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Vascular Streak Dieback of cacao in Southeast Asia and Melanesia: *in planta* detection of the pathogen and a new taxonomy

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ABSTRACT

Vascular Streak Dieback (VSD) disease of cacao (*Theobroma cacao*) in Southeast Asia and Melanesia is caused by a basidiomycete (*Ceratobasidiales*) fungus *Oncobasidium theobromae* (syn. =*Thanatephorus theobromae*). The most characteristic symptoms of the disease are green-spotted leaf chlorosis or, commonly since about 2004, necrotic blotches, followed by senescence of leaves beginning on the second or third flush behind the shoot apex, and blackening of infected xylem in the vascular traces at the leaf scars resulting from the abscission of infected leaves. Eventually the shoot apex is killed and infected branches die. In susceptible cacao the fungus may grow through the xylem down into the main stem and kill a mature cacao tree. Infections in the stem of young plants prior to the formation of the first 3–4 lateral branches usually kill the plant. Basidiospores released from corticioid basidiomata developed on leaf scars or along cracks in the main vein of infected leaves infect young leaves. The pathogen commonly infects cacao but there are rare reports from avocado. As both crops are introduced to the region, the pathogen is suspected to occur asymptomatically in native vegetation. The pathogen is readily isolated but cultures cannot be maintained. In this study, DNA was extracted from pure cultures of *O. theobromae* obtained from infected cacao plants sampled from Indonesia. The internal transcribed spacer region (ITS), consisting of ITS1, 5.8S ribosomal RNA and ITS2, and a portion of nuclear large subunit (LSU) were sequenced. Phylogenetic analysis of ITS sequences placed *O. theobromae* sister to *Ceratobasidium* anastomosis groups AG-A, AG-Bo, and AG-K with high posterior probability. Therefore the new combination *Ceratobasidium theobromae* is proposed. A PCR-based protocol was developed to detect and identify *C. theobromae* in

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plant tissue of cacao enabling early detection of the pathogen in plants. A second species of *Ceratobasidium*, *Ceratobasidium ramicola*, identified through ITS sequence analysis, was isolated from VSD-affected cacao plants in Java, and is widespread in diseased cacao collected from Indonesia.

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Introduction

Cacao (*Theobroma cacao*, Malvales) is native to the upper Amazon region of America (Bartley 2005) and is grown in many tropical countries. As the source of chocolate, cacao is an economically important crop wherever it is grown. Although the disease that results in the greatest losses in cacao worldwide is Black Pod, caused by *Phytophthora palmivora* or other species of *Phytophthora* in different regions (Bowers *et al*. 2001), each of the respective geographic areas of cacao production suffers from indigenous pathogens. In America the most important diseases are Witches' Broom and Frosty Pod Rot, caused by *Moniliophthora perniciosa* and *Moniliophthora roreri*, respectively (Bowers *et al*. 2001). In West and Central Africa Cocoa Swollen Shoot Virus, which has been spread to cacao from local forest trees, has long been a destructive pathogen and the indigenous oomycete *Phytophthora megakarya* causes up to 100 % crop loss through pod rot (Bowers *et al*. 2001; Tondje *et al*. 2007). In Southeast Asia, where Indonesia is the world's 3rd largest producer of cacao (Shapiro *et al*. 2008; ICCO 2010), the main constraints to cacao production are Black Pod and Stem Canker caused by *P. palmivora*, pod damage caused by the indigenous cocoa pod borer moth, *Conopomorpha cramerella* Snellen (Wardojo 1980; Lim 1992; Shapiro *et al*. 2008), and Vascular Streak Dieback (VSD), caused by *Oncobasidium theobromae* (Talbot & Keane 1971; Keane & Prior 1991).

VSD has been reported only from the region that extends from New Britain Island in Papua New Guinea westward to Kerala State in India and Hainan Island, China (Keane & Prior 1991). The symptoms of VSD were first described by Shaw (1962), and subsequently by Keane *et al*. (1972), Keane & Prior (1991), and Guest & Keane (2007). Wind dispersed basidiospores of the fungus released during periods of sustained rainfall and high humidity are the only source of inoculum, and thought to infect very young, unhardened leaves. After penetrating the leaf, the fungus colonizes xylem vessels, causing vascular browning in veins of the lamina, and then moves into the midrib and petiole, eventually reaching the branch. Leaf symptoms, including chlorosis of leaves with green spots; more commonly since about 2004, necrotic blotches (Guest & Keane 2007) appear 3–5 m after infection by which time about two new flushes of leaves have developed on the shoot. Leaf abscission usually occurs within a few days of the full development of the spotted chlorotic symptoms on the leaf, although leaves with the new necrotic symptoms cling much more tenaciously to the stem. As fungal colonisation continues through the branch or main shoot of a seedling, leaves on either side of the first affected leaf show the same symptoms until the entire branch or stem defoliates and dies. In severe cases on susceptible cacao, the pathogen grows into the main stem and can cause death of a whole tree; infections in the stem of plants younger than about 12 m (prior to the formation of the first lateral branches) usually kill the plant. When leaf fall occurs during extended periods of wet weather, the hyphae can emerge from the vascular traces in the leaf scar and form white corticioid basidiomata on and around the scar. Recently the fungus has been observed sporulating on cracks along the main vein of leaves with necrotic blotches that remain attached to the branches for extended periods (Purwantara *et al*. 2009). The fungus does not continue to sporulate or survive on dead branches.

While the fungus readily grows from infected tissue onto water agar, the organism cannot be maintained in pure culture and the sexual stage has only been induced under elaborate conditions in tissue culture in Malaysia (Lam *et al*. 1988), a result that could not be repeated in Papua New Guinea (Dennis 1991). The disease has been induced only by ejecting basidiospores directly onto seedlings from basidiomata collected from the field, or by preparing a spore suspension from field-collected basidiomata (Prior 1978, 1979). Thus Koch's postulates have not been fulfilled unequivocally. The basis for the conclusion that *O. theobromae* causes VSD is the intimate physical and epidemiological association between the fungus and the disease; however this needs further investigation in light of the changes in observed symptom expression associated with the disease since 2004. Moreover, in the absence of pure cultures *O. theobromae* has not been included in any phylogenetic analyses of the *Ceratobasidiales* (e.g. Gonzalez *et al*. 2001, 2006) and its systematic position is unknown. No cultures are known to have been derived from basidiospores of *O. theobromae*. Our identification of *O. theobromae* was confirmed by comparison of ITS sequences with those from cultures obtained from symptomatic cacao tissue by one of the coauthors (P.M.).

Cacao is the only common host of *O. theobromae* although rare infection of seedlings of avocado (*Persea americana*) has also been found (Anderson 1989; Dennis 1991). As both cacao and avocado are native to tropical America, the occurrence of VSD in these crops in Southeast Asia and Melanesia is treated as a new encounter (Keane & Prior 1991). Because *O. theobromae* (a teleomorph associated with fungi having a Rhizoctonia-like anamorph; Roberts 1999) is not known to occur in America, it seems likely that the fungus is associated with one or more native species of plants in its range of distribution, perhaps as an asymptomatic endophyte.

The objectives of this study were to determine the phylogenetic position of *O. theobromae* and to develop a protocol for detecting and identifying the pathogen in tissue of infected cacao. In this study, we describe a method for detecting *O. theobromae* in plant tissue using an ITS-based Polymerase Chain Reaction (PCR) protocol. We present the phylogeny of this

species based on ITS and large subunit (LSU) analysis of DNA extracted from pure cultures and infected plants. We also report the common occurrence of a related species of *Ceratobasidium* on cacao in Indonesia.

