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

M. Mbenoun[a], M. J. Wingfield[a], A. D. Begoude Boyogueno[a, c], F. Nsouga Amougou[c], S. Petchayo Tigang[c], G. M. ten Hoopen[c, d], C. V. Mfegue[c], L. Dibog[c], S. Nyassé[c], B. D. Wingfield[b], J. Roux[a*]

[a]Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Private bag X20, Pretoria 0028, South Africa

[b]Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Private bag X20, Pretoria 0028, South Africa

[c]Institut de Recherche Agricole pour le Développement (IRAD), B.P. 2067 Yaounde, Cameroon

[d]Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), UR106 Bioagresseurs: analyse et maîtrise de risque, B.P. 2572 Yaounde, Cameroon

[*]E-mail: jolanda.roux@fabi.up.ac.za

Abstract

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. In this study, we investigated the occurrence of fungi related to the Ceratocystisdaceae 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 *Th. 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 *Th. ethacetica* and *H. pycnanthi* produced extensive lesions after inoculation on branches of mature cacao trees, while *Th. ethacetica* also caused pod rot. Although their impact remains unknown, fungi in the Ceratocystidaceae and their nitidulid beetle vectors are common and likely contribute to the parasitic pressure in Cameroonian cacao agrosystems.

Keywords: Crop protection, *Ceratocystis*, emerging diseases, fungal pathogens, Nitidulid beetles

Introduction

Cacao (*Theobroma cacao* L.) 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 to 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*, and especially *P. megakarya* Brasier & M.J. Griffin, in Africa; frosty pod rot caused by *Moniliophthora roreri* H.C. Evans, Stalpers, Samson & Benny as well as witches' broom caused by *M. perniciosa* (Stahel) Aime & Phillips-Mora in Latin America, are spreading to new areas (Evans, 2007).

Cameroon produces approximately 220 000 metric tons 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 1800's. 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 1970's (Sonwa *et al.*, 2005; Mfegue, 2012) and, more recently, an upsurge in tree mortality associated with infections by *Lasiodiplodia theobromae* (Pat.) Criffon & Maubl. (Mbenoun *et al.*, 2008). While it might not be possible to predict the nature of a 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 (Garett *et al.*, 2006).

This study study considered fungi belonging to the recently revised ascomycete family Ceratocystidaceae (de Beer *et al.*, 2014). This diverse and widely distributed group of fungi (Wingfield *et al.*, 1993; 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., Moller and Devay, 1968b; 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* C. J. B. Engelbr. & T. C. Harr., 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. Al Adawi *et al.*, 2006; 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 (iv) 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 (Supplementary Material – Figure 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 (4°30′16.7″N, 11°4′44.9″E) was located in Bokito, a relatively dry forest-savanna transition zone at the north-west limit of the region. The farm was established in the early 1990's 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* (L.) Gaertn. Two stands (3°16′6.4″N, 11°14′29.9″E and 3°16′3.5″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* Engl. & Diels. Some banana and fruit trees were also present in these stands. The fourth stand (2°49′10.5″N, 11°7′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 1970's on land completely cleared of primary forest, using scattered, non-native *Cassia spectabilis* (DC.) Irwin & Barn. 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, *Terminalia superba* at Ngomedzap and *Cassia spectabilis* at Nkoemvone) were selected at each site and marked. Wounds (one on cacao and two on shade trees) were made on the stems of the trees following the technique described by Barnes *et al*. (2003). Wounds were established ~1.5 m above ground. Approximately 10 ×10 cm of bark was cut from the stems using masonry chisels to expose the cambium and a ~5 × 5 mm slit was made into the sapwood at the centre of the cleared cambium to expose the vascular tissue (Figure 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 two 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 discoloration 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 discoloration, 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, Norway) and covered with "artificial bark" (Lac Balsam, Germany).

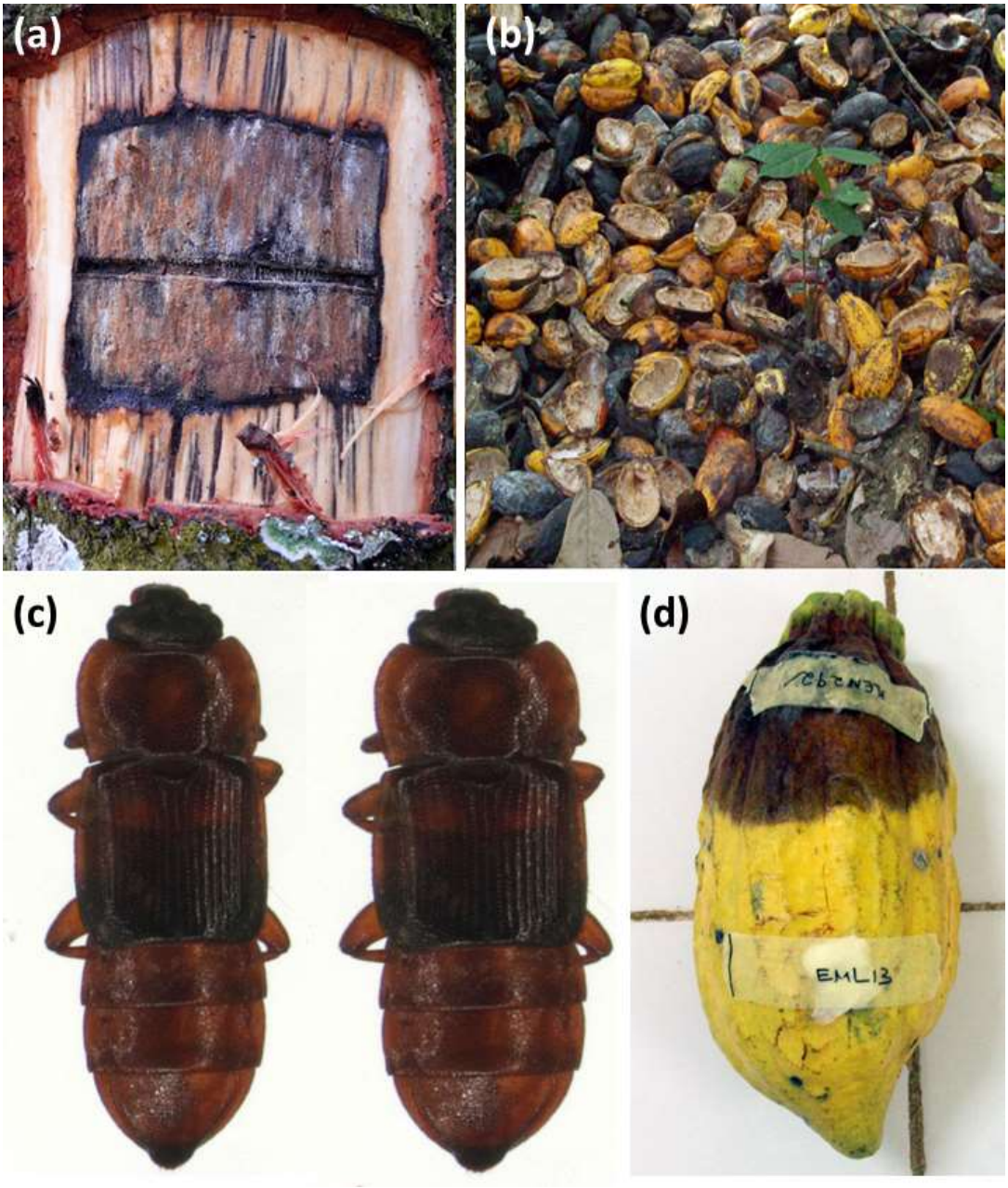

Figure 1 Substrates from which fungi were collected in cacao agroforests in Cameroon. (**a**) induced fresh wound (~10 × 10 cm) with a slit incised in the sapwood of a cacao tree after removal of the bark; (**b)** cacao pod husk heap; **(c**) *Brachypeplus* nitidulid beetles obtained from cacao stem 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.

