MYCOLOGICAL RESEARCH 112 (2008) 1335–1343

British Mycological Society promoting fungal science
journal homepage: www.elsevier.com/locate/mycres

Trichoderma martiale sp. nov., a new endophyte from sapwood of *Theobroma cacao* with a potential for biological control

Rogério E. HANADA[a], *T. de JORGE SOUZA*[b], *Alan W. V. POMELLA*[c], *K. Prakash HEBBAR*[d], *José O. PEREIRA*[e], *Adnan ISMAIEL*[f], *Gary J. SAMUELS*[f,*]

[a]*Instituto Nacional de Pesquisas da Amazônia (INPA/CPPF), Av. André Araujo, 2936, Petrópolis, Caixa Postal 478, 69083-000 Manaus, AM, Brazil*
[b]*Universidade Federal do Recôncavo da Bahia (UFRB/CCAAB), 44380-000 Cruz das Almas, BA, Brazil*
[c]*Sementes Farroupilha, Caixa Postal 90, 38702-054 Patos de Minas, MG, Brazil*
[d]*Mars, Hackettstown, NJ 07840, USA*
[e]*Universidade Federal do Amazonas (UFAM/FCA), Av. General Rodrigo Octávio Jordão Ramos, 3000, Aleixo, 69077-000 Manaus, AM, Brazil*
[f]*United States Department of Agriculture, ARS, Systematic Mycology and Microbiology Lab, Rm. 304, B-011A, Beltsville, Maryland 20705, USA*

ARTICLE INFO

Article history:
Received 19 December 2007
Received in revised form
29 May 2008
Accepted 11 June 2008
Corresponding Editor:
David L. Hawksworth

Keywords:
Brazil
Black pod disease
Cacao
Diversity
Hypocrea
Hypocreales
New species
Phytophthora
Plant disease
Systematics

ABSTRACT

The new species *Trichoderma martiale* was isolated as an endophyte from sapwood in trunks of *Theobroma cacao* (cacao, *Malvaceae*) in Brazil. Based on sequences of translation-elongation factor 1-alpha (*tef1*) and RNA polymerase II subunit (*rpb2*) *T. martiale* is a close relative of, and morphologically similar to, *T. viride*, but differs in the production of discrete pustules on corn meal–dextrose agar (CMD) and SNA, in having a faster rate of growth, and in being a tropical endophyte. This new species was shown, in small-scale, *in situ* field assays, to limit black pod rot of cacao caused by *Phytophthora palmivora*, the cause of black pod disease.

Introduction

Cacao (*Theobroma cacao*, *Malvaceae*), an understory tree native to the upper Amazon region of South America, suffers from severe losses due to pests and diseases everywhere it is cultivated (Bowers *et al.* 2001; Bartley 2005). As part of a search for novel biological control agents, Hanada (2006) isolated 147 cultures of fungi from sapwood of trunks and branches of *T. cacao* in the Brazilian states of Amazonas and Bahia. He assayed them for their potential to protect cacao pods from *Phytophthora palmivora*,

---

[*] Corresponding author.
E-mail address: gary.samuels@ars.usda.gov

0953-7562/$ – see front matter © 2008 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.mycres.2008.06.022

a major cause of black pod disease in South and Central America, and ultimately focused on one for further study, *Trichoderma* sp. ALF 247. When cacao pods were preinoculated with this culture and then challenged by *P. palmivora*, symptoms of the disease were reduced relative to control pods that were not inoculated with the *Trichoderma*. Hanada also demonstrated that germination of conidia of ALF 247 was not affected by the presence of copper hydroxide fungicide, and that conidia could survive on the pod surface for as long as 80 d. Because ALF 247 reduced disease severity, and because its conidia remained viable on the surface of pods and could resist copper fungicides, it was decided that the potential of this culture for biological control should be evaluated in field trials. We report those results here.

The culture ALF 247 was initially identified as *T. viride*, but phylogenetic analysis based on sequences of translation-elongation factor *tef1* (Jaklitsch *et al.* 2006, as VB2, G.J.S. 04-40) placed it close to, but phylogenetically distinct from *T. viride*. In the current work, we examine the taxonomy and phylogenetic relationships of ALF 247, and propose it as a new species, *T. martiale*.

Materials and methods

Isolation

Isolations were made from sapwood of the trunk and branches of a cultivated tree of *Theobroma cacao* in the Brazilian state of Bahia following Evans *et al.* (2003). Bark was removed from the tree using a sharp, surface-sterilized knife; and immediately five small pieces of the freshly revealed sapwood, each *ca* 25 mm^2, were removed with a flamed scalpel and placed in a Petri plate containing 20 ml potato–dextrose agar (PDA) with 25 μg ml^{-1} chloramphenicol and incubated at 25 °C in darkness. Individual fungi were recovered as they grew out of the wood.

