Aurifilum, a new fungal genus in the Cryphonectriaceae from *Terminalia* species in Cameroon

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Abstract

Native *Terminalia* spp. in West Africa provide a popular source of construction timber as well as medical, spiritual and social benefits to rural populations. Very little is, however, known regarding the diseases that affect these trees. During an investigation into possible diseases of *Terminalia* spp. in Cameroon, orange to yellow fungal fruiting structures, resembling those of fungi in the Cryphonectriaceae, were commonly observed on the bark of native *Terminalia ivorensis*, and on dead branches of non-native *Terminalia mantaly*. In this study the fungus was identified based on morphological features as well as DNA sequence data (ITS and β-tubulin) and its pathogenicity was tested on *T. mantaly* seedlings. Our results showed that isolates of this fungus represent a previously undescribed genus in the Cryphonectriaceae, which we describe as *Aurifilum marmelostoma* gen. et sp. nov. Pathogenicity tests revealed that *A. marmelostoma* is pathogenic on *T. mantaly*. These tests, and the association of *A. marmelostoma* with disease symptoms on *T. ivorensis*, suggest that the fungus is a pathogen of this important tree.

Introduction

The Cryphonectriaceae (Diaporthales) was described to include fungi belonging to the *Cryphonectria-Endothia* complex (Gryzenhout et al. 2006a). These fungi are characterized by a *Diaporthe*-type centrum and orange stromatic tissue in culture and on host tissue, as well as biochemical properties such as a pigment reaction with 3% KOH and lactic acid. While morphological features, such as the degree of development, type and color of stromatic tissues, color and length of perithecial necks, color and shape of conidiomata and ascospore septation are used to distinguish genera in the Cryphonectriaceae, species differentiation is primarily based on spore shape and size (Micales and Stipes 1987; Gryzenhout *et al*. 2009).

Ten genera have been described in the Cryphonectriaceae (Gryzenhout *et al*. 2009; Lumbsch and Huhndorf 2007), of which some, such as *Chrysoporthe* and *Cryphonectria*, accommodate virulent tree pathogens. For example, *Cryphonectria parasitica* is best known as the causal agent of chestnut blight that has devastated American chestnut trees (*Castanea dentata*) in North America (Anagnostakis 1987, 2001) and Europe (Heiniger and Rigling 1994). Examples of well-known pathogens in *Chrysoporthe* include *Chr. austroafricana* and *Chr. cubensis* (Gryzenhout *et al*. 2004) that have had a serious negative impact on *Eucalyptus* plantations in the tropics and subtropics,

causing stem cankers and tree death (Hodges 1980; Wingfield 2003).

Species in the Cryphonectriaceae have a worldwide occurrence and their hosts include native and introduced tree species (Gryzenhout *et al*. 2009). Although the total inventory of trees susceptible to infection by the Cryphonectriaceae is incomplete, more than 100 tree species in over 14 families have been reported as hosts (Gryzenhout *et al*. 2009). In Africa, the Cryphonectriaceae are well known on trees in the Myrtales. Hosts recorded include *Eucalyptus* spp. and *Syzygium* spp. (Myrtaceae) (Gibson 1981; Heath *et al*. 2006; Nakabonge *et al*. 2006a), *Heteropyxis canescens* (Heteropyxidaceae) (Nakabonge *et al*. 2006a), *Tibouchina* spp. (Melastomataceae) (Myburg *et al*. 2002; Nakabonge *et al*. 2006a) and *Terminalia ivorensis* (Combretaceae) (Ofosu-Asiedu and Cannon 1976). These plants occur naturally (*Syzygium* spp., *H. canescens*, *T. ivorensis*), are grown as a source of pulp and timber (*Eucalyptus* spp. and *T. ivorensis*) or are non-native species grown as ornamentals (*Tibouchina* spp.).

In West Africa, native species of *Terminalia*, such as *T. ivorensis* and *T. superba* are important for forestry. Timber products from *T. ivorensis* and *T. superba* are commercially popular and ranked third in the national round wood export business in Cameroon (Laird 1999). Moreover, they are widely grown as a plantation crop where they are established by direct planting or in the "taungya" system where food crops are grown together with them (Lamb and Ntima 1971; Norgrove and Hauser 2002). Various species of *Terminalia* also provide medical, spiritual and social benefits to rural people (Batawila *et al*. 2005; Kamtchouing *et al*. 2006; Thiombiano *et al*. 2006).

Despite the economic and sociological importance of *Terminalia* spp., little research has been conducted on the fungal diseases affecting these trees (Ofosu-Asiedu and Cannon 1976; Hodges and Ferreira 1981; Gryzenhout *et al*. 2005; Kamgan *et al*. 2008). As part of a larger project investigating diseases that affect *Terminalia* spp. in Africa, a survey was undertaken in Cameroon. Distinctive fungi with orange to yellow ascostromata resembling those of the Cryphonectriaceae were commonly observed in the bark of standing native *T. ivorensis*, and on dead branches of non-native *T. mantaly*. The objective of this study was to use DNA sequence and morphological comparisons to provide a taxonomic placement for this fungus. Furthermore, pathogenicity trials were performed to assess its potential ecological significance.

Materials and Methods

Survey and specimen collection

Surveys were conducted in the central and southern parts of Cameroon in December 2007. These regions are located in the fifth agro-ecological zone of the country (http://www.irad-cameroon.org/carte_us.php) where the vegetation and climatic conditions are characterized by humid forests with bimodal rainfall and relatively high temperatures, averaging 26°C. At the collection sites, native *T. ivorensis* is grown in plantations while *T. mantaly* is planted as ornamentals alongside city roads and in villages. Bark segments bearing fungal fruiting bodies were collected from trees showing signs of disease and transported to the laboratory.

For isolation, the ascostromata on the bark were cut horizontally with a scalpel under a dissecting microscope and ascospore masses were extracted with a sterile needle and transferred onto 2% malt extract agar (MEA) (2% malt extract, 1.5% agar; Biolab, Merck, Midrand, Johannesburg, S.A.). Single germ tubes developing from the spores were transferred to fresh Petri dishes containing MEA and incubated at 25°C. Pure cultures were deposited in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Duplicates of key isolates were also deposited with the Centaalbureau voor Schimmelcultures (CBS, Utrecht, Netherland), while bark specimens bearing fruiting bodies were

deposited with the National Collection of Fungi, Pretoria, South Africa (PREM).

DNA sequence comparisons

Mycelium was scraped from the surfaces of 10-day-old cultures of five isolates with a sterile scalpel and transferred to 1.5 µl Eppendorf tubes for freeze-drying. The freeze-dried mycelium was ground to a fine powder by shaking for 2 min at 30.0 1 s^{-1} frequency in a Retsch cell disrupter (Retsch Gmbh, Germany) using 2 mm-diameter metal beads. Total genomic DNA was extracted following the method of Möller *et al*. (1992) and the concentration of the resulting DNA was determined on a NanoDrop (ND-1000 uv/Vis spectrometer, NanoDrop Technologies, Wilmington, DE USA) version 3.1.0.