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 (Figure 1d) 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* L. and *Erythrophleum ivorense* A. Chev. (Tali), 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, Vastaburg, Michigan) were set up for ~12 hr 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 fungal structures, and baiting for *Ceratocystis* species using carrot slices (Moller & De Vay, 1968a). 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 pre-treated with streptomycin sulphate (Sigma, Steinheim, Germany). 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

slices, were transferred to 2% malt extract agar (MEA; Biolab, Midrand, South Africa), supplemented with ~0.01 g/L streptomycin sulphate (Sigma, Steinheim, Germany). Isolates were purified by sub-culturing 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 analyses.

Molecular characterization and phylogenetic analyses of fungal isolates

Fungal DNA extraction was conducted following the procedure described by Mbenoun *et al.* (2014a). 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 version 5 (Tamura *et al*., 2011) 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 17 isolates (Table 1) for which the internal transcribed spacer regions including the 5.8S rDNA of the ribosomal RNA cluster (ITS) and a portion of the translation elongation factor 1 alpha gene (*tef*1-α) were also sequenced. The protocols and procedures used for PCR and sequencing were the same as those described by Mbenoun *et al.* (2014a).

Three sequence datasets representing the ITS, *β-tub* and *tef*1-α loci were prepared for phylogenetic analyses. These included sequences for the 17 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* (Yuan & Mohammed) Z.W. de Beer, T.A. Duong & M.J. Wingf. were used to represent an outgroup

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

Species	Isolate no		Gene region / GeneBank accession no			Host	Geographic origin	Collector
			ITS	*β-tub*	*tef*1-α			
H. chlamydoformis	CMW36899					*Theobroma cacao*	Cameroun	M Mbenoun & J Roux
"	CMW36932	CBS 131674	KF769087	KF769109	KF769098	"	"	"
"	CMW37101					"	"	"
"	CMW37102	CBS 131675	KF769088	KF769110	KF769099	*Terminalia superba*	"	"
"	CMW37106	CBS 131673	KF769089	KF769111	KF769100	*T. cacao*	"	"
"	CMW37584		KF769090	KF769112	KF769101	"	Cameroun	"
H. cryptoformis	CMW36827		KC691463	KC691487	KC691511	*Combretum zeyheri*	South Africa	"
"	CMW36828	CBS 131279	KC691464	KC691488	KC691512	*Ziziphus mucronata*	"	"
H. decipiens	CMW25914	CBS 129737	HQ203219	HQ203236	HQ236438	*Eucalyptus maculata*	"	GN Kamgan & J Roux
"	CMW25918	CBS 129735	HQ203218	HQ203235	HQ236437	*E. cloeziana*	"	"
H. moniliformis	CMW4114	CBS 118151	AY528997	AY528986	AY529007	*Shizolobium parahyba*	Ecuador	MJ Wingfield
"	CMW9590	CBS 116452	AY431101	AY528985	AY529006	*E. grandis*	South Africa	J Roux
"	CMW36895	CBS 137247	KF769091	KF769113	KF769102	*T. cacao*	Cameroun	M Mbenoun & J Roux
"	CMW36908	CBS 137248	KF769092	KF769114	KF769103	"	"	"
"	CMW36919		KF769093	KF769115	KF769104	"	"	"
"	CMW37105					"	"	"
H. moniliformopsis	CMW9986	CBS 109441	AY528998	AY528987	AY529008	*E. obliqua*	Australia	ZQ Yuan
"	CMW10214	CBS 115792	AY528999	AY528988	AY529009	*E. sieberi*	"	MJ Dudzinski
H. pycnanthi	CMW36901	CBS 131671	KF769094	KF769116	KF769105	*T. cacao*	Cameroun	M Mbenoun & J Roux

"	CMW36910		KF769095	KF769117	KF769106	"	"	"
"	CMW36915					"	"	"
"	CMW36916	CBS 131672	KF769096	KF769118	KF769107	"	"	"
"	CMW36921		KF769097	KF769119	KF769108	"	"	"
"	CMW36933					"	"	"
H. oblonga	CMW23802		EU245020	EU244992	EU244952	*Acacia mearnsii*	South Africa	RN Heath
"	CMW23803	CBS 122291	EU245019	EU244991	EU244951	"	"	"
H. salinaria	CMW25911	CBS 129733	HQ203213	HQ203230	HQ236432	*E. maculata*	"	GN Kamgan & J Roux
"	CMW30703	CBS 129734	HQ203214	HQ203231	HQ236433	*E. saligna*	"	"
H. savannae	CMW173298		EF408553	EF408567	EF408573	*Ter. sericea*	"	"
"	CMW17300	CBS 121151	EF408551	EF408565	EF408572	*A. nigrescens*	"	"
H. sublaevis	CMW22444	CBS 122518	FJ151430	FJ151464	FJ151486	*Ter. ivorensis*	Ecuador	MJ Wingfield
"	CMW22449	CBS 122517	FJ151431	FJ151465	FJ151487	"	"	"
H. tribiliformis	CMW13011	CBS 115867	AY528991	AY529001	AY529012	*Pinus merkusii*	Indonesia	"
"	CMW13012	CBS 118242	AY528992	AY529002	AY529013	"	"	"
H. tyalla	CMW28917		HM071899	HM071909	HQ236448	*E. grandis*	Australia	GN Kamgan
"	CMW28920		HM071896	HM071910	HQ236449	"	"	"
H. ceramica	CMW15245	CBS 122299	EU245022	EU244994	EU244926	"	Malawi	RN Heath & J Roux
"	CMW15248	CBS 122300	EU245024	EU244996	EU244928	"	"	"

taxon. For all three datasets, alignments were generated using MAFFT (http://www.align.bmr.kyushu-u.ac.jp/mafft/online/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 performed using PAUP version 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 rescaled consistency (RC) indexes of the MPTs were calculated. ML analyses were performed online, using PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/). In both ML and MP reconstructions statistical support for branch nodes was determined by performing 1000 bootstrap analyses 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 putatively 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 (eight 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 three 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 wk old cultures of the isolates utilized 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 a Zeiss Axioskop microscope (Carl Zeiss Ltd, Germany), fitted with a HRc Axiocam digital camera and Axiovision 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 (FABI), University of Pretoria. In addition, for selected specimens representing each nitidulid morphotype from Cameroon, DNA was extracted using the prepGEM^{TM} Insect (zyGEM) DNA extraction kit, following the manufacturer's instructions. Subsequently, the mitochondrial cytochrome oxydase I (MT-CO1) gene region was amplified and sequenced using the universal primer combination Cl-J-2183: 5'-CAACATTTATTTTGATTTTTTGG-3'/TL2-N-3014: 5'-CCAATGCACTAATCTGCCATATTA-3' (Simon *et al*., 1994). PCR reactions were prepared in a 25 µL final volume, with MyTaq^{TM} (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 at 72 °C for 10 min. All other steps involved in the sequencing procedure were the same as those described by Mbenoun *et al.* (2014a). 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 detached cacao fruits in the laboratory. Each species was represented by two isolates. Cultures used to produce inoculum were maintained on MEA for 10 d 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 six 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, re-isolations were done by plating small pieces of symptomatic tissue, 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 TukeyHSD 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 d. Where rotting had developed, re-isolation of the inoculated fungus was attempted by transferring a piece of symptomatic fruit cortex 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 (Figure 1a–c) were typically characterized by a dark brown discoloration 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 pealed 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 discoloration developed from the slits, deeper into the sapwood. Cacao stem wounds were generally drier in Bokito, showing less severe internal discoloration as compared with those at the other two sites.

