seven different temperatures. These temperatures included 4 ºC and six temperatures between 10 ºC and 35 ºC at 5 ºC intervals. Growth was assessed after 7 d of incubation in the dark. Colony colour was assessed for the same isolates used as in the growth studies, grown on 2 % MEA for seven to 10 d at room temperature (25 ºC). The colour charts of Rayner (1970) were used for descriptions of colony colour.

Fifty measurements were made of all taxonomically informative characters for isolate CMW 11536 from *Eucalyptus* in South Africa. An additional ten measurements were made of these structures for isolates CMW 9998 and CMW 10000 and CMW 15054. The minimum, maximum, average and standard deviation (stdv) was calculated for the measurements of each structure and these are presented in this study as; (minimum-) stdv minus the mean – stdv plus the mean (-maximum).

RESULTS

Isolates

Twenty-five isolates obtainned from CMW that had been isolated from *Eucalyptus* trees were included in this study (Table 1). Fifteen of these originated from natural or artificially induced wounds on trees in three countries, South Africa, Thailand, and Indonesia. In addition, ten of

the isolates were from trees that are believed to have been killed by the fungus. The latter isolates were from Brazil, Congo, Uganda, and Uruguay.

PCR and sequencing reactions

Results were obtained for three separate datasets. The first provided a broad phylogenetic placement (i.e. Latin American or North American, Asian, and African clade) of the *C. fimbriata s. lat.* isolates from *Eucalyptus*. A more focussed analysis determined whether these isolates could be linked to any of the previously described species in the *C. fimbriata s.l.* complex that were obtained from *Eucalyptus*. Thereafter, the isolates from *Eucalyptus* apparently representing undescribed species were considered in combined as well as single gene trees generated from the sequence data for these isolates. This was to determine whether they could be grouped based on geographical origin.

Combined gene tree for all described species in the Ceratocystis fimbriata s.l. complex

Amplicons for the three gene regions were on average 500 bp for the ITS and βt-1 gene regions and 800 bp for the TEF1-α region (Table 1). The PHT for the data set including all described species in the *C. fimbriata s. lat.* complex, had a low value (P=0.01), but could be combined (Cunningham 1997).

Of the 1 989 characters in this dataset, 1 102 were constant, 45 were parsimony uninformative while 842 were parsimony informative. One hundred and forty two most parsimonious trees were obtained, of which one was selected for presentation (Figure 1). The tree topology was as follows: Tree length (TL) = 2054 steps, Consistency Index (CI) = 0.7, Retention Index (RI) = 0.9 and Rescaled Consistency (RC) = 0.6. Phylogenetic analyses revealed a clade specific for the isolates from *Eucalyptus* (Figure 1). Isolates in this large clade had high bootstrap (88 %) and Bayesian (88 %) support and included some substructure (Figure 1). The substructure in the large clade for the isolates from *Eucalyptus* was not strongly supported and these isolates were treated as reflecting a single group of genetically related, but not identical isolates. The closest phylogenetic relative of the isolates in the *Eucalyptus* clade was *C. colombiana* (van Wyk *et al.* 2010a).

The models obtained using MrModeltest2 were the HKY+I+G model for both the ITS and the TEF1-α genes and the GTR+G model for the βt-1 gene region. Including these models in the Bayesian analyses resulted in a burnin of 7000. These 7000 trees were discarded from the final analyses. The posterior probabilities obtained with the Bayesian analyses supported the bootstrap values obtained in PAUP (Figure 1).

Combined and separate gene trees for undescribed Ceratocystis fimbriata s.l. isolates from Eucalyptus

In the dataset for the combined gene regions, there were 1765 characters of which 1680 were constant, 31 were parsimony uninformative while 54 were parsimony informative. Twentyfour most parsimonious trees were obtained, one of which was selected for presentation (Figure 2). The tree topology was as follows: TL = 107 steps, CI = 0.8, RI = 0.9 and RC = 0.7. One well-supported clade (100 % bootstrap, 100 % Bayesian) was observed with high variation. Three clades that were supported within this large clade were also observed (Figure 2). The models obtained for this dataset were the HKY model for the ITS gene, the F81 model for the βt-1 gene region and the HKY+I model for the TEF1-α gene region. A burn-in of 1000 was obtained and these 1000 trees were discarded from the final analyses. The posterior probabilities obtained with the Bayesian analyses supported the bootstrap values obtained with PAUP (Figure 2).

Three well-supported clades were observed; the first included Asian (Indonesia and Thailand) and South American (Brazil and Uruguay) isolates; the second clade included African (Republic of Congo and South Africa) isolates while the third clade included African (Uganda) and Asian (Thailand) isolates. The previously described species, *C. colombiana*, grouped apart from these three clades (Figure 2).

Where the data were treated separately, the trees for the ITS, βT and TEF1-α gene regions had a different topology when compared with those for the combined gene regions (Figure 3). For the ITS gene tree, the same three clades emerged as in the combined dataset and included those for Asian and South American isolates, the African isolates and the African together with Asian isolates. However, only the African and Asian clade had strong support (97 %), the other two clades, African (63 %) and the Asian/South American (55 %) clades had weak support (Figure 3). In the case of the βt-1 gene tree, there was no support and all branches collapsed (Figure 3). For the TEF1-α gene tree, there were two small clades encompassing the South African isolates that had high and medium support (85 % and 65 % respectively), while the rest of the isolates grouped in a single clade with strong (85 %) support (Figure 3).

Where data for the *C. fimbriata s.l.* isolates were analysed in MEGA, the results showed that in the ITS gene region, the *C. fimbriata s.l.* isolates obtained from *Eucalyptus* were separated from *C. colombiana* by an average of 23 nucleotide differences (Table 2). Where isolates

from different countries were compared, there was also variation in the ITS with a maximum of 13 bp and average of 5 bp differences (Table 2).

Where isolates of *C. fimbriata s. lat.* from *Eucalyptus* were compared with *C. colombiana* in the βt-1 gene region, there were only 3bp differences between them (Table 2). Within the clade representing the *C. fimbriata s. lat.* group from *Eucalyptus*, there was only one base pair difference observed in the South African group and no differences between isolates from different countries (Table 2).

For the TEF1-α gene region, there were 21bp differences between the isolate from Brazil and *C. colombiana* and an average of 8 bp differences between *C. colombiana* and the other isolates from *Eucalyptus*. Only the single isolate from Brazil differed from the other isolates while no differences were observed between the isolates from the other countries. The allele networks drawn from the combined gene regions (ITS, βt-1 and TEF1-α) for the *C. fimbriata s. lat.* obtained from *Eucalyptus* revealed a single tree with high variation (Figure 4). There was no obvious geographic structure with regards to the origin of the eucalypt isolates. The previously described species, *C. colombiana*, formed a separate allele tree (Figure 4).

Culture characteristics and morphology

All isolates from *Eucalyptus* had a similar greenish olivaceous (33'''f) (Rayner 1970) colony colour. The cultures had a banana odour similar to that of many *Ceratocystis* species. The cultures all grew optimally at 30 ºC. No clear morphological differences could be observed between isolates from different countries (Table 3).

Isolate CMW 11536 from *Eucalyptus* in South Africa was chosen to represent the global collection of isolates obtained from *Eucalyptus*. Three additional isolates (CMW 9998, CMW 10000 and CMW 15054), also from South Africa, were chosen as additional specimens for description. Cultures of these isolates were grown on 2 % MEA, dried down and have been deposited with the National Collection of Fungi (PREM), Pretoria, South Africa. Living cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa and the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, The Netherlands.