Phenotypic characterization

Characterization of the phenotype of ALF 247 followed the procedures described in Jaklitsch *et al.* (2006). The growth rate on two media PDA (Difco, Becton & Dickinson, Sparks, MD) and SNA (low nutrient agar, Nirenberg 1976, 1.0 g KH_2PO_4, 1.0 g KNO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 1.0 L distilled water, 20.0 g agar without filter paper) at five temperatures (15, 20, 25, 30, 35 °C) was determined. Morphological characters were taken from SNA or corn meal–dextrose agar [CMD; cornmeal agar (Sigma, St Louis, MO), + 20 g l^{-1} dextrose] grown at 25 °C in alternating darkness 12 h and cool, white, fluorescent light 12 h ('intermittent light'). Measurements of microscopic structures were made in distilled water or 3 % potassium hydroxide. Colour standards were from Kornerup & Wanscher (1978). Cultures represented in Table 1 are preserved in the Centraalbureau voor Schimmelcultures and/or the culture collection of G.J. Samuels (BPI).

Molecular characterization: DNA extraction and sequencing methods

To obtain fresh mycelium for DNA extraction, the isolates were grown in potato–dextrose broth (Difco, Detroit, MI) in a 5 cm diam Petri dish for 3–5 d at 25 °C. The mycelial mat was dried using clean, absorbent, paper towels. The entire dried mycelial mat was then placed in a 1.5 ml Eppendorf tube for immediate DNA extraction. Extraction of the genomic DNA was done using Puregene™ Genomic DNA Isolation Kit (Gentra Systems, Minneapolis, MN).

The gene regions studied were RNA polymerase II subunit (*rpb2*) and translation elongation factor 1α (*tef1*). The primers for *rpb2* were fRPB2-5F (5′-GA(T/C)GA(T/C)(A/C)G(A/T)GATCA(T/C)TT(T/C)GG-3′), fRPB2-7cR (5′-CCCAT(A/G)GCTTG(T/C)TT(A/G)CCCAT-3′) (Liu *et al.* 1999). Primers for *tef1* were Ef728 (forward primer): 5′-CATCGAGAAGTTCGAGAAGG (Carbone & Kohn 1999); Tef1R: (reverse primer) 5′-GCCATCCTTGGGAGATACCAGC (Samuels *et al.* 2002).

PCR amplifications were performed in a total volume of 25 μl reaction, which contained: 2.5 μl of 10 × PCR Buffer (New England Biolabs, Ipswich, MA) with $MgCl_2$ for final concentration of 1.5 mM of 0.2 mM dNTPs, 0.2 μM of forward and reverse primers, 1.25 units Taq polymerase (New England Biolabs), and 10–50 ng genomic DNA. Double-distilled water was added to a total volume of 25 μl per reaction. The reactions were placed in 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 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. An additional 35 cycles followed, each consisting of 30 s denaturation at 94 °C, a 30 s annealing at 48 °C, and a 1 min extension at 72 °C, concluding with a 10 min extension at 72 °C. The resulting products were purified with ExoSAP-it kit (USB Corporation, Cleveland, OH) using the procedures provided by the company. Sequences were obtained using the BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Products were analysed directly on a 3100 DNA sequencer (Applied Biosystems). Both strands were sequenced for each locus using the primers used in producing the PCR products. For *rpb2* two additional internal primers RPB-432F (5′-ATGATCAACAGAGGYATGGA) and RPB-450R (5′-TCCATRCCTCTGTTTGATCAT) were used in sequencing reactions. Sequences were edited and assembled using Sequencher 4.1 (Gene Codes, Madison, WI). Clustal X 1.81 (Thompson *et al.* 1997) was used to align the sequences, followed by manual adjustment of the alignment using McClade version 3.06 software (Maddison & Maddison 2001). Sequences are deposited in GenBank (Table 1).

Phylogenetic analysis

Datasets of *tef1* and *rpb2* were combined and analysed using MP and Bayesian likelihood criteria. The MP analysis was performed in PAUP version b10 (Swofford 2002) using a heuristic search, with a starting tree obtained *via* 1K random stepwise addition sequences, tree bisection–reconnection (TBR) as the branch swapping algorithm and MULTREEES off. BS values were calculated with 500 replicates under the conditions described above.