Limited internal discoloration was observed on *Terminalia* shade trees wounded at Ngomedzap. In contrast, *Cassia* and *Ceiba* trees at Nkoemvone and Bokito respectively, showed no discoloration in the sapwood, although some mycelial growth and a few ascomata were observed on the exteriors of the wounds on *Ceiba* trees. The exposed cambium on *Cassia* trees was superficially covered with a black stain (Supplementary material – Figure S2), which could have prevented wound infection of on this host. Ascomata were observed on naturally damaged *Ter. 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 (Figure 1d) and samples were collected from them at Bokito and Ngomedzap. The endosperms of fresh pod husks were generally covered with a diverse fungal flora (Figure 1e), including species of Ceratocystidaceae and other ophiostomatoid fungi (Wingfield *et al.* 1993; Seifert *et al.* 2013).

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, 22 from Bokito and 10 from

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

Substrate	Study site	*Huntiella*			*Thielaviopsis*	
		H. chlamydoformis	***H. moniliformis***	***H. pycnanthi***	***Th. cerberus***	***Th. 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

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 *Ter. mantaly* trees alongside cacao plantations. In addition, two isolates were collected from two 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 analyses 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* Z.W. de Beer, T.A. Duong & M.J. Wingf. and *Thielaviopsis* Went, recently defined by de Beer *et al.* (2014). Isolates related to *Thielaviopsis* included *Th. cerberus* (Mbenoun, M.J. Wingf. & Jol. Roux) Z.W. de Beer, T.A. Duong & M.J. Wingf. and *Th. ethacetica* Went, previously identified by Mbenoun *et al.* (2014a), using a combination of comparative morphology, mating studies and multigene phylogenies. 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 analyses 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 (Figure 2), with strong statistical support (83–92%). Clade 1 included isolates representing *H. moniliformis* (Hedgc.) Z.W. de Beer, T.A. Duong & M.J. Wingf., while the other two clades were distinct from those delineated by known species used as references in the analyses. The two unnamed new clades were related to the *Huntiella* African lineage

(Mbenoun *et al.*, 2014b). Clade 2 had a basal position in the lineage and was most closely related to *H. decipiens* (Kamgan & Jol. Roux) Z.W. de Beer, T.A. Duong & M.J. Wingf., *H. salinaria* (Kamgan & Jol. Roux) Z.W. de Beer, T.A. Duong & M.J. Wingf. and *H. ceramica* (R.N. Heath & Jol. Roux) Z.W. de Beer, T.A. Duong & M.J. Wingf., while Clade 3 was monophyletic with *H.*

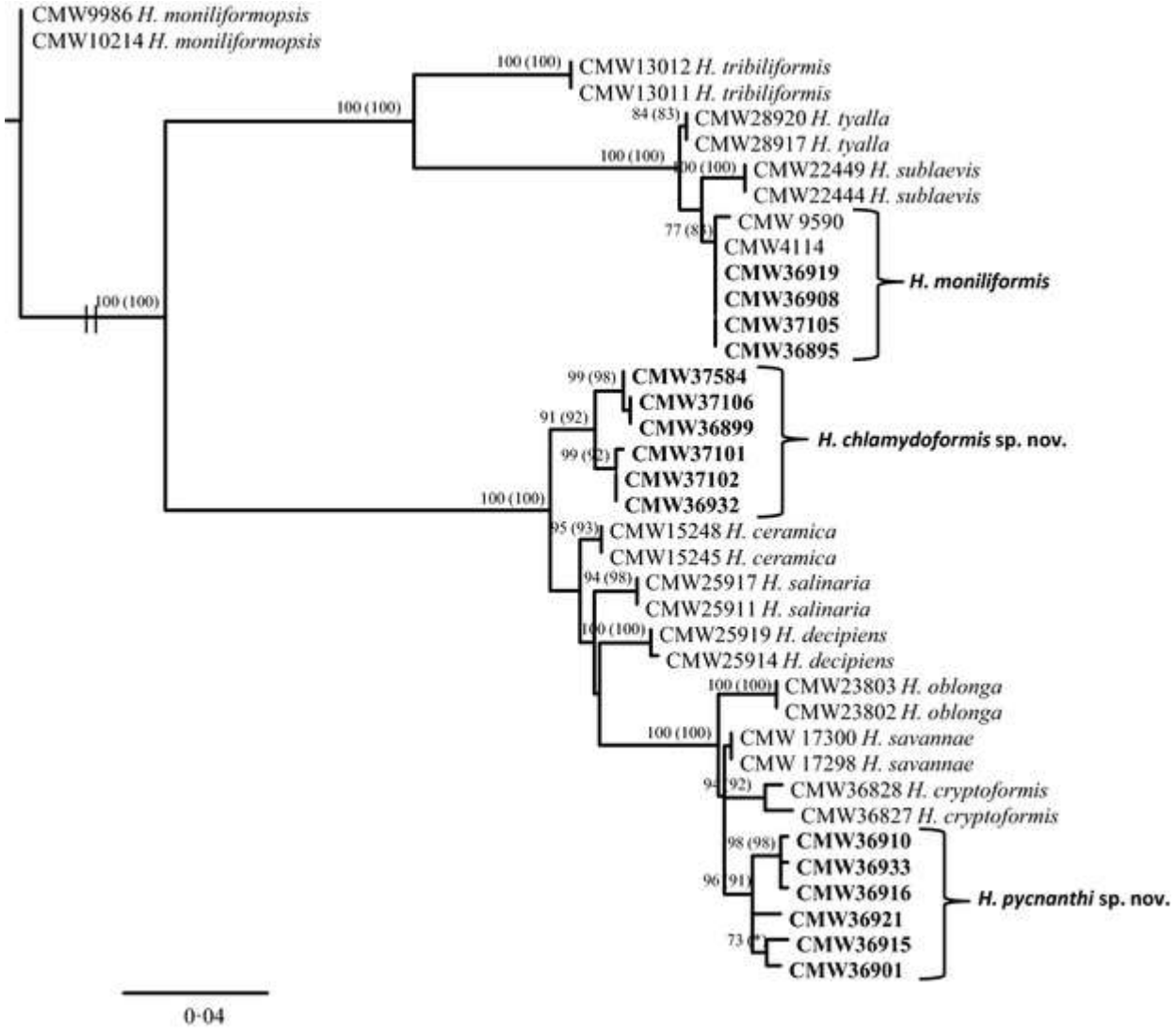

Figure 2 Phylogenetic tree derived from maximum likelihood analysis of combined ITS, *β-tub* and *tef*1-α gene sequences of *Huntiella* isolates from cacao agroforests in Cameroon together with sequences of selected reference species. Bootstrap support values ≥ 70 % are indicated next to branch nodes, including in brackets those from maximum parsimony analysis.

savannae (Kamgan & Jol. Roux) Z.W. de Beer, T.A. Duong & M.J. Wingf., *H. oblonga* (R.N. Heath & Jol. Roux) Z.W. de Beer, T.A. Duong & M.J. Wingf. and *H. cryptoformis* (Mbenoun & Jol. Roux) Z.W. de Beer, T.A. Duong & M.J. Wingf. The new clades showed substantial polymorphism (Figure 2), each including two statistically well-supported sub-clades. The phylogeny resulting from ML analyses was concordant with that from MP analyses in all respects (tree topology, relationship among taxa and statistical support) and generally produced stronger bootstrap values for branch nodes (Figure 2).

