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# Diversity and pathogenicity of the Ceratocystidaceae associated with cacao agroforests in Cameroon

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Knowledge of the diversity and ecology of plant pathogenic fungi in cacao agroforests and surrounding natural ecosystems can inform the development of sustainable management strategies for new cacao disease outbreaks. This study investigated the occurrence of fungi related to the Ceratocystidaceae and their nitidulid beetle vectors in cacao agroforests in Cameroon, under diverse agroecological conditions. The fungi and their vectors were collected from artificially induced stem wounds on cacao and associated shade trees. Collections were also made from abandoned cacao pod husks and other tree wounds within and around plantations. Fungal isolates were identified using DNA sequence-based phylogenies and morphological comparisons, and two representatives of each species were evaluated for pathogenicity on cacao. Five species of Ceratocystidaceae were recovered, including *Huntiella chlamydoformis* sp. nov., *H. pycnanthi* sp. nov. and *H. moniliformis*, as well as *Thielaviopsis cerberus* and *T. ethacetica*. The incidence of these fungi appeared to be influenced by the prevailing agroecological conditions. Nitidulid beetles in the genus *Brachypeplus* were found to be their most common insect associates on cacao. Both *T. ethacetica* and *H. pycnanthi* produced extensive lesions after inoculation on branches of mature cacao trees, while *T. ethacetica* also caused pod rot. Although their impact remains unknown, fungi in the Ceratocystidaceae and their nitidulid beetle vectors are common and probably contribute to the parasitic pressure in Cameroonian cacao agrosystems.

*Keywords:* *Ceratocystis*, crop protection, emerging diseases, fungal pathogens, nitidulid beetles

## Introduction

Cacao (*Theobroma cacao*) is affected by numerous pests and pathogens, and these represent the primary production constraint for this crop (Gotsch, 1997; Bowers *et al.*, 2001). In this regard, fungal pathogens have emerged as a serious threat to the sustainability of the global cacao industry (Bowers *et al.*, 2001; Ploetz, 2007), being responsible for losses estimated at 30–40% of potential annual world cocoa (cacao beans) production (http://www.icco.org/about-cocoa/pest-a-diseases.html). Cocoa yield losses due to fungal pathogens are also expected to increase substantially in the future. This is because the most destructive fungal diseases of cacao, including black pod disease (BPD) caused by various species of *Phytophthora*, especially *P. megakarya*, in Africa, frosty pod rot caused by *Moniliophthora roreri*, as well as witches' broom caused by *M. perniciosa* in Latin America, are spreading to new areas (Evans, 2007).

Cameroon produces *c.* 220 000 tonnes of cocoa per annum and is ranked fifth amongst the major world cocoa producers (www.worldcocoa.org). In Cameroon, cacao is generally cultivated by smallholder farmers in complex and diversified agroforestry systems, commonly intermingling cacao with fruit trees and other agricultural crops in thinned primary forests (Mbile *et al.*, 2009). These production systems add ecological and economic value (Rice & Greenberg, 2000; Mbile *et al.*, 2009), but they also provide suitable conditions for outbreaks of various pests and diseases (Schroth *et al.*, 2000).

The occurrence and impact of pests and pathogens of cacao has consistently increased in Cameroon since the crop was first introduced into the country in the late 1800s. In addition to severe mirid (Heteroptera: Miridae) damage recorded from the very early years of cacao cultivation, there has been a dramatic increase in yield losses due to BPD, resulting from the emergence of *P. megakarya* (native in Cameroon) in the 1970s (Sonwa *et al.*, 2005; Mfegue, 2012) and, more recently, an

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upsurge in tree mortality associated with infections by *Lasiodiplodia theobromae* (Mbenoun *et al.*, 2008). While it might not be possible to predict the nature of the next disease outbreak on cacao in Cameroon, the two cases mentioned above highlight the need for a better knowledge of the diversity of plant pathogenic fungi found naturally in surrounding ecosystems. This is also supported by changing environmental conditions and associated abiotic stress, which could increase cacao vulnerability to attacks by secondary or latent pathogens (Garrett *et al.*, 2006).

This study considered fungi belonging to the recently revised ascomycete family Ceratocystidaceae (de Beer *et al.*, 2014). This diverse and widely distributed group of fungi (Seifert *et al.*, 2013; de Beer *et al.*, 2014) includes numerous plant pathogens associated with fruit and tuber rot, canker and wilt diseases (Kile, 1993; Roux & Wingfield, 2009). These pathogens infect their hosts through wounds and are most commonly transmitted by insects, including nitidulid (Coleoptera: Nitidulidae) beetles (e.g. Hayslett *et al.*, 2008; Heath *et al.*, 2009). A number of species of Ceratocystidaceae have been reported to affect cacao, the best known of which is *Ceratocystis cacaofunesta*, a wilt pathogen that has been associated with substantial tree mortality in Central and South American cacao orchards (Engelbrecht *et al.*, 2007). There is also growing evidence to suggest that diseases of tree crops caused by fungi in the Ceratocystidaceae, and especially *Ceratocystis* species, are increasing worldwide (e.g. Roux & Wingfield, 2009; Wingfield *et al.*, 2013), justifying greater attention being paid to the diversity of these fungi globally.

Despite their obvious importance, the incidence and impact of the Ceratocystidaceae in Africa, especially Central and West Africa encompassing the cacao growing regions, is largely unknown. The aims of this study in cacao agroforests of Cameroon were thus to: (i) determine the occurrence of these fungi and their nitidulid vectors; (ii) characterize their species diversity; and (iii) assess their pathogenicity on cacao.

## Materials and methods

### Study sites

Field studies were undertaken during the cacao harvesting seasons, between October and November 2009 and 2010, at three sites in the Centre-South cacao growing basin of Cameroon (Fig. S1). This region is characterized by a tropical rainforest vegetation type, extending slightly into moist savanna grassland to the north. The climate is subequatorial, with an average temperature of 27°C and annual rainfall ranging from 1300 to 2000 mm and distributed over a bimodal seasonal pattern.

Four cacao stands were selected for the study and these were based on differences in age, ecological conditions and the cacao farming system being applied. One stand (04°30′16·7″N, 11°04′44·9″E) was located in Bokito, a relatively dry forest–savanna transition zone at the northwest limit of the region. The farm was established in the early 1990s on savanna and included various agricultural and food crops such as oil palm, banana and pineapple, within or alongside the cacao plot. It was slightly shaded by a few non-native fruit trees and indigenous *Ceiba pentandra*. Two stands (03°16′06·4″N, 11°14′29·9″E and 03°16′03·5″N, 11°14′50·4″E) were located in Ngomedzap, in the humid forest zone. Both plantations were over 60 years old, but with variable cacao tree ages. They were established in thinned primary forest with a high density and diversity of remnant forest shade trees dominated by *Terminalia superba*. Some banana and fruit trees were also present in these stands. The fourth stand (02°49′10·5″N, 11°07′57·9″E) was also located in the humid forest zone, at the Nkoemvone research station of the Institute of Agricultural Research for Development (IRAD), where several cacao genotype plots are maintained. The plots included in this study were being rejuvenated. They were planted in the 1970s on land completely cleared of primary forest, using scattered, non-native *Cassia spectabilis* as shade trees.

### Collections

Ten cacao trees (diameter at breast height ≥ 20 cm), at least 50 m apart, and three to five dominant shade trees (*Ceiba pentandra* at Bokito, *T. superba* at Ngomedzap and *Cassia spectabilis* at Nkoemvone) were selected at each site, marked and artificially wounded. Wounds were established *c.* 1·5 m above ground with one wound per tree on cacao and two per tree on shade trees. Approximately 10 × 10 cm of bark was cut from the stems using masonry chisels to expose the cambium and a *c.* 5 × 5 mm slit was made into the sapwood at the centre of the cleared cambium to expose the vascular tissue (Fig. 1a). At Nkoemvone, induced wounds were modified by lifting the bark at the top and bottom to create niches for visiting nitidulid beetles.

Artificially induced wounds were inspected after 2 weeks. Where nitidulid beetles had infested the wounds, the insects were collected using an aspirator and maintained in glass vials. Wound surfaces were examined using a ×10 magnification hand lens to determine the presence of fungal fruiting structures. The presence of internal discolouration in the sapwood was also determined by removing the surface wood and edges of wounds. Slices of wood and bark carrying fungal structures, or with discolouration, were collected in paper bags and transported to the laboratory for further study. After removing all visibly discoloured wood from the trees, wounds were treated with copper oxide (Nordox) and covered with 'artificial bark' (Lac Balsam).