Materials and methods

Isolation

Infected cacao stems and leaves were collected in Indonesia and sent to Beltsville, MD *via* express mail (under APHIS permit) by one of two packaging methods. In the first method, pieces of terminal branchlets with or without leaves and measuring to 30 cm in length were wrapped in plastic bags or plastic wrap. Branchlets were used for isolation of mycelium of *Oncobasidium theobromae* (*Ot*) and for isolation of DNA. In the second method, small pieces of twig, petiole or leaves were shipped to Beltsville in 3 mL cryovials containing sterile Tris–EDTA buffer (TRIS: 1 mM Tris, 0.1 mM EDTA, pH 7.4). These were used for direct isolation of DNA. Petri plates containing pure cultures of *Ot* on water agar were also sent *via* express mail but the cultures were never viable. Later in the project isolations were made in Indonesia onto water agar in 5-cm-diam plastic Petri plates following surface sterilisation (70 % ethanol (EtOH) for 2 min, washing in sterile distilled water for 2 min, blotting dry with sterile filter paper) of small pieces of freshly collected leaf laminae (with larger veins) or petioles which were then cut transversely into smaller pieces with a sterile scalpel to expose xylem tissue prior to plating; those isolation plates were sent immediately *via* express mail to Beltsville. Viable cultures of *Ot* were recovered from these Petri plates. All isolations made in Beltsville from infected plant tissue were conducted in a biological safety cabinet using the following protocol. Terminal branchlets of cacao trees with symptoms were cut into approximately 20-mm-long segments, soaked in 70 % EtOH for 2–3 min, then placed successively in 6 % bleach solution (40 % NaOCl) for 2 min, 70 % EtOH for 1 min, and distilled H_2O for at least 2 min. The bark was removed aseptically and stem segments were cut in half lengthwise to expose the vascular tissue. Small pieces of stem with vascular tissue were placed in 1.5 % water agar (Difco Laboratories, MI, USA) in 100-mm-diam plastic Petri plates, wrapped with Parafilm®, enclosed in a plastic box with wet paper towels (100 % humidity) and incubated at 25 °C. After 3 d, stem segments were examined for hyphal growth from the area of the vascular tissue in contact with the agar.

Slowly growing, hyaline, sterile, *Rhizoctonia*-like mycelium was suspected of being *Ot* (Fig 1A and D). These hyphae were examined microscopically for the distinctive characteristics of right angle branching and dolipore septa (Fig 1B and F) by removing a small piece of agar containing terminal branches of hyphae from the Petri plate and placing it in a drop of 3 % KOH on a glass slide. A cover slip was placed on the material, which was then crushed gently with the tip of a scalpel while observing with a dissecting microscope to keep the sample in the middle of the preparation. After the material was crushed, a small drop of phloxine 1 % (aq.) was placed at the edge of the cover slip and the mount was heated with a flame until it boiled, dissolving the agar and providing a flat preparation. The phloxine leaves a negative image of dolipore septa, which are most easily seen within a few minutes with phase contrast microscopy (Fig 1B and F). If dolipore septa were observed, a block of agar was placed on a fresh Petri plate with water agar and incubated as above. When there was visible growth in the new Petri plate a block of agar with mycelium was placed in a liquid medium for DNA extraction.

Attempts to stain nuclei of *Ot* using phenosafranine (Bartz *et al*. 2010), aniline blue, and trypan blue (Sneh *et al*. 1991) were not successful. Nuclei in hyphae of a second *Rhizoctonia*-like fungus that was isolated from cacao tissue and grew rapidly in culture, described below, were stained with phenosafranin (Sigma–Aldrich199648-1G, 0.1 % aq.) as follows: Blocks of agar 5 mm square were taken from the edge of an actively growing culture and placed *ca* 1 cm apart on a glass slide. A 22 × 30 mm cover slip was placed over the blocks and the slide was incubated in a Petri plate at 100 % humidity for a few days until mycelium grew onto the cover slip. The cover slip with mycelium was then inverted into a drop of phenosafranin and 3 % KOH, described above, on a clean glass microscope slide.

Extraction, amplification and sequencing of DNA

DNA was initially isolated from four pure cultures obtained from terminal branchlets of VSD-infected cacao in Indonesia (South Sulawesi; Fig 5, Table 1 SULAWESI 8, 10, 11, and 12). Mycelium was placed in Corticium Culture Medium (CCM; KH_2PO_4, 1.25 g, $CaNO_3$, 2.36 g, $MgSO_4 \cdot 7H_20$, 0.59 g, Maltose, 6.25 g, malt extract, 6.25 g, distilled water to make 1 L; Kotila 1929) in plastic Petri plates and incubated for up to 2 wk at 25 °C, or in 50 % coconut water (Goya® commercial 'young coconut' water with distilled water) in 5-cm-diam Petri dishes for approximately 10 d at 25 °C. Mycelium from these cultures was collected and dried by pressing between paper towels. DNA was extracted from the mycelium using ArchivePure DNA Cell/Tissue kit from 5 PRIME Inc. (Gaithersburg, MD, USA) following the manufacturer's protocol. In subsequent experiments, fungal DNA was extracted directly from leaves, woody tissue, and stems of cacao using the following procedure: Pieces of terminal branchlets with symptoms of VSD were cut vertically and the inner surfaces were scrapped using a sterile scalpel to reveal the darkened vascular tissue. The woody tissue or pieces of leaves were macerated in lysing buffer from the DNA kit in small vials. DNA was extracted from plant tissue as described above for isolation of DNA from pure culture. DNA was also extracted from asymptomatic tissue and used as a negative control. For this study, DNA from 12 pure cultures with hyphal characteristics of *Ot* as described by Talbot & Keane (1971) and Roberts (1999) was isolated (Table 1). DNA was isolated from an additional 54 samples of plant tissue with symptoms of VSD (Table 1).

The DNA extracted from pure cultures was used as the template for amplification of the internal transcribed spacer region (ITS), consisting of ITS1, 5.8S ribosomal RNA, and ITS2. Primers ITS5 and ITS4 (White *et al*. 1990) were used for amplification of ITS. A portion of the 28S nuclear LSU was also amplified using primers LROR and LR7 (Vilgalys & Hester 1990). The PCR mixture contained 10 μL of Quick-load