Mr Bayes 3.0b4 (Huelsenbeck & Ronquist 2001) was used to perform Bayesian analysis. The dataset was partitioned into two sets: *tef1* (1–642) and *rpb2* (643–1490). The evolution

Table 1 – Geographic origin and GenBank numbers of *Trichoderma* and *Hypocrea* cultures used in the study

Strain	Species	Geographic location	GenBank accession numbers		
			tef 1-α	Rpb2	ITS 1+2
DIS 328gi[a] (syn. CBS 120254, IMI 394148)	*Trichoderma* VB 1[b]	Ecuador	DQ315454		DQ307534
TR 21[a] (syn. ATCC 28038)	*Trichoderma* cf *viride* VB3[b]	USA, NC	AY376054	EU248595	AY380909
G.J.S. 90-95[a] (syn. IMI 352470)	*Trichoderma* cf *viride* VB3[b]	USA, VA	DQ307535	EU248596	DQ315455
ALF 247 (syn. G.J.S. 04-40, CBS 123052)	*T. martiale*	Brazil: BA	DQ307534	EU248597	DQ315454
G.J.S. 99-13 (syn. NRRL 6955)	*T. viride*	Finland	DQ288988	EU248598	DQ313155
CBS 101526	*T. viride*	Netherlands	AY376053	EU248599	AY376053
G.J.S. 04-21	*T. viride*	Sweden		EU252001	
TR 22 (syn. ATCC 28020)	*T. viride*	USA: WA	AY937449	EU252002	DQ109535
G.J.S. 04-369 (syn. CBS 119326)	*T. viride*	Austria	DQ307553		DQ323430
G.J.S. 98-16 (syn. CBS 240.63, ATCC 18652)	*T. viride*	UK	EU248620	EU252004	X93979
G.J.S. 99-14	*T. viride*	UK	EU248623	EU241494	EU263995
G.J.S. 97-271 (syn. BBA 70239)	*T. viride*	Denmark	AF348116	EU264003	DQ315456
G.J.S. 05-463	*T. viride*	UK	EU248621	EU252005	
G.J.S. 92-14 (syn. ICMP 16298)	*T. viride*	New Zealand	DQ288988	EU252006	DQ313155
G.J.S. 03-74	*T. scalesiae*	Galapagos Islands	DQ841726	EU252007	DQ841742
TR 5	*T. viridescens*	USA: OR	DQ307525	EU252008	DQ315443
ATCC 20898	*T. viridescens*	USA: NY	DQ307518	EU252009	DQ315434
CBS 274.79	*T. viridescens*	Austria	DQ307513	EU252010	DQ315428
G.J.S. 98-182	*T. viridescens*	Austria	DQ307511	EU252011	DQ315425
G.J.S. 89-142	*T. viridescens*	USA: NC	AY376049	AY376049	DQ109532, DQ315426
G.J.S. 94-9	*T. viridescens*	Taiwan	DQ307507		DQ315421
G.J.S. 99-86	*T. viridescens*	Australia: Victoria	DQ315421		DQ315432
G.J.S. 99-142	*T. viridescens*	Australia:	DQ307512		DQ315427
CBS 439.95	*T. viridescens*	UK	AY937413		DQ315439
CBS 142.95	*T. atroviride*	Slovenia	AF456891, AY376051	EU341801	AF456917
G.J.S. 96-32 (syn. CBS 112888, DAOM 231835)	*T. stilbohypoxyli*	Puerto Rico	AY376062	EU341805	AY380915
DIS 240m	*Trichoderma* cf. *stilbohypoxyli*	Ecuador	EU248622		EU263996
DIS 217i	*Hypocrea* cf. *rufa* VE[2]	Ecuador	DQ307549		DQ323420
G.J.S. 90-97	*H.* cf. *rufa* VE[2]	USA: NC	DQ307530	EU341808	DQ315449
G.J.S. 02-78	*T. intricatum*	Sri Lanka	EU248630	EU241505	EU264002
G.J.S. 93-20 (syn. CBS 112888, DAOM 231835)	*T. koningiopsis*	Cuba	DQ284966		DQ313140
DIS 205f (syn. IMI 385805, CBS 119068)	*T. koningioipsis*	Brazil	DQ288993		DQ323419
DAOM 222105	*T. koningiopsis*	Canada: ON	AY376042	EU341810	AY380901, DQ313146
DIS 326h (syn. IMI 393639, CBS 119070)	*T. koningioipsis*	Ecuador	DQ288997		DQ379015
DIS 229d (syn. IMI 391590, CBS 119069)	*T. koningiopsis*	Ecuador	DQ284971		DQ313143
G.J.S. 04-11	*T. koningiopsis*	USA: TX	DQ289009		DQ323421
G.J.S. 90-18 (syn. CBS 988.97)	*T. koningii*	USA: WI	DQ289007	EU248600	DQ323409
CBS 979.70	*T. koningii*	Netherlands	DQ288994	DQ641671	DQ323410
G.J.S. 99-202 (syn. ICMP 16288, CBS 119089)	*H. dorotheae*	New Zealand	DQ307536	EU248602	DQ313144, DQ313145
DAOM 230019	*T. erinaceus*	Thailand	AY750880	EU248603	DQ083009
DIS 7 (syn. IMI 393635)	*T. erinaceus*	Peru	DQ109547	EU248604	DQ109534
DAOM 166162	*T. strigosum*	USA: NC	AY750887	AF545552	Q083016
DIS 173k (syn. IMI 385999)	*T. strigosum*	Brazil	DQ109545	EU248606	DQ109531
G.J.S. 05-02	*T. ? strigosum*	Cameroon	EU248631	EU248607	EU263997
G.J.S. 01-257 (syn. CBS 115283)	*H. pezizoides*	Thailand	AY937438		DQ000632