During field visits, all substrates likely to harbour species of Ceratocystidaceae and nitidulid beetles present within or around the study sites were also examined and where appropriate, additional samples (plant material and insects) were collected from these substrates. These specifically included cacao pod husk piles (Fig. 1b) abandoned in the plantations by farmers after harvesting the beans in Bokito and Ngomedzap, as well as natural wounds on two tree species, *Terminalia mantaly* and *Erythrophleum ivorense*, growing alongside the cacao plots in Ngomedzap. These trees had been recently damaged by a wind storm. Furthermore, seven wind-oriented traps baited with dough and *Carpophilus hemipterus* pheromone lure (Great Lakes IMP) were set up for *c.* 12 h at each farm in Ngomedzap. This was in an attempt to capture flying nitidulid beetles from which fungi related to the Ceratocystidaceae could be isolated.

### Sample processing and fungal isolation

Sample processing for fungal isolation included incubation of plant materials under moist conditions to induce further development of

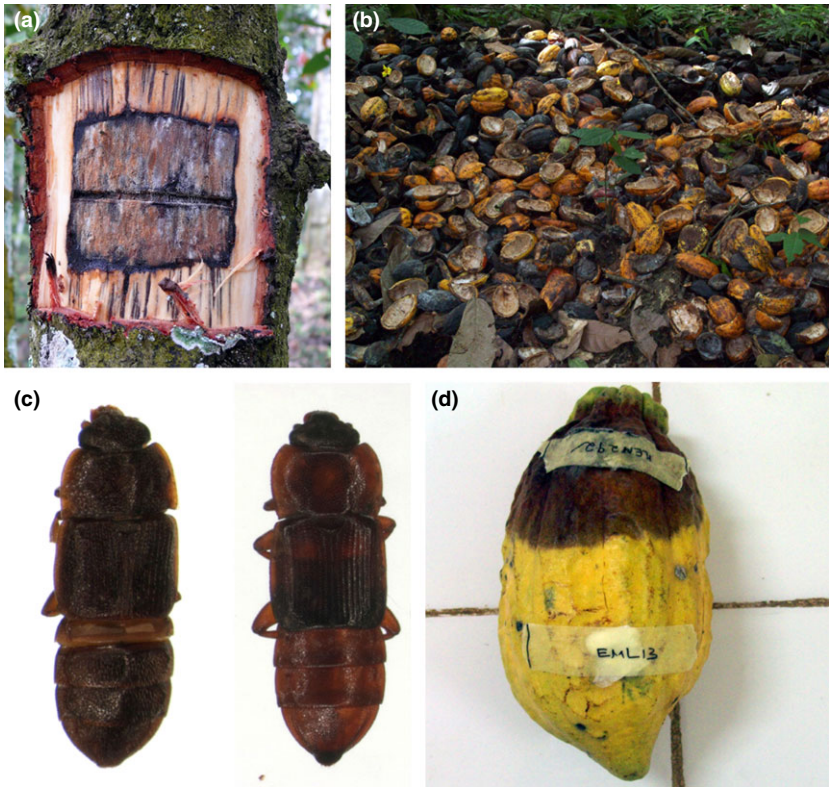


Figure 1 Substrates from which fungi were collected in cacao agroforests in Cameroon. (a) induced fresh wound (*c.* 10 × 10 cm) with a slit incised in the sapwood of a cacao tree after removal of the bark showing fungal growth and brown streaking extending from the wound; (b) cacao pod husk heap; (c) *Brachypeplus* nitidulid beetles obtained from cacao stem wounds and pod husks; (d) *Thielaviopsis ethacetica* (top, CMW35028) causing pod rot contrasting with *Thielaviopsis cerberus* (bottom, CMW35021) after inoculation onto a healthy cacao pod.

fungal structures, and baiting for *Ceratocystis* species using carrot discs. Pieces of plant material were placed in 90 mm Petri dishes lined with moistened tissue paper. Small chips were taken from the same material and wrapped between two carrot discs pretreated with streptomycin sulphate (Sigma). Isolation from nitidulid beetles was also attempted using carrot baiting. The insects were initially grouped into morphotypes, and five representatives of each morphotype were individually crushed onto the surface of carrot discs. The remaining specimens were kept in 90% ethanol for later identification.

Fungi were isolated from processed materials after 7–10 days. Mycelial strands or single masses of ascospores at the tips of ascomata that formed on the surfaces of cacao tissues or on the carrot discs, were transferred to 2% malt extract agar (MEA; Biolab), supplemented with *c.* 0·01 g $L^{-1}$ streptomycin sulphate (Sigma). Isolates were purified by subculturing from single hyphal tips, and they were grouped based on their morphotypes. When possible, two isolates, representing each morphotype, were retained per substrate for further analysis.

## Molecular characterization and phylogenetic analysis of fungal isolates

Fungal DNA extraction was conducted following the procedure described by Mbenoun *et al.* (2014). Sequence data were generated for the beta-tubulin (*β-tub*) gene region and used as a means of first level sorting of isolates. For this purpose, the *β-tub* sequences were aligned using the MUSCLE algorithm implemented in MEGA v. 5 (http://www.megasoftware.net/) and, subsequently, a neighbour joining (NJ) phylogenetic tree was constructed in MEGA. Four to six representatives from different sites and substrates were selected from each clade of the NJ tree for further identification. This led to the selection of 16 isolates (Table 1) for which the internal transcribed spacer regions including the 5·8S rDNA locus of the ribosomal RNA cluster (ITS) and a portion of the translation elongation factor 1 alpha gene (*tef1-α*) were also sequenced. The protocols and procedures used for PCR and sequencing were the same as those described by Mbenoun *et al.* (2014).

Three sequence data sets representing the ITS, *β-tub* and *tef1-α* loci were prepared for phylogenetic analysis. These included sequences for the 16 selected isolates from Cameroon generated in this study, as well as those retrieved from GenBank, representing closely related reference species identified using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (see Table 1 for GenBank accession numbers). Sequences of *Huntiella moniliformopsis* were used to represent an out-group taxon. For all three data sets, alignments were generated using MAFFT (http://mafft.cbrc.jp/alignment/server/) and edited in MEGA. Single gene alignments were thereafter concatenated into a super-alignment, which was then used for multigene phylogenetic inference (http://purl.org/phylo/treebase/phylows/study/TB2:S16363).

Two parallel multigene phylogenetic reconstructions were conducted, using the maximum likelihood (ML) and maximum parsimony (MP) approaches, respectively. MP analyses were

Table 1 *Huntiella* species and GenBank accession numbers of DNA sequences used for phylogenetic analyses

| Species | Isolate no. | | Gene region/GeneBank accession no. | | | Host | Geographic origin | Collector |
|---|---|---|---|---|---|---|---|---|
| | | | ITS | *β-tub* | *tef1-α* | | | |
| *H. ceramica* | CMW15245 | CBS 122299 | EU245022 | EU244994 | EU244926 | *Eucalyptus grandis* | Malawi | R. N. Heath & J. Roux |
| | CMW15248 | CBS 122300 | EU245024 | EU244996 | EU244928 | *E. grandis* | Malawi | R. N. Heath & J. Roux |
| *H. chlamydoformis* | CMW36899 | | | | | *Theobroma cacao* | Cameroon | M. Mbenoun & J. Roux |
| | CMW36932 | CBS 131674 | KF769087 | KF769109 | KF769098 | *T. cacao* | Cameroon | M. Mbenoun & J. Roux |
| | CMW37101 | | | | | *T. cacao* | Cameroon | M. Mbenoun & J. Roux |
| | CMW37102 | CBS 131675 | KF769088 | KF769110 | KF769099 | *Terminalia superba* | Cameroon | M. Mbenoun & J. Roux |
| | CMW37106 | CBS 131673 | KF769089 | KF769111 | KF769100 | *T. cacao* | Cameroon | M. Mbenoun & J. Roux |
| | CMW37584 | | KF769090 | KF769112 | KF769101 | *T. cacao* | Cameroon | M. Mbenoun & J. Roux |
| *H. cryptoformis* | CMW36827 | | KC691463 | KC691487 | KC691511 | *Combretum zeyheri* | South Africa | M. Mbenoun & J. Roux |
| | CMW36828 | CBS 131279 | KC691464 | KC691488 | KC691512 | *Ziziphus mucronata* | South Africa | M. Mbenoun & J. Roux |
| *H. decipiens* | CMW25914 | CBS 129737 | HQ203219 | HQ203236 | HQ236438 | *Eucalyptus maculata* | South Africa | G. N. Kamgan & J. Roux |
| | CMW25918 | CBS 129735 | HQ203218 | HQ203235 | HQ236437 | *Eucalyptus cloeziana* | South Africa | G. N. Kamgan & J. Roux |
| *H. moniliformis* | CMW4114 | CBS 118151 | AY528997 | AY528986 | AY529007 | *Shizolobium parahyba* | Ecuador | M. J. Wingfield |
| | CMW9590 | CBS 116452 | AY431101 | AY528985 | AY529006 | *E. grandis* | South Africa | J. Roux |
| | CMW36895 | CBS 137247 | KF769091 | KF769113 | KF769102 | *T. cacao* | Cameroon | M. Mbenoun & J. Roux |
| | CMW36908 | CBS 137248 | KF769092 | KF769114 | KF769103 | *T. cacao* | Cameroon | M. Mbenoun & J. Roux |
| | CMW36919 | | KF769093 | KF769115 | KF769104 | *T. cacao* | Cameroon | M. Mbenoun & J. Roux |
| | CMW37105 | | | | | *T. cacao* | Cameroon | M. Mbenoun & J. Roux |
| *H. moniliformopsis* | CMW9986 | CBS 109441 | AY528998 | AY528987 | AY529008 | *Eucalyptus obliqua* | Australia | Z. Q. Yuan |
| | CMW10214 | CBS 115792 | AY528999 | AY528988 | AY529009 | *Eucalyptus sieberi* | Australia | M. J. Dudzinski |
| *H. pycnanthi* | CMW36901 | CBS 131671 | KF769094 | KF769116 | KF769105 | *T. cacao* | Cameroon | M. Mbenoun & J. Roux |
| | CMW36910 | | KF769095 | KF769117 | KF769106 | *T. cacao* | Cameroon | M. Mbenoun & J. Roux |
| | CMW36915 | | | | | *T. cacao* | Cameroon | M. Mbenoun & J. Roux |
| | CMW36916 | CBS 131672 | KF769096 | KF769118 | KF769107 | *T. cacao* | Cameroon | M. Mbenoun & J. Roux |
| | CMW36921 | | KF769097 | KF769119 | KF769108 | *T. cacao* | Cameroon | M. Mbenoun & J. Roux |
| | CMW36933 | | | | | *T. cacao* | Cameroon | M. Mbenoun & J. Roux |
| *H. oblonga* | CMW23802 | | EU245020 | EU244992 | EU244952 | *Acacia mearnsii* | South Africa | R. N. Heath |
| | CMW23803 | CBS 122291 | EU245019 | EU244991 | EU244951 | *A. mearnsii* | South Africa | R. N. Heath |