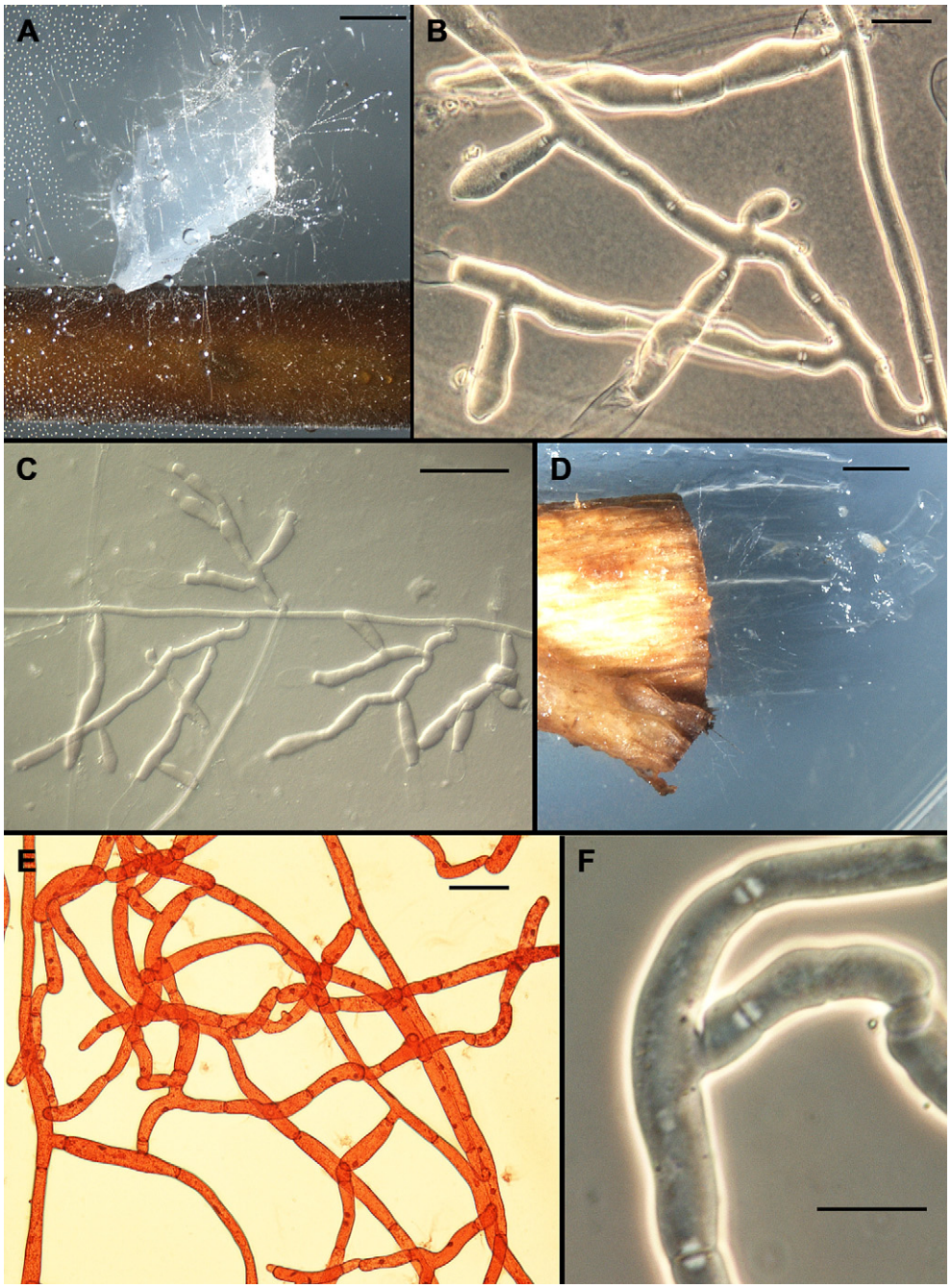

Fig 1 – (A–C). *Ceratobasidium theobromae*. (A) Mycelium growing from a piece of water agar onto a cacao stem on water agar. (B) Mycelium in water agar with typical branching at right angles and dolipore septa. (C) Hypha in water agar showing inflated hyphal cells. (D–F). *Ceratobasidium ramicola*. (D) Hyphae growing from the end of an infected piece of cacao stem onto water agar. (E) Hyphae showing binucleate nuclear condition and hyphal anastomoses; stained with phenosafranin. (F) Hypha showing dolipore septa. (B) and (F) stained in 1 % aq. (w v^{-1}) phloxine and viewed with phase contrast microscopy. Scale bars: A, D = 2 mm; B, E, F = 10 μm; C = 50 μm. A–C from Luwu (SULAWESI 12, Table 1), D–F from CBS 127104, Mamuju (SULAWESI 16, Table 1).

2× master mix (New England Biolabs, MA, USA), 1 μL of 25 mM $MgCl_2$, 0.5 μL of 10 mM both forward and reverse primers, and ~1–2 ng genomic DNA. Final volume was adjusted to 20 μL with deionized H_2O. The reactions were placed in a PTC-200 MJ Research thermo-cycler (Waltham, MA) using a touchdown program (Don *et al.* 1991). The touchdown PCR was initiated with a 2 min denaturation at 94 °C followed by initial 15 cycles of PCR amplification. The annealing temperature in the first amplification cycle was 65 °C, which was subsequently incrementally reduced by 1 °C per cycle over the next 15 cycles. Then an additional 35 cycles were performed, each consisting of 30 s denaturation at 94 °C, a 30 s annealing at 48 °C, and

Table 1 – Sources of cultures and isolated DNA of *Ceratobasidium theobromae* and *C. ramicola*.

Code	Identification	Country	Locality	Material	ITS 1 + 2	LSU
MAL 0	*C. theobromae*	Malaysia	Pahang	Terminal branchlet		
MAL 1	*C. theobromae*	Malaysia	Pahang	Terminal branchlet		
MAL 2	*C. theobromae*	Malaysia	Pahang	Terminal branchlet		
MAL 3	*C. theobromae*	Malaysia	Pahang	Terminal branchlet		
MAL 4	*C. theobromae*	Malaysia	Pahang	Terminal branchlet	HQ424248	
MAL 5	*C. theobromae*	Malaysia	Pahang	Terminal branchlet	HQ424249	
MAL 6	*C. theobromae*	Malaysia	Pahang	Terminal branchlet	HQ424250	
MAL 7	*C. theobromae*	Malaysia	Pahang	Terminal branchlet		
MAL 8	*C. theobromae*	Malaysia	Pahang	Terminal branchlet		
MAL 9	*C. theobromae*	Malaysia	Pahang	Terminal branchlet		
MAL 10	*C. theobromae*	Malaysia	Pahang	Terminal branchlet		
MAL 11	*C. theobromae*	Malaysia	Pahang	Terminal branchlet		
MAL 12	*C. theobromae*	Malaysia	Hilir Perak	Terminal branchlet		
MAL 13[1]	*C. theobromae*	Malaysia	Sabah	Terminal branchlet, leaf, petiole	FJ763575	
MAL 14[1]	*C. theobromae*	Malaysia	Sabah	Terminal branchlet, leaf, petiole	FJ763574	
SULAWESI 1	*C. theobromae*	Indonesia	South Sulawesi: Luwu	Terminal branchlet		
SULAWESI 2	*C. theobromae*	Indonesia	South Sulawesi: Luwu	Terminal branchlet		
SULAWESI 3	*C. theobromae*	Indonesia	South Sulawesi: Luwu	Terminal branchlet		
SULAWESI 4	*C. theobromae*	Indonesia	South Sulawesi	Terminal branchlet		
SULAWESI 5	*C. theobromae*	Indonesia	South Sulawesi	Leaf		
SULAWESI 6	*C. theobromae*	Indonesia	SW Sulawesi, Belopa	Terminal branchlet		
SULAWESI 7	*C. theobromae*	Indonesia	South Sulawesi: Luwu	Pure culture from terminal branchlet	HQ424244	
SULAWESI 8	*C. theobromae*	Indonesia	South Sulawesi: Luwu	Bark		
SULAWESI 9	*C. theobromae*	Indonesia	South Sulawesi: Pinrang	Terminal branchlet		
SULAWESI 10	*C. theobromae*	Indonesia	South Sulawesi: Luwu	Pure culture from terminal branchlet	HQ424246	HQ424242
SULAWESI 11	*C. theobromae*	Indonesia	South Sulawesi: Luwu	Pure culture from terminal branchlet	HQ424247	HQ424243
SULAWESI 12	*C. theobromae*	Indonesia	South Sulawesi: Luwu	Pure culture from terminal branchlet	HQ424245	
SULAWESI 13	*C. theobromae*	Indonesia	South Sulawesi: Wajo	Leaf		
SULAWESI 14	*C. theobromae*	Indonesia	NE Sulawesi, North Kolaka, Tanggeawo	Terminal branchlet		
SULAWESI 15	*C. theobromae*	Indonesia	South Sulawesi: Bone	Terminal branchlet		
JAVA 1[1]	*C. theobromae*	Indonesia	East Java: Jember	Terminal branchlet	FJ763573	
JAVA 2	*C. theobromae*	Indonesia	East Java, Batu Batu	Leaf		
JAVA 3	*C. theobromae*	Indonesia	East Java, Batu Batu	Leaf		
JAVA 4	*C. theobromae*	Indonesia	East Java, Batu Batu	Leaf		
JAVA 5	*C. theobromae*	Indonesia	East Java, Batu Batu	Leaf		
BALI 1	*C. theobromae*	Indonesia	Bali	Petiole		
BALI 2	*C. theobromae*	Indonesia	Bali	Petiole		
PAPUA 1	*C. theobromae*	Indonesia	Papua, West Papua	Petiole, restricted symptoms	HQ424251	
PAPUA 2	*C. theobromae*	Indonesia	Papua, West Papua	Midrib, leaf necrosis with vascular browning	HQ424252	
PAPUA 3	*C. theobromae*	Indonesia	Papua, West Papua	Terminal branchlet, healthy with mild green leaf spotting	HQ424253	
PAPUA 4	*C. theobromae*	Indonesia	Papua, West Papua	Petiole, healthy with mild green leaf spotting		
PAPUA 5	*C. theobromae*	Indonesia	Papua, West Papua	Petiole, with VSD symptoms		
PAPUA 6	*C. theobromae*	Indonesia	Papua, West Papua	Petiole, with VSD symptoms		
PAPUA 7	*C. theobromae*	Indonesia	Papua, West Papua	Petiole, with VSD symptoms		
VIETNAM 1	*C. theobromae*	Vietnam		Terminal branchlet	FJ763577	
VIETNAM 2	*C. theobromae*	Vietnam		Terminal branchlet	FJ763578	
VIETNAM 3	*C. theobromae*	Vietnam		Terminal branchlet	FJ763576	
JAVA 6	*C. ramicola*	Indonesia	Java, East Java: Kaliwining	Terminal branchlet		
JAVA 7	*C. ramicola*	Indonesia	Java, East Java: Kaliwining	Terminal branchlet		
JAVA 8	*C. ramicola*	Indonesia	Java, East Java: Kaliwining	Terminal branchlet		
JAVA 9	*C. ramicola*	Indonesia	Java, East Java: Kaliwining	Terminal branchlet		
JAVA 10	*C. ramicola*	Indonesia	Java, East Java: Kaliwining	Terminal branchlet	HQ424238	
SULAWESI 16	*C. ramicola*	Indonesia	West Sulawesi: Mamuju	Terminal branchlet	HQ424240	HQ424240
JAVA 11	*C. ramicola*	Indonesia	East Java: Kaliwining	Terminal branchlet	HQ424239	

