Chapter 5

Genetic structure of *Lasiodiplodia theobromae* and *L. pseudotheobromae* from native and non-native hosts in Cameroon

ABSTRACT

Lasiodiplodia theobromae and *L. pseudotheobromae* are common colonists of native *Terminalia* spp. and non-native *Theobromae cacao*, grown in a taungya intercropping system, in Cameroon. Studies have shown that these fungi are capable of causing disease on both hosts. These sister species are very closely related, but their populations have not been studied in the same geographic area, where introgression would be possible. In this study we determined the genetic diversity and structure of 126 isolates representing both *L. theobromae* and *L. pseudotheobromae,* collected from *T. cacao* and *Terminalia* spp. in Cameroon, using SSR markers. The objectives were to assess the integrity of the species boundaries, determine the population structure of the fungi, the level of gene flow between isolates from *Terminalia* spp. and *T. cacao* and the mode of reproduction of both species. Analysis of the SSR alleles showed clear genetic distinction between *L. theobromae* and *L. pseudotheobromae*, supporting their earlier distinction as sister species. Both *L. theobromae* and *L. pseudotheobromae* populations from Cameroon had high levels of gene diversity, moderate degrees of genotypic diversity, and high levels of gene flow between isolates from *T. cacao* and *Terminalia* spp. There was no evidence for geographic substructure in these populations. These *Lasiodiplodia* spp. are either native to the region, or have been introduced repeatedly or in large numbers. Despite evidence for abundant asexual reproduction, based on fruiting bodies and in repeated SSR genotypes, the SSR alleles were randomly associated in both species, suggesting outcrossing.

1. INTRODUCTION

Lasiodiplodia theobromae (Pat) Griffon and Maubl., the type species of the genus *Lasiodiplodia,* was first described from *Theobromae cacao* fruit in Ecuador in the late 1800s (Patouillard and Lagerheim 1892). It is recognized as an important plant pathogen of many woody and herbaceous hosts, especially in the tropics and subtropics (Punithalingam 1980; Sinclair and Lyon 2005). Symptoms of diseases associated with *L. theobromae* include dieback, canker, gummosis, leaf blight, root and collar rot of woody plants and agricultural crops (Punithalingam 1980; Sinclair and Lyon 2005). There are also reports of *L. theobromae* causing timber stain after felling (Apetorgbor *et al.* 2004). *L. theobromae* is predominantly a latent pathogen, frequently found as an endophyte in healthy plant tissue, but that can become a virulent pathogen when the host is weakened or stressed (Mullen *et al.*1991; Sinclair and Lyon 2005).

Subsequent to the description of *L. theobromae,* the taxonomy of species in *Lasiodiplodia* has undergone considerable change. Two routes were initially followed in the taxonomy of *Lasiodiplodia* spp. with dark, striate conidia. On the one hand isolates resembling this fungus, but originating from different hosts, were described as separate species (Punithalingam 1976). Alternatively, all isolates resembling *L. theobromae* based on colony and conidial morphology, were lumped under the name *L. theobromae* (Punithalingam 1976). Both these strategies lead to significant errors in the systematics of this plant pathogen. More recently, DNA sequence comparisons have shown that *L. theobromae* represents several unique lineages, for which names have been applied. Thus, 10 *Lasiodiplodia* species are presently recognised based on morphology and sequence data (Pavlic *et al.* 2004; Burgess *et al.* 2006a; Damm *et al.* 2007; Alves *et al.* 2008; Pavlic *et al.* 2008; Begoude *et al.* 2009a).

Lasiodiplodia pseudotheobromae A.J.L. Phillips, A. Alves & Crous. emerged from a recent separation of cryptic species originally identified as *L. theobromae* (Alves *et al.* 2008). The species is known from Africa, Europe and Latin America, where it has been described from forest and fruit trees. Growing evidence suggests that *L. pseudotheobromae,* like *L. theobromae,* has a worldwide distribution and a wide host range (Begoude *et al*. 2009a,b; Mehl *et al.* 2009; van der Walt 2008).

Both *L. theobromae* and *L. pseudotheobromae* have been reported from trees in Cameroon (Mbenoun *et al.* 2008; Begoude *et al*. 2009a,b). *L. theobromae* has been reported to cause die-back and death of *T. cacao* in the country (Mbenoun *et al.* 2008). Together with *L. pseudotheobromae* and many other species in the Botryosphaeriaceae, it was also recently reported from native and introduced *Terminalia* spp. in Africa (Begoude *et al*. 2009a,b). In both studies, *L. theobromae* and *L. pseudotheobromae* were common on *Terminalia* spp. and they were highly pathogenic in inoculation trials.

Theobromae cacao is native to South America (Purseglove 1968) and was introduced into West Africa towards the end of the 19th century (Havinden 1970). It has become one of the most important cash crops in Cameroon and other West African countries (Havinden 1970). Traditionally, *T. cacao* is planted in the shade of forest trees. Various timber and fruit trees are also intercropped with *T. cacao*. In Cameroon, some of the most popular timber trees planted as a shade crop for *T. cacao* include *T. ivorensis* and *T. superba*. These native tree species are used to establish a "taungya" agrisylvicultural system where the production of timber is combined with that of *T. cacao* (Lawson 1995; Norgrove and Hauser 2002).

Insect pests and pathogens are recognised as important constraints to the productivity of agroforestry systems (Epila 1986; Rao *et al.* 2000). Schroch *et al.* (2000), provided an extensive review of pests and diseases in agroforestry systems in the humid tropics and raised the fact that latent pathogens of one crop could move to other crops grown in association with it. The Botryosphaeriaceae provide an excellent example of latent pathogens of woody plants that move between hosts (Slippers and Wingfield 2007). Because native *Terminalia* spp. and non-native *T. cacao* trees occur in close association in plantations, it is possible that pathogens such as the Botryosphaeriaceae can move between these trees.

Very few studies have been conducted on the population genetics of fungi in the Botryosphaeriaceae (Burgess *et al.* 2004; Mohali *et al.* 2005; Burgess *et al.* 2006b). None of these studies have considered the structure of two closely related species from two different hosts occurring sympatrically. Because *L. theobromae* and *L. pseudotheobromae* are closely related, and occur on closely associated hosts, studying the genetic structure of these species in a taungya system will help to better understand the ecology of the interacting partners.

The aim of this study was to analyse the genetic diversity and structure of populations of *L. theobromae* and *L. pseudotheobromae* from non-native *T. cacao* and native *Terminalia* spp.

in Cameroon using polymorphic microsatellite DNA markers previously developed for *L. theobromae* by Burgess *et al*. (2003). The specific objectives were to: (i) test the integrity of species boundaries between *L. theobromae* and *L. pseudotheobromae,* (ii) determine the population structure of *L. theobromae* and *L. pseudotheobromae* from non-native *T. cacao* and native *Terminalia* spp. in Cameroon, (iii) determine the level of gene flow between isolates of these species from different hosts and (iv) determine the mode of reproduction of *L. theobromae* and *L. pseudotheobromae*.

2. MATERIALS AND METHODS

2.1. Fungal isolates

A total of 126 *L. theobromae* and *L. pseudotheobromae* isolates, collected from two different regions in Cameroon (Table 1), were used for population analyses in this study. Of these, 42 isolates were previously obtained from asymptomatic bark and branches of *Terminalia* spp. in December 2007 and January 2008 (Begoude *et al.* 2009b). The remaining 84 isolates were collected in November 2008 from *T. cacao* trees showing symptoms of dieback. All the collection sites occurred within an area of 250 km^2. One isolate per tree was selected to be used in the population genetic studies. For isolation of fungi from *T. cacao*, the technique described in Begoude *et al*. (2009a) was used. Single conidial cultures were prepared for all isolates and duplicates of these cultures are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

2.2. DNA extraction, PCR reactions and DNA sequencing

To identify isolates collected from *T. cacao*, conidial morphology and DNA sequence data for the Internal Transcribed Spacer regions (ITS) of the nrDNA, including the 5.8S operon, were used. Procedures and protocols for genomic DNA extraction and sequencing of representative isolates of the Botryosphaeriaceae from *T. cacao* were as described in Begoude *et al.* (2009a). The identity of isolates representing *L. theobromae* and *L. pseudotheobromae* was confirmed by comparing the ITS sequences of isolates obtained in

this study with corresponding sequences in GenBank for isolates CBS 164.96 and CMW 9074, representing *L. theobromae,* and isolates CBS 116459 and CBS 447.62, representing *L. pseudotheobromae.* 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 of 1000 replicates, tree bisection and reconnection (TBR) as branch swapping algorithms, and random taxon addition for the construction of maximum parsimonious trees. The support for branches of the most parsimonious trees was assessed with a 1000 bootstrap replication (Felsenstein 1985).

2.3. Simple sequence repeat (SSR)-PCR and GENESCAN analyses

Thirteen PCR-based SSR microsatellite markers (Burgess *et al.* 2003) were employed to study the population structure of *L. theobromae* and *L. pseudotheobromae* isolates. The PCR reactions and conditions were the same as those described by Burgess *et al.* (2003). The DNA concentration of the PCR products were measured visually against the intensity of a 100 bp marker (Roche Molecular Biochemicals) on 2 % agarose gels, exposed to Ultra-violet (UV) illumination.