Where growth in culture was characterised based on the average colony diameter (from the five inoculated plates) for the four selected *Eucalyptus* isolates from South Africa, after 7 d, limited growth was observed at 4 °C (8 mm), 10 °C (7 mm), 15 °C (19 mm) and 35 °C (10

mm). Intermediate growth was observed after 7 d at 20 °C (34 mm) and 25 °C (35 mm), while the optimum temperature for growth in culture was 30 °C at which isolates reached an average of 39 mm diam after 7 d.

TA XONOMY

Isolates of the *Ceratocystis* from *Eucalyptus,* originating from many different countries, were phylogenetically distinct from all other *Ceratocystis* species residing in the *C. fimbriata s.l.* clade. They also formed distinct phylogenetic groups based on geographic origin and might be found to represent distinct taxa in the future. For the present, those isolates from South Africa, which also had a morphology different to all described species from *Eucalyptus* (Table 4) are described as representing a novel taxon.

Ceratocystis eucalypticola M. van Wyk & M.J. Wingf., **sp. nov.** (Figure 5)
MB512397
Etymology: The name refers to *Eucalyptus* on which the fungus occurs.

All species of *Ceratocystis* from *Eucalyptus* are phylogenetically distinct. Colonies of *C. eucalypticola* are typically green colonies, relatively slow growing, and have a fruity banana odour.

Ascomatal bases dark brown to black, globose, un-ornamented (105–)140–186(–222) µm wide, (118–)146–184(–216) µm high. *Ascomatal necks* dark brown to black at bases becoming lighter towards the apices, (274–)376–464(–499) µm long, apices (14–)16–20(–22) µm wide, bases (19–)25–33(–42) µm wide. *Ostiolar hyphae* divergent, (39–)45–59(–66) µm long. *Ascospores* hyaline, hat-shaped in side view, invested in sheath, 3–5 µm long, 4–6 µm wide without sheath, 5–7(–8) µm wide including sheath. *Anamorph* thielaviopsis-like, conidiophores of two types: *Primary conidiophores* phialidic, flask-shaped, (58–)77–113(–131) µm long, (3–)4–6 µm wide at the bases, 4–6(–7) µm wide at broadest points and 3–5 µm wide at apices. *Secondary conidiophores* flaring or wide mouthed, (43–)60–100(–143) µm long, (3–)4–6(–7) µm wide at bases and (4–)5–7(–8) µm wide at apices. *Primary conidia* cylindrical in shape (14–)16–22(–25) µm long, 3–5 µm wide. *Secondary conidia*, barrelshaped, abundant, (6–)7–9(–12) µm long, 4–6(–7) µm wide. *Chlamydospores*, scarce, hair brown (17’’’’i), globose to subglobose (10–)11–13(–15) µm long, 8–10(–11) µm wide.

Habitat: Wounded and diseased *Eucalyptus*.

Known distribution: South Africa.

Material examined: **South Africa**: Kwa-Zulu Natal, KwaMbonambi, isolated from artificially wounded *Eucalyptus*, M. van Wyk & J. Roux, PREM60168, CMW11536, CBS124016 (Dec. 2002). **South Africa,** Mpumalanga, Sabie, isolated from artificially wounded *Eucalyptus* trees, M. van Wyk & J. Roux, PREM60169, CMW9998, CBS124017 (July 2002); **South Africa,** Mpumalanga, Sabie, isolated from artificially wounded *Eucalyptus* trees, M. van Wyk & J. Roux, PREM60170, CMW10000, CBS124019 (July 2002)..

DISCUSSION

Isolates of *Ceratocystis fimbriata s. lat.* collected from *Eucalyptus* in Brazil, Indonesia, Republic of Congo, South Africa, Thailand, Uganda, and Uruguay were shown to be phylogenetically related. These included isolates taken from wounds on trees and also those that were associated with trees dying as result of infection by the fungus. Although all isolates from *Eucalyptus* resided in a single large clade, there was a high degree of diversity among them. It is thus possible that they represent a number of different cryptic species that cannot be resolved. For the present, those isolates from South Africa are provided with the name *C. eucalypticola* here. Future studies should seek to include additional isolates from *Eucalyptus* as well as to include sequences for gene regions not considered in this study, and that might discriminate more clearly between species in the *C. fimbriata s.l.* complex. Currently, the group is unified based on a specific host and relatively strong phylogenetic similarity. In this respect, it also provides the foundation for further studies including a suite of isolates that would be difficult to obtain.

The species of *Ceratocystis* most closely related to *C. eucalypticola* is *C. colombiana*. *Ceratocystis colombiana* is a pathogen of coffee trees (Marin *et al.* 2003) as well as numerous other hosts including indigenous crops in Colombia. Although the two species are phylogenetically related, they are ecologically distinct and are not likely to be confused.

Ceratocystis eucalypticola is one of a number of species in the *C. fimbriata s. lat.* complex to be described from *Eucalyptus* trees. Other species from this host include; *C. atrox* (Van Wyk *et al.* 2007) and *C. corymbiicola* (Kamgan Nkuekam *et al.* 2012) from Australia, *C. pirilliformis* (Barnes *et al.* 2003b) from Australia and South Africa, *C. neglecta* (Rodas *et al.* 2007) from Colombia, *C. fimbriatomima* (Van Wyk *et al.* 2008) from Venezuela, and *C. zombamontana* (Heath *et al.* 2009) from Malawi. All of these species from *Eucalyptus* can be

distinguished from each other based on phylogenetic inference and they have some morphological features that can be used to recognise them.

Morphologically, the specimens of *C. eucalypticola* cited here resemble species in the *C. fimbriata s. lat.* complex. The fungus has the typical green colony colour, is relatively slow growing, and has a fruity banana odour. *Ceratocystis eucalypticola* can be distinguished from other species in the *C. fimbriata s. lat.* complex in that they occur on *Eucalyptus* and based on differences in size of some diagnostic characters for this group of fungi.

Ceratocystis eucalypticola includes isolates only from wounds on trees in South Africa in the absence of disease, but is very closely related to isolates that originated from dying trees and that have been shown to be pathogenic (Laia *et al.* 1999, Roux *et al.* 2000, 2001, 2004). The species is also closely related to isolates that were collected from wounds on trees in countries other than South Africa where a *Ceratocystis* disease on *Eucalyptus* has not been seen. *Eucalyptus* death associated with *C. eucalypticola* has never been found in South Africa although trees dying of unknown causes are thought to have died due to infection by this fungus, which can be difficult to isolate. The fungus collected from wounds on trees has also been shown to be pathogenic in greenhouse inoculation trials (Roux *et al.* 2004, Van Wyk *et al.* 2010b).

Isolates of *C. eucalypticola* from South Africa represent a clonal population (Van Wyk *et al.* 2006b) and it was most likely introduced into the country. It is thus intriguing that *Eucalyptus* death associated with this fungus has not been seen. This might be due to planting stock susceptible to *C. eucalypticola* not having occurred in the country, or that conditions for infection were not suitable. Alternatively, it is possible that trees dying of unexplained causes might have been killed by *C. eucalypticola*, even though the fungus was not isolated from them. This is a question that is currently being pursued, particularly linked to unexplained *Eucalyptus* death in South Africa and where *Ceratocystis* cultures emerge from isolations.

REFERENCES

Barnes I., Gaur A., Burgess T., Roux J., Wingfield B.D. & Wingfield M.J. (2001) 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., Wingfield B.D., O'Neil M. & Wingfield M.J. (2003a) *Ceratocystis fimbriata* infecting *Eucalyptus grandis* in Uruguay. *Australasian Plant Pathology* **32**: 361–355.

Barnes I., Roux J., Wingfield M.J., Old K.M. & Dudzinski M. (2003b) *Ceratocystis pirilliformis,* a new species from *Eucalyptus nitens* in Australia*. Mycologia* **95**: 865–871.

Clement M., Posada D. & Crandall K. (2000) TCS: a computer program to estimate gene genealogies*. Molecular Ecology* **9**: 1657–1660.