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 *tef*1-α genes could either discriminate only Clades 3 or Clade 2 respectively, showing from four to 14 fixed nucleotide changes (Supplementary material – Figure S3). 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 sub-clades 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 d 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 reminisecent 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 other *Huntiella* species, aging 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* (Mbenoun *et al.*, 2014a; de Beer *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 d 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

Etymology

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

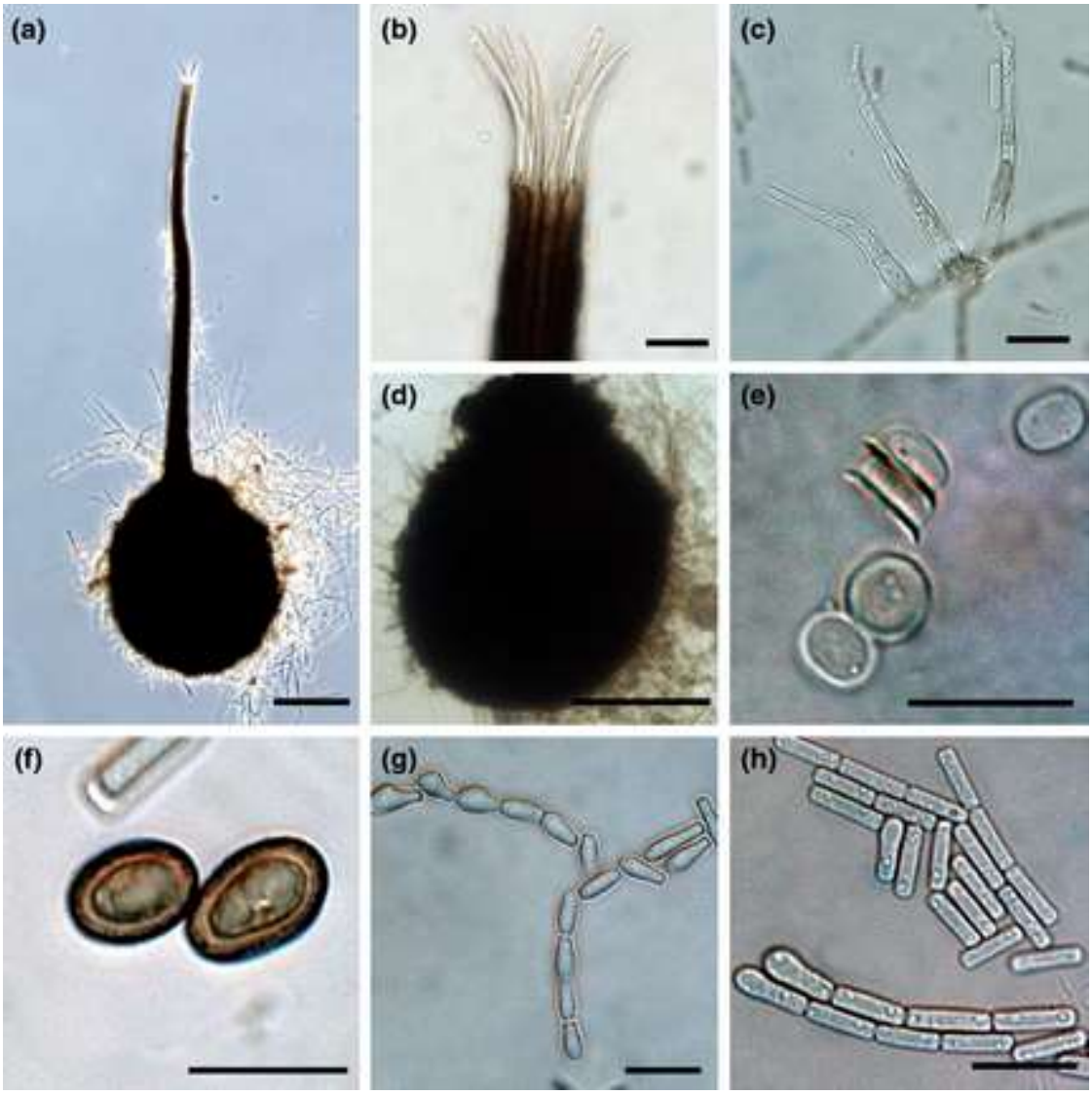

Figure 3 Morphological characteristics of *Huntiella chlamydoformis* sp. nov. (**a**) ascomata with globose base and extended neck; (**b**) tip of ascomatal neck showing divergent ostiolar hyphae; (**c**) monophialidic conidiophores; (**d**). ascocarp base showing bulbous collar structure at neck base; (**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.

Description

Colonies on MEA fast growing, reaching 50–61 mm in diameter within 3 d, 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, cucullate 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 (3°16′3.5″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 (3°16′3.5″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 (4°30′16.7″N, 11°4′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 (2°49′10.5″N, 11°7′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 aging 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). *H. chlamydoformis* can also be distinguished from closely related species by its relatively larger ascomata. The *tef*1-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* (Welw.) Warb in Cameroon.