PCR products were multiplexed for GENESCAN analysis based on the approximate sizes of the PCR products and type of fluorescent label attached to the primer. Each sample mix contained 1 µl of combined DNA, 0.14 µl 1x loading buffer and 1 µl internal standard GENESCAN-500 LIZ (Applied Biosystems, Warrington, UK). Fluorescent-labelled SSR-PCR products were separated on an ABI Prism 3100 sequencer (Applied Biosystems). Allele sizes were determined by comparing the mobility of the SSR products with that of the LIZ internal size standard using a combination of the GENESCAN 2.1 analysis software (Applied Biosystems) and GENOMAPPER V3.5 (Applied Biosystems).

2.4. Statistical analyses

Isolates that contained the same alleles at each locus potentially represented clones. The inclusion of clonal multicopies can strongly distort estimates of population genetic

parameters (Frantz *et al.* 2006). Therefore, duplicates of each multilocus genotype were discarded from the analyses to provide a clone-corrected dataset.

2.4.1. Bayesian clustering analyses

The software programme Structure version 2.2 (Pritchard *et al*. 2000; Falush *et al*. 2003) was used to infer the population structure of all isolates, without any *a priori* knowledge of population subdivision, using a Bayesian model-clustering algorithm. This algorithm assumes a model in which there are *K* populations or clusters, in which a set of allele frequencies at each locus characterized each population. Individuals in the same sample are probabilistically distributed to *K* clusters, or jointly to two or more clusters if their genotypes indicate that they are admixed, regardless of their region or host origin. Parameter estimation considers that populations are at Hardy-Weinberg equilibrium and that the marker loci are unlinked and at linkage equilibrium. The model with admixture was applied in all simulations as this model is recommended for situations where little is known about the existence of admixture (Falush *et al.* 2003). Priors were assumed uniform for the vectors of proportions q_i of the individual *i*'s genome deriving from each cluster. Iteration parameters were set to 950 000 Monte Carlo Markov Chain (MCMC) iterations preceded by a burn-in period of 50 000 iterations and 20 independent simulations were performed to test for the consistency of the results. The number of clusters, *K,* was varied from 1 to 10. Individuals were assigned to a single cluster when their proportion of ancestry in that cluster was greater than 80 %. This threshold was determined after analyzing the distribution of mean ancestry coefficients for each *K*.

2.4.2. Gene and genotypic diversity

Single alleles were assigned a different letter for each of the loci. For each isolate, a data matrix of multistate characters, each state corresponding to a different locus, was compiled with the polymorphic loci, thus providing each isolate with a haplotype. A number was assigned to each haplotype and the equation $\hat{G} = 1/\Sigma p_i^2$, developed by Stoddart and Taylor (1988), was applied to estimate the genotypic diversity ($\hat{G}$). In this equation, p_i stands for the observed frequency of the i^{th} phenotype. The maximum percentage of genotypic diversity (G_{max}), obtained from the equation $G_{max} = \hat{G}/N \times 100$ (where N is the population size), was used to compare the genotypic diversities between populations (Chen *et al*. 1994). Allelic

frequency, as well as the number of alleles at each locus, was calculated and gene diversity determined, using the program POPGENE version 1.31 (Yeh *et al.* 1999) based on the equation $H = 1-\Sigma x_k^2$, where x_k is the frequency of the k^{th} allele (Nei 1973). Chi-square tests for differences in allele frequencies were calculated for each locus across clone-corrected collections. The free software Programme Multilocus version 1.2 (Agapow and Burt 2000) available at http://www.agapow.net/software/multilocus/ was used to plot the genotypic diversity against the number of loci with 1000 resampling repetitions, in order to determine whether the isolates and microsatellite markers used were sufficient to recover the maximum genotypic diversity.

2.4.3. Genetic differentiation and gene flow

The genetic differentiation among populations was assessed in POPGENE, using Nei's (1973) G_{ST} statistic, which varies between zero and one. POPGENE was also used to estimate the number of migrants (N_m) exchanged among the populations for each generation from the estimate of G_{ST} where $N_m = 0.5(1-G_{ST})/G_{ST}$ (McDermott and McDonald, 1993). Populations that are completely genetically isolated would have N_m values of zero and G_{ST} values tending towards one (Hartl and Clark 1989). The software programme GENALEX 6.2 (Peakall and Smouse 2006) was used to analyse the molecular variance (AMOVA) among populations of *Lasiodiplodia* spp. from *T. cacao* and *Terminalia* spp. at different locations.

2.4.4. Linkage disequilibrium

The multilocus linkage disequilibrium for each clone-corrected population was tested with the Index of Association (I_A) (Maynard-Smith *et al.* 1993). The Index of Association provides information related to whether two different individuals which possess the same allele at one locus, will more likely possess the same allele at another locus (Fournier and Giraud 2008). The tests were performed on a data matrix of multistate characters using the program Multilocus (Agapow and Burt 2000). For any pair of individuals, the number of loci at which they differ was calculated and its variance was compared with that expected ($I_A = 0$). There is no linkage disequilibrium when the observed data fall within the distribution range of the recombined data and the population is most likely clonal if the observed data falls outside the distribution range with a significant P value ($P < 0.05$).

3. RESULTS

3.1. Fungal isolates

A total of 16 *L. theobromae* isolates and 26 *L. pseudotheobromae* isolates were obtained from *T. ivorensis* and *T. superba* in a previous study (Begoude *et al*. 2009b). An additional 84 isolates resembling species of *Lasiodiplodia* were collected from *T. cacao* in the present study.

Lasiodiplodia isolates from *T. cacao* were identified to species level using DNA sequence data for the ITS and 5.8S gene regions. The ITS dataset comprised 114 sequences of which 97 originated from *Terminalia* spp. and *T. cacao* and 17 sequences were retrieved from GenBank. Of the 486 characters present in the ITS sequence data set, 34 were parsimony informative. The MP analyses generated two trees with identical topology (Tree length (TL) = 129, Consistency index (CI) = 0.698, Retention index (RI) = 0.839, Rescaled consistency index (RC) = 0.585). These analyses revealed that all 97 isolates from *Terminalia* spp. and *T. cacao* belonged to the clades accommodating either *L. theobromae* (Bootstrap support (BS) = 55 %) or *L. pseudotheobromae* (BS = 77 %) (Figure 1). Of the isolates from *T. cacao*, 54 represented *L. theobromae* and 33 *L. pseudotheobromae*.

3.2. Microsatellite PCR amplification

Eleven of the 13 microsatellite primer pairs developed previously for *L. theobromae* by Burgess *et al*. (2003) successfully amplified DNA markers for *L. theobromae* from Cameroon. Primers las13&14 and las31&32 produced multiple bands even after numerous attempts to optimize them. Among the primers that yielded successful PCR products, six were monomorphic for *L. theobromae* in Cameroon.

Nine of the 13 microsatellites primers previously developed for *L. theobromae* by Burgess *et al*. (2003) successfully amplified DNA markers in Cameroonian *L. pseudotheobromae*. Primers las13&14, las31&32 and las37&38 produced multiple bands, whereas primers las23&24 failed to amplify a product from isolates of *L. pseudotheobromae*. Among the primers that yielded successful PCR products, four were monomorphic for *L. pseudotheobromae*.

Products of two monomorphic primer pairs, las17&18 and las33&34 had identical alleles (249 and 276, respectively) among isolates of both fungal species. The product amplified with primers las3&4 was monomorphic within the species, but distinct between them (351 and 361 for isolates of *L. pseudotheobromae* and *L. theobromae*, respectively). Products from primer pair las35&36 was monomorphic among isolates of *L. pseudotheobromae* (387), but contained two alleles (376 and 379) amongst isolates of *L. theobromae*. PCR products from primer pairs las15&16, las27&28 and las29&30, which were monomorphic among isolates of *L. theobromae*, were polymorphic among isolates of *L. pseudotheobromae*. Overall, seven primers were polymorphic among isolates of both species (Figure 2) and five primers were polymorphic among isolates of only one of the species (Table 2 & 3).

3.3. Statistical analyses

3.3.1. Bayesian clustering analyses

The Bayesian inference of the population structure was performed with 21 unique haplotypes representing all the multilocus genotypes inferred with seven polymorphic loci among isolates of *L. theobromae* and *L. pseudotheobromae*. These samples included isolates from *Terminalia* spp. and *T. cacao* from all the locations sampled. The distribution of the maximum likelihood was the highest for $K = 2$ with an assignment rate value of 98.8 % (Figure 3). The first cluster included all the genotypes of isolates representing *L. pseudotheobromae* while the second cluster consisted of genotypes of *L. theobromae*. There was no subdivision in the population according to either host or location. Separate investigation of the population structure within each species, showed that the distribution of the maximum likelihood was the highest for $K = 1$, suggesting a high degree of admixture which suggests that neither the host nor the geographic location considered influenced the population structure within the species. This analysis confirmed the classification of *L. theobromae* and *L. pseudotheobromae* as distinct sister species, but did not show any differentiation within species according to host or geographic location.