Cunningham C.W. (1997) Can three incongruence tests predict when data should be combined? *Molecular Biology and Evolution* **14**: 733–740.

Engelbrecht C.J.B. & Harrington T.C. (2005) Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. *Mycologia* **97**: 57–69.

Feirreira E.M., Harrington T.C., Thorpe D.J. & Alfenas A.C. (2010) Genetic diversity and interfertility among highly differentiated populations of *Ceratocystis fimbriata* in Brazil. *Plant Pathology* **59**: 721–735.

Glass N.L. & 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.

Heath R.N., Wingfield B.D., Wingfield M.J., Meke G., Mbaga A. & Roux J. (2009) *Ceratocystis* species on *Acacia mearnsii* and *Eucalyptus* spp. in eastern and southern Africa including six new species. *Fungal Diversity* **34**: 41–67.

Jacobs K., Bergdahl D.R., Wingfield M.J., Halik S., Seifert K.A., Bright D.E. & Wingfield B.D. (2004) *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* **108**: 411–418.

Johnson J.A., Harrington T.C. & Engelbrecht C.J.B. (2005) Phylogeny and taxonomy of the North American clade of the *Ceratocystis fimbriata* complex. *Mycologia* **97**: 1067–1092.

Kamgan Nkuekam G., Wingfield M.J., Mohammed C., Carnegie A.J., Pegg G.S. & Roux J. (2012) *Ceratocystis* species, including two new species associated with nitidulid beetles on *Eucalyptus* in Australia. *Antonie van Leeuwenhoek* **101**: 217–241.

Katoh K., Misawa K., Kuma K. & Miyata T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**: 3059–3066.

Kile G.A. (1993) Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In: *Ceratocystis and Ophiostoma: taxonomy, ecology and pathogenicity* (MJ Wingfield, KA

Seifert, K, Webber, J.A. (eds): 173–183. St Paul, MN: American Phytopathological Society Press.

Laia M.L., Alfenas A.C. & Harrington T.C. (1999) Isolation, detection in soil, and inoculation of *Ceratocystis fimbriata*, causal agent of wilting, dieback and canker in *Eucalyptus*. In: *Proceedings of the 12th Biennial Conference of the Australasian Plant Pathology Society, 27–30 September* (L Morin, ed): 77. Canberra: *Australasian Plant Pathology* Society.

Marin M., Castro B., Gaitan A., Preisig O., Wingfield B.D. & Wingfield M.J. (2003) Relationships of *Ceratocystis fimbriata* isolates from Colombian coffee-growing regions based on molecular data and pathogenicity. *Journal of Phytopathology* **151**: 395–400.

Moller W.J. & DeVay J.E. (1968) Carrot as a species-selective isolation medium for *Ceratocystis fimbriata*. *Phytopathology* **58**: 123–124.

Nylander J.A.A. (2004) *MrModeltest v2.* [Program distributed by the author.] Uppsala: Evolutionary Biology Centre, Uppsala University.

Old K.M., Wingfield M.J. & Yuan Z.Q. (2003) *A manual of diseases of Eucalypts in South-East Asia.* Jakarta: Centre for International Forestry Research.

Rayner R.W. (1970) *A Mycological Colour Chart*. Kew: Commonwealth Mycological Institute.

Rodas C., Roux J., Van Wyk M., Wingfield B.D. & Wingfield M.J. (2007) *Ceratocystis neglecta* sp. nov., infecting *Eucalyptus* trees in Colombia. *Fungal Diversity* **28**: 73–84.

Ronquist F. & Huelsenbeck J.P. (2003) *MrBayes 3:* Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Roux J., Coutinho T.A., Munjuni Byabashaija D. & Wingfield M.J. (2001) Diseases of plantation *Eucalyptus* in Uganda. *South African Journal of Science* **97**: 16–18.

Roux J., Van Wyk M., Hatting H. & Wingfield M.J. (2004) *Ceratocystis* species infecting stem wounds on *Eucalyptus grandis* in South Africa. *Plant Pathology* **53**: 414–421.

Roux J. & Wingfield M.J. (2009) *Ceratocystis* species: emerging pathogens of non-native plantation *Eucalyptus* and *Acacia* species. *Southern Forests* **71**: 115–120.

Roux J., Wingfield M.J., Wingfield B.D., Bouillett J.P. & Alfenas A.C. (2000) A serious new disease of *Eucalyptus* caused by *Ceratocystis fimbriata* in Central Africa. *Forest Pathology* **30**: 175–184.

Swofford D.L. (2002) *PAUP*: phylogenetic analysis using parsimony (*and other methods).* Version 4. Sunderland, MA:Sinauer Associates.

Tamura K., Dudley J., Nei M. & Kumar S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596–1599.

Van Wyk M., Pegg G., Lawson S. & Wingfield M.J. (2007) *Ceratocystisatrox* sp. nov. associated with *Phoracanthta acanthocera* infestations on *Eucalyptus* in Australia. *Australian Journal of Plant Pathology* **36**: 407–414.

Van Wyk M., Roux J., Barnes I., Wingfield B.D. & Wingfield M.J. (2006a) Molecular phylogeny of the *Ceratocystis moniliformis* complex and description of *C. tribilliformis* sp. nov. *Fungal Diversity* **21**: 181–201.

Van Wyk M., Van der Merwe N.A., Roux J., Wingfield B.D., Kamgan G.N. & Wingfield M.J. (2006b) Population biology of *Ceratocystis fimbriata* from *Eucalyptus* trees in South Africa. *South African Journal of Science* **102**: 259–263.

Van Wyk M., Wingfield B.D., Marin M., Wingfield M.J. (2010a) Two new *Ceratocystis* species infecting coffee, cacao, citrus and native trees in Colombia. *Fungal Diversity* **40**: 103–117.

Van Wyk M., Wingfield B.D. & Wingfield M.J. (2010b) Comparison of procedures to evaluate the virulence of *Ceratocystis fimbriata sensu lato* isolates from *Eucalyptus* in South Africa. *Southern Forests* **72**: 57–62.

Van Wyk M., Wingfield B.D., Mohali S. & Wingfield M.J. (2008) *Ceratocystis fimbriatomima,* a new species in the *C. fimbriata sensu lato* complex isolated from *Eucalyptus* trees in Venezuela. *Fungal Diversity* **34**: 173–183.

White T.J., Bruns T., Lee S. & Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a sequencing guide to methods and applications* (MA Innis, DH Gelfand, JJ Sninsky, TJ White, eds): 315–322. San Diego: Academic Press.

Wingfield M.J., Slippers B., Hurley B.P., Coutinho T.A., Wingfield B.D. & Roux J. (2008) Eucalypt pests and diseases: growing threats to plantation productivity. *Southern Forests* **70**: 139–144.

Table 1. Isolates of *Ceratocystis fimbriata s.l.* used in this study.