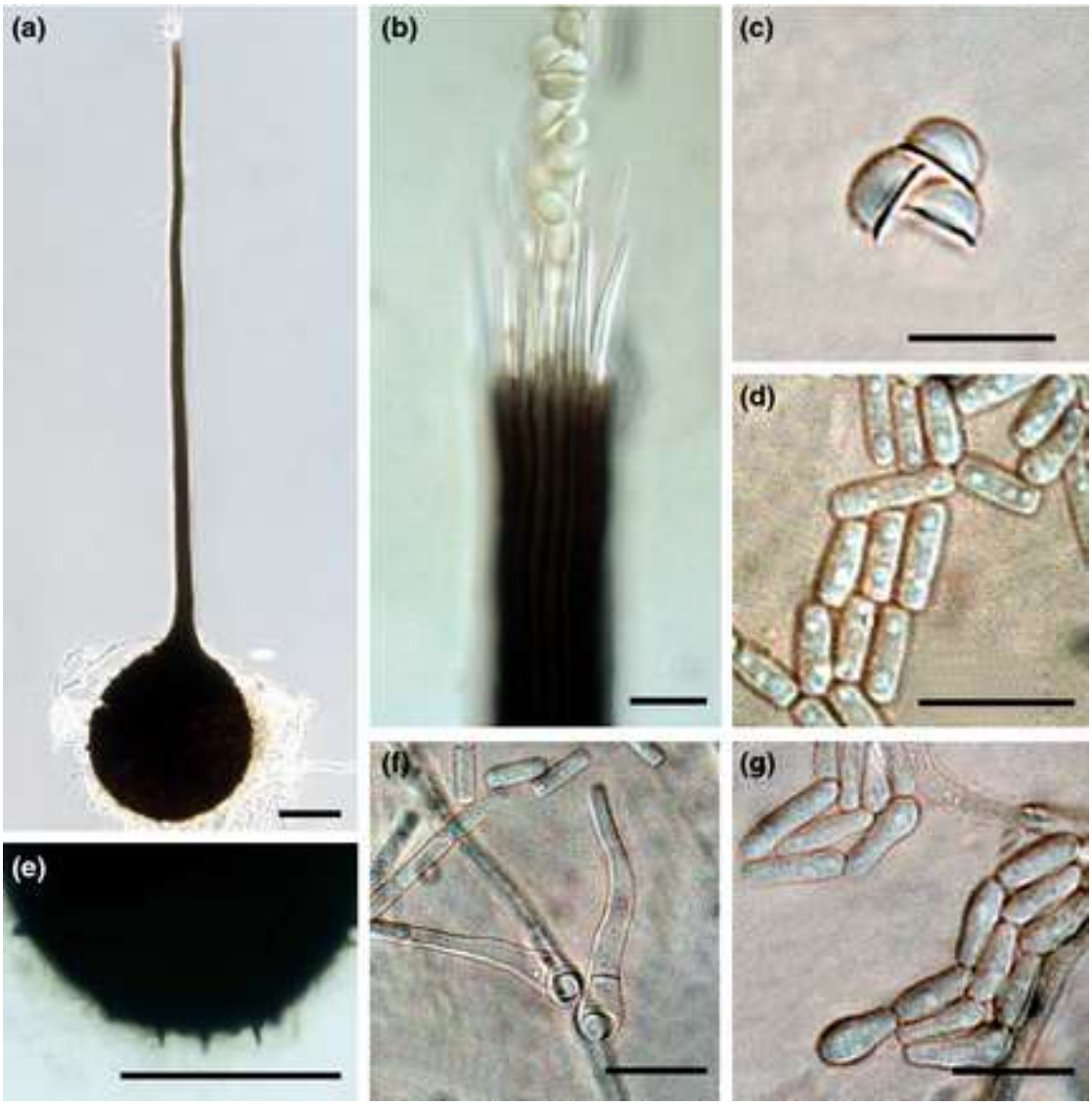

Figure 4 Morphological characteristics of *Huntiella pycnanthi* sp. nov. (**a**) ascomata with globose base and extended neck; (**b**) tip of ascomatal neck with divergent ostiolar hyphae extruding ascospores in string; (**c**) ascospores in basal and side (hat-shaped) views; (**d**) bacilliform primary 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.

Description

Colonies on MEA fast growing, reaching 61–75 mm in diameter within 3 d, 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 (4°30′16.7″N, 11°4′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 (4°30′16.7″N, 11°4′44.9″E), on wounded stem of *Theobroma cacao,* December 2009, coll. M. Mbenoun, living cultures, CMW36910, CMW36915; Ngomedzap (3°16′3.5″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 (2°49′10.5″N, 11°7′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 "Theobroame", "Pycnanthi" and "Davidsonii" forms as distinct from the original "Typica" from. 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. *H. 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. *H. 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* (*Th. cerberus* and *Th. ethacetica*). Of the three sites investigated in this study, Bokito had the greatest species richness, including all five species as compared to four and three for Ngomedzap and Nkoemvone respectively. *Th. cerberus* was recovered only at Bokito and *Th. ethacetica* was not found at Nkoemvone. *Huntiella* species were predominant on

tree wounds, whereas *Th. 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 (Figure 5). *H. pycnanthi* and *H. moniliformis* were the most and least prevalent respectively at Bokito, contrasting with Ngomedzap and Nkoemvone where *H. moniliformis* was 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, *Th. cerberus* from two trees at Bokito and *Th. ethacetica* from one tree at Ngomedzap.

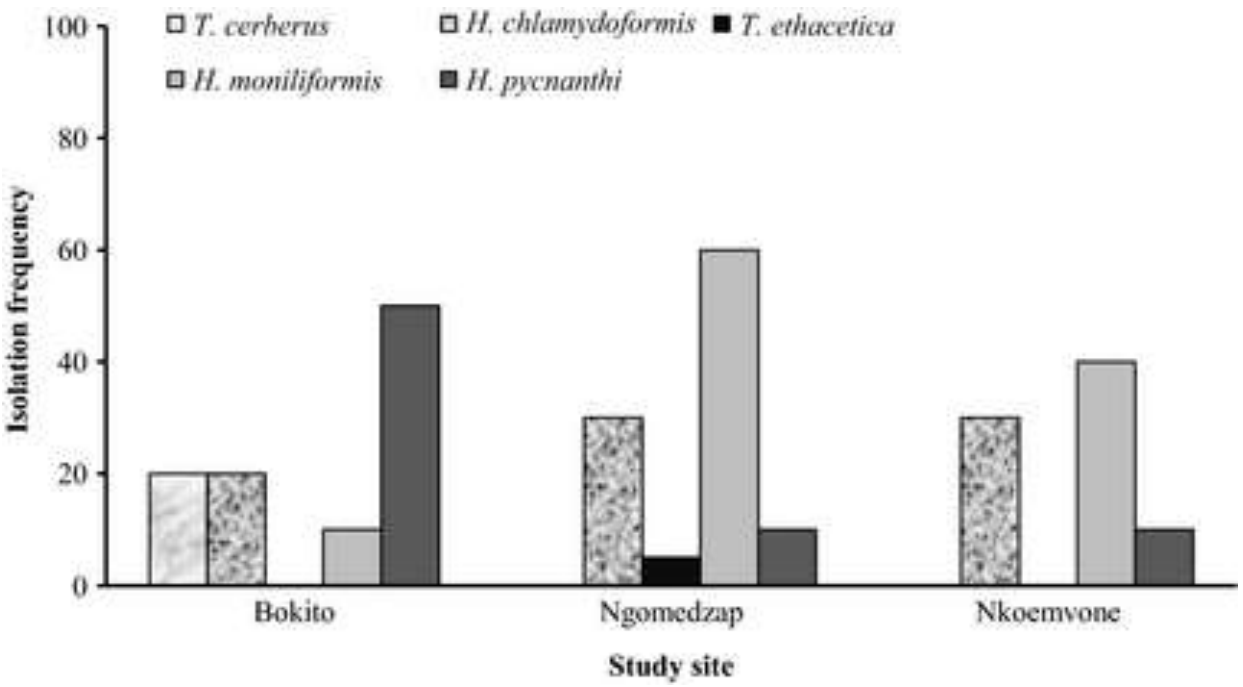

Figure 5 Incidence of the Ceratocystidaceae in cacao agroforests across study sites based on isolation frequencies on artificially induced wounds on cacao stems.

Identification of nitidulid beetles

Nitidulid beetles morphologically resembling *Brachypeplus* species 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 (Supplementary material – FigureS4) showed that the collected insects represented at least three species of *Brachypeplus* (GenBank accessions: KF769077, KF769078, KF769079, KF769080, KF769081, KF769082). Other less commonly occuring 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 morpho-type (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 *Th. cerberus*, CMW35018 (from cacao pod husk) and CMW35028 (from cacao stem wound) for *Th. 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 cambium lesion length (CLL) variance between the two replicated trials (χ^2 = 0.3427; df = 1; *P* = 0.5583). Hence, all CLL data could be pooled-analyzed. In contrast, the null hypothesis of homogeneous variance between trials was rejected (χ^2 = 13.3176; df = 1; *P* = 0.0026) when the test was applied to bark lesion lengths (BLL). Therefore, BLL data for the replicated trials were analyzed 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 = 18.37; df = 10; *P* < 2e-16) and TukeyHSD 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 (*Th. 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, Figure 7).

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 one (F = 56.02; df = 10; *P* < 2e-16) and trial two (F = 43.94; df = 10; *P* < 2e-16). All isolates produced significantly longer lesion as compared to the control and isolates CMW35028 (*Th. ethacetica*) and CMW36966 (*H. pycnanthi*) were consistently the most aggressive (Table 3). Re-isolations 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. The inoculated fungi were, however, never isolated associated with the control inoculations.

Table 3 Average lesion lengths on bark (BLL) and cambium (CLL) caused by species of Ceratocystidaceae on cacao branches six weeks post inoculation. Values followed by the same letters are not significantly different based on TukeyHSD multiple range test.