3.3.2. Gene diversity

Sixty-nine isolates of *L. theobromae* from *Terminalia* spp. and *T. cacao* were analyzed at five polymorphic loci (Table 2). The number of alleles ranged from two to four per locus. A total of 13 alleles were produced across populations from *Terminalia* spp. and *T. cacao*, of which nine alleles were observed across isolates from *Terminalia* spp. whereas all 13 alleles were observed among isolates from *T. cacao*. Four unique alleles, with low frequency (8-24 %), were observed in isolates from *T. cacao*. The mean total gene diversity (*H*), calculated using the allele frequencies across all isolates of *L. theobromae* was 0.46, which was similar to the gene diversity observed in isolates from *T. cacao* and higher that the gene diversity observed in isolates from *Terminalia* spp. (Table 2).

Fifty three isolates of *L. pseudotheobromae* from *Terminalia* spp. and *T. cacao* were analyzed at five polymorphic loci after clone correction of populations (Table 3). The number of alleles ranged from two to six per locus. A total of 14 alleles were identified across isolates from both hosts of which 13 alleles were observed among isolates from *Terminalia* spp. and 12 alleles were observed in isolates from *T. cacao*. Two unique alleles, with low frequency (16 %), were observed in isolates from *Terminalia* spp. and only one unique allele, with low frequency (12 %), was observed in isolates originating from *T. cacao*. The mean total gene diversity across all isolates of *L. pseudotheobromae* was 0.445, which was similar to the gene diversity observed in isolates from each host (Table 3).

3.3.3. Genotypic diversity

Among the 69 isolates of *L. theobromae,* 26 different multilocus genotypes were discriminated. Among these genotypes, 19 were unique to the sampled localities (three in Nkong, six in Mbalmayo and ten in Nkoemvone) whereas seven genotypes representing 60.9 % of the isolates collected were shared among the three localities (Figure 4). Where *Terminalia* spp. and *T. cacao* occurred in the same area, such as in Mbalmayo, of 14 genotypes found in the area, three genotypes representing 65.6 % of the isolates collected were shared between both hosts.

When considering isolates of *L. theobromae* from different hosts separately, six genotypes were found in isolates from *Terminalia* spp. and 25 genotypes were found in isolates from *T.*

cacao (Table 4). Among these genotypes, only one genotype (33.3 %) was unique to *Terminalia* spp. whereas 16 genotypes (64 %) were unique to the *T. cacao* population. The percentage of shared genotypes between *Terminalia* spp. and *T. cacao* represented 19 % of all the genotypes observed. The most common genotypes accounted for 14.5 % of the population and the genotypes occurring only once were very abundant in isolates from *T. cacao* (Table 4). Overall, moderate values were generated for the genotypic diversities in each population, ranging from 28.57 % on the *Terminalia* spp. to 32.12 % for isolates from *T. cacao*. The moderate value of genotypic diversity obtained from these populations was probably due to the occurrence of only a few single isolate genotypes (12 and 23.2 %, respectively for each host).

Ten different multilocus genotypes were detected amongst the 57 isolates of *L. pseudotheobromae* from *Terminalia* spp. and *T. cacao*. Among these genotypes, three were unique to the localities of Mbalmayo (one) and Nkoemvone (two), whereas seven genotypes representing 87.7 % of the isolates collected were shared among the three sampled locations (Figure 4). Where isolates were collected from both *Terminalia* spp. and *T. cacao* in the same locality, such as Mbalmayo and Nkoemvone, of seven genotypes obtained in Mbalmayo, four were shared between both hosts and represented 90 % of the total isolates collected in Mbalmayo. Two out of nine genotypes obtained in Nkoemvone, representing 65.2 % of the isolates collected in the area, were shared between *Terminalia* spp. and *T. cacao.*

When considering isolates from different hosts, regardless of their locality of origin, six different genotypes of *L. pseudotheobromae* were found in isolates from *Terminalia* spp. and eight genotypes were found in isolates from *T. cacao* (Table 5). Among these genotypes, two genotypes (33.3 %) were unique to *Terminalia* spp. whereas four genotypes (50 %) were unique to the *T. cacao* population. The percentage of shared genotypes between *Terminalia* spp. and *T. cacao* represented 40 % of all the genotypes observed for *L. pseudotheobromae*. The most common genotypes accounted for 30.2 % of the population and the genotypes occurring only once were very few in both the *Terminalia* spp. and the *T. cacao* populations (Table 5). The overall genotypic diversities calculated for each population were low, ranging from 17.57 % on *Terminalia* spp., to 15.97 % for the *T. cacao* population. This is most probably due to the occurrence of few single isolate genotypes (7.6 and 6.5 %, respectively for each host genus).

In both *L. theobromae* and *L. pseudotheobromae* populations, from single hosts as well as those that occur on both *Terminalia* spp. and *T. cacao*, the genotypic diversity was plotted against the number of loci. The graph obtained reached a plateau in the case of *L. pseudotheobromae,* whereas the graph approached a plateau in the case of *L. theobromae* (Figure not shown). This suggested that the genotypic diversity calculated from the isolates and microsatellite markers used in this study was sufficient to characterize the populations of both species.

3.3.4. Genetic differentiation and gene flow

The measure of genetic differentiation between populations of *L. theobromae* from *Terminalia* spp. and *T. cacao* reflected a lack of substructuring in the *L. theobromae* population. The values obtained for χ^2 tests revealed no significant differences ($P > 0.05$) in allele frequencies at any loci for populations from either the *Terminalia* spp. or *T. cacao* (Table 6). These results were further confirmed by very low G_{ST} values corresponding to 0.046, indicating that most of the gene diversity is found within the subpopulations (*Terminalia* spp. and *T. cacao*). This was also true when comparing populations of *L. theobromae* from different hosts at different locations. Consequently, a low level of differentiation exists in populations of *L. theobromae* from *Terminalia* spp. and *T. cacao*. The number of migrants (N_m) exchanged between populations for each generation was estimated at 10.47.

Similar to *L. theobromae,* the measure of genetic differentiation between populations of *L. pseudotheobromae* from *Terminalia* spp. and *T. cacao* showed a lack of substructuring. The values obtained for χ^2 tests revealed no significant differences ($P > 0.05$) in allele frequencies at any loci for either the *Terminalia* spp. or *T. cacao* populations of *L. pseudotheobromae* (Table 6). There was only 3.5 % genetic diversity distributed between populations from *Terminalia* spp. and *T. cacao* and no difference was observed after comparing populations of *L. pseudotheobromae* from different hosts at different locations. This indicated that most of the genetic variation is distributed within each subpopulation. Therefore, a low level of differentiation also exists in populations of *L. pseudotheobromae* from *Terminalia* spp. and *T.*

cacao. The number of migrants (N_m) exchanged between populations for each generation was estimated at 13.83.

3.3.5. Linkage disequilibrium

The Index of association (I_A) calculated for populations of *L. theobromae* and *L. pseudotheobromae* were -0.153 ($P = 0.99$) and 0.069 ($P = 0.4$), respectively. These values did not significantly deviate from the expected value when there is no linkage disequilibrium. This suggests that alleles are randomly associated, as would be expected for outcrossing populations.

4. DISCUSSION

Genetic diversity analyses for *L. theobromae* and *L. pseudotheobromae* in Cameroon showed that there is no gene flow between these recently diverged sister species. This supports the previous segregation of *L. pseudotheobromae* from *L. theobromae* based on divergence in sequences of their nuclear genes, ITS and *tef 1-*α (Alves *et al.* 2008). Both *L. theobromae* and *L. pseudotheobromae* have a worldwide distribution and they share similar biological and ecological characteristics. Since their description as cryptic species, *L. pseudotheobromae* and *L. theobromae* have not been studied in areas where they occur on the same host or environment, which is where possible hybridization of two such closely related fungal genets might be expected (Shardl and Craven 2003).

A number of lines of evidence supported the distinction of *L. theobromae* and *L. pseudotheobromae*. Of the 22 alleles that were detected, 11 and six were unique to populations of *L. pseudotheobromae* and *L. theobromae*, respectively. A number of these were fixed in either species, and any recombination between them would have shared these alleles. The five alleles that were shared between these species occurred in significantly different frequencies. The alleles shared between populations of *L. theobromae* and *L. pseudotheobromae* in this study possibly remain from an ancestral species, which would be expected in such closely related species (Carbone and Kohn 2004). The structure inferred from a Bayesian clustering algorithm confirmed the most likely number of clusters amongst

all isolates to be two. All the individuals representing *L. theobromae* and *L. pseudotheobromae* clustered separately in either of these two clusters.