The oligonucleotide primer pairs ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) (White *et al*. 1990), Bt1A (5′ TTCCCCCGTC TCCACTTCTTCATG 3′) and Bt1B (5′ GACGAGATCGTTCATGTTGAACTC 3′), Bt2A (5′ GGTAACCAAATCGGTGCTGCTTT C 3′) and Bt2B (5′ ACCCTCAGTGTAGTGACC CTTGGC 3′) (Glass and Donaldson 1995) were used to amplify and sequence the internal transcribed spacer (ITS) regions (including the complete 5.8S) and the β-tubulin 1 and 2 (β-tub) gene regions respectively. A "hot start" polymerase chain reaction (PCR) was carried out in an Icycler thermal cycler (BIO-RAD, Hercules, CA, USA) to amplify a 25 µl PCR reaction mixture containing 0.5 µl of each primer (10 mM), 2.5 µl dNTPs (10 mM), 4 µl of 10 mM $MgCl_2$, 2.5 µl of 10 mM reaction buffer (25 mM), 1 U of Taq polymerase (Roche Diagnostics GmbH, Mannheim, Germany), 60–100 ng/µl of DNA and 13.5 µl of sterile SABAX water. The amplification conditions were an initial denaturation at 96°C for 1 min, followed by 35 cycles of 30 s at 94°C, annealing for 1 min at 54°C, extension for 90 s at 72°C and a final elongation step of 10 min at 72°C. The PCR amplification products were separated by electrophoresis on 2% agarose gels stained with ethidium bromide in a 1× TAE buffer and visualized under UV light.

The amplified PCR fragments were cleaned using 6% Sephadex G-50 fine mini spin-columns (Sigma, Steinhein, Germany) following the manufacturer's instructions. Thereafter, 25 amplification cycles were carried out for each sample on an Icycler thermal cycler to generate sequences in both the forward and reverse directions using 10 µl mixes. Each mix contained 1 µl reaction buffer, 2 µl ready reaction buffer (Big dye), 1 µl primer (10 mM), 3 µl of the PCR product and 3 µl Sabax water. The reaction cycles had the following parameters: one step at 96°C for denaturation of the double stranded DNA (10 s), followed by an annealing step at 50°C (5 s) and primer extension at 60°C (4 min). The BigDye Terminator v 3.1 Cycle sequencing Kit (PE Applied Biosystems) was used for sequencing reactions, following the manufacturer's protocols, on an ABI PRISM 3130 × l genetic analyzer using Pop 7 polymer (Applied Biosystems, Foster City, California, USA).

The sequences of the isolates from *Terminalia* spp. were edited using MEGA version 4 (Tamura *et al*. 2007). For the phylogenetic analyses, DNA sequences from this study were compiled into a matrix using the dataset produced by Gryzenhout *et al*. (2009) as a template (TreeBase number: S2003 Matrix M3737). The matrix was aligned using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) version 6 (Katoh *et al*. 2005). The aligned sequences were transferred to PAUP version 4.0b10 (Swofford 1998) where a final manual alignment was made.

Phylogenetic analyses were run for each of the gene region datasets separately, as well as for a combined ITS and β-tub data set. In the analyses, gaps were treated as a fifth character (NEWSTATE) and all characters were unordered and of equal weight. The phylogenetic analyses for all the datasets were performed using the maximum parsimony (MP) option, with trees generated by heuristic searches with random stepwise addition in 1000 replicates, tree bisection and

reconnection (TBR) as branch swapping algorithm, and random taxon addition for the construction of MP trees. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. MAXTREES was set to auto-increase in all analyses. Sequences of two isolates of Diaporthe ambigua (Gryzenhout *et al*. 2009), which belong to the Diaporthaceae, another family in the Diaporthales (Castlebury *et al*. 2002; Rossman *et al*. 2007), were used as outgroups in all analyses, following examples of previously published data sets (Gryzenhout *et al*. 2009). The outgroup was monophyletic in the phylogenetic analyses. The support of the branches for the most parsimonious trees was assessed with a 1000 bootstrap replications (Felsenstein 1985). Other measures noted were tree length, consistency index, rescaled consistency index, and retention index (Hillis and Huelsenbeck 1992). A partition homogeneity test of 500 replicates was conducted in PAUP to assess the possibility of combining the ITS and β-tub data sets.

Bayesian analyses using the Markov Chain Monte Carlo (MCMC) method were performed to ascertain the topology of trees obtained with PAUP. Before launching the Bayesian analyses, the best nucleotide substitution models for each dataset were separately determined with MrModelTest version 2.2 (Nylander 2004) and included in each partition in MrBayes v3.1.2. (Huelsenbeck and Ronquist 2001). HKY + I + G were chosen as best-fitting model for both the ITS and β-tub datasets. The MCMC analyses, with four chains, started from random tree topology and lasted one million generations. Trees were saved every 100th generation. The burn-in number was graphically estimated from the likelihood scores and trees outside this point were discarded in the analyses. The consensus trees were constructed in MEGA version 4 and posterior probabilities were assigned to branches after 50% majority rule.

Morphology

A small piece of an original bark specimen bearing fruiting structures from which the fungal isolates were obtained was cut and boiled in water for 1 min to rehydrate the cells (Myburg *et al*. 2004; Gryzenhout *et al*. 2005). The structures were then broken from the bark and sections (12 µm thick) were made with a Leica CM1100 cryostat (Setpoint Technologies, Johannesburg, South Africa) at −20°C. Sections were mounted on microscope slides in 85% lactic acid. Stromata were also crushed on microscope slides in 3% KOH in order to study the asci, ascospores, conidia, conidiophores and conidiogenous cells. Morphological features of fruiting bodies were photographed with a HRc Axiocam and accompanying Axiovision 3.1 software (Carl Zeiss Ltd., München, Germany). For the holotype specimen, 50 measurements of each structure mentioned above were taken, whereas 20 measurements were made for the paratypes. These measurements were recorded as the extreme in brackets and the range calculated as the mean of the overall measurements plus and minus the standard deviation.

The physiognomy of fungal colonies was described from cultures grown on 2% MEA at 25°C under near UV-light for 2 weeks. Colony colours of the isolates were recorded using the color notations of Rayner (1970). Growth studies for isolates growing on 2% MEA in the dark was performed by measuring the daily growth at 5°C intervals ranging from 10 to 35°C for five replicates of two isolates (ex-holotype isolate CMW28290, ex-paratype isolate CMW28285).

Pathogenicity

Seedlings of native species of *Terminalia* were not available for pathogenicity tests and these were consequently performed only on non-native *T. mantaly* trees. Pathogenicity experiments were carried out on 1-year-old *T. mantaly* plants grown in the Yaoundé Urban Council nursery, Cameroon. The trees were maintained in 15 cm diameter plastic bags and watered daily. At the time of inoculation, the average stem diameter of the trees was approximately 10 mm. For inoculations, isolates were grown on 2% MEA for 10 days prior to inoculation.