Fungal species	Isolate	CLL	BLL1	BLL2
Th. cerberus	CMW 35021	50.17±22.85d	11.00±2.35cd	10.11±0.60cd
	CMW35024	49.63±25.85d	11.00±1.41cd	9.78±0.97cd
Th. ethacetica	CMW 35018	60.05±34.16cd	10.30±0.82d	10.22±0.83cd
	CMW 35028	137.42±46.65a	29.40±3.84a	22.78±3.53a
H. chlamydoformis	CMW 36899	68.53±42.68cd	11.20±1.75cd	9.67±0.50cd
	CMW 37104	58.42±22.15cd	13.00±4.70c	11.00±1.41cd
H. moniliformis	CMW 36922	58.74±32.58cd	10.70±1.34d	11.56±2.55c
	CMW 36895	49.53±20.87d	11.40±1.90cd	11.56±1.24c
H. pycnanthi	CMW 36917	75.67±34.09c	11.40±1.17cd	10.67±0.71cd
	CMW 36933	106.37±35.82b	15.90±2.18b	14.56±2.79b
Control		23.00±12.97e	10.00±1.49d	9.56±0.73d

BBL1: Average bark lesion length for the first trial
BBL2: Average bark lesion length for the second trial

Fruit inoculations

Most of the isolates inoculated onto mature cacao pods did not result in lesions. Only isolate CMW35028 (*Th. ethacetica*) caused rotting of inoculated pods (Figure 8). This fungus was consistently re-isolated 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 include two species of *Thielaviopsis* (*Th. cerberus* and *Th. ethacetica*) and three species of *Huntiella* (*H. chlamydoformis*, *H. moniliformis* and *H. pycnanthi*), two of which are described as new. No species related to the genus *Ceratocystis*, well-known to accommodate destructive plant pathogens (Kile, 1993; Engelbrecht & Harrington, 2005; Roux & Winfield, 2009; van Wyk *et al.*, 2013), including *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 *Th. ethacetica* and *H. pycnanthi* sp. nov. emerging as potentially harmful 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 that region include a study by Dade (1928), who first described the sexual state of *Th. 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 *Py. angolensis* in Cameroon. The application of DNA-based tools has now made it possible to recognise 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 *Th. paradoxa* in the list of pathogenic fungi affecting cacao in Ghana (Anonymous, 1996).

Among the five fungal species recovered from cacao agroforests in this study, *H. pycnanthi* and *Th. ethacetica* showed substantial ability to cause lesions on cacao. These species both induced extensive discoloration 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, *Th. 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 pathogenic (e.g., Tarigan *et al.*, 2010, Kamgan Nkuekam *et al.*, 2012b).

Differences observed in the incidence of the Ceratocystidaceae between plantations could be linked to variation in prevailing agro-ecological 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. *H. 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 and in comparison to *H. moniliformis* that 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, *Th. cerberus*, a fungus predominantly known from oil palm (Mbenoun *et al*., 2014a) was found only in Bokito, where this crop occurred alongside cacao. In contrast, most of the isolates of *Th. 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 no pod husks occurred. This suggests that abandoned pod husk piles contribute to increase the inoculum of *Th. ethacetica* in cacao plantations, as was shown for *Phytophthora* spp. causing BPD (Bowers *et al.* 2001; Guest 2006). Moreover, we found that pod husk piles also provide suitable breeding conditions for nitidulid beetles that have been shown to transmit *P. palmivora* (Bult.) Bult. 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 (Moller & DeVay, 1968b; 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*., 2012a). In particular, Heath *et al.* (2009) showed that *B. depressus* is one of the principal overland vectors of the wattle wilt pathogen, *C. albifundus* M.J. Wingf., de Beer & M.J. Morris, 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, thus, needs to be further investigated. This could, for instance, involve assessing whether *Th. 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 agri-silvicultural activities involving extensive tree wounding such as pruning, thinning and coppicing. In the interim, our study has shown that applying the well-known good management practices for pests and diseases of cacao (ICCO, 2009; Dropdata, 2012), including the maintenance of adequate levels of shade in plantation, 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.

Acknowledgements

We acknowledge the technical staff of the NKoemvone IRAD research station and Essomo Ngomba for their invaluable assistance during field studies. We are grateful to Ohono Emile, Essomba Cosmas and Mebenga René for allowing us to work on their farms. This study was funded by the NRF-DST Centre of Excellence in Tree Health Biotechnology (CTHB) of South Africa and the Department of Corporate International Relations of the University of Pretoria through a postgraduate "study abroad grant" awarded to the first author.

References

Al Adawi AO, Deadman ML, Al Rawahi AK, Al Maqbali YM, Al Jahwari AA, Al Saadi BA, Al Amri IS, Wingfield MJ, 2006. Aetiology and causal agents of mango sudden decline disease in the Sultanate of Oman. *European Journal of Plant Pathology* **116**, 247–254.

Anonymous, 1996. Checklists of crop plant diseases in Ghana [http://ghana.ipm-info.org/list_diseases]. Last accessed 12 December 2014.

Barnes I, Roux J, Wingfield BD, Dudzinski MJ, Old KM, Wingfield MJ, 2003. *Ceratocystis pirilliformis*, a new species from *Eucalyptus nitens* in Australia. *Mycologia* **95**, 865–871.

Bowers JH, Bailey BA, Hebbar PK, Sanago S, Lumsdem RD, 2001. The impact of plant diseases on world chocolate production. *Plant Health Progress* Online [http://www.plantmanagementnetwork.org/pub/php/review/cacao/] doi:10.1094/PHP-2001-0709-01-RV.Introduction.

Cristobal BD, Hansen AJ, 1962. Un hongo semjante a *Ceratocystis moniliformis* en cacao en Costa Rica. *Turrialba* **12**, 46–47.

Dade HA, 1928. *Ceratostomella paradoxa*, the perfect stage of *Thielaviopsis paradoxa* (de Seynes) von Höhnel. *Transactions of the British Mycological Society* **13**, 184–194, plates X–XII.

de Beer ZW, Duong TA, Barnes I, Wingfield BD, Wingfield MJ, 2014. Redefining *Ceratocystis* and allied genera. *Studies in Mycology* **79**, 187–219.

Dropdata, 2012. Cocoa pests and diseases: Best managements practices [http://www.dropdata.org/cocoa/icm_bkp.htm]. Last accessed 13 December 2014.

Engelbrecht CJB, Harrington TC, 2005. Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. *Mycologia* **97**, 57–69.

Engelbrecht CJ, Harrington TC, Alfenas A, 2007. Ceratocystis wilt of cacao — A disease of increasing importance. *Phytopathology* **97**, 1648–1649.

Evans HC, 1973. Invertebrate vectors of *Phytophthora palmivora*, causing black pod disease of cocoa in Ghana. *Annals of Applied Biology* **75**, 331–345.

Evans HC, 2007. Cacao diseases — The trilogy revisited. *Phytopathology* **97**, 1640–1643.

Garrett KA, Dendy SP, Frank EE, Rouse MN, Travers SE, 2006. Climate change effects on plant disease: genomes to ecosystems. *Annual Review of Phytopathology* **44**, 489–509.

Gotsch N, 1997. Cocoa crop protection: an expert forecast on future progress, research priorities and policy with the help of the Delphi survey. *Crop Protection* **16**, 227–233.

Guest D, 2006. Black pod: Diverse pathogens with a global impact on cocoa yield. *Phytopathology* **97**, 1650–1653.

Hayslett M, Juzwik J, Moltzan B, 2008. Three *Colopterus* beetle species carry the Oak wilt fungus to fresh wounds on red Oak in Missouri. *Plant Disease* **92**, 270–275.