Delimitating boundaries between sister species with low levels of genetic divergence is challenging. Two methods are used in species delimitation, one of which encompasses tree-based approaches that delimit species as historical lineages (Goldstein and DeSalle 2000). The other method includes non tree-based analyses where information regarding the level of gene flow is the main basis to determine boundaries between species (Sites and Marshall 2003; 2004). Application of microsatellite markers, which fall within the latter category, represents a powerful tool to demarcate barriers to gene flow between individuals of closely related fungal species (Fisher *et al.* 2000; 2002). Using microsatellites, it is possible to screen multilocus genotypes for many individuals rapidly. Consequently, the significant genetic distinction between *L. theobromae* and *L. pseudotheobromae* using a non tree-based method with microsatellite markers, provides strong additional support for their previous separation based on phylogenetic analyses of ITS and *tef 1-α* sequence data (Alves *et al.* 2008).

No evidence of host or geography linked population structure was observed for either *L. theobromae* or *L. pseudotheobromae* in Cameroon. Agro-ecology in Cameroon is subdivided into five zones based on vegetation and climatic conditions (http://www.irad-cameroon.org/carte_us.php). The collection sites in this study occurred within zone five, characterized by humid forests with bimodal rainfall. The maximum distance between collection sites was ~200 km. Consequently, there is no effective barrier that could restrict dispersal of fungal propagules between locations. The hosts are fairly continuous over the distances studied, providing a possible explanation for the connectedness of the populations. Endophytic Botryosphaeriaceae infections of woody hosts are thought to develop over time by horizontal transmission through wind- or water-dispersed spores (Arnold *et al.* 2003a,b; Slippers and Wingfield 2007). Movement of infected material would provide another explanation of genetic similarity between populations. When establishing cacao in the shade of thinned forest trees, farmers obtain seedlings from a centralized seedling distributor (Sonwa 2002). This could have contributed to the spread of the pathogens as endophytes over large areas. The population of *L. theobromae* and *L. pseudotheobromae* on *T. cacao* and native *Terminalia* spp. appeared to be totally integrated, suggesting that the movement of

these pathogens between the hosts may be symmetrical (Hayden *et al.* 2007; Fournier and Giraud 2008).

We detected no restriction in the movement of *L. theobromae* and *L. pseudotheobromae* on *Terminalia* spp. and *T. cacao*. Most alleles were found on both hosts, and in similar frequencies. Both *L. theobromae* and *L. pseudotheobromae* commonly occur in the tropics and subtropics on a wide diversity of hosts with no observed specialization (Mohali *et al.* 2005; Alves *et al.* 2008), so this result is not unexpected. Many cases of shared genotypes were also observed on both *Terminalia* spp. and *T. cacao*, possibly representing clonal lineages. These occurred either in the same field within the location or among locations (over scales of a few metres to 200 km). Both populations displayed a high allelic diversity and almost all of them were shared between isolates from *T. cacao* and *Terminalia* spp. As explained above, this could reflect natural spread through asexual conidia in a step-wise manner, or by direct transport of infected material from a central location. The non-limited genetic exchange between populations on *T. cacao* and *Terminalia* spp. could increase the gene diversity because gene flow introduces new alleles into populations that are part of the same genetic neighborhood (McDonald and Linde 2002a).

Our results showed high genetic diversity of microsatellite loci in populations of both *L. theobromae* and *L. pseudotheobromae* from non-native *T. cacao* and native *Terminalia* spp. in Cameroon. In the *L. theobromae* population, the number of alleles and genotypes observed in isolates from *T. cacao* was higher than that found in isolates from native *Terminalia* spp., resulting in a higher genetic diversity for isolates from *T. cacao*. This could, however, be explained by the larger number of isolates collected from *T. cacao*. Native populations or populations closest to their centre of origin generally have high levels of genetic diversity while introduced populations often exhibit lower levels of diversity (McDonald and Linde 2002a; Stukenbrock *et al.* 2006). At the time of its description, *L. pseudotheobromae,* was known from few hosts and a very limited geographic distribution (Alves *et al.* 2008). This distribution is expanding markedly and is beginning to reflect the worldwide distribution of *L. theobromae* (Begoude *et al*. 2009a,b; Mehl *et al.* 2009; van der Walt 2008). The wide distribution, both geographically and in host relationships, of *L. theobromae* and *L. pseudotheobromae* complicates speculation regarding their likely origin since multiple introductions through anthropological action and movement between various hosts could strongly influence their genetic diversity.

Analyses of the mode of linkage disequilibrium amongst alleles at the SSR loci in populations of *L. theobromae* and *L. pseudotheobromae* from *Terminalia* spp. and *T. cacao* suggest that both species undergo regular sexual reproduction (Milgroom 1996). However, despite this evidence of sexual reproduction, sexual states for this group of fungi are rarely seen. *Botryosphaeria rhodina* (Berk. & M.A. Curtis) Arx [which is no longer a valid name following Crous *et al.* (2006)] is frequently reported as the sexual state of *L. theobromae.* However, since Stevens (1925; 1926) determined *B. rhodina* as the teleomorph of *L. theobromae*, this connection has not been confirmed. The recent description of a number of species that were previously confused with *L. theobromae* sheds doubt on the accuracy of those discoveries. Although the purported sexual state might be known for *L. theobromae*, there is no evidence for such a state in *L. pseudotheobromae.*

Both *L. theobromae* and *L. pseudotheobromae* exhibit a genetic structure with a moderate degree of genotypic diversity. The level of genotypic diversity obtained for *L. theobromae* isolates from both *Terminalia* spp. and *T. cacao*, suggests that a high level of recombination takes place in this fungus. These results are different from those of Mohali *et al.* (2005) who found very low levels of recombination for *L. theobromae* from *Pinus* sp., *Acacia* sp. and *Eucalyptus* sp. in Venezuela. Populations of *L. pseudotheobromae* were characterized based on a small number of single isolate genotypes. Indeed, 60 % of the total genotypes occurred more than once and the proportion of the most common genotype (30.2 %) was high, resulting in a clonal fraction of 82.4 %. These results indicate the presence of a high proportion of widely distributed clonal genotypes across both *Terminalia* spp. and *T. cacao,* despite some evidence of recombination. Although similar observations were made for *L. theobromae,* the frequency of recombination was higher than that in *L. pseudotheobromae*.

Fungi with both sexual and asexual reproduction pose the highest risk of pathogen evolution because they receive benefits from both modes of reproduction (McDonald and Linde 2002a,b). Thus, the fit combination of alleles generated through regular recombination with the highest level of fitness could be increased rapidly through asexual reproduction (Ciampi *et al.* 2008). This risk is increased in cases where there is high gene flow over large areas (as we observed here), because fitter genotypes can quickly spread. Therefore, *L. theobromae* and *L. pseudotheobromae* correspond to the highest category of evolutionary risk as defined by McDonald and Linde (2002a,b). Together with data from previous studies showing the pathogenicity of these fungi, they clearly pose a significant threat to both native *Terminalia*

spp., and introduced *T. cacao*. Given their wide host and geographic ranges, this is most likely also true for other native and non-native hosts growing in close proximity to each other.

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Table 1. Source of *Lasiodiplodia theobromae* and *Lasiodiplodia pseudotheobromae* isolates in Cameroon.

Region	Location	Host	No of isolates	
			L. theobromae	*L. pseudotheobromae*
Centre	Mbalmayo	*Terminalia ivorensis*	16	22
		Theobroma cacao	16	8
	Nkong	*T. cacao*	9	4
South	Nkoemvone	*T. cacao*	28	18
		Terminalia superba	-	5

Table 2. Allele frequencies at 5 loci for *Lasiodiplodia theobromae* populations collected from *Terminalia* spp. and *Theobroma cacao* in Cameroon.

Locus	Allele length	Allele configuration	*Terminalia* spp.	*T. cacao*
las21-22	383	A	-	0.24
	388	B	1	0.76
las23-24	454	A	-	0.08
	458	B	0.333	0.48
	461	C	0.667	0.36
	463	D	-	0.08
las25-26	417	A	0.333	0.2
	420	B	0.5	0.48
	421	C	0.167	0.32
las35-36	376	A	0.333	0.4
	379	B	0.667	0.6
las37-38	117	A	1	0.8
	135	B	-	0.2
No isolates			**16**	**53**
No Alleles			**9**	**13**
No. Unique Alleles			**0**	**4**
Polymorphic loci			**3**	**5**
H			**0.3**	**0.484**

Table 3. Allele frequencies at 5 loci for *Lasiodiplodia pseudotheobromae* populations collected from *Terminalia* spp. and *Theobroma cacao* in Cameroon

Locus	Allele length	Allele configuration	*Terminalia* spp.	*T. cacao*
las15-16	351	A	0.833	0.625
	353	B	0.167	0.375
las21-22	383	A	0.833	0.625
	388	B	0.167	0.375
las25-26	415	A	0.333	0.25
	417	B	0.667	0.75
Las27-28	458	A	0.167	-
	463	B	0.167	0.375
	466	C	0.167	-
	471	D	0.167	0.125
	474	E	0.333	0.375
	477	F	-	0.125
Las29-30	180	A	0.833	0.875
	188	B	0.167	0.125
No isolates			**26**	**31**
No Alleles			**13**	**12**
No. Unique Alleles			**2**	**1**
Polymorphic loci			**5**	**5**
H			**0.41**	**0.44**

Table 4. Genotype estimation from multilocus profiles generated from 5 SSR loci for *Lasiodiplodia theobromae*.