(continued)

Table 1 (continued)

| Species | Isolate no. | | Gene region/GeneBank accession no. | | | Host | Geographic origin | Collector |
|---|---|---|---|---|---|---|---|---|
| | | | ITS | *β-tub* | *tef1-α* | | | |
| *H. salinaria* | CMW25911 | CBS 129733 | HQ203213 | HQ203230 | HQ236432 | *E. maculata* | South Africa | G. N. Kamgan & J.Roux |
| | CMW30703 | CBS 129734 | HQ203214 | HQ203231 | HQ236433 | *Eucalyptus saligna* | South Africa | G. N. Kamgan & J.Roux |
| *H. savannae* | CMW173298 | | EF408553 | EF408567 | EF408573 | *Terminalia sericea* | South Africa | G. N. Kamgan & J.Roux |
| | CMW17300 | CBS 121151 | EF408551 | EF408565 | EF408572 | *Acacia nigrescens* | South Africa | G. N. Kamgan & J.Roux |
| *H. sublaevis* | CMW22444 | CBS 122518 | FJ151430 | FJ151464 | FJ151486 | *Terminalia ivorensis* | Ecuador | M. J. Wingfield |
| | CMW22449 | CBS 122517 | FJ151431 | FJ151465 | FJ151487 | *T. ivorensis* | Ecuador | M. J. Wingfield |
| *H. tribiliformis* | CMW13011 | CBS 115867 | AY528991 | AY529001 | AY529012 | *Pinus merkusii* | Indonesia | M. J. Wingfield |
| | CMW13012 | CBS 118242 | AY528992 | AY529002 | AY529013 | *P. merkusii* | Indonesia | M. J. Wingfield |
| *H. tyalla* | CMW28917 | | HM071899 | HM071909 | HQ236448 | *E. grandis* | Australia | G. N. Kamgan |
| | CMW28920 | | HM071896 | HM071910 | HQ236449 | *E. grandis* | Australia | G. N. Kamgan |

CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW, culture collection, Forestry and Agricultural Biotechnology Institute, FABI, Pretoria, South Africa.

performed using PAUP v. 4.0b10* (Swofford, 2002) and involved only parsimony-informative characters, including gaps as a fifth character state. All characters were assigned the same weight and considered unordered. Trees were generated via a heuristic tree search with 1000 random stepwise addition replicates and tree-bisection-reconstruction (TBR) branch-swapping. All equally most parsimonious trees (MPTs) were saved. The tree length (TL), consistency (CI), retention (RI) and re-scaled consistency (RC) indices of the MPTs were calculated. The partition homogeneity test (PHT) was used to test for incongruence between individual gene partitions, using 1000 heuristic search replicates. ML analyses were performed online, with PHYML 3.0 (http://www.atgc-montpellier.fr/phyml/), applying the TN93 nucleotide substitution model with estimated gamma shape parameter and proportion of invariable sites. This model was selected as a good fit for all three single genome regions as well as the concatenated alignment data sets, based on the Akaike information criterion implemented in JMODELTEST v. 2.1.3 (Darriba *et al.*, 2012). In both ML and MP reconstructions, statistical support for branch nodes was determined by performing 1000 bootstrap analysis replicates.

Unnamed clades with significant statistical support emerging from multigene phylogenetic analyses were considered as putatively undescribed taxa. For these candidate new taxa, the relationships with their closest known relatives, based on single gene loci, was investigated. This was achieved using unrooted MP analyses in MEGA, and made it possible to also determine the number of fixed nucleotide changes between the new taxa and their closest relatives.

## Growth studies and culture morphology

For each of the putative new fungal species identified in the phylogenetic analyses, two representative isolates from different sites were chosen and used in growth studies at different temperatures. Temperature optima, minima and maxima were determined for the fungi by measuring colony diameters at six different temperatures, ranging from 10 to 35°C at 5°C intervals. Five replicate plates were prepared for each isolate at each temperature, by transferring agar plugs (8 mm in diameter) from the margins of actively growing cultures to the centres of 90 mm Petri dishes containing fresh, sterile 2% MEA. Plates were incubated at the test temperatures in the dark for 3 days. Measurements were taken along two perpendicular axes centred on the plugs, and averages and standard deviations were computed. Optimum, minimum and maximum temperature intervals were determined from growth curves.

Morphological descriptions were made using 1–2-week-old cultures of the isolates used for the growth studies, maintained at their optimum growth temperatures. Descriptions of colony colour were based on the mycological colour charts of Rayner (1970). Fungal structures were examined and described using an Axioskop microscope (Carl Zeiss Ltd), fitted with a HRc Axiocam digital camera and AXIOVISION v. 3.1 particle sizing software. Slides for microscopy were prepared by placing relevant structures in 85% lactic acid. Where possible, 50 measurements were taken for each taxonomically informative character for holotype specimens, and 10 measurements for each of two paratypes of new species. Species means and standard deviation values were computed for each character. These measurements are presented as the extremes in brackets and the range represented by the mean over all holotype and paratype measurements, plus or minus the standard deviations.

Isolates selected as holotypes as well as one or two paratypes of new species were deposited as living cultures with the Centraalbureau voor Schimmelcultures (CBS), the Netherlands and dried down replicate specimens were deposited with the National Collection of Fungi (PREM), Pretoria, South Africa.

## Incidence of the Ceratocystidaceae in cacao fields

After identification, the number of isolates representing each fungal species collected from the various sites and substrates was determined. The isolation frequency from cacao stem wounds was then calculated for each study site. This was done in order to assess the possible influence that agroecological

conditions and management practices may have on the incidence of the Ceratocystisdaceae in cacao plantations.

### Identification of nitidulid beetles

Identification of insects was primarily based on morphology, facilitated by comparing specimens with those in the collection of nitidulid beetles maintained at the Forestry and Agricultural Biotechnology Institute, University of Pretoria. In addition, for selected specimens representing each nitidulid morphotype from Cameroon, DNA was extracted using the prepGEM Insect (zyGEM) DNA extraction kit, following the manufacturer's instructions. Subsequently, the mitochondrial cytochrome oxidase I (*MT-CO1*) gene region was amplified and sequenced using the universal primer combination Cl-J-2183: 5′-CAA CATTTATTTTGATTTTTTGG-3′/TL2-N-3014: 5′-CCAATGC ACTAATCTGCCATATTA-3′ (Simon *et al.*, 1994). PCR reactions were prepared in a 25 μL final volume, with MyTaq (Bioline) DNA polymerase and reaction buffer, following the manufacturer's instructions. The thermal cycling conditions included 5 min at 96°C for initial denaturation, 35 cycles of 60 s at 94°C, 60 s at 50°C and 90 s at 72°C, and final extension for 10 min at 72°C. All other steps involved in the sequencing procedure were the same as those described by Mbenoun *et al.* (2014). *MT-CO1* sequences were compared based on NJ algorithms in MEGA and evaluated against the GenBank database using NCBI-BLAST.