Heath RN, Wingfield MJ, van Wyk M, Roux J, 2009. Insect associates of *Ceratocystis albifundus* and patterns of association in a native savanna ecosystem in South Africa. *Environmental Entomology* **38**, 356–364.

ICCO, 2009. Overview of best practices in cocoa production [http://www.icco.org/about-us/international-cocoa-agreements/cat_view/30-related-documents/32-consultative-board-on-the-world-cocoa-economy.html] Last accessed 13 December 2014.

Kamgan Nkuekam G, Wingfield MJ, Mohammed C, Carnegie AJ, Pegg GS, Roux J, 2012a. *Ceratocystis* species, including two new species associated with nitidulid beetles, on eucalypts in *Australia*. *Antonie van Leeuwenhoek* **101**, 217–241.

Kamgan Nkuekam G, Wingfield MJ, Roux J, 2012b. *Ceratocystis* species, including two new taxa, from *Eucalyptus* trees in South Africa. *Australian Plant Pathololog*y **42**, 283–311.

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

Konam JK, Guest DI, 2004. Role of beetles (Coleoptera: Scolytidae and Nitidulidae) in the spread of *Phytophthora palmivora* pod rot of cocoa in Papua New Guinea. *Australian Plant pathology* **33**, 55–59.

Luc M, 1952. *Ophiostoma moniliforme* (Hedgc.) H. et P. Syd. et ses diverses formes. *Revue de Mycologie* **17**, 10–16.

Mbenoun M, Momo Zeutsa EH, Samuels G, Nsouga Amougou F, Nyasse S, 2008. Dieback due to *Lasiodiplodia theobromae*, a new constraint to cocoa production in Cameroon. *Plant Pathology* **57**, 381.

Mbenoun M, de Beer ZW, Wingfield MJ, Wingfield BD, Roux J, 2014a. Reconsidering species boundaries in the *Ceratocystis paradoxa* complex, including a new species from oil palm and cacao in Cameroon. *Mycologia* **106**, 757–784.

Mbenoun M, Wingfield MJ, Begoude Boyoguneno AD, Wingfield BD, Roux J, 2014b. Molecular phylogenetic analyses reveal three new *Ceratocystis* species and provide evidence for geographic differentiation of the genus in Africa. *Mycological Progress* **13**, 219–240.

Mbile P, Ngaunkam P, Besingi M, Nfoumou C, Degrande A, Tsobeng A, Sado T, Menimo T, 2009. Farmer management of cocoa agroforests in Cameroon: impacts of decision scenarios on structure and biodiversity of indigenous tree species. *Biodiversity* **10**, 12–19.

Mfegue CV, 2012. *Origine et mécanismes de dispersion des populations de Phytophthora megakarya, pathogène du cacaoyer au Cameroun*. Montpellier, France : Centre International d'Etudes Supérieures en Sciences Agronomiques – Montpellier SupAgro, Ph.D. thesis.

Moller WJ, De Vay JE, 1968a. Carrot as species-selective isolation medium for *Ceratocystis fimbriata*. *Phytopathology* **58**, 123–126.

Moller WJ, DeVay JE, 1968b. Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* **58**, 1499–1508.

Morgan-Jones G, Booth C, Pirozynski A, 1967.*CMI descriptions of pathogenic fungi and bacteria, Set No 15*. Kew, Surrey, England: Commonwealth Mycological Institute.

Ploetz RC, 2007. Cacao diseases: important threats to chocolate production worldwide. *Phytopathology* **97**, 1634–139.

Rayner RW, 1970. A mycological colour chart. Kew, Surrey UK: Commonwealth Mycological Institute and British Mycological Society.

Rice RA, Greenberg R, 2000. Cacao cultivation and the conservation of biological diversity. *AMBIO* **29**, 167–173.

Roux J, Wingfield MJ, 2009. *Ceratocystis* species: emerging pathogens of non-native plantation *Eucalyptus* and *Acacia* species. *Southern Forests* **71**, 115–120.

Roux J, Wingfield MJ, 2013. *Ceratocystis* species on the African continent, with particular reference to *C. albifundus*, and African species in the *C. fimbriata* sensu lato species complex. In: Seifert KA, de Beer ZW, Wingfield MJ, eds. *The ophiostomatoid fungi: expanding frontiers*. Utrecht, the Netherlands: CBS biodiversity series, vol. 12, 131–138.

Schroth G, Krauss U, Gasparotto L, Duarte Aguilar JA, Vohland K, 2000. Pests and diseases in agroforestry systems of the humid tropics. *Agroforestry Systems* **50**, 199–241.

Seifert KA, de Beer ZW, Wingfield MJ, eds, 2013. *The ophiostomatoid fungi: expanding frontiers*. Utrecht, The Netherlands: CBS Biodiversity Series, vol. 12.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P, 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* **87**, 651–701.

Sonwa DJ, Weise S, Adesina A, Nkongmeneck AB, Tchatat M, Ndoye O, 2005. Production constraints on cocoa agroforestry systems in West and Central Africa: The need for integrated pest management and multi-institutional approaches. *The Forestry Chronicle* **81**, 345–349.

Swofford DL, 2002. *Phylogenetic analysis using parsimony (*and other methods), Versoin 4.* Sunderland, Massachusetts, USA: Sinauer Associates.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S, 2011 MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731–2739.

Tarigan M, van Wyk M, Roux J, Tjahjono B, Wingfield MJ, 2010. Three new *Ceratocystis* spp. in the *Ceratocystis moniliformis* complex from wounds on *Acacia mangium* and *A. crassicarpa*. *Mycoscience* **51**, 53–67.

van Wyk M, Wingfield BD, Wingfield MJ, 2013. *Ceratocystis* species in the *Ceratocystis fimbriata* complex. In: Seifert KA, de Beer ZW, Wingfield MJ, eds. *The ophiostomatoid fungi: expanding frontiers*. Utrecht, the Netherlands: CBS biodiversity series, vol. 12, 65–73.

Wingfield MJ, Seifert KA, Webber JF, eds, 1993. *Ceratocystis* and *Ophiostoma: Taxonomy, Ecology and Pathogenicity.* St. Paul, Minnesota, USA: APS Press.

Wingfield MJ, Roux J, Wingfield BD, Slipppers B, 2013. *Ceratocystis* and *Ophiostoma*: international spread, new associations and plant health. In: Seifert KA, de Beer ZW, Wingfield MJ, eds. *The Ophiostomatoid fungi: expanding frontiers*. Utrecht, the Netherlands: CBS Biodiversity Series, vol. 12, 191–200.