To inoculate trees, wounds were made on the stems, ~10 cm above soil level, by removing the outer bark with a 5 mm diameter cork-borer. A 5 mm-diameter plug bearing mycelium of each isolate was placed into each wound, with the mycelium facing the cambium, and wrapped with a strip of Parafilm (Pechiney Plastic Packaging, Chicago, USA) to prevent desiccation and cross contamination. The trees were divided into two separate blocks and within each block, six trees arranged in a completely randomized design, were used for each isolate. The entire trial was repeated once. For the control inoculations, a sterile MEA plug was used. After 6 weeks, the lengths of the lesions in the cambium were measured to obtain an indication of the virulence of the isolates tested. Single fruiting bodies were removed from the necrotic tissue and these were placed on MEA to produce cultures and to confirm the cause of the lesions. As no significant differences were noticed between the two pathogenicity tests ($P > 0.05$), the data for all isolates were pooled in a single dataset for analyses. Variation in lesion lengths was assessed through a one-way analysis of variance (ANOVA) using SAS (SAS systems, version 8.2; SAS Institute).

Results

Survey and specimen collection

Terminalia trees at three sites, in two regions, in southern and central Cameroon were inspected. A total of seven trees were found with fruiting bodies of the unknown fungus. On living standing *T. ivorensis*, disease symptoms included cankers on the tree trunks (Fig. 1a), cracked bark containing yellow to orange fruiting structures (Fig. 1b) and necrotic cambium including dead wood. On *T. mantaly*, cankers covered with abundant fruiting bodies were observed on the trunks of dead trees and on senescing branches (Fig. 1c). Samples were obtained from one *T. mantaly* tree in Yaoundé, five *T. ivorensis* trees in Mbalmayo in the Central Region, and one *T. mantaly* tree in Kribi in the Southern Region of Cameroon (Fig. 2).

DNA sequence comparisons

Five isolates resembling the Cryphonectriaceae, collected from *Terminalia* spp. in Cameroon, were selected for DNA sequencing (Table 1). Sequencing resulted in fragments of ~600 bps for the ITS and ~550 bps for each of the β-tub gene regions. BLAST searches against the NCBI (www.blast.ncbi.nlm.nih.gov) data base confirmed that the isolates collected represented species in the Cryphonectriaceae and indicated that isolates from Cameroon were most closely related to *Microthia havanensis*.

Data sets containing ITS and β-tub sequences were compiled using sequences obtained from isolates of Cryphonectriaceae in Cameroon and those obtained from Gryzenhout *et al*. (2009). These data sets comprised a total of 36 isolates each (Table 1), five isolates obtained from *Terminalia* spp. and 31 sequences that were used in the monograph of the Cryphonectriaceae (Gryzenhout *et al*. 2009) that represents the most complete database of sequences for this family.

Of the 592 characters present in the ITS data set, 250 were parsimony-informative. The MP analyses generated 60 identical trees (TL = 568, CI = 0.697, RI = 0.876, RC = 0.611). Isolates from *Terminalia* spp. grouped in a single, well supported clade, distinct from all other recognized genera of the Cryphonectriaceae. A consensus tree generated through Bayesian analyses of the ITS data confirmed the uniqueness of the isolates from Cameroon.

Fig. 1. Symptoms of infection by *Aurifilum marmelostoma* on *Terminalia* spp. in Cameroon. **a** canker on the basal parts of a *Terminalia ivorensis* trunk; **b** bark cracks containing yellow to orange fruiting structures; **c** orange stromata on bark of *Terminalia mantaly.*

In the analyses of the β-tub data set, sequences consisted of 991 characters with 486 parsimony informative characters of which 316 came from ambiguous portions representing introns (one for the β-tub 1 and two for the β-tub 2 gene region). The MP analyses yielded four most parsimonious trees (TL = 1382, CI = 0.614, RI = 0.834, RC = 0.512). The tree generated from the β-tub data (figure not shown) also separated the isolates from *Terminalia* spp. into a well supported clade, distinct from all the known genera in the Cryphonectriaceae. The tree obtained after Bayesian analyses confirmed results obtained from the MP analyses, suggesting that isolates from Cameroon represent a distinct genus and species. In a separate analysis of β-tub sequences without the ambiguous portions representing the introns (384 characters), a similar tree topology with high statistical support was obtained (tree not shown). However, the analysis of β-tub sequences containing the introns were preferred as it provided more informative characters.
Concordance among the ITS and β-tub datasets was confirmed by the results of the partition homogeneity test (P = 0.002) suggesting a lack of conflict between these gene genealogies, and they were thus combined. A total of 1583 bases were generated for the combined ITS and β-tub data sets. Of these, 735 characters were parsimony informative. After heuristic searches, one most parsimonious tree of 1980 steps (CI = 0.628, RI = 0.839, RC = 0.527; TreeBase Accession No: SN4451) was obtained (Fig. 3). The consensus tree obtained from the combined analysis of ITS and β-tub sequences showed that isolates of the unknown fungus from *Terminalia* spp. formed a well

supported clade (Bayesian Posterior Probability (BPP)/Bootstrap support (BS): 1/100). This clade is distinct from other phylogenetically related genera in the Cryphonectriaceae (Gryzenhout *et al*. 2009).

Fig. 2. Map of Cameroon showing sites where *Aurifilum marmelostoma* was collected from *Terminalia ivorensis* and *Terminalia mantaly*.

Morphology

Consistent with DNA sequence data, the fruiting bodies from *Terminalia* spp. showed typical microscopic characteristics of members of the Cryphonectriaceae (Table 2). These fruiting structures were characterized by distinct orange stromatic tissue, *Diaporthe*-type centra (Gryzenhout *et al*. 2006a) and a pigment that turns purple and yellow in culture and host tissue when treated with 3% KOH and lactic acid respectively (Castlebury *et al*. 2002; Rossman *et al*. 2007; Gryzenhout *et al*. 2009). The teleomorph fruiting structures were abundant on the bark, while conidiomatal structures were only occasionally seen on the bark specimens.

The fungus on *Terminalia* spp. in Cameroon resembled those species in the Cryphonectriaceae that have uniformly orange fruiting bodies as opposed to those with different colors between their anamorph and teleomorph states, such as species of *Chrysoporthe* (Gryzenhout *et al*. 2009). However, it had a number of features distinguishing it from all other Cryphonectriaceae. The most obvious of these characteristics were present in the anamorph. Conidiomata of the fungus from Cameroon were broadly convex, and thus wider than similar structures of *Amphilogia* and *Rostraureum* (Table 2). The presence of darkened ostiolar openings at the apex of the conidiomata was also unique to the fungus. Long paraphyses, or seemingly sterile cells (<90 μm) (Walker *et al*. 1985; Venter *et al*. 2002), were observed between conidiophores, similar to those found for conidiomata of *Holocryphia* and *Microthia* (Gryzenhout *et al*. 2006b).

Table 1. Isolates of the Cryphonectriaceae used in this study.