Species	Isolate no.	GenBank accession no.	Host	Area
C. acaciivora	CMW22563	EU588656, EU588636, EU588646	*Acacia mangium*	Indonesia
C. acaciivora	CMW22564	EU588657, EU588637, EU588647	*Acacia mangium*	Indonesia
C. albifundus	CMW4068	DQ520638,EF070429,EF070400	*Acacia mearnsii*	RSA
C. albifundus	CMW5329	AF388947,DQ371649,EF070401	*Acacia mearnsii*	Uganda
C. atrox	CMW19383 CBS120517	EF070414,EF070430,EF070402	*Eucalyptus grandis*	Australia
C. atrox	CMW19385 CBS120518	EF070415,EF070431,EF070403	*Eucalyptus grandis*	Australia
C. cacaofunesta	CMW15051 CBS152.62	DQ520636,EF070427,EF070398	*Theobroma cacao*	Costa Rica
C. cacaofunesta	CMW14809 CBS115169	DQ520637,EF070428,EF070399	*Theobroma cacao*	Ecuador
C. caryae	CMW14793 CBS114716	EF070424,EF070439,EF070412	*Carya cordiformis*	USA
C. caryae	CMW14808 CBS115168	EF070423,EF070440,EF070411	*Carya ovate*	USA
C. colombiana	CMW9565 CBS121790	AY233864,AY233870,EU241487	*Soil*	Colombia
C. colombiana	CMW5751 CBS121792	AY177233,AY177225,EU241493	*Coffea Arabica*	Colombia
C. colombiana	CMW9572	AY233863,AY233871,EU241488	*Mandarin*	Colombia
C. curvatq	CMW22442 CBS122603	FJ151436,FJ151448,FJ151470	*Eucalyptus deglupta*	Colombia
C. curvata	CMW22435 CBS122604	FJ151437,FJ151449,FJ151471	*Eucalyptus deglupta*	Colombia
C. diversiconidia	CMW22445 CBS123013	FJ151440,FJ151452,FJ151474	*Terminalia ivorensis*	Colombia
C. diversiconidia	CMW22446	FJ151443,FJ151455,FJ151477	*Terminalia ivorensis*	Colombia
C. ecuadoriana	CMW22092 CBS124020	FJ151432,FJ151444,FJ151466	*Eucalyptus deglupta*	Colombia
C. ecuadoriana	CMW22093 CBS124021	FJ151433,FJ151445,FJ151467	*Eucalyptus deglupta*	Colombia
C. eucalypticola	**CMW4797**	**FJ236733,FJ236793,FJ236763**	***Eucalyptus* sp.**	**Congo**
C. eucalypticola	**CMW4799**	**FJ236734,FJ236794,FJ236764**	***Eucalyptus* sp.**	**Congo**
C. eucalypticola	**CMW4902**	**FJ236715,FJ236775,FJ236745**	***Eucalyptus* sp.**	**Brazil**
C. eucalypticola	**CMW5312**	**FJ236731,FJ236791,FJ236761**	***Eucalyptus* sp.**	**Uganda**
C. eucalypticola	**CMW5313**	**FJ236732,FJ236792,FJ236762**	***Eucalyptus* sp.**	**Uganda**
C. eucalypticola	**CMW7764**	**FJ236726,FJ236786,FJ236756**	***Eucalyptus* sp.**	**Uruguay**
C. eucalypticola	**CMW7765**	**FJ236727,FJ236787,FJ236757**	***Eucalyptus* sp.**	**Uruguay**
C. eucalypticola	**CMW7766**	**FJ236728,FJ236788,FJ236758**	***Eucalyptus* sp.**	**Uruguay**
C. eucalypticola	**CMW7767**	**FJ236729,FJ236789,FJ236759**	***Eucalyptus* sp.**	**Uruguay**
C. eucalypticola	**CMW7768**	**FJ236730,FJ236790,FJ236760**	***Eucalyptus* sp.**	**Uruguay**
C. eucalypticola	**CMW9998 CBS124017**	**FJ236721,FJ236781,FJ236751**	***Eucalyptus* sp.**	**South Africa**
C. eucalypticola	**CMW10000 CBS124019**	**FJ236722,FJ236782,FJ236752**	***Eucalyptus* sp.**	**South Africa**
C. eucalypticola	**CMW11536 CBS124016**	**FJ236723,FJ236783,FJ236753**	***Eucalyptus* sp.**	**South Africa**
C. eucalypticola	**CMW12663**	**FJ236724,FJ236784,FJ236754**	***Eucalyptus* sp.**	**South Africa**
C. eucalypticola	**CMW14631**	**FJ236744,FJ236804,FJ236774**	***Eucalyptus* sp.**	**Indonesia**
C. eucalypticola	**CMW14632**	**FJ236743,FJ236803,FJ236773**	***Eucalyptus* sp.**	**Indonesia**
C. eucalypticola	**CMW15054 CBS124018**	**FJ236725,FJ236785,FJ236755**	***Eucalyptus* sp.**	**South Africa**
C. eucalypticola	**CMW16008**	**FJ236735,FJ236795,FJ236765**	***Eucalyptus* sp.**	**Thailand**
C. eucalypticola	**CMW16009**	**FJ236736,FJ236796,FJ236766**	***Eucalyptus* sp.**	**Thailand**
C. eucalypticola	**CMW16010**	**FJ236737,FJ236797,FJ236767**	***Eucalyptus* sp.**	**Thailand**
C. eucalypticola	**CMW16034**	**FJ236739,FJ236799,FJ236769**	***Eucalyptus* sp.**	**Thailand**

C. eucalypticola	**CMW16035**	**FJ236738,FJ236798,FJ236768**	***Eucalyptus* sp.**	**Thailand**
C. eucalypticola	**CMW18572**	**FJ236740,FJ236800,FJ236770**	***Eucalyptus* sp.**	**Indonesia**
C. eucalypticola	**CMW18577**	**FJ236742,FJ236802,FJ236772**	***Eucalyptus* sp.**	**Indonesia**
C. eucalypticola	**CMW18591**	**FJ236741,FJ236801,FJ236771**	***Eucalyptus* sp.**	**Indonesia**
C. fimbriata s.s	CMW15049 CBS141.37	DQ520629,EF070442,EF070394	*Ipomaea batatas*	USA
C. fimbriata s.s.	CMW1547	AF264904,EF070443,EF070395	*Ipomaea batatas*	Papua New Guinea
C. fimbriatomima	CMW24174 CBS121786	EF190963,EF190951,EF190957	*Eucalyptus* sp.	Venezuela
C. fimbriatomima	CMW24176 CBS121787	EF190964,EF190952,EF190958	*Eucalyptus* sp.	Venezuela
C. laurium	CMW25434 CBS122512	EU881906,EU881894,EU881900	*Styrax benzoin*	Indonesia
C. laurium	CMW25435 CBS122606	EU881907,EU881895,EU881901	*Styrax benzoin*	Indonesia
C. manginecans	CMW13851 CBS121659	AY953383,EF433308,EF433317	*Mangifera indica*	Oman
C. manginecans	CMW13852 CBS121660	AY953384,EF433309,EF433318	*Hypocryphalus mangifera*	Oman
C. neglecta	CMW17808 CBS121789	EF127990,EU881898,EU881904	*Eucalyptus* sp.	Colombia
C. neglecta	CMW18194 CBS121017	EF127991,EU881899,EU881905	*Eucalyptus* sp.	Colombia
C. obpyriformis	CMW23807 CBS122608	EU245004,EU244976,EU244936	*Acacia mearnsii*	South Africa
C. obpyriformis	CMW23808 CBS122511	EU245003,EU244975,EU244935	*Acacia mearnsii*	South Africa
C. papillata	CMW8857	AY233868,AY233878,EU241483	*Annona muricata*	Colombia
C. papillata	CMW8856 CBS121793	AY233867,AY233874,EU241484	*Citrus lemon*	Colombia
C. papillata	CMW10844	AY177238,AY177229,EU241481	*Coffea Arabica*	Colombia
C. pirilliformis	CMW6569	AF427104,DQ371652,AY528982	*Eucalyptus nitens*	Australia
C. pirilliformis	CMW6579 CBS118128	AF427105,DQ371653,AY528983	*Eucalyptus nitens*	Australia
C. platani	CMW14802 CBS115162	DQ520630,EF070425,EF070396	*Platanus occidentalis*	USA
C. platani	CMW23918	EF070426,EF070397,EU426554	*Platanus* sp.	Greece
C. polychroma	CMW11424 CBS115778	AY528970,AY528966,AY528978	*Syzygium aromaticum*	Indonesia
C. polychroma	CMW11436 CBS115777	AY528971,AY528967,AY528979	*Syzygium aromaticum*	Indonesia
C. polyconidia	CMW23809 CBS122289	EU245006,EU244978,EU244938	*Acacia mearnsii*	South Africa
C. polyconidia	CMW23818 CBS122290	EU245007,EU244979,EU244939	*Acacia mearnsii*	South Africa
C. populicola	CMW14789 CBS119.78	EF070418,EF070434,EF070406	*Populus* sp.	Poland
C. populicola	CMW14819 CBS114725	EF070419,EF070435,EF070407	*Populus* sp.	USA
C. smalleyi	CMW14800 CBS114724	EF070420,EF070436,EF070408	*Carya cordiformis*	USA
C. smalleyi	CMW26383 CBS114724	EU426553,EU426555,EU426556	*Carya cordiformis*	USA
C. tanganyicensis	CMW15991 CBS122295	EU244997,EU244969,EU244929	*Acacia mearnsii*	Tanzania
C. tanganyicensis	CMW15999 CBS122294	EU244998,EU244970,EU244939	*Acacia mearnsii*	Tanzania
C. tsitsikammensis	CMW14276 CBS121018	EF408555,EF408569,EF408576	*Rapanea melanophloeos*	South Africa
C. tsitsikammensis	CMW14278 CBS121019	EF408556,EF408570,EF408577	*Rapanea melanophloeos*	South Africa
C. variospora	CMW20935	EF070421 EF070437 EF070409	*Quercus alba*	USA