(*continued on next page*)

Table 1 – (continued)

Code	Identification	Country	Locality	Material	ITS 1 + 2	LSU
CBS 133.82 extype culture of *C. ramicola*	*C. ramicola*	USA	Florida	Leaf	DQ278931	
PAPUA 8	*C. ramicola*	Indonesia	Papua, West Papua	Petiole, leaf necrosis without vascular browning		
PAPUA 9	*C. ramicola*	Indonesia	Papua, West Papua	Midrib. leaf necrosis without vascular browning		
PAPUA 10	*C. ramicola*	Indonesia	Papua, West Papua	Midrib. leaf necrosis without vascular browning		
PAPUA 11	*C. ramicola*	Indonesia	Papua, West Papua	Midrib. leaf necrosis without vascular browning		
PAPUA 12	*C. ramicola*	Indonesia	Papua, West Papua	Twig, leaf necrosis with vascular browning		
PAPUA 13	*C. ramicola*	Indonesia	Papua, West Papua	Midrib, leaf necrosis with vascular browning		
JAVA 12	*C. ramicola*	Indonesia	Java, East Java, Kaliwining	Terminal branchlet		
C. sp. (outgroup)	*C.* sp.	Brazil			GQ892615	

a 1 min extension at 72 °C concluding with a 10 min extension at 72 °C.

Amplified products (amplicons) were visualized on 1 % agarose gel stained with ethidium bromide. Following amplifications, amplicons were treated enzymatically using the Exosap-it kit from USB (Cleveland, OH, USA) following the manufacturer's protocol and sequenced using BigDye Terminator v3.1 chemistry in an automated DNA sequencer (ABI 3510, Applied Biosystems, CA, USA). The sequences were assembled using Sequencher 4.9 (Gene Codes, WI, USA).

Development of Oncobasidium theobromae *specific primers*

Oligonucleotide primers were designed from a unique region in the ITS sequence of *Ot*. ITS sequences for *Ot* were subjected to a basic local alignment search tool (BLAST) using the BLAST sequence analysis program available at GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of the initial 30 hits were retrieved in FASTA format and aligned with the ITS of *Ot* using Clustal X Multiple Sequence Alignment Program v. 2.0.11 with default settings. The alignment file was refined manually using MacClade 4.08 (Maddison & Maddison 2005). Based on an examination of the alignment file, forward (Than_ITS1: 5′-GAGTCTTGG CAGTTGCTG-3′) and reverse primers (Than_ITS2: 5′-AGAAGCGGTCATCT GTA-3′) were developed to amplify a 550 bp fragment of ITS. The primer sequences were compared against existing sequences in GenBank. Results of BLAST search showed primer Than-ITS1 to be very specific and no identical sequence was found in any other species of fungus or plant. No match was found for primer Than_ITS1; primer Than_ITS2 sequence was found in other species of fungi but especially in *Rhizoctonia*. Primer specificity was tested using DNA from four closely related strains of *Ceratobasidium* representing four different anastomosis groups (AG-A, AG-D, AG-K, AG-Q shown in Fig 4) as DNA template in PCR (Fig 2). DNA extracted from plants exhibiting symptoms of VSD as described above was amplified using the specific primers and no amplification occurred when DNA was extracted from asymptomatic (healthy) cacao plants. The PCR mixture and programs were as

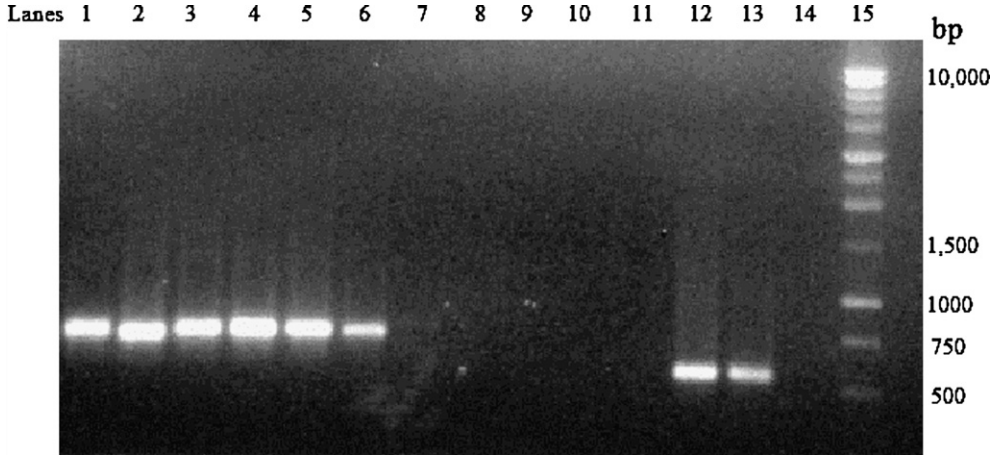

Fig 2 – Analysis of amplified PCR products on 1 % agarose gel stained with ethidium bromide and viewed under UV light. Lanes 1–7 produced by primers ITS5 and ITS4 (White *et al.* 1990) using DNA from closely related taxa. 1. binucleate *Rhizoctonia* anastomosis group K (AG-K), 2. binucleate *Rhizoctonia* AG-A, 3. binucleate *Rhizoctonia* AG-D, 4. binucleate *Rhizoctonia* AG-Q, 5. *C. theobromae* SULAWESI 10, 6. *C. theobromae* SULAWESI 11 7. Control (no DNA). Lanes 8–14 DNA amplified with *C. theobromae* specific primers Than_ITS1 and Than_ITS2. Lane 15 contained 1 kb molecular marker (Promega).

described above for PCR using genomic DNA of pure fungal cultures. DNA extracted from asymptomatic cacao plants was extracted and used as negative control.

The sensitivity of the method for detecting *Ot* was tested using serial dilutions of total genomic DNA from pure cultures in TE buffer (Fig 3). The concentration of genomic DNA was measured spectrophotometrically at A_{260} and extinction coefficient of 50 μg mL^{-1} with an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany). The lowest concentration of genomic DNA in a PCR reaction that produced an amplified product was determined.

Phylogenetic analysis

For the purpose of examining the phylogenetic position of *Oncobasidium theobromae* to closely related species in *Ceratobasidium* and *Thanatephorus*, the ITS and LSU sequences obtained from pure cultures were subjected to BLAST search analysis. Sequences having the greatest similarity to those of the pure cultures were retrieved and alignment files for each locus were prepared and refined manually in MacClade (Maddison & Maddison 2005). The ITS and LSU sequences were used to infer phylogenetic trees using maximum parsimony and Bayesian criteria. Maximum parsimony was performed using PAUP (Swofford 2002) with a heuristic search, 1000 random sequence addition, gaps considered as missing, tree bisection-reconnection- (TBR) branch swapping, and MulTrees off. The first 10 trees were used as starting trees in a second heuristic search with MulTrees on. Support for the clades was assessed with 1000 bootstrap replicates. Clades with bootstrap values of 70 % or greater were considered as significantly supported by the data (Hillis & Bull 1993). For analysis of ITS and LSU sequences, isolates in the genus *Botryobasidium* were used as outgroup taxa based on the study of Moncalvo *et al.* 2006 that placed *Botryobasidium* as a sister group to the Clade of *Ceratobasidium* and *Thanatephorus*.