### Pathogenicity trials

The pathogenicity of all fungal species recovered in this study was assessed using inoculations on branches of mature cacao trees in the field and on detached cacao fruits in the laboratory. Each species was represented by two isolates. Cultures used to produce inoculum were maintained on MEA for 10 days at room temperature. Inoculations were performed by making wounds on the various plant organs using a sterile 7 mm cork borer to remove the bark on branches, or outer cortex of fruit tissue. Subsequently, 7 mm diameter agar plugs taken from the margins of the fungal cultures were used to fill in the wounds, mycelium facing the cambium or inside of the fruit. Sterile MEA was used as a negative control. Inoculated wounds were covered with Parafilm (or with humidified cotton wool for fruits) to prevent desiccation and contamination.

Field inoculation of cacao branches were conducted at Ngomedzap, in the same cacao stands where some of the isolates had been collected. Ten cacao trees were selected and one lignified branch (10–15 mm in diameter) per tree was inoculated for each treatment. Inoculated branches were cut from the trees after 6 weeks, and lesion lengths were measured. These included lesions on the surface of the bark and those on the cambium after removing the bark. To confirm the association of observed lesions with the fungal strains used, reisolations were done by plating small pieces of tissue displaying symptoms, taken from the edges of lesions on randomly selected branches, onto MEA. Resultant isolates were identified based on morphology.

The field inoculation trial was repeated once. The aggressiveness of isolates tested was compared by submitting cambium lesion lengths (CLL) and bark lesion lengths (BLL) to one-way analyses of variance (ANOVA) and Tukey's HSD multiple range test. Prior to these analyses, the Bartlett test of homogeneity of variances was performed to assess whether data from the two replicated experiments could be combined. All statistical analyses were performed using R statistical software (http://www.R-project.org/).

Two independent fruit inoculation experiments were carried out in the laboratory, applying a completely randomized design. Each experiment included 22 mature pods from the same cacao cultivar, and two inoculations were made per pod at the stylar and peduncular ends. Inoculated pods were incubated at room temperature in plastic boxes lined with moist paper. They were examined for signs of rotting after 10 days. Where rotting had developed, reisolation of the inoculated fungus was attempted by transferring a piece of fruit cortex with symptoms from the margin of the lesion onto MEA, and the resultant isolates were identified based on morphology.

## Results

### Field observations and collections

Two weeks after they were established, artificially induced stem wounds had developed symptoms of fungal infection, with variable levels of severity. Wounds on cacao (Fig. 1a) were typically characterized by a dark brown discolouration on the surface of the exposed cambium. Abundant mycelial growth covered the wounds and ascomata resembling those formed by species of Ceratocystidaceae were observed in and around the central slit in the sapwood on some trees. When the mycelial mat was peeled from the surface of the wounds, substantial brown streaking of the sapwood was noted. The streaks extended vertically, reaching up to 20 cm in length. More extensive internal discolouration developed from the slits, deeper into the sapwood. Cacao stem wounds were generally drier in Bokito, showing less severe internal discolouration compared with those at the other two sites.

Limited internal discolouration was observed on *Terminalia* shade trees wounded at Ngomedzap. In contrast, *Cassia* and *Ceiba* trees at Nkoemvone and Bokito, respectively, showed no discolouration in the sapwood, although some mycelial growth and a few ascomata were observed on the exterior of the wounds on *Ceiba* trees. The exposed cambium on *Cassia* trees was superficially covered with a black stain, which could have prevented deep fungal colonization of the sapwood on this host. Ascomata were observed on naturally damaged *T. mantaly* and *E. ivorense* trees growing alongside the cacao plots at Ngomedzap. Wood samples carrying abundant, fresh mycelium and ascomata were collected from these trees for fungal isolation and identification.

Abundant fungal growth was observed on abandoned pod husks and samples were collected from them at Bokito and Ngomedzap. The endosperms of fresh pod husks were generally covered with a diverse fungal flora, including species of Ceratocystidaceae and other ophiostomatoid fungi.

Nitidulid beetles were observed on most of the substrates examined in this study. These insects (larval and adult stages) were especially abundant in cacao pod husk piles where they appeared to find ideal conditions for breeding. Nitidulid beetles were also found colonizing

stem wounds on cacao where they appeared shortly after the wounds were made. At Nkoemvone, large numbers (up to 15 per wound) of nitidulid beetles were recovered under bark flaps on cacao, while only two to three individuals (representing a different morphotype) were found on some wounded *Cassia* trees. No insects were collected from wounds on *Ceiba* trees and only a small number of insects were caught in the traps (<3 per trap, on average).

In total, 83 fungal isolates related to the Ceratocystidaceae were collected from cacao agroforests (Table 2). These included 53 isolates from Ngomedzap, 20 from Bokito and 10 from Nkoemvone. Of these, 49 isolates were obtained from 22 artificially wounded cacao trees, 16 isolates from abandoned cacao pod husks in three plantations, eight isolates from four wounded *Terminalia* shade trees, and eight isolates from naturally damaged *E. ivorense* and *T. mantaly* trees alongside cacao plantations. In addition, two isolates were collected from 2 of 35 nitidulid beetles caught infesting cacao pod husks and stem wounds. No fungal isolates related to the Ceratocystidaceae were obtained from wounded *Cassia* trees, and while some ascomata were observed on *Ceiba* trees, these fungi could not be isolated from this host.

### Phylogenetic analysis of fungal isolates

Based on general morphological characteristics, all fungal isolates collected in this study could broadly be placed in either of two genera, *Huntiella* and *Thielaviopsis*. Isolates related to *Thielaviopsis* included *Thielaviopsis cerberus* and *Thielaviopsis ethacetica*, identified in a previous study by Mbenoun *et al.* (2014). Isolates related to *Huntiella* were of unknown identity and only these were included in phylogenetic reconstructions.

The concatenated alignment matrix for *Huntiella* isolates included 38 sequences and 1575 characters. There were 222 parsimony-informative characters and MP analysis of these resulted in 15 MPTs of 323 steps (CI = 0·84, RI = 0·97, RC = 0·82). The Cameroonian isolates were resolved into three main clades (Fig. 2), with good statistical support (83–92%). Clade 1 included isolates representing *H. moniliformis*, while the other two clades were distinct from those delineated by known species. The two unnamed new clades were related to the *Huntiella* African lineage. Clade 2 had a basal position in the lineage and was most closely related to *H. decipiens*, *H. salinaria* and *H. ceramica*, while Clade 3 was monophyletic with *H. savannae*, *H. oblonga* and *H. cryptoformis*. The new clades showed substantial polymorphism (Fig. 2), each including two statistically supported subclades. The phylogeny resulting from ML analysis was concordant with that from MP analysis in all respects (tree topology, relationship among taxa and statistical support) and generally produced stronger bootstrap values for branch nodes (Fig. 2). However, the PHT ($P < 0{\cdot}001$) indicated significant conflicts among individual gene data sets.

When considered separately, each of the gene loci used in this study could discriminate the two unnamed clades from Cameroon, initially identified using a combined multigene data set. However, these markers showed variable resolution between the new taxa and their respective closest related species. The ITS produced no resolution, whereas the *β-tub* and *tef1-α* genes could either discriminate only Clade 3 or Clade 2, respectively, showing from 4 to 14 fixed nucleotide changes (Fig. S2). It also emerged that the polymorphism observed within Clade 2 and Clade 3 in the multigene tree was supported only by these same respective genes, prohibiting further consideration of their subclades as potential cryptic species based on available data.