Species	Isolates number[a]	Host	Origin	Collector	Genbank accession numbers[b]
Amphylogia gyrosa	CMW 10469	*Elaeocarpus dentalus*	New Zealand	G.J. Samuels	AF452111, AF525707, AF525714
	CMW 10740	*E. dentalus*	New Zealand	G.J. Samuels	AF452112, AF525708, AF525715
Aurapex penicillata	CMW 10030	*Miconia theazeans*	Colombia	C.A. Rodas	AY214311, AY214239, AY214275
	CMW 10035	*M. theazeans*	Colombia	C.A. Rodas	AY214313, AY214241, AY214277
Auriflum marmelostoma	**CMW 28285**	*Termindia mantaly*	Cameroon	D. Begoude and J. Roux	FJ882855, FJ900585, FJ900590
	CMW 28288	*T. ivorensis*	Cameroon	D. Begoude and J. Roux	FJ882856, FJ900586, FJ900591
	CMW 28289	*T. ivorensis*	Cameroon	D. Begoude and J. Roux	FJ890495, FJ900587, FJ900592
	CMW 28290	*T. ivorensis*	Cameroon	D. Begoude and J. Roux	FJ890496, FJ900588, FJ900593
	CMW 28592	*T. mantaly*	Cameroon	D. Begoude and J. Roux	FJ890497, FJ900589, FJ900594
Celoporthe dispersa	CMW 9976	*Syzygium cordatum*	South Africa	M. Gryzenhout	DQ267130, DQ267136, DQ267142
	CMW 9978	*S. cordatum*	South Africa	M. Gryzenhout	AY214316, DQ267135, DQ267141
Chrysoporthe austroafricana	CMW 2113	*Eucalyptus grandis*	South Africa	M.J. Wingfield	AF046892, AF273067, AF273462
	CMW 9327	*Tibouchina granulosa*	South africa	M.J. Wingfield	AF273473, AF273060, AF273455
Chrysoporthe cubensis	CMW 10639	*Eucalyptus grandis*	Colombia	C.A. Rodas	AY263419, AY263420, AY263421
	CMW 10669	*Eucalyptus* sp.	Republic of Congo	J. Roux	AF535122, AF535124, AF535126
Chrysoporthella hodgesiana	CMW 10625	*Tibouchina semidecandra*	Colombia	R. Arbelaez	AY956970, AY956979, AY956980
	CMW 10641	*T. semidecandra*	Colombia	R. Arbelaez	AY692322, AY692326, AY692325
Cryphonectria parasitica	CMW 7048	*Quercus vaginiana*	USA	F.F. Lombard	AF368330, AF273076, AF273470
	CMW 13749	*Castanea mollisima*	Japan	Unknown	AY697927, AY697943, AY697944
Cryphonectria japonica	CMW 13742	*Quercus grosseserrata*	Japan	T. Kobayashi	AY697936, AY697961, AY697962
	CMW 13747	*Q. serrata*	Japan	T. Kobayashi	AY697937, AY697963, AY697964
Cryhonectria radicalis	CMW 10455	*Castanea dendata*	Italy	A. Biraghi	AF452113, AF525705, AF525712
	CMW 10477	*Quercus suber*	Italy	M. Orsenigo	AF368328, AF368347, AF368346
Cryptodiaporthe corni	CMW 10526	*Cornus alternifolia*	USA	S. Redlin	DQ120762, DQ120769, DQ120770
Diaporthe ambigua	CMW 5288	*Malus domestica*	South Africa	W.A Smit	AF543817, AF543819, AF543821
	CMW 5587	*M. domestica*	South Africa	W.A. Smit	AF543818, AF543820, AF543822
Endothia gyrosa	CMW 2091	*Quercus palustris*	USA	R.J. Stipes	AF046905, AF368337, AF368336
	CMW 10442	*Q. palustris*	USA	R.J. Stipes	AF368326, AF368339, AF368338
Holocryphia eucalypti	CMW 7036	*Eucalyptus* sp.	South Africa	I. van der Westhuizen	AF232878, AF368341, AF368340
	CMW 7037	*Eucalyptus delegatensis*	Australia	K.M. Old	AF232880, AF368343, AF368342
Microthia havanensis	CMW 14550	*Eucalyptus saligna*	Mexico	C.S. Hodges	DQ368735, DQ368741, DQ368742
	CMW 11301	*Myrica faya*	Azores	C.S. Hodges and D.E. Gardner	AY214323, AY214251, AY214287
Rostraureum tropicale	CMW 9971	*Terminalia ivorensis*	Ecuador	M.J. Wingfield	AY167425, AY167430, AY167435
	CMW10796	*T. ivorensis*	Ecuador	M.J. Wingfield	AY167428, AY167433, AY167438
Ursiculum fallax	CMW 18115	*Cocoloba uvifera*	USA	C.S. Hodges	DQ368756, DQ368760, DQ368761
	CMW 18119	*C. uvifera*	USA	C.S. Hodges	DQ368755, DQ368758, DQ368759

Isolates marked in bold represent those obtained from *T. ivorensis* and *T. mantaly* in Cameroon

[a] *CMW* Research collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa

[b] Accession numbers given as sequences from the ITS region, and two regions from the β-tubulin genes respectively