	CBS114715			
C. variospora	CMW20936	EF070422,EF070438,EF070410	*Quercus robur*	USA
	CBS114714			
C. virescens	CMW11164	DQ520639,EF070441,EF070413	*Fagus americanum*	USA
C. virescens	CMW3276	AY528984,AY528990,AY529011	*Quercus robur*	USA
C. zombamontana	CMW15235	EU245002,EU244974,EU244934	*Eucalyptus* sp.	Malawi
C. zombamontana	CMW15236	EU245000,EU244972,EU244932	*Eucalyptus* sp.	Malawi

Table 2. The number of differences observed between the sequences of *C. eucalypticola* sp. nov. from Brazil, South Africa, Uruguay, Uganda, Congo, Thailand, and Indonesia and *C. Colombiana*.

Country	**Brazil**	**South**	**Uruguay**	**Uganda**	**Congo**	**Thailand**	**Indonesia**	***C. colombiana***
Gene region		**Africa**						
ITS								
Brazil	-	9	0	6	13	0	0	*16*
South Africa	9	**8**	6	6	0	4	9	*22*
Uruguay	0	6	**4**	7	9	0	0	*15*
Uganda	6	6	7	**0**	9	0	7	*22*
Congo	13	0	9	9	**0**	6	11	*24*
Thailand	0	4	0	0	6	**7**	0	*15*
Indonesia	0	9	0	7	11	0	**1**	*16*
C. colombiana	16	22	15	22	24	15	16	***0***
BT								
Brazil	-	0	0	0	0	0	0	*2*
South Africa	0	**1**	0	0	0	0	0	*2*
Uruguay	0	0	**0**	0	0	0	0	*2*
Uganda	0	0	0	**0**	0	0	0	*2*
Congo	0	0	0	0	**0**	0	0	*2*
Thailand	0	0	0	0	0	**0**	0	*2*
Indonesia	0	0	0	0	0	0	**0**	*2*
C. colombiana	2	2	2	2	2	2	2	***0***
EF								
Brazil	-	13	9	12	12	12	12	*18*
South Africa	13	**7**	0	0	0	0	0	*5*
Uruguay	9	9	**9**	0	0	0	0	*5*
Uganda	12	0	0	**7**	0	0	0	*4*
Congo	12	0	0	0	**0**	0	0	*5*
Thailand	12	0	0	0	0	**1**	0	*5*
Indonesia	12	0	0	0	0	0	**5**	*5*
C. colombiana	*18*	*5*	*5*	*4*	*5*	*5*	*5*	***0***

Table 3. Morphological comparison of two representative isolates each of *C. eucalypticola* sp. nov. from Brazil, republic Congo, Indonesia, South Africa, Thailand, Uganda and Uruguay. Ten measurements were taken of each structure and the (minimum-) average minus standard deviation - average plus standard deviation and (-maximum) was given below.

Characteristic / Country	Indonesia	South Africa	Thailand	Uruguay
Ascomatal bases				
Shape	Globose	Globose	Globose	Globose
Length	(125-)162-199(-200)	(120-)142-190(-202)	(188-)190-197(-200)	(144-)170-197(-200)
Width	(143-)173-193(-200)	(132-)143-193(-216)	(154-)177-199(-212)	(141-)164-184(-197)
Ascomatal necks				
Length	(390-)400-450(-470)	(372-)392-460(-486)	(354-)370-400(-424)	(354-)368-386(-409)
Width (bases)	(24-)25-35(-40)	(24-)25-35(-42)	(24-)25-35(-39)	(23-)26-32(-38)
Width (apices)	(15-)16-18(-20)	(15-)16-20(-22)	(16-)17-19(-20)	(15-)16-22(-25)
Ostiolar hyphae				
Shape	Divergent	Divergent	Divergent	Divergent
Length	(36-)43-53(-63)	(39-)40-52(-62)	(33-)35-39(-41)	(38-)41-51(-53)
Ascospores				
Length	3-5	3-5	3-4	3-4
Width (excluding sheath)	4-6	4-6	4-6	4-6
Width (including sheath)	5-8	5-7(-8)	5-7	6-7
Primary phialides				
Length	(69-)70-100(-134)	(73-)76-114(-131)	(67-)76-96(-100)	(73-)75-83(-88)
Width (bases)	4-6	4-6	4-6	2-4
Width (broadest point)	4-6	4-6	6-8	4-5
Width (apices)	3-5	3-5	3-5	3-4
Secondary phialides				
Length	(60-)70-100(-143)	(64-)69-109(-143)	(63-)68-77(-99)	(69-)72-96(-109)
Width (bases)	3-6	3-6	5-6	3-6
Width (apices)	5-7	5-7	4-8	6-8
Primary conidia				
Length	(13-)19-20(-24)	(15-)18-24(-25)	(10-)13-17(-18)	(10-)11-15(-18)
Width	4-5	4-5	3-4	2-3
Secondary conidia				
Length	6-8	6-8	6-8	(7-)9-11
Width	5-8	5-7	5-8	6-8
Chlamydospores				
Shape	Globose/Subglobose	Globose/Subglobose	Globose/Subglobose	Globose/Subglobose
Length	10-15	10-13	12-15	(6-)7-11(-13)
Width	8-13	8-10	10-13	(5-)7-11(-12)

Table 4. Morphological comparison of all previously described species in the *C. fimbriata s.l.* species complex obtained on *Eucalyptus* trees compared to *C. eucalypticola sp. nov.*