GENOTYPES	*Terminalia* spp.	*T. cacao*
AAAAA	2	4
AABAA	5	5
ABABA	1	
ABBBA	3	3
ABBAA	4	1
ABCAA	1	3
BAAAA		1
BABBA		1
BABBB		1
BBBBA		1
BABAA		3
BBAAA		1
ACBAA		1
ACCAA		1
AAABA		1
AABBA		5
AABAB		1
AACBA		5
AACAA		4
AACAB		1
ABAAA		1
ABBAB		1
ABCBA		4
ABCAB		1
ADBBA		1
ADCBA		1
N	**16**	**53**
N(g)	**6**	**25**
$\hat{G}$	**4.751**	**17.024**
$\hat{G}$ (%)	**28.57**	**32.12**

N, number of isolates

N(g), number of genotypes
$\hat{G}$, Genotypic diversity (Stoddart & Taylor 1988)

$\hat{G}$(%), % max diversity

Table 5. Genotype estimation from multilocus profiles generated from 5 SSR loci for *Lasiodiplodia pseudotheobromae.*

GENOTYPES	*Terminalia* spp.	*T. cacao*
AAAAA	1	
AABBA	6	5
AABCA	5	8
AABCB	6	2
ABBDA	1	
BAAEA	7	9
AABFA		1
ABBCA		3
BBAEA		3
BBBEA		1
N	**26**	**31**
N(g)	**6**	**8**
Ĝ	**4.56**	**4.95**
Ĝ (%)	**17.57**	**15.97**

N, number of isolates

N(g), number of genotypes

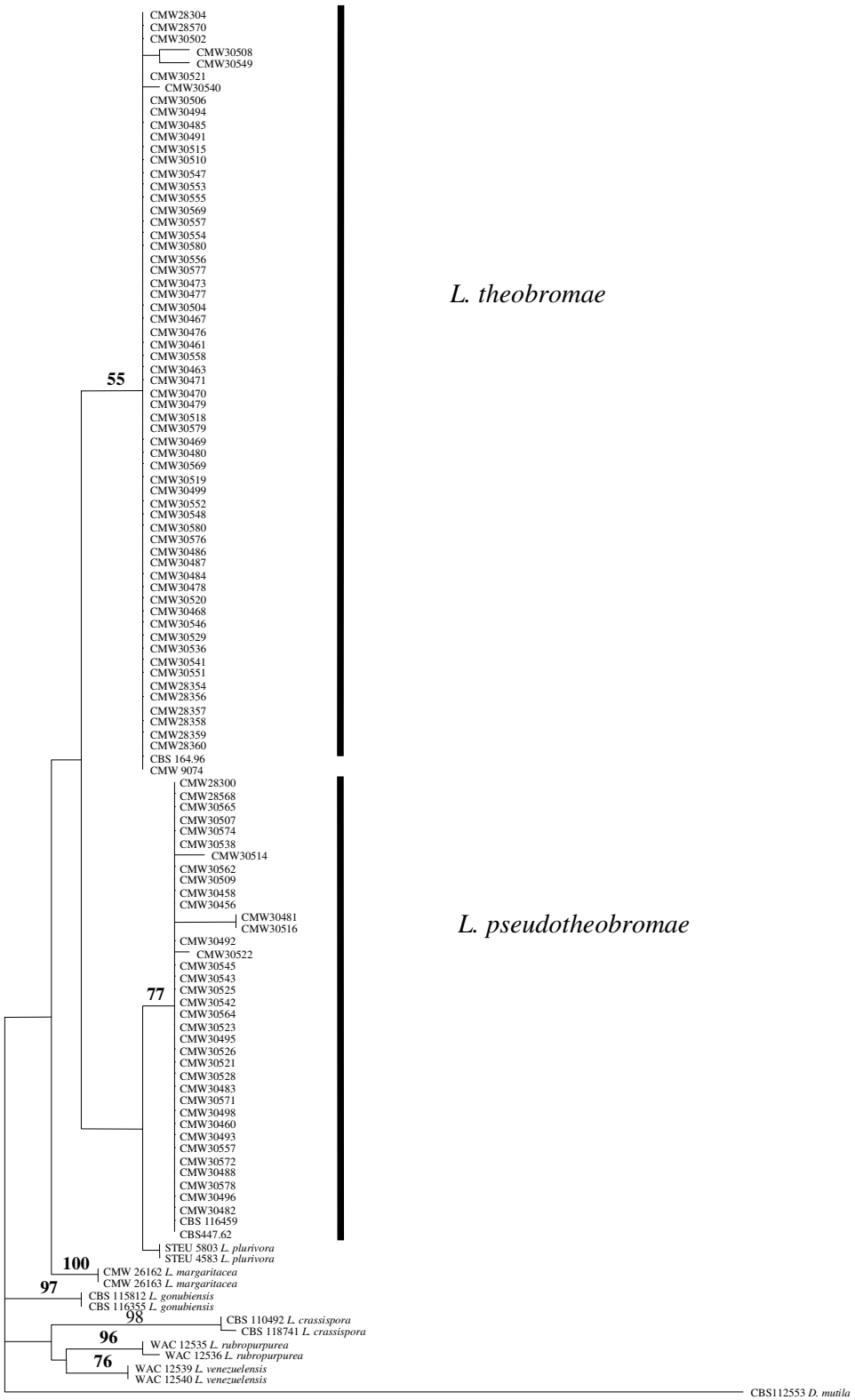
Ĝ, Genotypic diversity (Stoddart & Taylor 1988)

Ĝ(%), % max diversity

Table 6. Gene diversity (*H*) for the 5 polymorphic SSR loci across clone corrected populations of *Lasiodiplodia theobromae* and *L. pseudotheobromae* in Cameroon.

	Gene diversity (*H*)				*L. theobromae*		*L. pseudotheobromae*	
	L. theobromae		*L. pseudotheobromae*					
Loci	*Terminalia* spp.	*T. cacao*	*Terminalia* spp.	*T. cacao*	χ^2	df	χ^2	df
las15-15			0.28	0.47			0.7	1
las21-22	0.00	0.36	0.28	0.47	1.8	1	0.7	1
las23-24	0.44	0.63			2.3	3		
las25-26	0.61	0.63	0.44	0.37	0.8	2	0.1	1
las27-28			0.78	0.69			3.9	5
las29-30			0.28	0.22			0.04	1
las35-36	0.44	0.48			0.1	1		
las37-38	0.00	0.32			1.4	1		
N	6	25	6	8				
Mean	0.30	0.48	0.41	0.44				

Figure 1. Maximum parsimony phylogram of *Lasiodiplodia theobromae* and *L. pseudotheobromae* from this study obtained with sequences of ITS. Bootstrap support (%) from 1000 replications is given on the branches.


CMW28304
CMW28570
CMW30502
CMW30508
CMW30549
CMW30521
CMW30540
CMW30506
CMW30494
CMW30485
CMW30491
CMW30515
CMW30510
CMW30547
CMW30553
CMW30555
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CMW30471
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CMW30518
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CMW30484
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CMW30520
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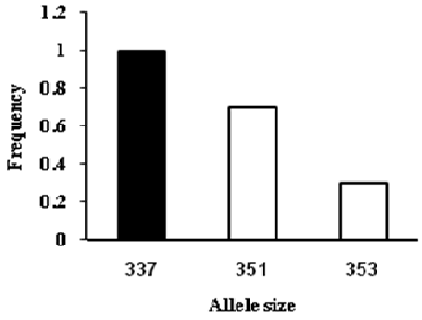
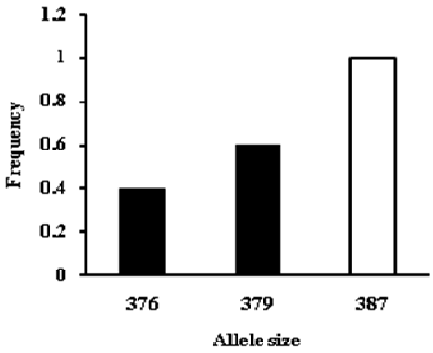
CMW30546
CMW30529
CMW30536
CMW30541
CMW30551
CMW28354
CMW28356
CMW28357
CMW28358
CMW28359
CMW28360
CBS 164.96
CMW 9074
L. theobromae
CMW28300
CMW28568
CMW30565
CMW30507
CMW30574
CMW30538
CMW30514
CMW30562
CMW30509
CMW30458
CMW30456
CMW30481
CMW30516
CMW30492
CMW30522
CMW30545
CMW30543
77
CMW30525
CMW30542
CMW30564
CMW30523
CMW30495
CMW30526
CMW30521
CMW30528
CMW30483
CMW30571
CMW30498
CMW30460
CMW30493
CMW30557
CMW30572
CMW30488
CMW30578
CMW30496
CMW30482
CBS 116459
CBS447.62
L. pseudotheobromae
STEU 5803 L. plurivora
STEU 4583 L. plurivora
100
CMW 26162 L. margaritacea
CMW 26163 L. margaritacea
97
CBS 115812 L. gonubiensis
CBS 116355 L. gonubiensis
98
CBS 110492 L. crassispora
CBS 118741 L. crassispora
96
WAC 12535 L. rubropurpurea
WAC 12536 L. rubropurpurea
76
WAC 12539 L. venezuelensis
WAC 12540 L. venezuelensis
CBS112553 D. mutila
0.5 changes

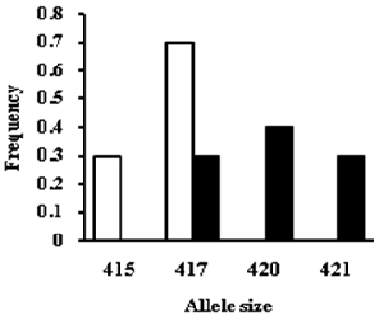
Figure 2. Distribution of alleles showing the size of PCR product at seven microsatellite loci for *Lasiodiplodia pseudotheobromae* (white) and *L. theobromae* (black).