Supplementary materials

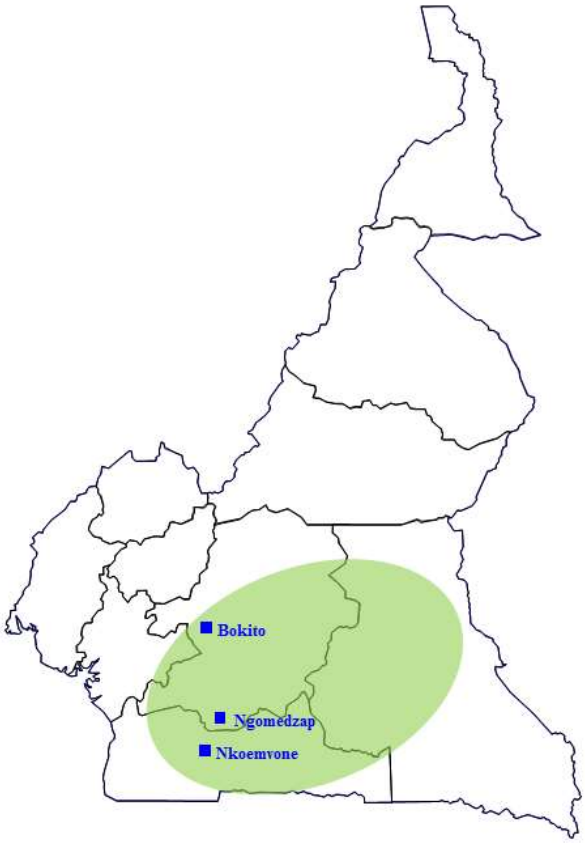

Figure S1 Map of Cameroon showing the Centre-South cacao growing region (shaded) and the sites involved in this study. The map was adapted from Efombagn *et al*. (2008)

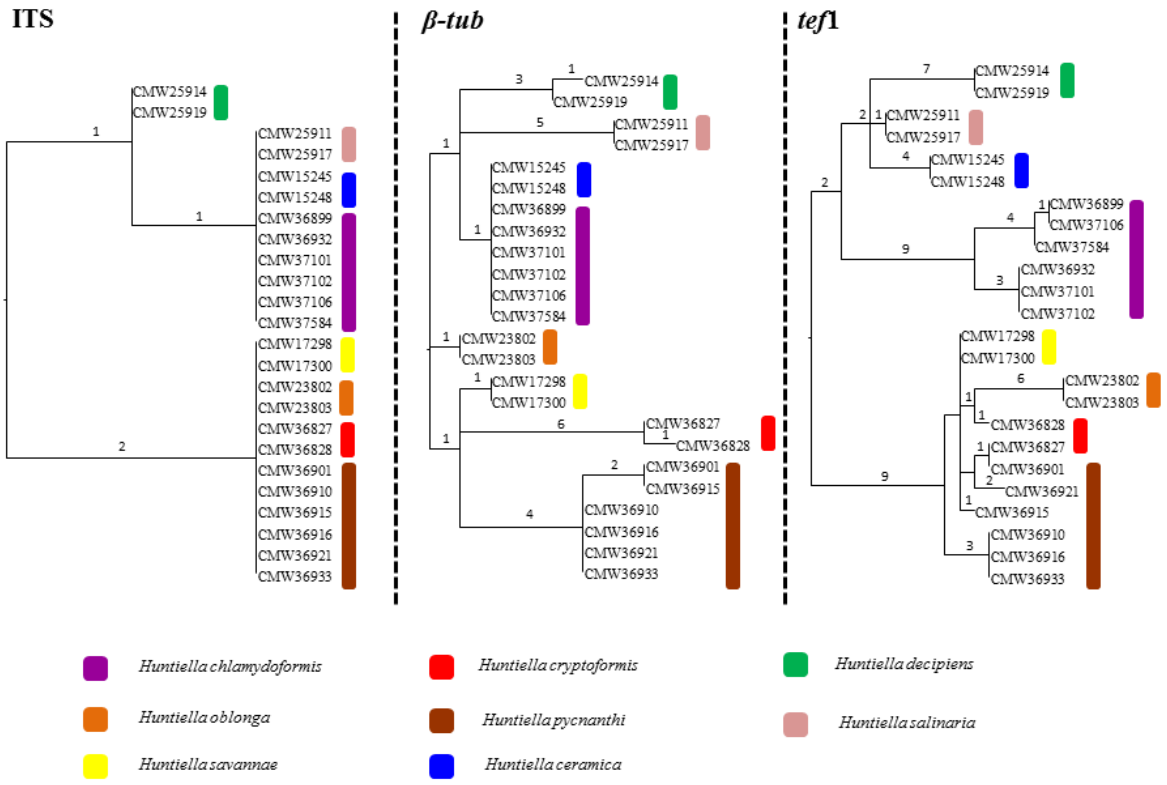

Figure S2 Phylogenetic relationships between the new *Huntiella* species discovered in cacao agroforests in Cameroon and their closest known relatives based on single gene loci. The number of corresponding nucleotide substitutions is indicated above tree branches.

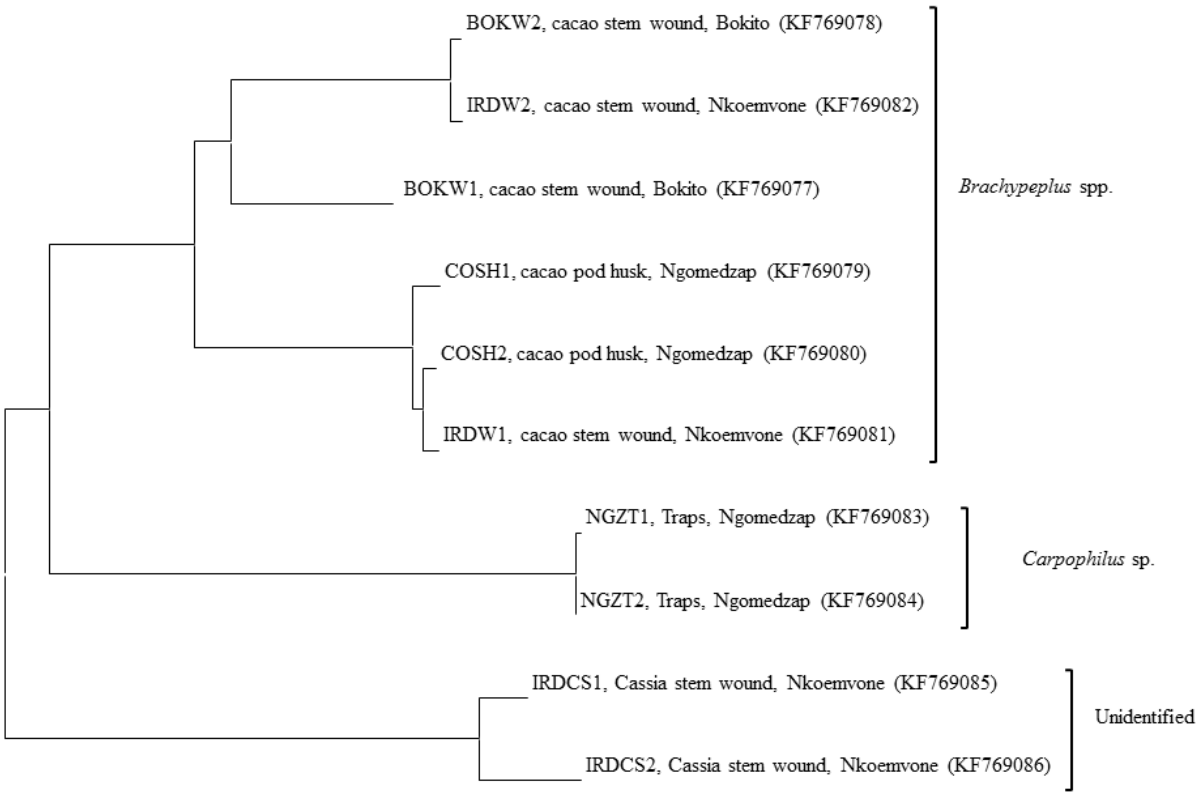

Figure S3. Neighbour joining phylogenetic tree of nitidulid beetles found in cacao agroforests in Cameroon based on MT-CO1 gene sequences. GenBank accession numbers of sequences are provided in parentheses.

TreeBase information

http://purl.org/phylo/treebase/phylows/study/TB2:S16363?x-access-code=55dd6cfb410c327cb75d39b1c1969b11&format=html

http://purl.org/phylo/treebase/phylows/study/TB2:S16363