Character / Species	*C. atrox*	*C. eucalypticola*	*C. fimbriatomima*	*C. neglecta*	*C. colombiana*	*C. pirilliformis*
Ascomatal bases						
Shape	Globose	Globose	Globose	Globose	Globose	Obpyriform
Length	(120-)140-180(-222)	(105-)140-186(-222)	(142-)173-215(-234)	(173-)202-244(-281)	(140-)177-237(-294)	145-216(-279)
Width	(120-)150-178(-200)	(118-)146-184(-216)	(145-)178-225(-255)	(153-)178-228(-250)	(140-)177-237(-294)	115-186(-206)
Ascomatal necks						
Length	(270-)310-400(-460)	(274-)376-464(-499)	(446-)660-890(-1070)	(691-)745-840(-889)	(375-)448-560(-676)	372-683(-778)
Width (bases)	(21-)26-34(-40)	(19-)25-33(-42)	(28-)32-42(-47)	(27-)31-39(-46)	(24-)27-35(-43)	18-33(-40)
Width (apices)	(13-)14-16(-19)	(14-)16-20(-22)	(16-)18-24(-28)	(14-)16-20(-22)	(12-)14-18(-19)	12-21(-25)
Ostiolar hyphae						
Shape	Divergent	Divergent	Divergent	Divergent	Divergent	Convergent
Length	(18-)20-26(-28)	(39-)45-59(-66)	(40-)49-61(-68)	(35-)41-49(-54)	(28-)38-46(-52)	N/A
Ascospores						
Length	3-4	3-5	2-4	3-6	3-4	4-6
Width (excluding sheath)	3-4	4-6	4-6	4-7	(3-)4-6(-7)	3-5
Width (including sheath)	4-6	5-7(-)8	5-7	5-8	6-8(-11)	3-5
Primary phialides						
Length	(78-)87-151(-218)	(58-)77-113(-131)	(49-)60-94(-122)	(75-)80-114(-152)	(58-)65-83(-106)	62-147(-216)
Width (bases)	5-7(-13)	(3-)4-6(-7)	4-7	(4-)5-7(-8)	4-6(-8)	N/A
Width (broadest point)	4-7	4-6(-7)	5-9	5-9	(3-)6-8(-9)	N/A
Width (apices)	4-9	3-5	3-5	(3-)4-6(-7)	3-5(-6)	N/A
Secondary phialides						
Length	(39-)43-57(-66)	(43-)60-100(-143)	Absent	(38-)48-76(-89)	(42-)49-71(-85)	N/A
Width (bases)	5-7(-9)	(3-)4-6(-7)	Absent	(3-)5-7(-8)	(4-)5-7	N/A
Width (apices)	4-6)-7)	(4-)5-7(-8)	Absent	(3-)5-7(-8)	(5-)6-8	N/A
Primary conidia						
Length	(9-)11-15(-17)	(14-)16-22(-25)	(14-)20-28(-31)	(11-)15-27(-30)	(12-)16-24(-29)	12-25(-33)
Width	3-5	3-5	3-5	(3-)5-6	4-6	2-5
Secondary conidia						
Length	(7-)8-12(-14)	(6-)7-9(-12)	Absent	(6-)10-11	9-14	4-6
Width	(5-)6-8(-9)	4-6(-7)	Absent	(4-)5-7(-9)	6-8(-11)	3-5
Chlamydospores						
Shape	Absent	Globose/Subglobose	Subglobose	Globose	Globose	Oval
Length	Absent	(10-)11-13(-15)	(6-)10-14(-15)	(8-)10-12(-13)	11-14	8-12(-13)
Width	Absent	8-10(-11)	(6-)7-11(-12)	(9-)10-14(-16)	11-15(-17)	5-8(-10)
Reference	Van Wyk *et al.* 2007	This study	Van Wyk *et al.* 2008	Rodas *et al.* 2008	Van Wyk *et al.* 2010a	Barnes *et al.* 2003

Figure 1. Phylogenetic tree based on the combined sequences of the ITS, βt and EF1-α gene regions for *C. eucalypticola sp. nov.* and other described species in the *C. fimbriata s.l.* complex. *Ceratocystis virescens* represents the out-group taxon. Bootstrap values are indicated at the branch nodes and Bayesian values in brackets.

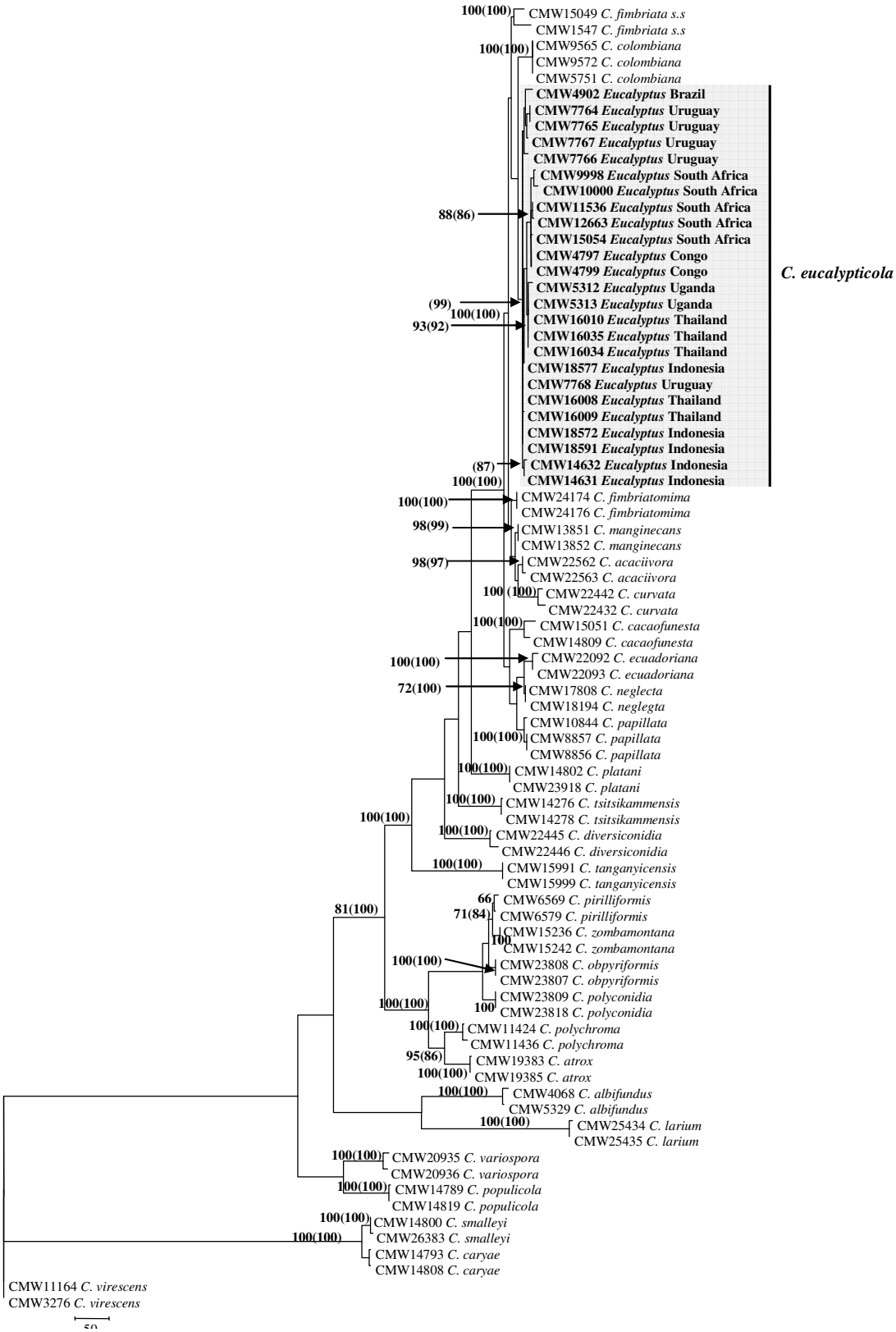


Figure 2. A phylogenetic tree for the combined sequences of the ITS, BT and EF1-α gene regions, including only the undescribed *C. fimbriata s.l.* isolates with *Eucalyptus* as their host. The closely related species *C. colombiana* is included in this analysis Bootstrap support is indicated at the branch nodes while Bayesian support is indicated in brackets.