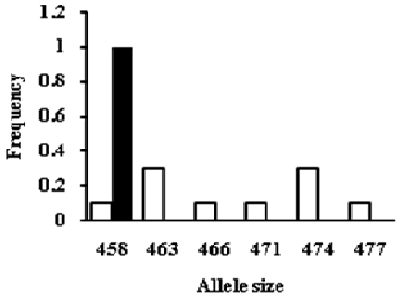
Las 15&16



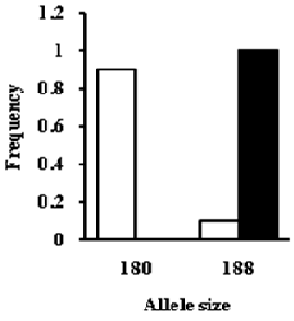
Las 35&36



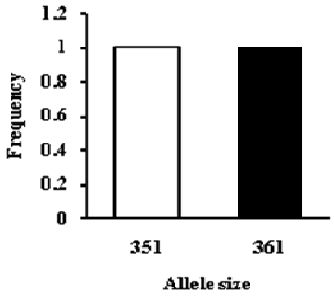
Las 25&26



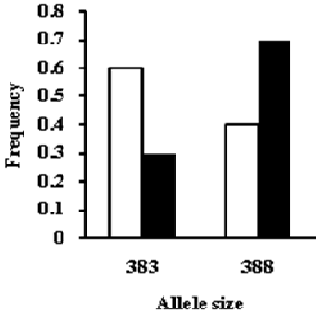
Las 27&28



Las 29&30

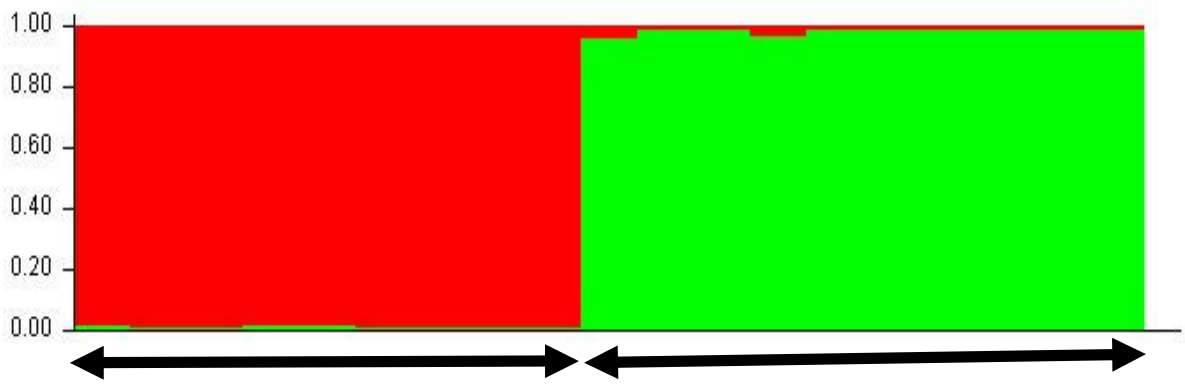


Las 3&4



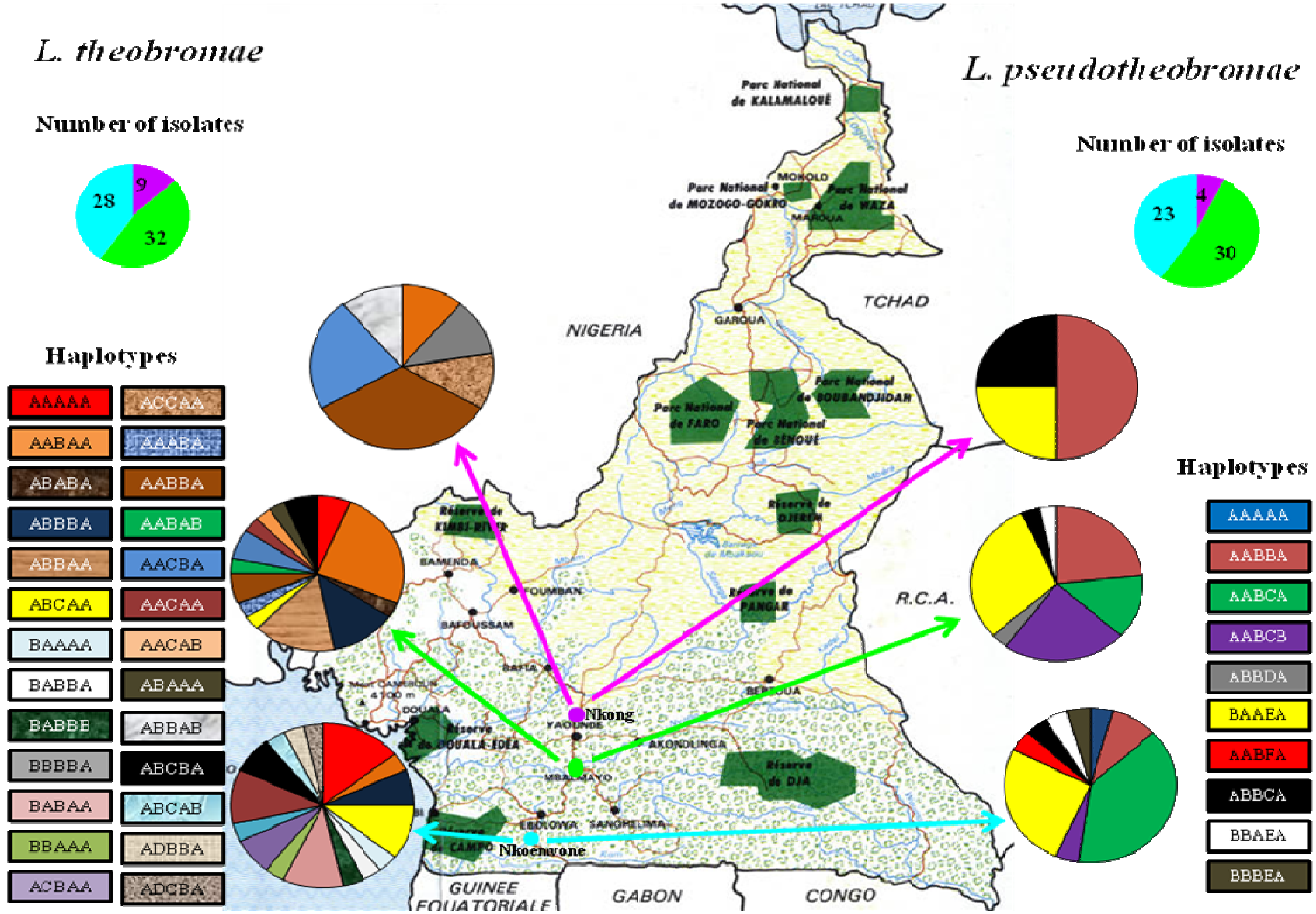
Las 21&22

Figure 3. Bayesian assignment of individuals into two clusters. Red : *Lasiodiplodia pseudotheobromae*; Green: *Lasiodilpodia theobromae*.



Lasiodiplodia pseudotheobromae *Lasiodiplodia theobromae*

Figure 4. Map of collecting sites and distribution of the 26 and 10 haplotypes of *Lasiodiplodia theobromae* and *L. pseudotheobromae,* respectively among the three locations. Each pie chart linked with arrows represents a collecting site and its haplotypes indicated by different color. The pie charts topping the left and right sides represent the number of isolates of *L. theobromae* and *L. pseudotheobromae,* respectively collected per locality.


L. theobromae
Number of isolates
9
28
32
Haplotypes
AAAAA
AABAA
ABABA
ABBBA
ABBAA
ABCAA
BAAAA
BABBA
BABBB
BBBBA
BABAA
BBAAA
ACBAA
ACCAA
AAABA
AABBA
AABAB
AACBA
AACAA
AACAB
ABAAA
ABBAB
ABCBA
ABCAB
ADBBA
ADCBA
L. pseudotheobromae
Number of isolates
4
23
30
Haplotypes
AAAAA
AABBA
AABCA
AABCB
ABBDA
BAAEA
AABFA
ABBCA
BBAEA
BBBEA
NIGERIA
TCHAD
R.C.A.
GUINEE
EQUATORIALE
GABON
CONGO
Nkong
Nkoemvone

Chapter 6

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 *T. ivorensis*, and on dead branches of non-native *T. 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.