### Culture and morphological characteristics

Isolates in Clade 2 grew optimally at 25°C, covering 56–61 mm within 3 days on MEA, whereas no growth was observed at 10 and 35°C. They developed fluffy colonies with aerial mycelium changing from hyaline to hazel (17'''b) with age. Hyphae were smooth, without constriction at the septa. Where ascomata had formed, they were generally distributed in a stellar fashion around the centre of the colony. The general structure of the ascomata was reminiscent of *H. decipiens*, including a relatively thick collar plate connecting the ascomatal necks and bases. The surfaces of ascomatal bases were rough, but sharp conical spines known for *H. decipiens* and other *Huntiella* species were not observed. Unlike any

Table 2 Number of isolates and substrates from which species of Ceratocystidaceae were collected in cacao agroforests in Cameroon

| | | *Huntiella* | | | *Thielaviopsis* | |
|---|---|---|---|---|---|---|
| Substrate | Study site | *H. chlamydoformis* | *H. moniliformis* | *H. pycnanthi* | *T. cerberus* | *T. ethacetica* |
| Cacao stem wounds | Bokito | 4 | 2 | 7 | 4 | – |
| | Nkoemvone | 3 | 5 | 2 | – | – |
| | Ngomedzap | 6 | 10 | 4 | – | 2 |
| Cacao pod husks | Bokito | – | – | – | – | 3 |
| | Ngomedzap | 4 | – | – | – | 9 |
| *Terminalia* stem wounds | Ngomedzap | 3 | 5 | – | – | – |
| Other tree wounds | Ngomedzap | 3 | 3 | – | – | 2 |
| Nitidulid beetles | Ngomedzap | – | – | – | – | 2 |

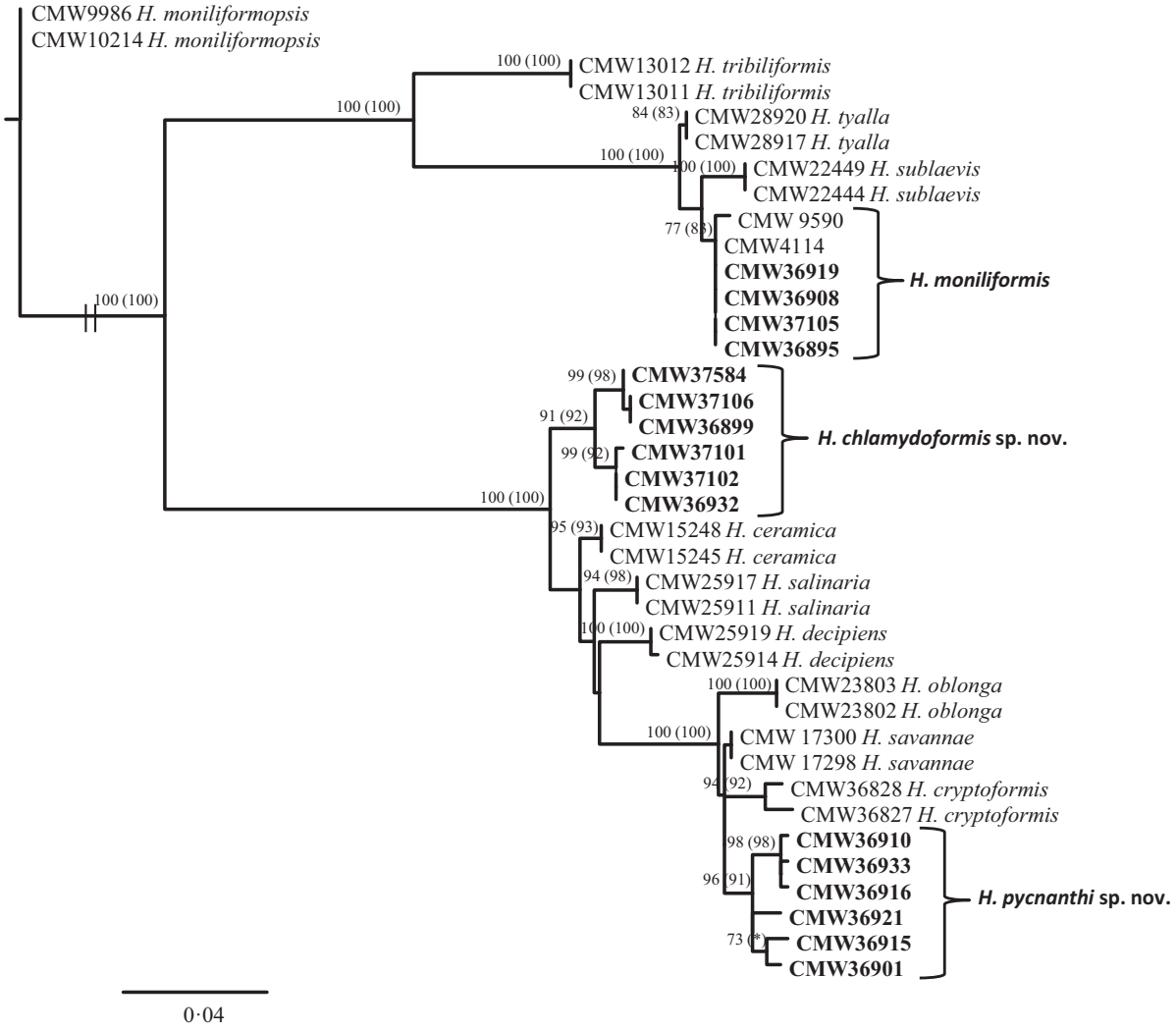


Figure 2 Phylogenetic tree derived from maximum likelihood analysis of combined ITS, *β-tub* and *tef1-α* gene sequences of *Huntiella* isolates from cacao agroforests in Cameroon together with sequences of selected reference species. Bootstrap support values ≥70% are indicated next to branch nodes, including those from maximum parsimony analysis in brackets.

other *Huntiella* species, ageing cultures of isolates in Clade 2 formed thick-walled chlamydospores. These structures appeared to evolve from transformation of mycelial cells. This distinguishes them from aleurioconidia, which are formed basipetally from specialized conidiogenous cells in other genera of Ceratocystidaceae such as *Ceratocystis*, *Chalaropsis* and *Thielaviopsis* (de Beer *et al.*, 2014; Mbenoun *et al.*, 2014).

The optimal growth temperature for isolates in Clade 3 was 30°C. The average colony diameter was 61–75 mm after 3 days at 30°C on MEA. Substantial residual growth was noted at 35°C, but no growth at 10°C. Cultures had fluffy, aerial mycelium, changing from hyaline or white to smoke grey (21''''d) with age. Hyphae were smooth and granular, without constrictions at the septa. Ascomata were randomly distributed on the agar plates and their bases were more globoid and lighter in colour than those of closely related species. The disciform collar structures connecting the ascomatal necks and bases, common to most species in the African lineage of *Huntiella*, was absent in isolates of Clade 3.

## Taxonomy

Based on their nucleotide divergence, as revealed by phylogenetic analyses, combined with some distinct morphological characteristics, the Cameroonian *Huntiella* isolates from cacao agroforests and residing in Clade 2 and Clade 3 represent two previously undescribed species. They are described here respectively as *H. chlamydoformis* sp. nov. and *H. pycnanthi* sp. nov.

***Huntiella chlamydoformis*** **Mbenoun & Jol. Roux sp. nov.**, Figure 3.

MycoBank MB 807094

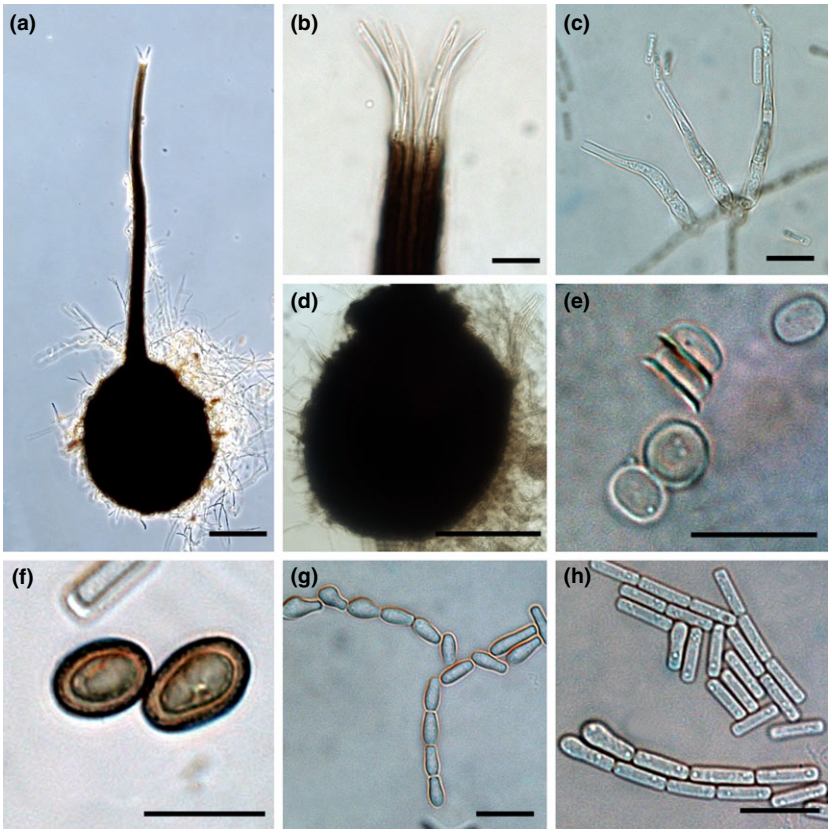


Figure 3 Morphological characteristics of *Huntiella chlamydoformis* sp. nov. (a) ascomata with globose base and extended neck; (b) tip of ascomatal neck showing divergent ostiolar hyphae; (c) monophialidic conidiophores; (d) ascocarp base showing bulbous collar structure at neck base; (e) ascospores in side (hat-shaped) and basal views; (f) thick-walled chlamydospores; (g) variform secondary conidia; (h) rectangular primary conidia. Scale bars: a, d = 100 μm; b, c, e, f, g, h = 10 μm.