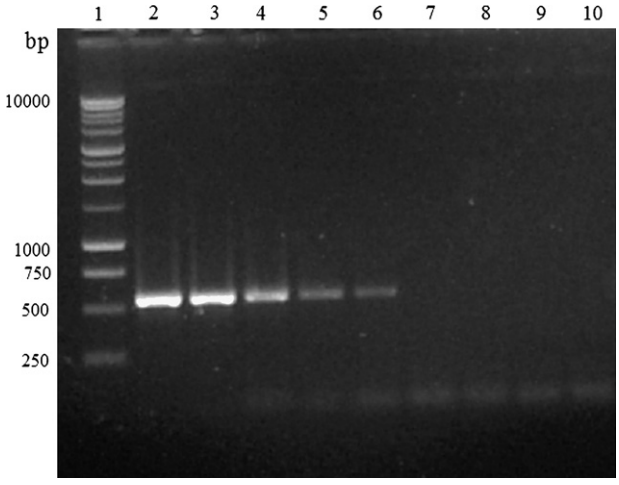

Fig 3 – Agarose gel (1 %) showing PCR sensitivity range for detecting *Ceratobasidium theobromae*. Lane 1: 1 kb ladder from Promega, lanes 2–10: 5 μL PCR performed with 80, 8, 8×10^{-1}, 8×10^{-2}, 8×10^{-3}, 8×10^{-4}, 8×10^{-5}, 8×10^{-6}, and 0 ng of genomic DNA extracted from pure culture, respectively. The gel was stained with ethidium bromide and viewed under UV light.

Bayesian analyses were performed as implemented in Mr Bayes v3.1.2 (Ronquist & Huelsenbeck 2003). Modeltest v3.7 (Posada & Crandall 1998) was used to determine the appropriate likelihood substitution model for each locus in the Bayesian analyses. Model F81 and GTR + I + G were selected for ITS and LSU, respectively. Two concurrent analyses of four chains were both run for one million generations, ensuring analyses were not trapped in a local optimum, using random starting tree and sampled every 100 generations. The burn in phase was obtained based on the *x*–*y* plot of generation times *vs.* –log likelihood of the trees and the initial trees before stationarity in log likelihood was reached were discarded. The remaining trees were used for obtaining a 50 % majority-rule consensus tree with consensus index representing posterior probabilities (pp) of the clades. Support for clades was considered significant when the pp value was 0.95 or higher (Leache & Reeder 2002).

A second basidiomycete fungus with hyphal characteristics of *Rhizoctonia*, *Ceratobasidium ramicola*, was isolated from cacao in the course of our research and subjected to further investigation. This fungus was identified through comparison of ITS sequences of pure cultures obtained during this study with the ITS sequence for *C. ramicola* in GenBank. The diversity of *O. theobromae* and *C. ramicola* was studied. For this purpose an ITS sequence alignment file was prepared that contained all ITS sequences for both species whether from pure culture or DNA isolated directly from VSD-infected cacao plant materials as well as ITS sequences deposited in GenBank. A *Ceratobasidium* species was selected as an outgroup taxon. The dataset was analysed using maximum parsimony as described earlier and support for clades was assessed with bootstrap values obtained from 1000 replicates.

Testing for molecular clock for Oncobasidium theobromae *haplotypes*

An alignment containing ITS sequences of *O. theobromae* from Vietnam, Malaysia, and Indonesia and Papua was prepared (Fig 5). Modeltest v3.7 (Posada & Crandall 1998) was used to determine the appropriate likelihood substitution model. Using the substitution model found by Modeltest and likelihood criterion in PAUP, two maximum likelihood trees were constructed: one rooted and the other unrooted. The difference in log likelihood scores of the two trees ($2\Delta L$, Statistic *Chi* square) was determined, where *L* is the negative log likelihood score of the trees with degree of freedom equal to taxon numbers minus 2. The *P* value was determined using a *Chi* square interactive calculator available at http://www.fourmilab.ch/rpkp/experiments/analysis/chiCalc.html. A *P* value of 0.05 or less was considered significant to reject the molecular clock.

Results

Identification of Oncobasidium theobromae

Twelve pure cultures with hyphal characteristics of *Ot* were obtained from cacao (Fig 1). Sequences of *Ot* obtained from VSD-infected cacao trees in Malaysia, Indonesia (Java), and Vietnam were previously deposited in GenBank (National

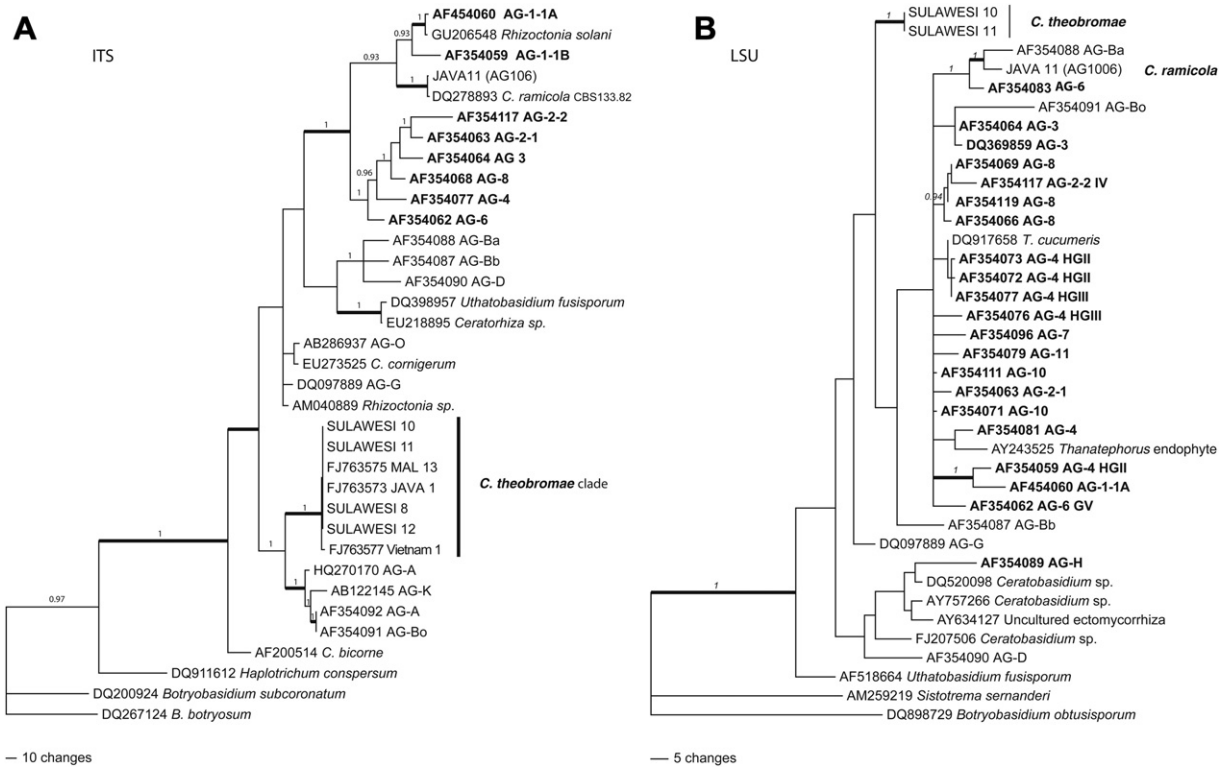

Fig 4 – (A and B) Phylogeny of *Ceratobasidium theobromae* and closely related species based on ITS (A) and LSU (B) regions of rDNA. Trees were obtained using Bayesian analyses. Unnamed GenBank accession numbers in bold refer to *Thanatephorus*; otherwise *Ceratobasidium*. Numbers on the branches refer to Bayesian posterior probabilities ≥ 0.9. Thickened branch lines refer to bootstrap values ≥70 %. For each tree *Botryobasidium* sp. was used as outgroup. Scale bar represents expected substitutions per site.

Center of Biotechnology Information (NCBI), www.ncbi.nlm.nih.gov) (P.M., A.P., Jaffar & P.K., unpubl.). The ITS sequences from four of our new Indonesian isolates (SULAWESI 8, 10–12; Fig 5, Table 1) were identical to each other and to sequences deposited in GenBank from Malaysia and Indonesia and differed by four base pairs (bp) from ITS sequences of *Ot* collected from infected cacao trees in Vietnam. As there is no extype culture of *Ot* we conclude that this fungus constantly associated with symptoms of VSD on cacao is *Ot*.