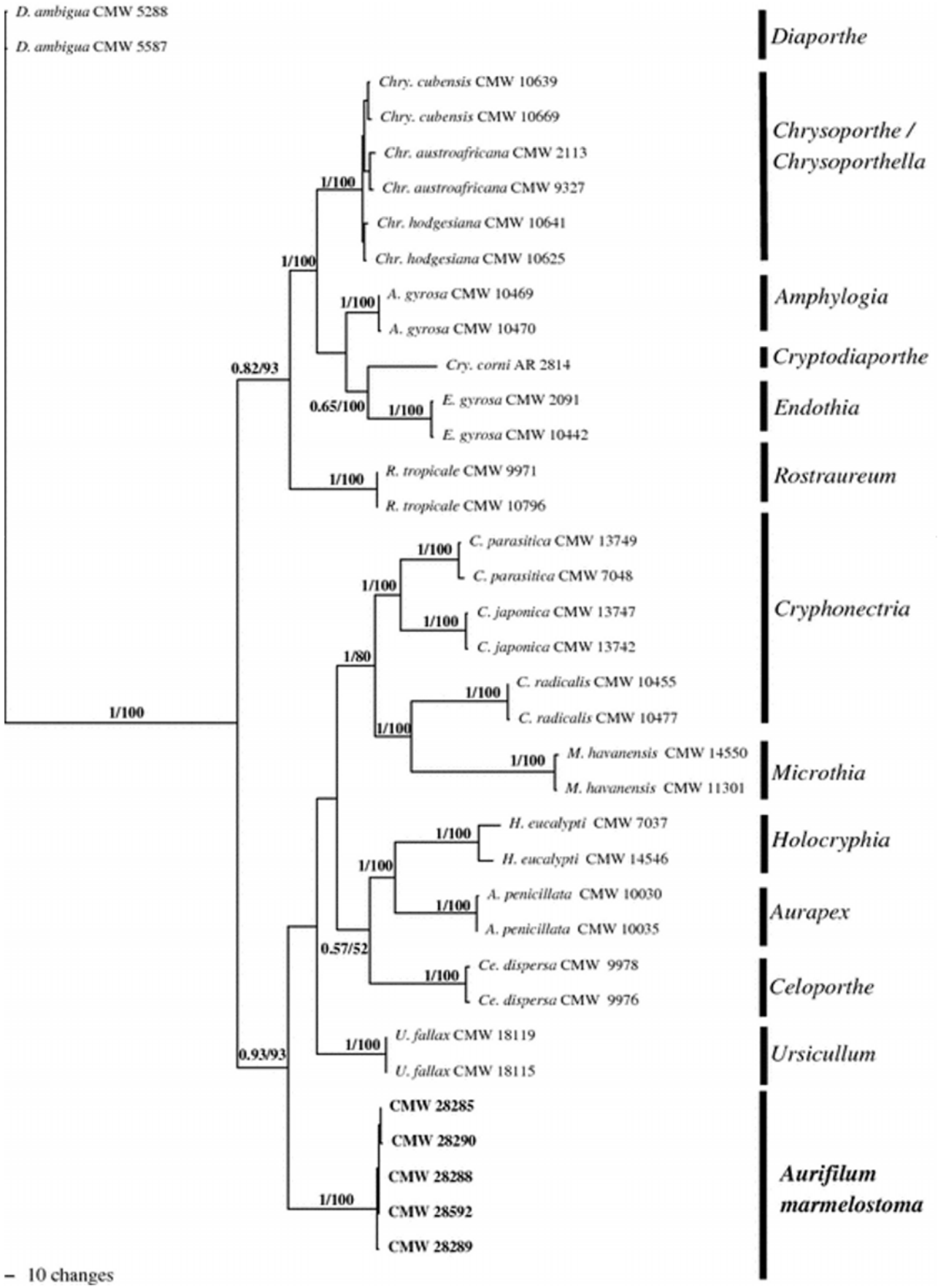

Fig. 3. Most parsimonious tree obtained from MP analyses of the combined ITS and β-tub sequence data of the Cryphonectriaceae. Posterior probabilities followed by Bootstrap support (%) from 1000 replications are given on the branches (BPP/BS). Isolates marked in bold represent those obtained from *Terminalia* spp. Scale "10 changes" reflects the graphical amount of nucleotide change

between two sequences since their divergence from the common ancestor.

Table 2. Morphological characteristics of genera in the Cryphonectriaceae with uniformly orange ascostromata, compared with those of *Aurifilum*.

Morphological characteristics		*Amphilogia*	*Cryphonectria*	*Endothia*	*Holocryphia*	*Microthia*	*Rostraureum*	*Urticulum*	*Aurifilum*
Teleomorph	Structure of ascostromata	Pulvinate, erumpent, slightly immersed to superficial	Large, pulvinate, erumpent, semi-immersed	Large, pulvinate to clavate, erumpent, superficial	Pulvinate, semi-immersed	Large, pulvinate, erumpent, semi-immersed	Pulvinate, erumpent, immersed to semi-immersed	Not known	Large, pulvinate to pyriform, semi-immersed
	Ascospore shape	Hyaline, ellipsoidal to fusoid	Hyaline, ellipsoidal to fusoid	Hyaline, cylindrical	Hyaline, cylindrical	Hyaline, ellipsoidal to fusoid	Hyaline, ellipsoidal to fusoid	Not known	Hyaline, ellipsoidal to fusoid
	Ascospore septation	1-3 septate	One septate	Aseptate	Aseptate	One septate	One septate	Not known	One septate
Anamorph	Structure of conidiomata	Conical, superficial	Pulvinate, erumpent, semi-immersed	Pulvinate, erumpent, superficial,	Pulvinate, erumpent, semi-immersed	Pulvinate, erumpent, semi-immersed	Clavate to rostrate	Pyriform or rostrate, superficial	Broadly convex
	Conidiomatal neck	Absent	Absent	Absent	Absent	Absent	Present	Present	Absent, ostiolar opening darkened
	Conidiomatal stromatic tissue	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma	Of different textum type	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma
	Paraphyses	Absent	Absent	Absent	Present	Present	Absent	Absent	Present

Gryzenhout *et al*. (2005), provided details of morphological characteristics of African specimens (IMI 187898 and IMI 288729) of Cryphonectriaceae obtained from *T. ivorensis* in Ghana and Kenya, respectively. The same specimens were also considered in this study. Conidiomata of the Ghanaian specimen were characterized by orange, pulvinate conidiomata without elongated necks, similar to the conidiomata of the Cameroonian specimens that are orange and broadly convex. The stromatic ascostromata of specimens from both Ghana and Kenya resemble the Cameroonian isolates. The similarity of specimens from Cameroon, Ghana and Kenya was, furthermore, supported by spore characteristics (one septate, fusoid to ellipsoid ascospores and minute, cylindrical conidia) and overlapping spore dimensions. The presence of conidiophores was not mentioned for specimens from Ghana and Kenya (Gryzenhout *et al*. 2005) and they were not present in specimens examined in this study.

Taxonomy

Comparisons of DNA sequences and morphology of the fungus from Cameroon with the genera in the Cryphonectriaceae revealed that the fungus from Cameroon represents a previously undescribed genus in the family. A new genus and the linked species are described as follows.

Aurifilum Begoude, Gryzenh. & Jol. Roux, gen. Nov.

Etymology—The name is derived from the Latin *Aureus* (golden) and *filum* (thread) referring to the orange, confluent stromata found in the cracks on the bark of infected trees.

Ascostromata magna, plerumque sub cortice vel erumpentia, pulvinata vel pyriformia, subimmersa. Ascosporae hyalinae, fusoideae vel ellipsoideae, septo singulo mediano.

Conidiomata ascomatorum partes pro loculis conidialibus vel structuris solitariis, aurantiaca cum apertura ostiolari, sine collo atrato, late convexa, subimmersa. Conidiophorae cylindricae hyalinae non septatae, cellulae conidiogenae phialidicae basibus inflatis apicibus attenuatis, cellulae nonnullae cylindricae steriles, paraphysibus similes. Conidia minuta hyalina cylindrica vel allantoidea, non septata. Species typical *A. marmelostoma* Begoude *et al*.

Ascostromata large, usually beneath or erumpent through bark, pulvinate to pyriform, semi-immersed, orange, upper region eustromatic, lower region pseudostromatic, pseudoparenchymatous to prosenchymatous tissue. Perithecia valsoid, embedded in stroma, fuscous black, bases globose to

subglobose, necks emerge at stromatal surface with black ostioles, surround with orange stromatal tissue to form papillae. Asci fusoid to ellipsoidal, floating freely in perithecial cavity, unitunicate with non-amyloid, refractive apical ring. Ascospores hyaline, fusoid to ellipsoidal, one median septum.