*Etymology*
The name refers to the chlamydospores formed by this species, a feature not common in the genus *Huntiella*.

*Description*
Colonies on MEA fast growing, reaching 50–61 mm in diameter within 3 days, optimum temperature for growth 25°C, no growth at 10°C and 35°C. Mycelium fluffy and superficial, initially hyaline to white, turning to hazel (17'''b) with age. Hyphae smooth without constrictions at septa. Ascomata with bulbous bases and long necks, formed superficially on substrate, generally distributed in a stellar fashion around the centre of the colony. Ascomatal bases dark brown, globose or subglobose, (174–)204–298(–382) × (155–)180–264(–363) μm. Ascomatal necks dark brown, erect or slightly curled, forming a bulbous collar at the junction with ascomatal bases (372–)486–714(–896) μm long, (12–)15–20(–23) μm wide at apices and (30–)34–42(–49) μm wide at bases. Ostiolar hyphae hyaline, divergent, (21–)28–43(–51) μm long. Asci not observed. Ascospores accumulating in creamy to yellow droplets at the tips of ascomatal necks, aseptate, cuculate in side view, (5–)6–8 μm wide and 2–3 μm high. Conidiophores occurring solitary or aggregated in small bundles and arising laterally from hyphae, hyaline, monophialidic, (17–)23–33(–45) μm long, 1–3 μm wide at apices and (2–)3–4(–5) μm wide at bases. Primary conidia hyaline, aseptate, rectangular, (5–)6–9(–12) × 1–3 μm. Secondary conidia hyaline, smooth, aseptate, variable in shape, (4–)6–8(–10) × 2–4(–5) μm. Chlamydospores cacao brown, thick-walled, aseptate, obovoid, (7–)9–11(–15) × (4–)5–7(–8) μm. Aleurioconidia not observed.

*Type*
Holotype of *Huntiella chlamydoformis*: CAMEROON, Centre region, Ngomedzap (03°16′03·5″N, 11°14′50·4″ E), on wounded stem of *Theobroma cacao*, December 2009, coll. M. Mbenoun & J. Roux, PREM 60837 (PREM), culture ex-holotype CMW36932 = CBS 131674.

*Additional specimens examined*
CAMEROON, Centre region, Ngomedzap (03°16′03·5″ N, 11°14′50·4″E), on wounded stem of *Terminalia superba*, December 2009, coll. M. Mbenoun & J. Roux, dry specimen PREM 60838, living culture CMW37102 = CBS 131675; on wounded stem of *Theobroma cacao*,

December 2009, coll. M. Mbenoun & J. Roux, living culture, CMW37101; Centre Region, Bokito (04°30′16·7″N, 11°04′44·9″E), on wounded stem of *Theobroma cacao*, December 2009, coll. M. Mbenoun & J. Roux, living culture, CMW37106; South region, Nkoemvone, IRAD research station (02°49′10·5″N, 11°07′57·9″E), on wounded stem of *Theobroma cacao*, December 2009, coll. M. Mbenoun, dry specimen PREM 60836, living culture CMW37584 = CBS 131673; on wounded stem of *Theobroma cacao*, December 2009, coll. M. Mbenoun, living culture, CMW36899.

Notes: The most distinctive morphological characteristic of *H. chlamydoformis* is the production of chlamydospores in ageing cultures. In the literature treating *Huntiella*, these structures are mentioned only in the C.M.I. descriptions of *H. moniliformis* (Morgan-Jones *et al.*, 1967). *Huntiella chlamydoformis* can also be distinguished from closely related species by its relatively larger ascomata. The *tef1-α* was the only gene that could differentiate *H. chlamydoformis* and *H. ceramica*. The two species had identical sequences at the ITS and *β-tub* loci.

***Huntiella pycnanthi* Mbenoun & Jol. Roux sp. nov.**, Figure 4

MycoBank MB 807095

*Etymology*

The name refers to the morphological similarities between this species and the fungus treated by Luc (1952) as a morphological variant of *H. moniliformis*, and isolated from logs of *Pycnanthus angolensis* in Cameroon.

*Description*

Colonies on MEA fast growing, reaching 61–75 mm in diameter within 3 days, optimum temperature for growth 30°C, substantial growth at 35°C but no growth at 10°C. Mycelium fluffy and superficial, initially hyaline to white turning smoke grey (21’’’’d) with age. Hyphae smooth and granular, without constrictions at septa. Ascomata perithecial with bulbous bases and long necks, formed superficially on substrate, randomly distributed. Ascomatal bases light brown, globose to subglobose, (145–)182–270(–339) × (137–)187–259(–259) μm, ornamented with scattered conical spines. Ascomatal necks dark brown, erect or slightly curled, disciform structure connecting ascomatal bases and necks absent, (391–)529–817(–1045) μm long, (15–)17–23(–27) μm wide at apices and (33–)37–47(–58) μm wide at bases, ostiolar hyphae hyaline, divergent, (30–)39–51(–67) μm long. Asci not observed. Ascospores accumulating in creamy to yellow droplets at the tips of ascomatal necks, aseptate, cucullate in side view, (5–)6–7(–8) μm wide and 3–4 μm high. Conidiophores occurring mostly solitary and arising laterally from hyphae, hyaline, monophialidic (16–)23–35(–51) μm long, 2–3 μm wide at apices and 3–4(–5) μm wide at bases. Primary conidia hyaline, aseptate, cylindrical, (4–)5–7(–11) × 1–2 μm. Secondary conidia hyaline, aseptate, oblong to cylindrical, (4–)5–7(–9) × 2–4(–5) μm. Aleurioconidia and chlamydospores not observed.

*Type*

Holotype of *Huntiella pycnanthi*: CAMEROON, Centre Region, Bokito (04°30′16·7″N, 11°04′44·9″E), on wounded stem of *Theobroma cacao*, December 2009, coll. M. Mbenoun & J. Roux, PREM 60835 (PREM), culture ex-holotype CMW36916 = CBS 131672.

*Additional specimens examined*

CAMEROON, Centre Region, Bokito (04°30′16·7″N, 11°04′44·9″E), on wounded stem of *Theobroma cacao*, December 2009, coll. M. Mbenoun, living cultures, CMW36910, CMW36915; Ngomedzap (03°16′03·5″N, 11°14′50·4″E), December 2009, on wounded stem of *Theobroma cacao*, coll. M. Mbenoun & J. Roux, living cultures, CMW36921, CMW36933; South Region: Nkoemvone, IRAD research station (02°49′10·5″N, 11°07′57·9″E), on wounded stem of *Theobroma cacao*, December 2009, coll. M. Mbenoun, dry specimen PREM 60834, living culture CMW36901 = CBS 131671.

Notes: Luc (1952) identified four morphological variants of *Huntiella moniliformis* (then treated as *Ophiostoma moniliforme*) that he named the ‘Theobromae’, ‘Pycnanthi’ and ‘Davidsonii’ forms as distinct from the original ‘Typica’ form. The description made of the Pycnanthi form matches closely with the characteristics of *H. pycnanthi*. The two fungi might represent the same species, occurring within the same geographic range. *Huntiella pycnanthi* differs from its closest relatives by the structure of its ascomata, which are characterized by lighter bases and the absence of the disciform collar structure connecting the ascomatal bases and necks. *Huntiella pycnanthi* is phylogenetically best-circumscribed by the *β-tub* gene while the ITS shows no difference with *H. cryptoformis*, *H. oblonga* and *H. savannae*.

## Diversity and incidence of the Ceratocystidaceae in cacao fields

The diversity of Ceratocystidaceae recovered from cacao agroforests in Cameroon (Table 2) encompassed five species in two genera, *Huntiella* (*H. chlamydoformis*, *H. moniliformis* and *H. pycnanthi*) and *Thielaviopsis* (*T. cerberus* and *T. ethacetica*). Of the three sites investigated in this study, Bokito had the greatest species richness, including all five species compared to four and three for Ngomedzap and Nkoemvone, respectively. *Thielaviopsis cerberus* was recovered only at Bokito and *T. ethacetica* was not found at Nkoemvone. *Huntiella* species were predominant on tree wounds, whereas *T. ethacetica* was most abundant on cacao pod husks and scarce on wounded trees.