ITS sequences of eight of the 12 pure cultures (JAVA 6–12, SULAWESI 16; Fig 5, Table 1) had greater than 96.5 % identity compared to the ITS sequence deposited for the extype culture of *Ceratobasidium ramicola* (CBS 133.82, DQ2789310). This species of *Ceratobasidium* was identified from an additional six cacao samples from one location in Papua (Indonesia) using specifically designed *C. ramicola*-specific primers (not presented in this study).

Isolation and hyphal characteristics

Oncobasidium theobromae does not produce asexual or sexual spores in agar culture; it is recognized by distinctive features of the mycelium (Fig 1A–C) that include: broad (4.5–7.5 μm), slowly growing, white, sterile arachnoid hyphae; typically branching at right angles and having a small constriction at the point of departure of the branch and dolipore septum near the branch, and lack of clamp connections.

The strains identified as *Ceratobasidium ramicola* were readily distinguished from *Ot* by mycelium that quickly covered the surface of a 9-cm-diam Petri plate containing common media such as potato dextrose agar (PDA). Unlike *Ot*, cultures of this species can be preserved in 20 % glycerol at −80 °C. *Ceratobasidium ramicola* was isolated from cacao with symptoms of VSD in the Indonesian provinces of Papua, East Java, and West Sulawesi. Hyphae of these strains lack clamp connections (Fig 1D–F), are somewhat narrower (4.2–6.7 μm) than hyphae of *Ot*, and can anastomose. Hyphae of both *C. ramicola* (Fig 1E) and *Ot* are binucleate (Talbot & Keane 1971).

Phylogenetic analysis of sequence data

The ITS dataset contained 35 sequences obtained from GenBank for isolates deposited as *Thanatephorus* or *Ceratobasidium* representing different anastomosis groups (AG). The dataset also contained three sequences of *Botryobasidium* and one *Uthatobasidium*. The latter two genera have been shown to be sister to *Ceratobasidium* and *Thanatephorus* (Moncalvo *et al.* 2006; Rodriguez-Carres *et al.* 2010) and *Botryobasidium botryosum* was used as outgroup to root the tree. The dataset contained a total of 823 characters of which 138 ambiguously aligned characters were excluded from analysis. The remaining 685 had 222 parsimony informative characters representing 32 % of the total characters included in the analyses. The trees obtained by both parsimony and Bayesian methods were essentially

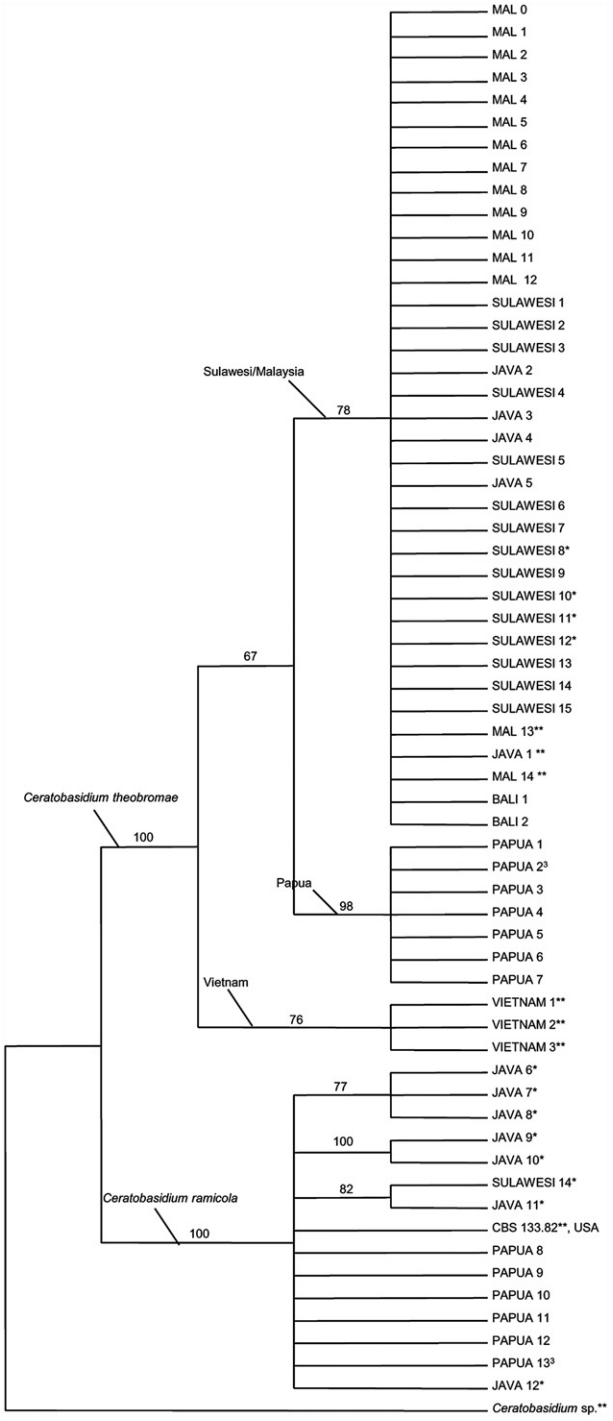

Fig 5 – Strict consensus phylogenetic tree based on ITS sequence showing diversity of *Ceratobasidium theobromae* and *C. ramicola* isolated from *Theobroma cacao*. The tree was constructed using parsimony analysis with consistency index Ci = 0.94, retention index = 0.99, steps = 153, bootstrap values above 50 % from 1000 replicates are above branches. * = sequences derived from pure cultures. ** = sequences obtained from GenBank. The Codes are explained in Table 1.

identical and therefore only the tree based on Bayesian analysis is presented (Fig 4A). Bootstrap values obtained from parsimony analyses are also presented on the Bayesian tree. All isolates deposited in GenBank as *Thanatephorus* that were included in this study formed a monophyletic clade that was supported by both pp and BS values (Fig 4A). However, isolates of *Ceratobasidium* did not form a monophyletic group suggesting that the genus is paraphyletic. This result was consistent with the study of Gonzalez *et al.* (2001) where *Ceratobasidium* was not resolved as monophyletic based on ITS and LSU sequence data. The four pure cultures of *Ot* (SULAWESI 8, 10, 11, and 12) isolated in this study were identical to sequences deposited for *Ot* from Malaysia and Java. However, there is a slight difference between these and GenBank sequences deposited for isolates from Vietnam. All *Ot* isolates formed a separate clade within the *Ceratobasidiaceae* that was highly supported by pp and BS. The *Ot* clade had a sister relationship with a clade of *Ceratobasidium* strains in anastomosis groups AG-A, AG-Bo, and AG-K, which were originally described from Asia but subsequently identified in the US.

One strain (JAVA 11), isolated from diseased cacao, had high identity (97 % with two nucleotide differences) to the extype culture of *Ceratobasidium ramicola* (CBS 133.82), and these two strains were resolved within the *Thanatephorus* clade. However, sequence analyses of additional protein coding regions of the genome are needed to better substantiate our findings.

The LSU dataset contained 38 taxa of *Botryobasidium*, *Ceratobasidium*, *Uthatobasidium*, and *Thanatephorus*. In this analysis we included sequences from two of our pure cultures of *Ot* (SULAWESI 10 and 11) because there are no deposited LSU sequences for this species in GenBank. The dataset contained 909 characters of which 113 were parsimony informative. The Bayesian tree (Fig 4B) did not provide resolution for genera of the *Ceratobasidiaceae*, including *Oncobasidium*.

These results indicate that *Ot* belongs to the family *Ceratobasidiaceae*. Consistent with the observation of Gonzalez *et al.* (2001) that *Ceratobasidium* is more diverse than *Thanatephorus* our results show a paraphyletic *Ceratobasidium* and a monophyletic *Thanatephorus*. *Oncobasidium theobromae* does not cluster with *Thanatephorus*, but it is closely allied to *Ceratobasidium* AG's A, Bo, and K, leading us to conclude that *Ot* is a unique species of *Ceratobasidium*. This is further supported by the presence of binucleate hyphal cells (Talbot & Keane 1971; Roberts 1999), a characteristic that defines *Ceratobasidium* (Gonzalez *et al.* 2001).

A new combination for *O. theobromae* in *Ceratobasidium* is indicated as:

Ceratobasidium theobromae (P.H.B. Talbot & Keane) Samuels et Keane, comb. nov.