1. 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 *Cryphonectria* and *Chrysoporthe,* 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. cubensis* and *Chr. austroafricana* (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) (Ofusu-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 (Ofusu-Asiedu and Cannon 1976; Hodges and Fereira 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.

2. MATERIALS AND METHODS

2.1. 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. ivorensi*s 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).

2.2. DNA extraction and 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 $1s^{-1}$ 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 (10mM), 2.5 µl dNTPs (10mM), 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 1x 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 1µl 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 3130xl 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 2001) 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.

2.3. 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 two 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).

2.4. 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 one-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 six 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).

3. RESULTS

3.1. 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 standing *T. ivorensis*, disease symptoms included cankers on the tree trunks (Figure 1a), cracked bark containing yellow to orange fruiting structures (Figure 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 (Figure 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 (Figure 2).

3.2. 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.

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 analyses 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 (Figure 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).

3.3. 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).

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.

3.4. 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) x 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) x 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) x 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) x 1.0–1.5 (–2.5) 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 one 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.

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* when sectioned longitudinally, 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***

3.5. Pathogenicity

Six weeks after inoculation, all isolates of *A. marmelostoma* yielded visible stem cankers on *T. mantaly* trees (Figure 5). Analysis of variance showed that lesion lengths on the cambium for all isolates were significantly different ($P < 0.0001$) to those associated with the negative control (Figure 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 lesions produced by all the isolates, and these provided the basis to confirm the association of *A. marmelostoma* with the lesions resulting from inoculation. In contrast, all control lesions were surrounded by callus, with no necrotic lesions, indicating a healed wound.

4. 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.

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, the presence of the 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 *Chrysoporthe*, *Celoporthe* and *Holocryphia,* were reported infecting trees in the Combretaceae, Heteropyxidaceae, Melastomataceae and the Myrtaceae (all Myrtales) in the sub-Saharan part of the continent (Ofusu-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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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
Aurifilum marmelostoma	**CMW 28285**	*Terminalia 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	CMW 2113	*Eucalyptus grandis*	South Africa	M.J. Wingfield	AF046892, AF273067, AF273462

Species	Isolates number [a]	Host	Origin	Collector	Genbank accession numbers [b]
austroafricana	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

Species	Isolates number [a]	Host	Origin	Collector	Genbank accession numbers [b]
	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
Ursicullum fallax	CMW 18115	*Cocoloba uvifera*	USA	C.S. Hodges	DQ368756, DQ368760, DQ368761
	CMW 18119	*C. uvifera*	USA	C.S. Hodges	DQ368755, DQ368758, DQ368759

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

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

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

	Morphological characteristics	*Amphilogia*	*Cryphonectria*	*Endothia*	*Holocryphia*	*Microthia*	*Rostraureum*	*Ursicullum*	*Aurifilum*
Teleomorph	**Structure of ascostroma**	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 textura type	Prosenchyma and pseudoparenchyma	Prosenchyma and pseudoparenchyma

Morphological characteristics	*Amphilogia*	*Cryphonectria*	*Endothia*	*Holocryphia*	*Microthia*	*Rostraureum*	*Ursicullum*	*Aurifilum*
Paraphyses	Absent	Absent	Absent	Present	Present	Absent	Absent	**Present**

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


a
b
c

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

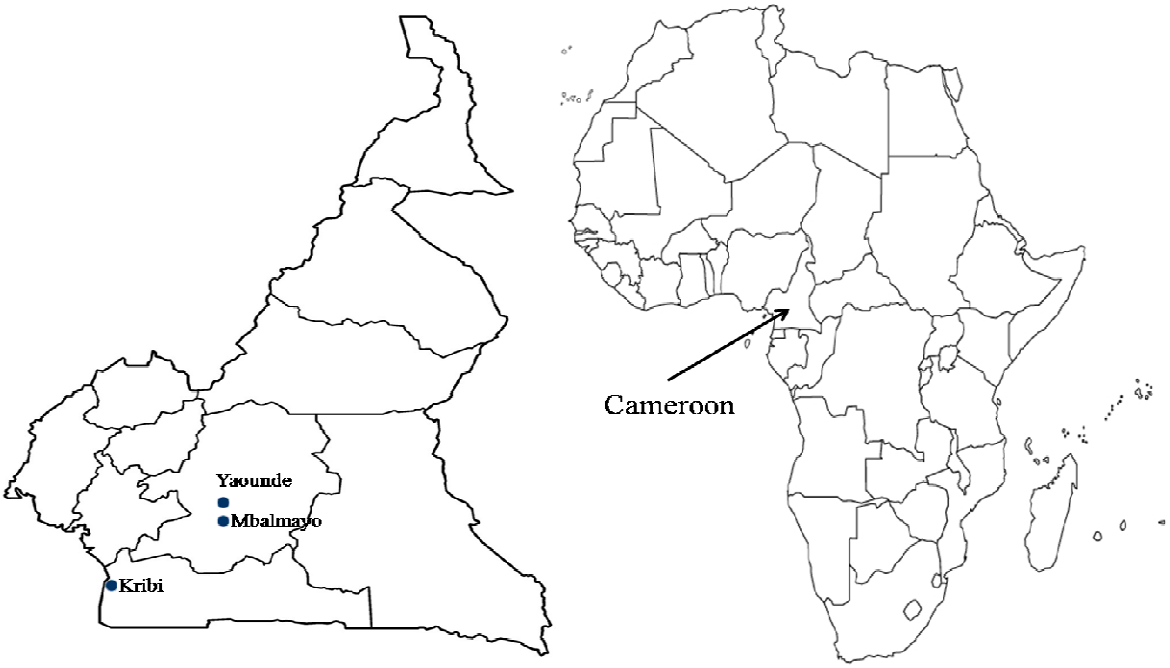

Yaounde
Mbalmayo
Kribi
Cameroon

Figure 3. Most parsimonious tree obtained from MP analyses of the combined ITS and BT 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.