The occurrence of Ceratocystidaceae on cacao stem wounds ranged from 50% at Nkoemvone, to 60% at Bokito and 80% at Ngomedzap. Variation was also observed among sites regarding the incidence of the five species recovered on cacao stem wounds (Fig. 5). *Huntiella pycnanthi* and *H. moniliformis* were the most and least prevalent, respectively, at Bokito, contrasting with Ngomedzap and Nkoemvone where *H. moniliformis* was

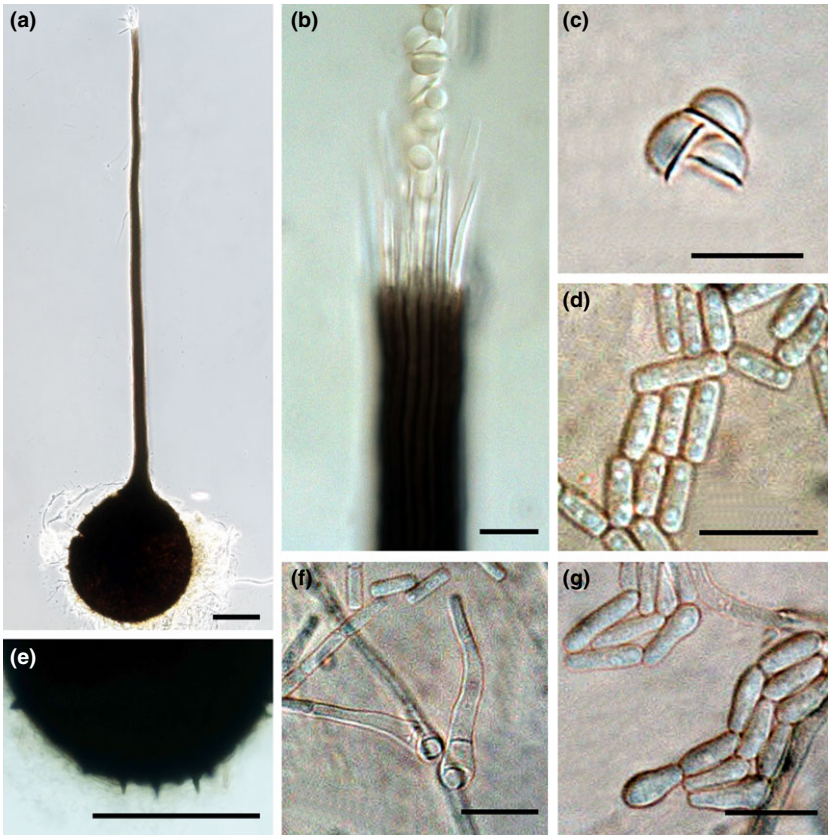


Figure 4 Morphological characteristics of *Huntiella pycnanthi* sp. nov. (a) ascomata with globose base and extended neck; (b) tip of ascomatal neck with divergent ostiolar hyphae extruding ascospores in string; (c) ascospores in basal and side (hat-shaped) views; (d) bacilliform primary conidia; (e) conical spines on the surface of ascocarp base; (f) monophialidic conidiophores; (g) variform secondary conidia. Scale bars: a, e = 100 μm; b, c, d, f, g = 10 μm.

dominant and *H. pycnanthi* rare. The only species that occurred widely across sites was *H. chlamydoformis*. Each of the *Thielaviopsis* species was found on cacao stem wounds only at one site, *T. cerberus* from two trees at Bokito and *T. ethacetica* from one tree at Ngomedzap.

### Identification of nitidulid beetles

Nitidulid beetles morphologically resembling *Brachypeplus* species (Fig. 1c) were the most common insects associated with species of Ceratocystidaceae in cacao agroforests. These insects were collected from artificially induced stem wounds and other naturally occurring wounds on trees, as well as cacao pod husks. DNA sequence variation of the *MT-CO1* gene (Fig. S3) showed that the collected insects represented at least three species of *Brachypeplus* (GenBank accessions: KF769077, KF769078, KF769079, KF769080, KF769081, KF769082). Other less commonly occurring nitidulid beetles found in cacao agroforests included *Carpophilus* species (GenBank accessions: KF769083, KF769084) from wind-oriented traps and wood cracks on *E. ivorense*, and one unidentified morphotype (GenBank accessions: KF769085, KF769086) under bark flaps on *Cassia* trees. DNA sequences of closely related nitidulid species were not found in GenBank and the insects collected in Cameroon could not be further identified.

### Pathogenicity tests

Two isolates for each identified fungal species were evaluated for pathogenicity on cacao. These included CMW35021 and CMW35024 (from cacao stem wounds) for *T. cerberus*, CMW35018 (from cacao pod husk) and CMW35028 (from cacao stem wound) for *T. ethacetica*, CMW36899 (from cacao stem wound) and CMW37104 (from *Terminalia* stem wound) for *H. chlamydoformis*, CMW36922 and CMW36895 (from cacao stem wounds) for *H. moniliformis*, as well as CMW36917 (from cacao stem wound) and CMW36933 (from cacao pod husk) for *H. pycnanthi*.

#### *Branch inoculations*

The Barlett's chi-squared test showed no significant difference in CLL variance between the two replicated trials ($\chi^2 = 0{\cdot}34$; df = 1; $P = 0{\cdot}56$). Hence, all CLL data could be pooled for analysis. In contrast, the null hypothesis of

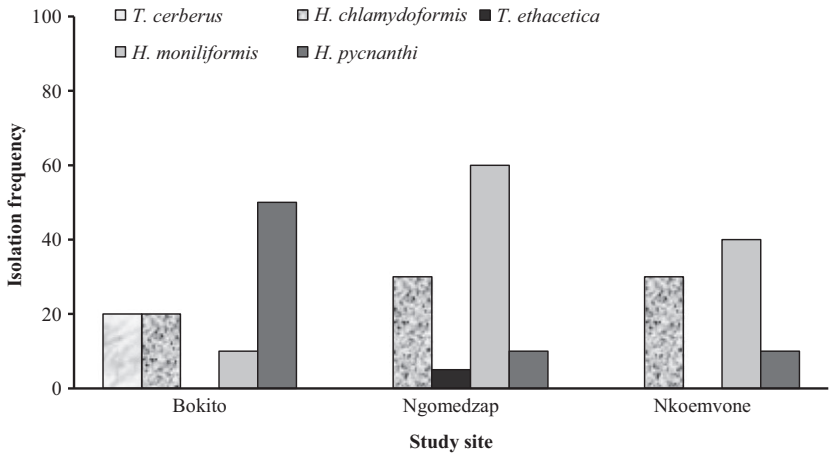


Figure 5 Incidence of Ceratocystidaceae species (*Thielaviopsis* and *Huntiella*) in cacao agroforests across study sites based on isolation frequencies on artificially induced wounds on cacao stems.

homogeneous variance between trials was rejected ($\chi^2 = 13{\cdot}32$; df = 1; $P = 0{\cdot}0026$) when the test was applied to BLL. Therefore, BLL data for the replicated trials were analysed separately.

All isolates used in inoculation trials induced distinct lesions in the cambium and sapwood of lignified cacao branches. Results of ANOVA of CLL data ($F = 18{\cdot}37$; df = 10; $P < 2e{-}16$) and Tukey's HSD test (Table 3) showed that there were significant differences in virulence between and among fungal species recovered from cacao agroforests. All isolates induced significantly longer lesions than the sterile agar used for the controls (Table 3). Overall, isolate CMW35028 (*T. ethacetica*), from artificially induced stem wounds on cacao, was the most aggressive, followed by isolate CMW36933 (*H. pycnanthi*) from a cacao pod husk (Table 3).

The lesions measured in the bark were generally shorter than those measured in the sapwood (Table 3). ANOVA showed significant variation in the BLL data for both trial 1 ($F = 56{\cdot}02$; df = 10; $P < 2e{-}16$) and trial 2 ($F = 43{\cdot}94$; df = 10; $P < 2e{-}16$). All isolates produced significantly longer lesions compared to the control, and isolates CMW35028 (*T. ethacetica*) and CMW36933 (*H. pycnanthi*) were consistently the most aggressive (Table 3). Reisolations from the lesions on the inoculated branches resulted in the recovery of inoculated fungi in some cases, but it was generally hampered by other faster growing fungi, especially those related to the Botryosphaeriaceae. However, the inoculated fungi were never isolated associated with the control inoculations.

*Fruit inoculations*

Most of the isolates inoculated onto mature cacao pods did not result in lesions. Only isolate CMW35028 (*T. ethacetica*) caused rotting of inoculated pods (Fig. 1d). This fungus was consistently reisolated from the margins of rotting fruit tissues.