≡*Oncobasidium theobromae* P.H.B. Talbot & Keane, Aust J Bot 19: 203. 1971.

≡*Thanatephorus theobromae* (P.H.B Talbot & Keane) P. Roberts, *Rhizoctonia*-forming fungi. A taxonomic guide p. 101. 1999.

For descriptions of this species see Talbot & Keane (1971) and Roberts (1999, as *Thanatephorus*).

Detection of Ceratobasidium theobromae *from cacao*

Twelve collections were sent to Beltsville from Indonesia (11) or Malaysia (one). These included six collections of terminal branchlets and leaves wrapped in plastic, and six collections

of plant tissue in TRIS buffer. The samples wrapped in plastic contained a variable number of pieces of plant material. Typically there were 100 samples in separate TRIS buffer vials for each collection. All sampling was done in the early months of 2009; the number of trees sampled was not recorded. At the end of the project, twenty 5-cm-diam Petri plates containing pieces of cacao leaf lamina or petioles plated onto water agar were also received. Approximately 400 DNA extractions were made from the plant tissue samples, among which 50 were positive for *C. theobromae* based on the PCR-based primers and assay developed in this study. Of the 20 Petri plates, hyphae characteristic of *C. theobromae* were seen in six and the remaining 14 plates were overgrown by other fungi. *Ceratobasidium theobromae* was confirmed in the six cases where typical mycelium was seen, and it was detected in 11 of the 14 'overgrown' Petri plates.

Our method was able to detect 8 pg of *C. theobromae* DNA from pure cultures (Fig 3). However, the sensitivity of the assay may be reduced by the presence of plant carbohydrates and proteins because cacao wood releases copious amounts of a viscous sugary material that potentially can interfere with DNA isolation and precipitation. This was a common problem with material that had been shipped in plastic vials containing TRIS buffer. Frequent changes (three changes each followed by incubation at 4 °C for 24 h) of the buffer prior to extraction improved isolation of DNA of *C. theobromae* from plant tissue (data not shown). Despite the high sensitivity of the method, *C. theobromae* was not detected in any of the cacao samples collected in Soppeng and only once from Pinrang (both South Sulawesi) or Kaliwining (East Java), all areas in which new symptoms of VSD have occurred in recent years (Purwantara *et al.* 2009). There was little difference in the ability to detect *C. theobromae* in tissue wrapped in plastic when compared to tissue shipped in TRIS buffer. *Ceratobasidium theobromae* was recovered from 5–30 % of the samples from most of the locations. The rate of detection was highest from the isolations made onto water agar in Petri plates from freshly collected material in Indonesia, even when there was no physical evidence of *C. theobromae* and the plates were overgrown by other species of filamentous fungi such as *Colletotrichum* or *Botryosphaeria*.

Ceratobasidium ramicola was isolated in pure culture from samples collected from cacao plants in East Java and South Sulawesi. Subsequently we determined that this fungus was common in Papua along with *C. theobromae*, and in two cases (PAPUA 3, PAPUA 13) both species were detected in the same sample of wood. *Ceratobasidium ramicola* was also isolated from cacao planted in widely separated areas of Indonesia (Papua, East Java, South Sulawesi).

Diversity of Ceratobasidium theobromae

Limited geographic sampling of diseased cacao was undertaken. This included presumed VSD-infected plants collected in Indonesian provinces of Bali (Lesser Sunda Islands), South Sulawesi (Sulawesi Island), Papua (Western New Guinea Island), and East Java (Java Island), and in Malaysia. GenBank sequences of *C. theobromae* (as *Oncobasidium theobromae*) from Indonesia (Java), Malaysia (Sabah State), and Vietnam were also included. All the ITS sequences of pure cultures of *C. theobromae* and ITS sequences obtained from infected cacao tissue including the six ITS sequences of *C. theobromae* from GenBank were subjected to phylogenetic analysis with parsimony (Fig 5 and Table 1). The dataset consisted of 63 strains (47 strains of *C. theobromae*, 15 strains of *Ceratobasidium ramicola*, and one outgroup). *Ceratobasidium* sp. GenBank GQ892615 was selected as outgroup as it was the most closely related accession to *C. theobromae*. The data had 847 characters, 712 were constant, 26 parsimony-uninformative and 109 parsimony informative. The tree (Fig 5) suggests three geographically distinct ITS haplotypes for the 47 strains of *C. theobromae*, respectively Vietnam (haplotype 1), Malaysia/Indonesia (haplotype 2: Java, Sabah, South Sulawesi), and Indonesia (Papua, haplotype 3). The respective groups were supported with bootstrap values greater than 70 % and did not share any polymorphisms. Testing for molecular clock, which determines mutation rate consistency of the haplotypes in the ITS region as described in the Materials and methods section, found a *P* value of 0.055, indicating that the clock was not rejected and an age for each of the nodes can be estimated. The uncorrected genetic distances between these haplotypes were 0.007, 0.013, and 0.015 for haplotype 1 *vs.* 2, haplotype 2 *vs.* 3, and haplotype 1 *vs.* 3, respectively. Using a substitution rate of 2.5×10^{-9} per site per year provided by Takamatsu & Matsuda (2004), the separation time between Vietnam and Malaysia/Indonesia was estimated at 1.5 million years whereas that between Vietnam and Papua at 3 million years. In the phylogeny (Fig 5) haplotype 1 clustered basally, suggesting that haplotypes 2 and 3 in this study may have originated in Vietnam.

Discussion

The VSD pathogen was originally described in the monotypic genus *Oncobasidium*, which was distinguished from both *Ceratobasidium* and *Thanatephorus* on the basis of basidial morphology (Talbot & Keane 1971). The colonization only of xylem vessels of cacao and formation of sexual fruiting structures (basidia and basidospores) on leaf scars of cacao following the emergence of hyphae from xylem vessels exposed by the abscission of infected leaves is uncharacteristic of pathogenic fungi that belong to the *Ceratobasidiales*. *Ceratobasidium theobromae* is also distinctive in being a nearly obligate parasite (biotroph) with minimal growth on standard nutrient media. A similar type of host dependence has been observed for certain isolates of *Ceratobasidium* and *Thanatephorus* from orchids (Andersen & Rassmussen 1996). The species is not known to have a *Rhizoctonia* anamorph. Andersen (1996) has suggested that sclerotia are of limited taxonomic utility for fungi with *Rhizoctonia* anamorphs and Roberts (1999) did not accept the argument, attributed by him in error to Talbot & Keane (1971), that pathogenic *Thanatephorus*-like species lacking sclerotia should all be referred to *Oncobasidium*, and those isolates that were nonpathogenic and lack sclerotia are *Uthatobasidium*. Therefore, he synonymized *Oncobasidium* with *Thanatephorus*, and transferred *Oncobasidium theobromae* to that genus. Results from Gonzalez *et al.* (2001) and here suggest that *Ceratobasidium* is paraphyletic and *Thanatephoprus* is monophyletic. Our phylogenetic analysis of two ribosomal

genes gives moderate support for the derivation of *O. theobromae* from within *Ceratobasidium* and that at least for now the appropriate genus for this fungus is *Ceratobasidium*. Thus *Oncobasidium* is placed in synonymy of *Ceratobasidium*. Placement of *O. theobromae* in *Ceratobasidium* is further supported by the fact that hyphae of *C. theobromae* are usually binucleate whereas hyphae of *Thanatephorus* are multinucleate (Talbot & Keane 1971; Roberts 1999) and wider. *Uthatobasidium* was erected to include nonpathogenic strains of *Thanatephorus*; thus would not be appropriate for *C. theobromae*. The best placement for *O. theobromae* is in *Ceratobasidium*, but additional studies are needed with additional genes to determine the relationship of the cacao isolates to other fungi with *Rhizoctonia* anamorphs. Clearly the quasi obligately biotrophic life style of this fungus poses a challenge to the better definition of its phylogenetic placement.