Conidiomata part of ascomata as conidial locules or as solitary structures, orange, without a neck, tissue around ostiolar opening darkened, broadly convex, semi-immersed, uni- to multilocular, even to convoluted lining, tissue mostly prosenchymatous with pseudoparenchymatous tissue towards the margin depending on the developmental stage of the structures. Conidiophores cylindrical, aseptate, hyaline, conidiogenous cells phialidic with inflated bases and attenuated apices, some cylindrical cells sterile similar to paraphyses. Conidia minute, hyaline, cylindrical to allantoid, aseptate, exuded through ostioles as orange droplets or tendrils.

Aurifilum marmelostoma Begoude, Gryzenh. & Jol. Roux, sp. nov. MB 513488 Fig. 4

Etymology—The word “marmelo” is Greek for confectionary cooking practice using quinces with honey and from which the jam known as marmalade is derived. The name refers to the darkened stomatal (stoma = mouth) opening of the conidiomata giving the impression that they are covered with jam.

Ascostromata in cortice gregaria vel singula, saepe in rimis confluentia, mediocria vel magna, 300.0–830.0 µm supra corticem 760.0–1050.0 µm diametro crescentia, plerumque sub cortice vel erumpentia, subimmersa, pulvinata vel pyriformia, aurantiaca. Ascosporae hyalinae, fusoideae vel ellipticae, septo singulo mediano apice attenuata (9.0−) 10.0–12.0 (−13.5) × 3.0–4.0 (−4.5) µm.

Conidiomata ascomatarum partes pro loculis conidialibus vel structuris solitariis, aurantiaca, sine collis, apertura ostiolari atrata, late convexa, subimmersa, usque ad 660.0 µm supra superficiem corticis et 660.0 µm lata. Conidia minuta hyalina cylindrica vel allantoidea non septata (3.0−) 3.5–4.5 (−5.0) × 1.0–1.5 (−2.5) µm, pro guttulis vel cirrhis aurantiacis per aperturam in superficie stromatis exsudata.

Ascostromata on bark gregarious or single, often confluent in cracks, medium to large, ascostromata extending 300.0–830.0 µm high above the bark, 760.0–1050.0 µm diam, usually beneath or erumpent through bark, semi-immersed, pulvinate to pyriform, orange, upper region eustromatic, lower region pseudostromatic, pseudoparenchymatous to prosenchymatous tissue. Perithecia valsoid, up to nine per stroma, embedded in stroma at irregular levels with bases touching host tissue, fuscous black, bases globose to subglobose, 190.0–310.0 µm diam, perithecial walls 170.0–275.0 µm thick. Perithecial necks periphysate, black, 30.0–100.0 µm wide, emerging at stromatal surface as black ostioles, surrounded with orange stromatal tissue to form papillae, textura porrecta, extended necks up to 550.0 µm long. Asci fusoid to ellipsoidal, floating freely in the perithecial cavity, unitunicate with non-amyloid, refractive apical rings, non-stipitate, 8-spored, (44.5−) 47.0–53.5 (−61.0) × (7.0−) 7.5–9.0 (−10.5) µm. Ascospores hyaline, fusoid to ellipsoidal, one median septum with tapered apex, (9.0−) 10.0–12.0 (−13.5) × 3.0–4.0 (−4.5) µm.

Conidiomata part of ascomata as conidial locules or as solitary structures, orange, necks absent, tissue around ostiolar openings darkened, broadly convex, semi-immersed, up to 660.0 µm above the bark surface and up to 600.0 µm in diam, uni- to multilocular, even to convoluted lining, locule 80.0–300.0 µm diam, tissue mostly prosenchymatous with pseudoparenchyma towards the margin depending on the developmental stage of the structure. Conidiophores cylindrical, aseptate, hyaline, (8.5−) 15.5–41.5 (−58.5) µm long, conidiogenous cells phialidic, sometimes with inflated bases, collarettes inconspicuous with attenuated apexes, (2.0−) 2.5–3.5 (−4.5) µm wide, long sterile cylindrical cells similar to paraphyses present, (22.5−) 33.5–66.0 (−89.0) × 2.5–3.5 (−4.0) µm.

Conidia minute, hyaline, cylindrical to allantoid, aseptate, exuded through opening at stromatal surface as orange droplets or tendrils, (3.0−) 3.5–4.5 (−5.0) × 1.0–1.5 (−2.5) µm.