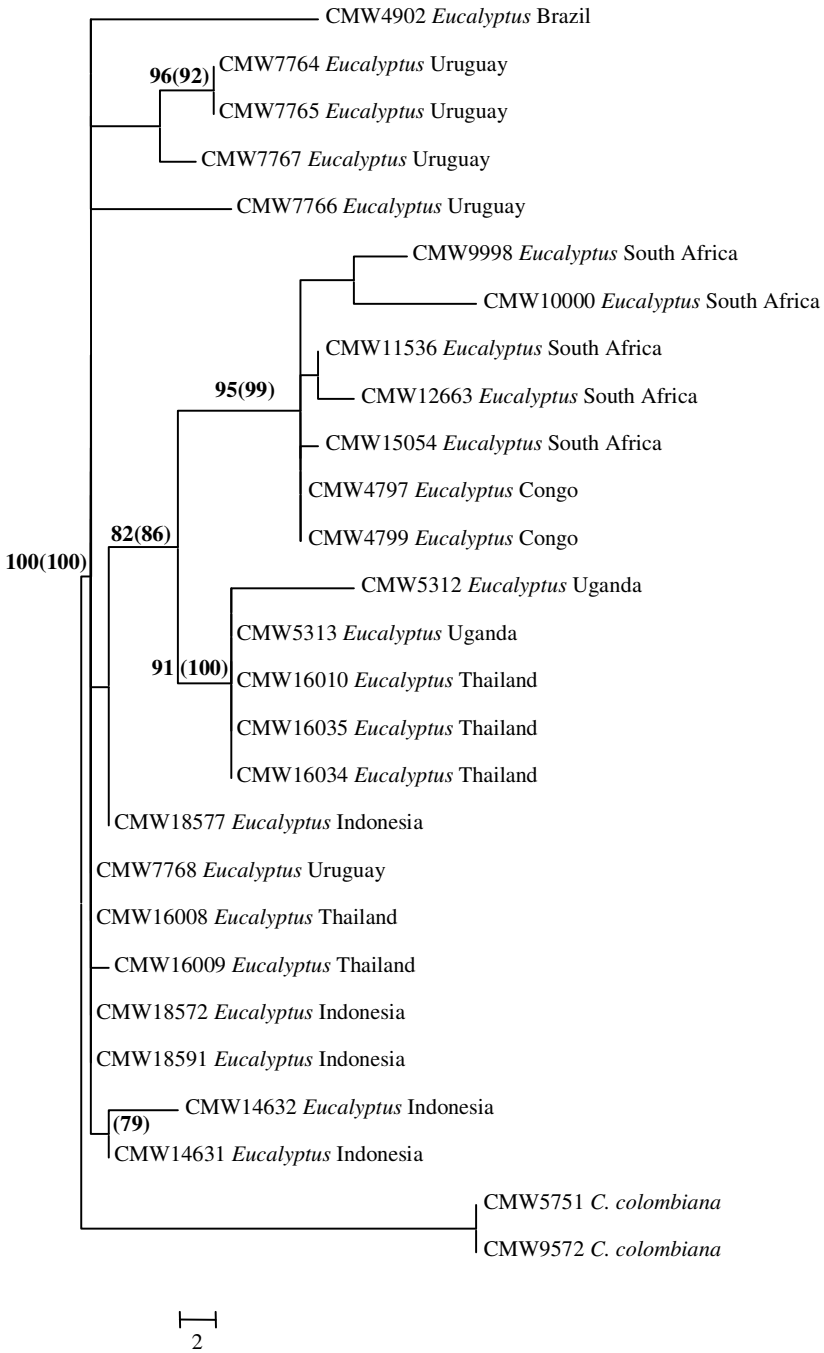
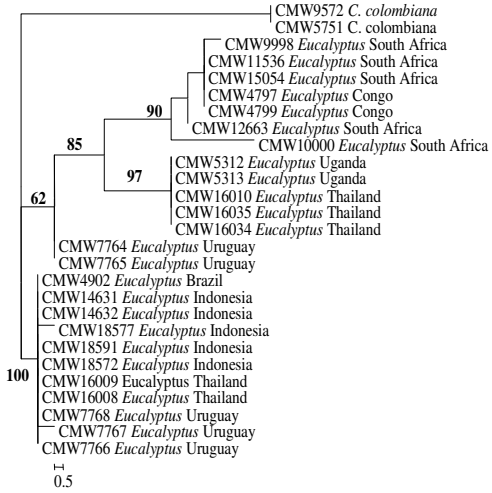
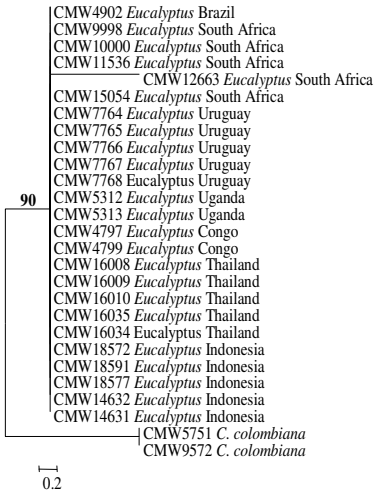


Figure 3. Three phylograms each representing a single gene region (ITS, βt and EF1-α, top to bottom) for the undescribed isolates from *Eucalyptus* representing *C. fimbriata s.l.* showing low variation in the three separate gene regions as well as no support for the sub-clades observed in the combined gene trees. No outgroup was assigned to this dataset.





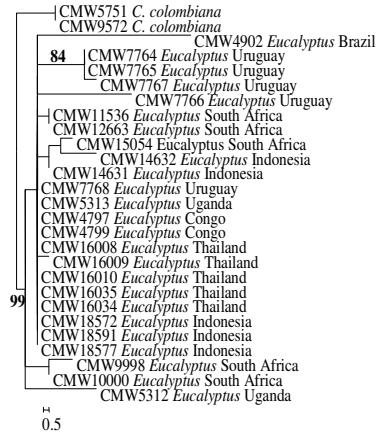


Figure 4. Allele networks obtained from the three combined gene regions (ITS, βt and EF1-α) for all *C. eucalypticola sp. nov.* isolates as well as *C. colombiana.* The species *C. colombiana* is represented as highly different to the *Eucalyptus* isolates due to the fact that it formed a separate allele tree. The *C.fimbriata s.l.* isolates from *Eucalyptus* all formed one allele tree with high levels of variation within the tree.