## Discussion

Results of this study to consider the diversity of the Ceratocystidaceae in cacao agroforests revealed that at least five species of these fungi are commonly found in this ecosystem in Cameroon. These included two species of *Thielaviopsis* (*T. cerberus* and *T. ethacetica*) and three species of *Huntiella* (*H. chlamydoformis*, *H. moniliformis* and *H. pycnanthi*), two of which were described as new. No species related to the genus *Ceratocystis*, well known to accommodate destructive tree pathogens such as *C. cacaofunesta* that causes ceratocystis wilt of cacao in Latin America (Engelbrecht *et al.*, 2007), were found. Inoculation experiments revealed variable levels of pathogenicity among the recovered fungal species, with *T. ethacetica* and *H. pycnanthi* emerging as potential cacao pathogens.

This study represents the first extensive consideration of species of Ceratocystidaceae on cacao in Cameroon, and more broadly in Africa. Previous reports of these fungi on cacao in this region include a study by Dade (1928), who first described the sexual state of *T. paradoxa* on cacao pod husks in Ghana. Luc (1952), subsequently, described a 'Theobromae' form of *H. moniliformis* on cacao roots in Madagascar. In the same publication, he also described a 'Pycnanthi' form of the same fungus on *P. angolensis* in Cameroon. The application of DNA-based tools has now made it possible to recognize a much richer diversity of the Ceratocystidaceae in Africa (Roux & Wingfield, 2013) and elsewhere (de Beer *et al.*, 2014 and references therein). However, there is little information regarding the impact of these fungi on cacao health in Africa, other than the inclusion of *T. paradoxa* in the list of pathogenic fungi affecting cacao in Ghana (http://ghana.ipm-info.org/list_diseases.htm).

Among the five fungal species recovered from cacao agroforests in this study, *H. pycnanthi* and *T. ethacetica*

Table 3 Average lesion lengths on bark (BLL) and cambium (CLL) caused by species of Ceratocystidaceae on cacao branches 6 weeks post-inoculation

| Fungal species | Isolate | CLL | BLL1[a] | BLL2[b] |
|---|---|---|---|---|
| *Thielaviopsis cerberus* | CMW 35021 | 50·17 ± 22·85 d[c] | 11·00 ± 2·35 cd | 10·11 ± 0·60 cd |
| | CMW35024 | 49·63 ± 25·85 d | 11·00 ± 1·41 cd | 9·78 ± 0·97 cd |
| *Thielaviopsis ethacetica* | CMW 35018 | 60·05 ± 34·16 cd | 10·30 ± 0·82 d | 10·22 ± 0·83 cd |
| | CMW 35028 | 137·42 ± 46·65 a | 29·40 ± 3·84 a | 22·78 ± 3·53 a |
| *Huntiella chlamydoformis* | CMW 36899 | 68·53 ± 42·68 cd | 11·20 ± 1·75 cd | 9·67 ± 0·50 cd |
| | CMW 37104 | 58·42 ± 22·15 cd | 13·00 ± 4·70 c | 11·00 ± 1·41 cd |
| *Huntiella moniliformis* | CMW 36922 | 58·74 ± 32·58 cd | 10·70 ± 1·34 d | 11·56 ± 2·55 c |
| | CMW 36895 | 49·53 ± 20·87 d | 11·40 ± 1·90 cd | 11·56 ± 1·24 c |
| *Huntiella pycnanthi* | CMW 36917 | 75·67 ± 34·09 c | 11·40 ± 1·17 cd | 10·67 ± 0·71 cd |
| | CMW 36933 | 106·37 ± 35·82 b | 15·90 ± 2·18 b | 14·56 ± 2·79 b |
| Control | | 23·00 ± 12·97 e | 10·00 ± 1·49 d | 9·56 ± 0·73 d |

[a]BBL1: average bark lesion length for the first trial.

[b]BBL2: average bark lesion length for the second trial.

[c]Values followed by the same letters are not significantly different based on Tukey's HSD multiple range test.

showed substantial ability to cause lesions on cacao. These species both induced extensive discolouration and streaking of the vascular tissue on lignified cacao branches, similar to those developing from induced wounds on cacao stems in the field. In addition, *T. ethacetica* was shown to cause fruit rot. Although *H. moniliformis* has previously been reported killing cacao trees in Costa Rica (Cristobal & Hansen, 1962), the isolates used in inoculation tests in this study, as well as those representing *H. chlamydoformis* sp. nov., showed little pathogenicity on cacao. This is consistent with most prior knowledge of species in the genus *Huntiella* (previously treated in the *Ceratocystis moniliformis* complex), which are generally considered not to be serious pathogens (e.g. Tarigan *et al.*, 2010; Kamgan Nkuekam *et al.*, 2012).

Differences observed in the incidence of the Ceratocystidaceae between plantations could be linked to variation in prevailing agroecological conditions and management practices, including local average temperatures, humidity and shade levels in cacao fields. In this regard, relatively smaller lesions developed from cacao stem wounds in Bokito, which has a hotter and drier climate. These conditions, coupled with the limited shade on the farm, could have caused the wounds to dry rapidly, impeding further development and colonization by the fungi of the exposed wood tissues. *Huntiella pycnanthi* was most commonly isolated from cacao tree wounds in Bokito. This was consistent with the fact that this fungus grows best at higher temperatures, whilst *H. moniliformis* was more prevalent in Ngomedzap and Nkoemvone where cooler conditions prevail.

Besides controlling the shade levels, other management practices that could influence the incidence of the Ceratocystidaceae in cacao fields include the identity of plants associated with cacao and the handling of pod husks after harvesting beans. For example, *T. cerberus*, a fungus predominantly known from oil palm (Mbenoun *et al.*, 2014) was found only in Bokito, where this crop occurred alongside cacao. In contrast, most of the isolates of *T. ethacetica* were collected from abandoned pod husk piles and only two isolates, from the same tree in Ngomedzap, came from cacao stem wounds. Interestingly, this fungus was not recovered at Nkoemvone where pod husks did not occur. This suggests that abandoned pod husk piles contribute to increase the inoculum of *T. ethacetica* in cacao plantations, as was shown for *Phytophthora* spp. causing BPD (Bowers *et al.*, 2001; Guest, 2006). Moreover, it was found that pod husk piles also provide suitable breeding conditions for nitidulid beetles that have been shown to transmit *Phytophthora palmivora* in cacao fields (Evans, 1973; Konam & Guest, 2004). These insects are also well known for their important role in the dispersal of tree pathogens related to the Ceratocystidaceae (e.g. Hayslett *et al.*, 2008; Heath *et al.*, 2009).

*Brachypeplus* species were the most common nitidulid beetles found in association with species of Ceratocystidaceae in cacao agroforests, but other nitidulids such as *Carpophilus* species also occurred in these ecosystems. *Brachypeplus* species have previously been reported in association with members of the Ceratocystidaceae (Heath *et al.*, 2009; Kamgan Nkuekam *et al.*, 2012). In particular, Heath *et al.* (2009) showed that *Brachypeplus depressus* is one of the principal overland vectors of the wattle wilt pathogen, *Ceratocystis albifundus*, in South Africa. In the present study, it emerged based on molecular data that the same *Brachypeplus* species visit both cacao stem wounds and abandoned pod husk piles in Cameroon. This highlights the possible role that these insects could play in the transmission of diseases between these substrates.

Although evidence from this study suggests that some species of Ceratocystidaceae occurring in cacao agroforests in Cameroon have the potential to cause disease on cacao, these fungi are not recognized among the pathogens affecting this tree crop in the country. The real impact of the Ceratocystidaceae on cacao health and productivity therefore needs to be further investigated.

This could, for instance, involve assessing whether *T. ethacetica* is associated with significant additional fruit losses other than that caused by *P. megakarya*. Species of Ceratocystidaceae could also be investigated in connection with tree mortality, especially as it occurs often following agrisilvicultural activities involving extensive tree wounding such as pruning, thinning and coppicing. In the interim, this study has shown that applying the well-known good management practices for pests and diseases of cacao (http://www.dropdata.org/cocoa/icm_bkp.htm), including the maintenance of adequate levels of shade in plantations, an informed selection of intercropping systems and a proper handling of discarded pod husks can help reduce the incidence of the Ceratocystidaceae. This would reduce opportunities for new diseases caused by these fungi to emerge in cacao agrosystems.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

**Figure S1.** Map of Cameroon showing the Centre-South cacao growing region (shaded) and the sites involved in this study. The map was adapted from Efombagn *et al.* (2008).

**Figure S2.** Phylogenetic relationships between the new *Huntiella* species discovered in cacao agroforests in Cameroon and their closest known relatives based on single gene loci. The number of corresponding nucleotide substitutions is indicated above tree branches.

**Figure S3.** Neighbour joining phylogenetic tree of nitidulid beetles found in cacao agroforests in Cameroon based on *MT-CO1* gene sequences. GenBank accession numbers of sequences are provided in parentheses.