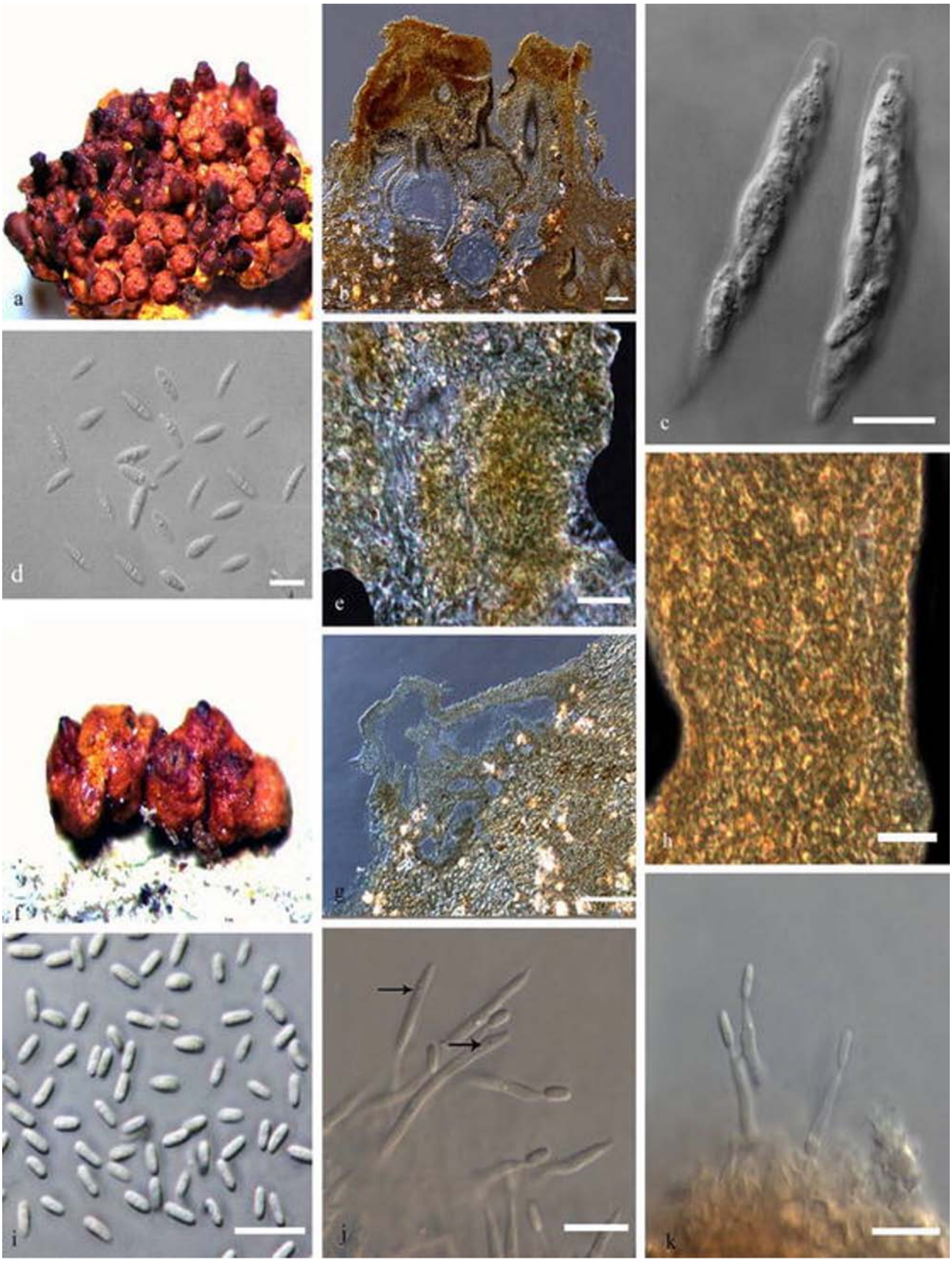

Fig. 4. Fruiting structures of *Aurifilum marmelostoma*. **a** orange ascostromata on bark; **b** vertical section through ascostromata; **c** ascus; **d** ascospores; **e** stromatic tissue of ascostromata; **f** conidiomata showing a black ostiolar opening; **g** vertical section though conidiomata;, **h** stromatic

tissue of conidiomata; **i** conidia; **j** conidiophores and sterile paraphyses (arrows); **k** conidiophores. Scale bars: **a**, **d**, **f**, **i**, **j**, **k** = 10 μm; **c**, **e**, **g**, **h** = 20 μm; **b** = 50 μm.

Cultural characteristics—mycelium fluffy, slightly aerial, creamy white to pale luteous. Conidiomata produced occasionally on old cultures. Optimum temperature of growth 25–30°C, covering the 90 mm diameter Petri plate after 1 week in the dark. No growth at 10 and 35°C.

Hosts—*Terminalia mantaly* H. Perrier, *Terminalia ivorensis* A. Chev.

Distribution—Cameroon: Kribi, Mbamalyo and Yaounde.

Specimens examined: Cameroon, Mbamalyo, bark of *Terminalia ivorensis*, Dec 2007, D. Begoude and J. Roux, PREM 60256—holotype, CMW28290/CBS124928 ex-type culture.

Additional specimens: Cameroon, Yaoundé, bark of dead branches of *Terminalia mantaly*, Dec 2007, D. Begoude and J. Roux. PREM 60257—Paratype, living cultures CMW28285/CBS124929; Mbalmayo: isolated from bark of *Terminalia ivorensis*, Dec 2007, D. Begoude and J. Roux. Paratype, living cultures CMW28288/CBS124930, CMW28592, CMW28289. The specimens, IMI 288729 and IMI 187898 obtained from *Terminalia* spp. in Ghana and Kenya, respectively, were also used, but could not be sequenced.

To facilitate identification of this genus, the dichotomous key presented by Gryzenhout *et al*. (2009) was updated to include this fungus. In this key, *A. marmelostoma* is compared with all members of the Cryphonectriaceae including those with dark fruiting bodies.

This key is based on characteristics of both the anamorph and teleomorph (key follows below).

Pathogenicity

Six weeks after inoculation, all isolates of *A. marmelostoma* yielded visible stem cankers on *T. mantaly* trees (Fig. 5). Analysis of variance showed that brown lesion lengths on the cambium for all isolates were significantly different ($P < 0.0001$) to those associated with the negative control (Fig. 5). Isolate CMW28290 from *T. ivorensis*, was the most virulent and produced significantly longer lesions in the cambium than the other isolates. Orange-colored fruiting bodies were observed on the brown lesions produced by all the isolates, and these provided the basis to confirm the association of *A. marmelostoma* with the brown lesions resulting from inoculation. In contrast, all control inoculations did not result in any brown lesions, with all of them having developed callus tissue and sealing of the inoculation wound.

Discussion

The Cryphonectriaceae was described as a family to accommodate fungal species previously treated in the *Cryphonectria-Endothia* complex (Gryzenhout *et al*. 2006a). This study records the discovery of a previously unknown genus in the Cryphonectriaceae with a single species. Comparisons of DNA sequences of isolates representing all the genera in the Cryphonectriaceae suggest that this fungus represents a new taxon, for which the name *Aurifilum marmelostoma* was provided.

1a.	Orange conidiomata	2
1b.	Black conidiomata	9
2a.	Conidiomata pulvinate, ascospores septate or aseptate	3
2b.	Conidiomata conical or rostrate or pyriform or convex, with or without a neck, ascospores septate	6
3a.	Ascospores septate	4
3b.	Ascospores aseptate	5
4a.	Stromata strongly developed, erumpent, semi-immersed, usually no paraphyses	***Cryphonectria***
4b.	Stromata small to medium, semi-immersed to superficial, paraphyses present	***Microthia***
5a.	Stromata strongly developed, large, erumpent, mostly superficial, numerous conidial locules, no paraphyses in conidial locules	***Endothia***
5b.	Stromata small to medium, semi-immersed, few conidial locules or one convoluted locule, paraphyses in conidial locules	***Holocryphia***
6a.	Conidiomata with necks, ascospores single septate	7
6b.	Conidiomata without necks, ascospores septate	8
7a.	Conidiomata rostrate, white sheath of tissue surrounding perithecial necks when sectioned longitudinally	***Rostraureum***
7b.	Conidiomata rostrate to pyriform with large base, neck attenuated or not, teleomorph still unknown	***Ursicollum***
8a.	Conidiomata conical without attenuated necks, uniformly orange, ascospores 1 to 3-septate	***Amphilogia***
8b.	Conidiomata convex, with blackened ostiolar openings, ascospores 1-septate	***Aurifilum***
9a.	Conidiomata uniformly black	9
9b.	Conidiomata with orange neck, teleomorph still unknown	***Aurapex***
10a.	Conidiomata pulvinate to pyriform with attenuated neck, base tissue of *textura globulosa*, perithecial necks long and covered with dark tissue	***Chrysoporthe***
10b.	Conidiomata pulvinate or conical, occasionally with short necks, base tissue prosenchymatous, perithecial necks short and of same color as stroma	***Celoporthe***