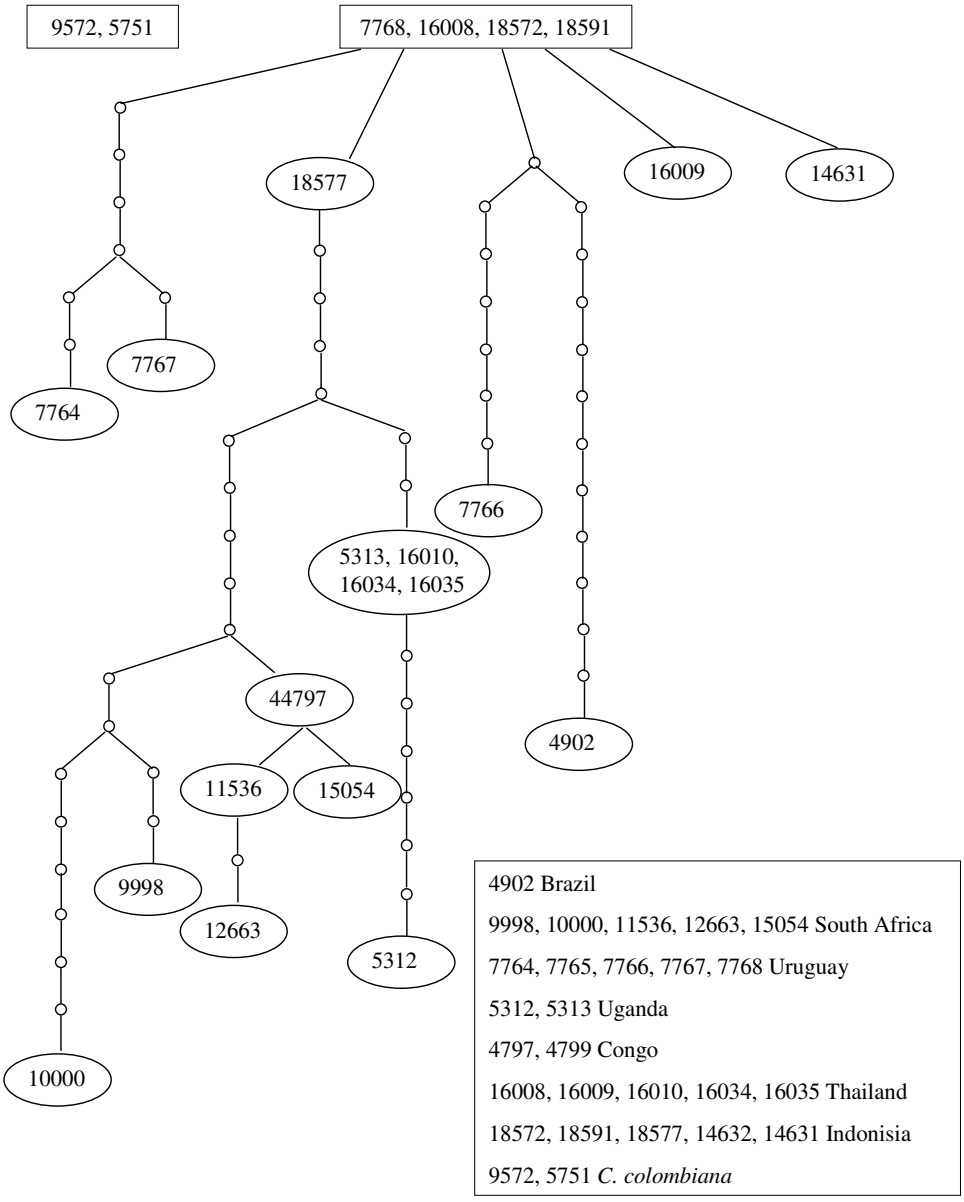
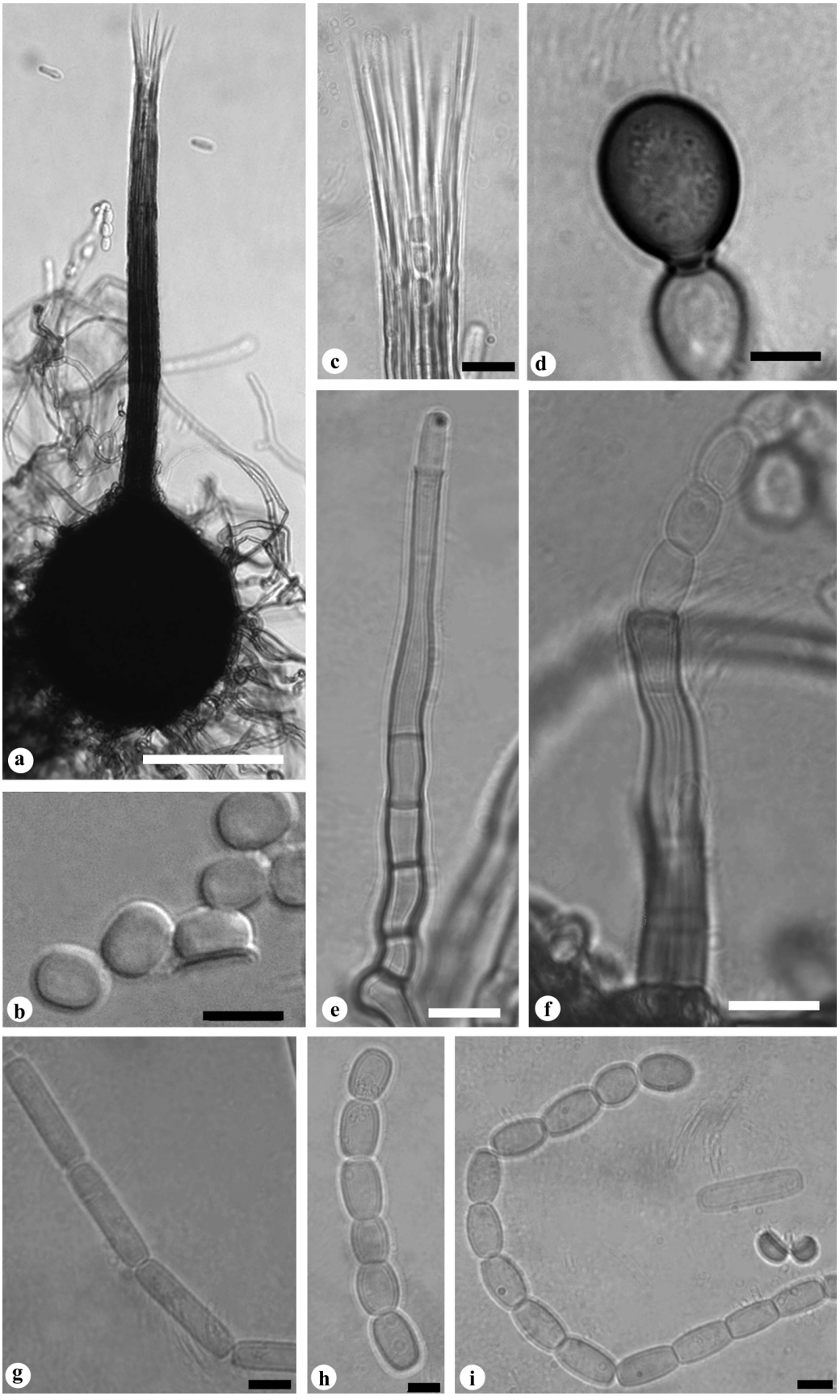


Figure 5. Morphological characteristics of *Ceratocystis eucalypticola.* **a.** Ascomata with globose base. **b.** Hat-shaped (in side view) and cucullate (in top view) ascospores. **c.** Divergent ostiolar hyphae **d.** Dark, globose to sub-globose chlamydospore. **e.** Primary conidiophore, flask-shaped phialide, producing cylindrical conidia. **f.** Tubular shaped secondary conidiophore, producing a chain of barrel-shaped conidia. **g.** Chain of cylindrical conidia. **h.** Chain of barrel-shaped conidia. **i.** A chain of barrel-shaped conidia, two hat-shaped ascospores and a cylindrical conidium. Bars; **a.** = 100 µm, **b, f-i** = 5 µm, **c-e** = 10 µm.



SUMMARY

About a hundred and twenty years ago *Ceratocystis fimbriata* was described as the etiological agent of root rot of sweet potato in the USA. Subsequently, and during the course of many years, *C. fimbriata* was isolated from numerous hosts on every continent in the world (except for Antarctica). There was considerable debate amongst interested mycologists regarding the taxonomic placement of this fungal species and it continued unabated for at least a Century. As new and more powerful techniques were applied to resolve the taxonomic status of *C. fimbriata sensu lato* and its relatives, it became increasingly clear that this fungus represented a complex of species. Most recently, a molecular phylogeny based on DNA sequence comparisons emerged and this has strongly influenced the work presented in this thesis. Subsequent to the description of species distinct from *C. fimbriata*, *C. fimbriata*, *C. moniliformis* and *C. coerulescens* were shown to include three discrete complexes of species, each including species clearly defined and characterised by similar morphology ecology and close phylogenetic relatedness. Thus, studies presented in this thesis include descriptions of *Ceratocystis* spp., which would previously have been treated as *C. fimbriata.* The *C. monilifomis* species complex has also been expanded via the description of several new species. Studies presented in this thesis present a foundation for a complete revision of *Ceratocystis* as well as the species included in the genera that it currently encompasses.