The only known common host for *C. theobromae* is *Theobroma cacao*, although avocado was reported as a host and presented similar disease symptoms to those found on cacao (Anderson 1989; Dennis 1991). The known geographic distribution of this fungus is Southeast Asia and Melanesia, from the island of New Britain east as far as Kerala (India), Thailand, Vietnam, and Hainan Island. The molecular clock estimation that we present here suggests that *C. theobromae* has been present in the region for a much longer time than cacao, which was first introduced *via* the Philippines in the 17th Century. VSD was first detected in Papua New Guinea and Malaysia in the 1960s (Shaw 1962; Keane & Prior 1991). Given the nearly obligately parasitic nature of *C. theobromae,* it has been hypothesized that the fungus has one or more native hosts whose distribution parallels the distribution of the disease on cacao (Keane & Prior 1991). The only known related fungi indigenous to the region are the *Rhizoctonia*-like endomycorrhizae that are commonly associated with orchids growing in the same geographic areas as cacao (Athipunyakom *et al.* 2004). In contrast to *C. theobromae*, orchid endomycorrhizal fungi are usually saprobic, although some orchid mycorrhizae are difficult to culture and are not competitive saprobes. It is likely that *C. theobromae* is sporulating undetected on a host plant in the rainforests of the region. The prospect of finding basidiomata in rainforests has proven very daunting because they are indistinct, short-lived and formed only sporadically (at least on cacao), and the VSD epidemics occurred on commercially grown cacao in Papua New Guinea and Malaysia for more than a decade before the fungus was observed sporulating on diseased plants. The PCR protocol developed in this study provides a rapid and reliable technique to detect the pathogen in cacao and possibly on putative native hosts on which it may cause minimal disease symptoms. The fact that the fungus was detected in 5–30 % of plant samples that were sent suggests either that there is a high rate of misdiagnosis of the disease or that the fungus is present but below the level of detection. A perhaps more reliable method of detecting *C. theobromae* was found when the pathogen was allowed to grow out from pieces of putatively infected plant material placed on water agar; even though many contaminating fungi obscured the *C. theobromae*, the PCR protocol was able to detect it with high reliability. This PCR method can also be used to further study the variability of the pathogen and limit spread of the disease by detecting the pathogen in cacao germplasm, which may not show signs of the pathogen and/or symptoms of infection for 3–5 m (Guest & Keane 2007).

The spread of the disease is *via* basidiospores discharged near midnight following the wetting of basidiomata by late-afternoon rain and is limited by the short life span and low numbers of the basidiospores (Keane 1981). The fungus is not transmitted in seed and has a very low probability of transfer in budwood (Keane & Prior 1991). Although VSD is common on the island of New Britain, it is not present 70 km to the east on the neighbouring island of New Ireland even though cacao is widely grown there (Keane & Prior 1991). The evidence supports the hypothesis that *C. theobromae* was repeatedly transferred from an indigenous host(s) to cacao planted in the widely separated areas of cacao plantings throughout the region. A long evolutionary history of the fungus with an indigenous host gives the possibility of many genetic lineages throughout the region. In view of this, it is surprising that until 2004 the distinctive symptoms on cacao (the chlorosis of leaves with green spots) were uniform throughout the region. The recent report of different disease symptoms in several areas of Southeast Asia (Purwantara *et al.* 2009) may be linked to transfer of new lineages of the fungus or to the occurrence of *Ceratobasidium ramicola* (or other pathogen(s)) on cacao reported here. However, it is difficult to explain the more-or-less simultaneous change in symptoms in such widely separated locations (Vietnam, Java, Sulawesi, Papua, Papua New Guinea) by a change in the genotype of the pathogen, which on the balance of probabilities is likely to be rare and therefore to occur in one location. Although the haplotypes that we report here may not be related to the development of new symptoms, the introduction of any haplotypes to new areas of established cacao could potentially result in that cacao being a 'new encounter' for that haplotype. Purwantara *et al.* (2009) observed morphological and biological differences in *C. theobromae* and our phylogenetic results add another dimension of distinction, all of which reinforces the need for extreme caution in distributing cacao seedlings or budding material in the area.

A second species of *Ceratobasidium*, *C. ramicola*, has not previously been reported from cacao but it has been reported as an orchid endomycorrhizal fungus in the region (Athipunyakom *et al.* 2004). Our identification of *C. ramicola* is based on shared sequence identity with CBS 133.82, the exholotype culture. *Ceratobasidium ramicola* was originally described from epiphytic growth on leaves of *Pittosporum tobira* in Florida (Tu *et al.* 1969) and was synonymized under *Ceratobasidium cornigerum* (Bourdot) by Roberts (1999). The morphologically defined species *C. cornigerum* is paraphyletic because strains identified as *C. cornigerum* fall into several different anastomosis groups (Burpee *et al.* 1980; Rodriguez-Carres *et al.* 2010) and the species has a wide reported host association and geographic distribution (Farr & Rossman 2011). While the identity of *C. cornigerum* s. str. must await epitypification, we identify our cacao strains as *C. ramicola* based on DNA sequence identity to its extype strain. The ITS data presented here for the extype strain of *C. ramicola* clearly distinguish it from the morphologically similar *C. theobromae*. Rinehart *et al.* (2007) and M.A.C., (unpubl.) considered *C. ramicola* to be most closely related to AG-U of *Ceratobasidium* (Rinehart *et al.* 2007), a group that is characterized by having binucleate hyphal cells.

Ceratobasidium ramicola has a wide distribution on cacao in Indonesia, including the province of Papua, and was isolated from apparently healthy woody tissues as well as from terminal branchlets and petioles showing the variant symptoms of VSD (i.e. leaves having large necrotic lesions rather than chlorosis with distinct green islands). All the isolates from cacao in Papua showed the necrotic symptoms and yet some were identified based on ITS DNA sequence analysis as *C. ramicola* and some as *C. theobromae*. All isolates from cacao in Bali were obtained from plant tissue with necrotic symptoms and yet all were identified as *C. theobromae*. This represents the first report of VSD from Bali. The hyphal characteristics of *C. ramicola* when it first emerges from cacao tissue are indistinguishable from *C. theobromae*. Because neither is known to produce basidiospores in pure culture, it could be easy to confuse the two species and thus *C. ramicola* could have been overlooked as *C. theobromae*. However, subsequent growth of the two fungi on an agar culture medium is very different. We have not been able to maintain cultures of *C. theobromae*. These results are consistent with previous research (Keane & Prior 1991). Musa (1983) reported that cultures isolated from cacao in Malaysia and identified as *O. theobromae* were able to grow on coconut water agar, which we have not been able to reproduce with samples from Indonesia. This suggests either that *C. theobromae* in Malaysia has different and less stringent nutritional requirements than populations of the species found elsewhere, or that a fungus other than *C. theobromae* was the subject of that research. In contrast to *C. theobromae*, *C. ramicola* grows prolifically on standard nutrient media and can be preserved in 20 % glycerol at −80 °C. Currently, we do not know whether *C. ramicola* is a pathogen of cacao but we note that it was commonly isolated from cacao in East Java (Kaliwining), one of the areas cited by Purwantara *et al.* (2009) as experiencing new disease symptoms. It was isolated from cacao in two of eight samples obtained from plants with VSD symptoms. Pure cultures of this *Ceratobasidium* were isolated from the vascular tissue of wood of diseased cacao in South Sulawesi and East Java. *Ceratobasidium ramicola* represents a species complex that includes pathogens of azalea and rose in Japan and the USA, respectively (Hyakumachi *et al.* 2005; Rinehart *et al.* 2007) and more recently on carrot in Japan (Tomo Misawa, Shiro Kuninaga & Takashi Toda, unpubl.).

While VSD can be devastating, causing the loss of cacao seedlings and even mature trees, the disease can be managed by, (1) the use of moderately resistant cultivars (Keane & Prior 1991; Efron *et al.* 2002) including types that have maintained their resistance since they were first selected in Papua New Guinea in the 1960s and still appear to be resistant despite the change in symptoms (Purwantara *et al.* 2009), and (2) good cultural practices, including the selection of planting locations based on appropriate distances from older diseased cacao, covering of plants in nurseries to exclude inoculum, pruning of cacao trees to open the canopy and maintain a lower humidity, and regular removal of infected branches. Identification of indigenous hosts would assist in VSD management, which must also include monitoring for the presence of *C. theobromae* and *C. ramicola* before distributing cacao seedlings or budwood for propagation. In the present work, we present a user-friendly protocol that should make this monitoring practical and easy. Research is in progress to determine what effect, if any, *C. ramicola* has on cacao and its possible relationship to the emergence of new symptoms of VSD.

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