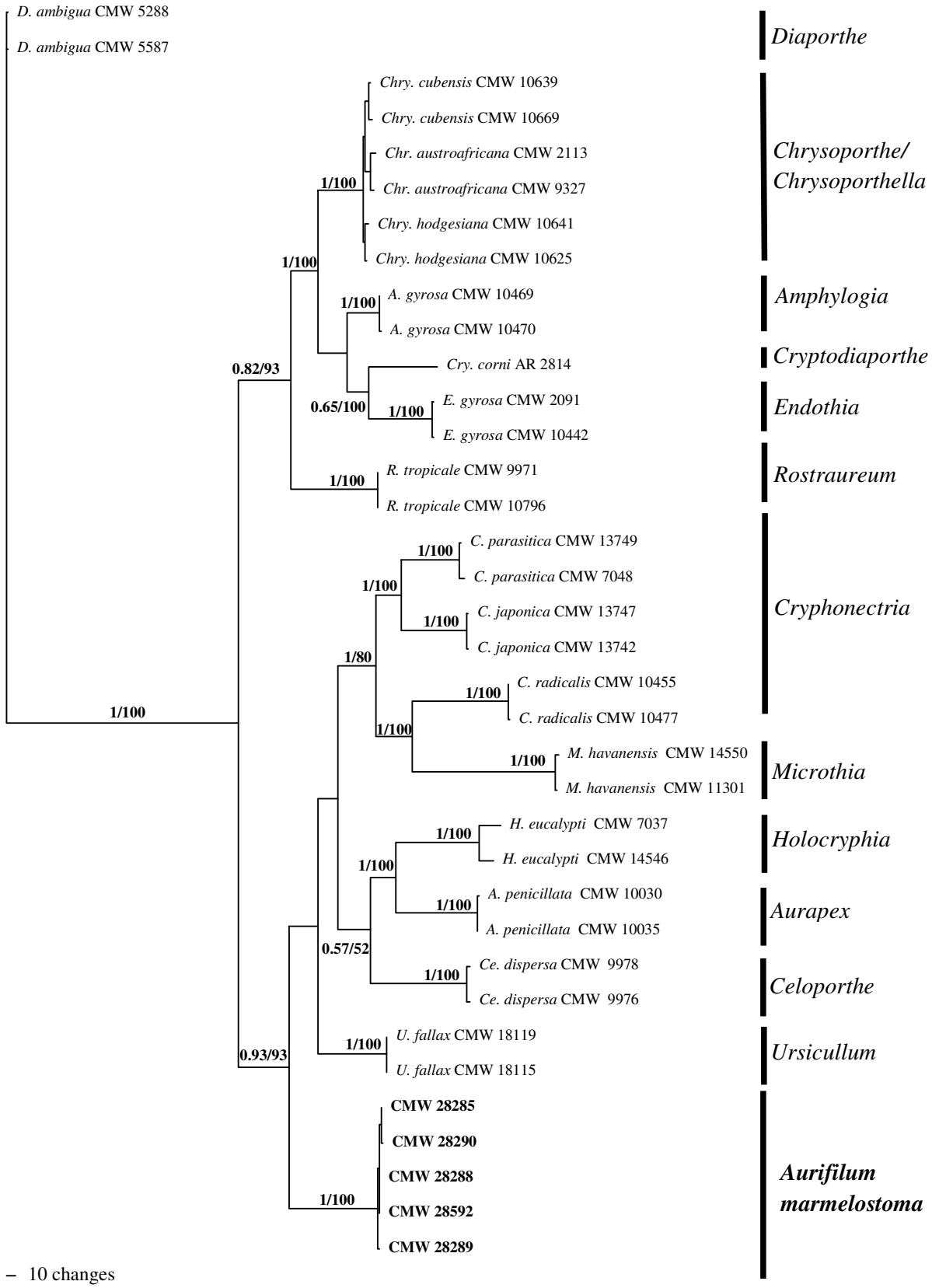

D. ambigua CMW 5288
D. ambigua CMW 5587
Chry. cubensis CMW 10639
Chry. cubensis CMW 10669
Chr. austroafricana CMW 2113
Chr. austroafricana CMW 9327
Chry. hodgesiana CMW 10641
Chry. hodgesiana CMW 10625
A. gyrosa CMW 10469
A. gyrosa CMW 10470
Cry. corni AR 2814
E. gyrosa CMW 2091
E. gyrosa CMW 10442
R. tropicale CMW 9971
R. tropicale CMW 10796
C. parasitica CMW 13749
C. parasitica CMW 7048
C. japonica CMW 13747
C. japonica CMW 13742
C. radicalis CMW 10455
C. radicalis CMW 10477
M. havanensis CMW 14550
M. havanensis CMW 11301
H. eucalypti CMW 7037
H. eucalypti CMW 14546
A. penicillata CMW 10030
A. penicillata CMW 10035
Ce. dispersa CMW 9978
Ce. dispersa CMW 9976
U. fallax CMW 18119
U. fallax CMW 18115
CMW 28285
CMW 28290
CMW 28288
CMW 28592
CMW 28289
1/100
1/100
1/100
1/100
1/100
0.82/93
0.65/100
1/100
1/100
1/100
1/100
1/100
1/80
1/100
1/100
1/100
1/100
1/100
0.57/52
1/100
1/100
0.93/93
1/100
Diaporthe
Chrysoporthe/
Chrysoporthella
Amphylogia
Cryptodiaporthe
Endothia
Rostraureum
Cryphonectria
Microthia
Holocryphia
Aurapex
Celoporthe
Ursicullum
Aurifilum
marmelostoma
– 10 changes

Figure 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 = 20 µm; b = 50 µm.

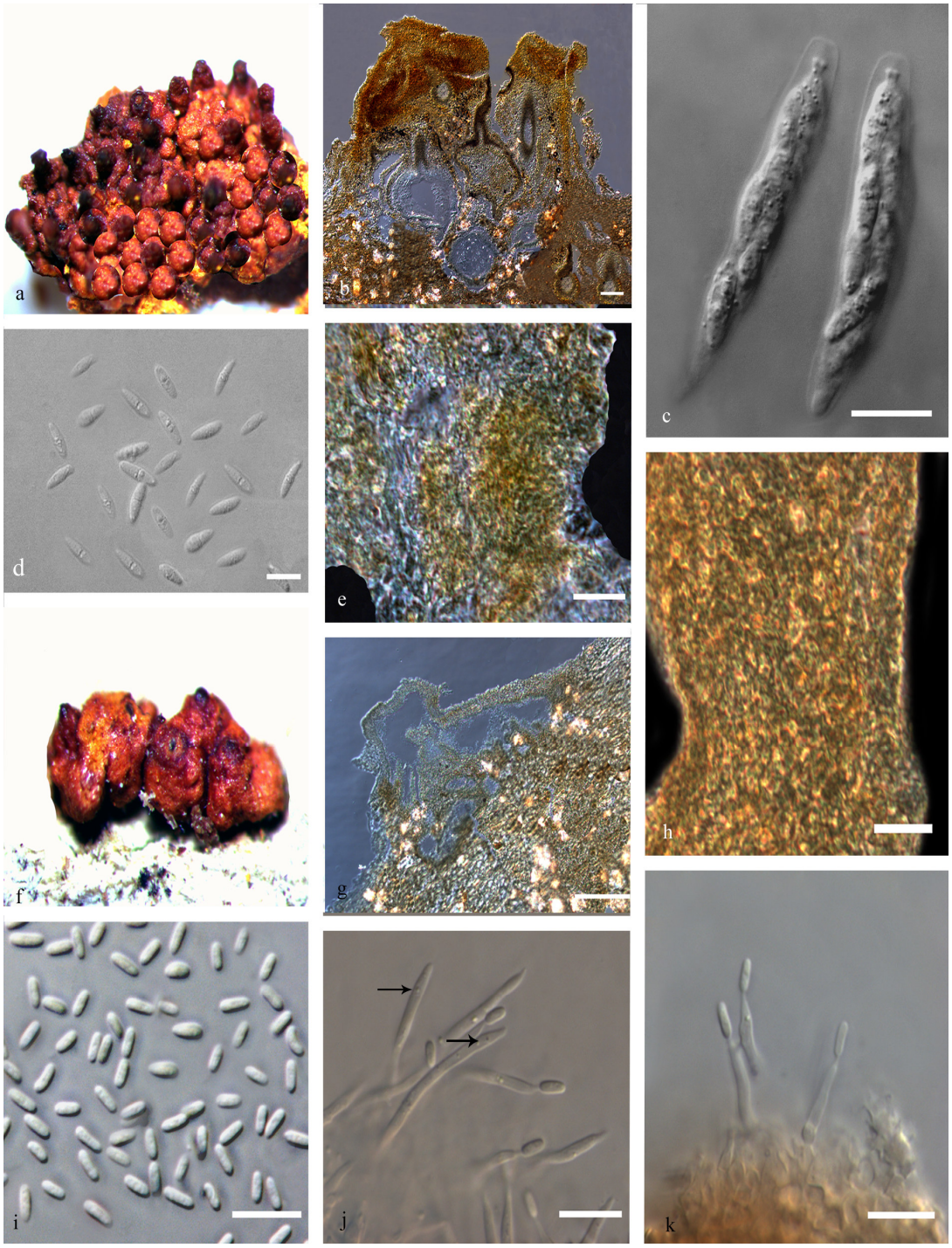
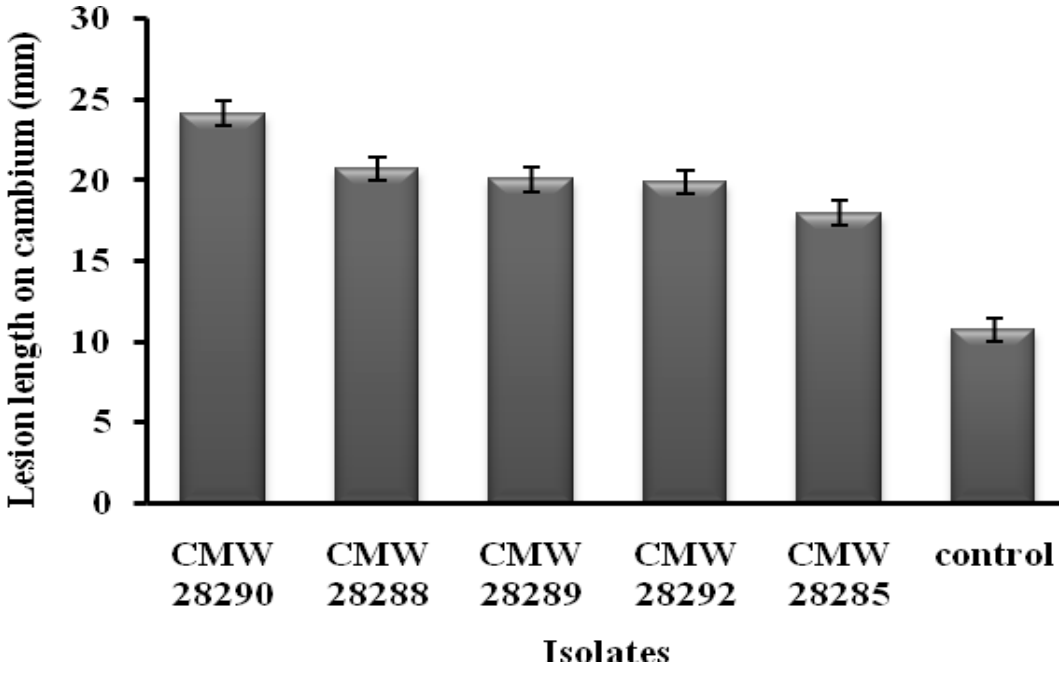

a
b
c
d
e
f
g
h
i
j
k

Figure 5. Mean lesion lengths (mm) on the cambium for each *A. marmelostoma* isolate six weeks after inoculation on *T. mantaly* ($P < 0.0001$).


Lesion length on cambium (mm)
30
25
20
15
10
5
0
CMW 28290
CMW 28288
CMW 28289
CMW 28292
CMW 28285
control
Isolates