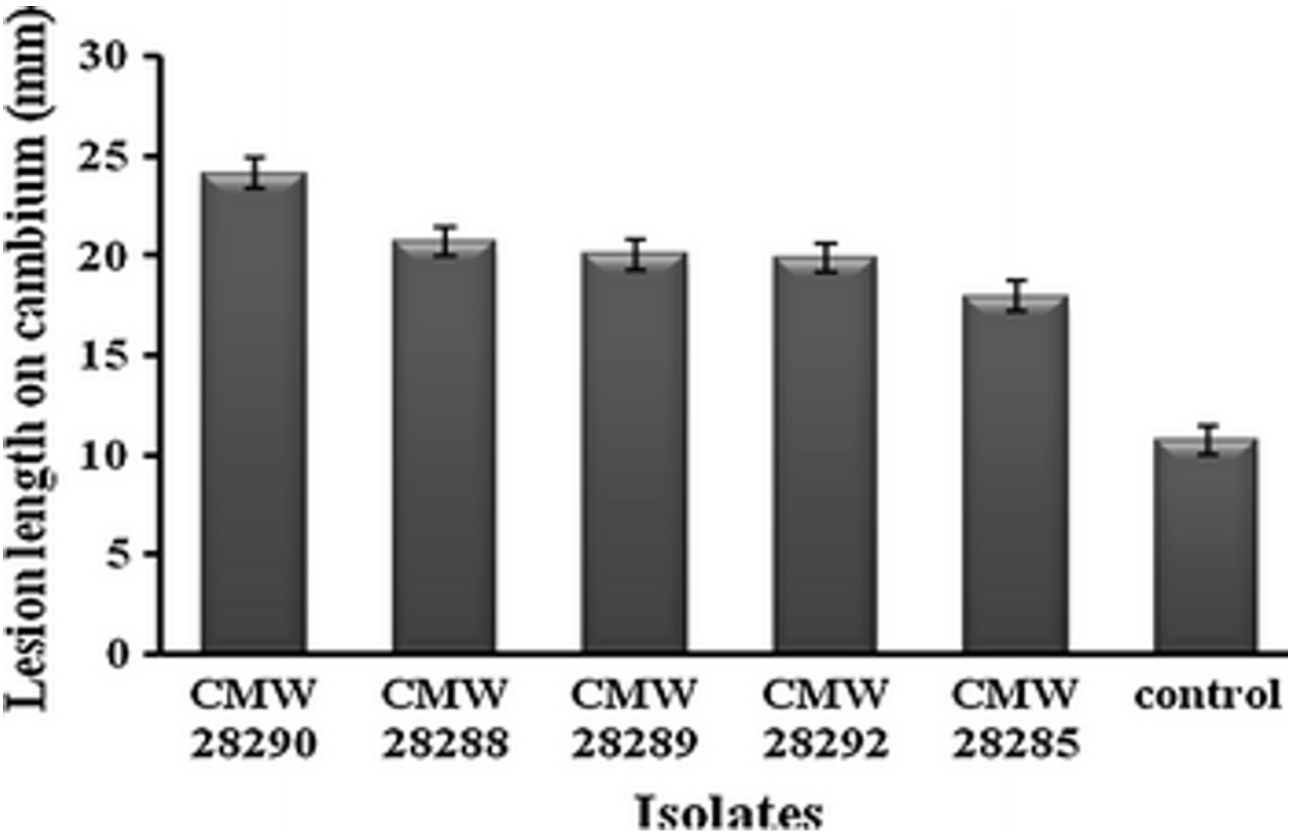

Fig. 5. Mean lesion lengths (mm) on the cambium for each *Aurifilum marmelostoma* isolate 6 weeks after inoculation on *Terminalia mantaly* ($P < 0.0001$).

Aurifilum marmelostoma shares characteristics with several taxa in the Cryphonectriaceae, especially those with uniformly orange fruiting bodies. While the teleomorph state was especially similar to those of other Cryphonectriaceae, a suite of characters in the anamorph of *A. marmelostoma* differentiate this species. The broadly convex conidiomata without necks were similar but wider than conidiomata of *Amphilogia* and *Rostraureum*, and different in shape from the conidiomata of *Cryphonectria*, *Endothia*, *Holocryphia* and *Microthia* that have pulvinate structures. Furthermore, the ostiolar openings of *A. marmelostoma* were often darkened while this has not been observed for any other anamorph in the Cryphonectriaceae. The presence of long cylindrical cells, similar to paraphyses (Walker *et al*. 1985; Venter *et al*. 2002), provides additional characteristic to differentiate the anamorph of *A. marmelostoma* from the anamorphs of morphologically similar Cryphonectriaceae.

The morphological comparison of specimens previously obtained from *T. ivorensis* in Ghana and Kenya with those isolated from *Terminalia* spp. in Cameroon revealed some similarities in their teleomorph states. However, long sterile cells, or paraphyses, present in anamorphs of the Cameroonian specimens, were not observed in the other African specimens. Because molecular evidence to support morphological findings is not available, it is difficult to provide a conclusive taxonomic position to the specimens associated with *T. ivorensis* in the other African countries.

Aurifilum marmelostoma produced more ascostromata on the bark of *Terminalia* spp. than asexual fruiting structures. This inconsistency in the production of sexual and asexual fruiting structures is well-known in other species of the Cryphonectriaceae. For example, *Chr. austroafricana* in South Africa produces ascostromata on native *Syzygium* spp., but rarely produces these structures on non-native *Eucalyptus* spp. (Van Heerden and Wingfield 2001; Heath *et al*. 2006; Nakabonge *et al*. 2006b). However, in countries such as Malawi, Mozambique and Zambia, *Chr. austroafricana* produced both sexual and asexual structures on *Syzygium* spp. and *Eucalyptus* spp. (Nakabonge *et al*. 2006b). Surveys in other regions and on hosts other than *Terminalia* spp. will thus be necessary

to determine whether the production of more sexual than asexual structures is consistent regardless of host or location. This is important since it is the anamorph structures that distinguish *A. marmelostoma* from related genera and species of Cryphonectriaceae with orange fruiting bodies.

Results of the pathogenicity trials showed that all isolates were pathogenic to young *T. mantaly* trees. This result and the consistent association of A. marmelostoma with disease symptoms on *T. ivorensis* suggest that the fungus is a pathogen of *Terminalia* trees. However, pathogenicity tests on *T. ivorensis* will be needed to provide conclusive evidence of its impact and threat to these important native trees. Furthermore, *A. marmelostoma* caused lesions on *T. mantaly* during nursery inoculations, but on mature trees the fungus was present only on cut and dying branches lying on the ground.

Members of the Cryphonectriaceae are well known to occur on Myrtales in Africa. Prior to this study, three genera, including *Celoporthe*, *Chrysoporthe* and *Holocryphia*, were reported infecting trees in the Combretaceae, Heteropyxidaceae, Melastomataceae and the Myrtaceae (all Myrtales) in the sub-Saharan part of the continent (Ofosu-Asiedu and Cannon 1976; Gibson 1981; Myburg *et al*. 2002; Roux *et al*. 2003; Heath *et al*. 2006; Nakabonge *et al*. 2006b). Although members of the Cryphonectriaceae have been reported from Africa regularly (Gibson 1981; Conradie *et al*. 1990; Myburg *et al*. 2002; Gryzenhout *et al*. 2003; Roux *et al*. 2003, 2005; Nakabonge *et al*. 2006a, b), it is clear that their geographical and host distribution on the continent deserves further study.

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