(*continued on next page*)

Table 1 – (*continued*)

Strain	Species	Geographic location	GenBank accession numbers		
			tef 1-α	Rpb2	ITS 1+2
DIS 321j	*T. hamatum*	Ecuador	EU248624	EU248609	EU263998
DIS 326f	*T. hamatum*	Ecuador	EU248625	EU248610	EU263999
DIS 65g	*T. hamatum*	Ecuador	EU248626	EU248611	EU264000
DAOM 167057	*T. hamatum*	Canada	F456911, AY750893	AF545548	Z48816
DAOM 166162	*T. pubescens*	USA: NC	AY750887	AF545552	DQ083016
G.J.S. 05-328	*T. asperellum*	Cameroon	EU248627	EU248614	EU264001
G.J.S. 04-105	*T. asperellum*	Vietnam	EU248628	EU248615	
G.J.S. 05-226	*T. asperellum*	Cameroon	EU248629	EU248616	
TR 3 (syn. CBS 433.97, ATCC 204424, BBA 70684, NBRC 101777)	*T. asperellum*	USA: MD	AF456907, AY376058	EU248617	X93981
G.J.S. 05-302	*T. asperellum*	Cameroon	EU248632	EU264004	
G.J.S. 90-22 (syn. IMI 393966)	*T. harzianum*	USA: WI	AF443933	AY391925	AF443915
G.J.S. 02-76 (syn. CBS 114232, ATCC MYA-3221, DAOM 232830)	*H. catoptron*	Sri Lanka	AY737726	AY391900	AY737766

The reported growth rate is the average of three replications of the growth trial over three successive weeks. Continuous measurements are reported as the means plus and minus the standard deviation of 30 measured units, the extremes in brackets.

a DIS, TR, and G.J.S. cultures are in the culture collection of the USDA-ARS, Systematic Mycology and Microbiology Laboratory (BPI).

b From Jaklitsch *et al.* (2006).

model for each set was determined separately using Modeltest 3.7 (Posada & Crandall 1998). For both loci Modeltest selected the General Time-reversible (GTR+G+I, nst = 6) model under the output strategy of Akaike Information Criterion (AIC).

Bayesian analysis was started from a random tree using the program's default values for the PPs. Metropolis-coupled MCMC (MCMCMC) sampling was performed with four chains, three heated and one cold, these were run for 10M generations and a single tree was sampled randomly every 100th generation. Isolates G.J.S. 90-22 (*T. harzianum*, IMI 393966) and G.J.S. 02-76 (*Hypocrea catoptron*, CBS 114232) were used as outgroups and tree branch was saved. Post run plot of likelihood scores *versus* generation number was used to determine the burn-in phase of the run. The first 2K trees were discarded (as burn-in) and the rest (8001 trees) were pooled into PAUP and a 50 % majority-rule consensus tree was obtained with the support values for each branch constituting their PPs. Clades with PPs of $\geq$ 95 % were considered as significantly supported by the data (Leache & Reeder 2002).

Field assay

To prepare inoculum for field assay, ALF 247 was grown 7 d on PDA. Conidia were scraped from the plates into sterile distilled water and the suspension was adjusted to 10^7 conidia ml^{-1}. *Phytophthora palmivora* isolate 611 from a collection maintained at CEPLAC (Brazilian Cacao Research Institute) was the pathogen used in all experiments. It was grown on carrot agar medium (200 g carrots sliced, boiled for 1 h in 500 ml distilled water, passed through a fine sieve, and 15 g agar was added to the broth. The volume was made up to 1 l and autoclaved at 121 °C for 20 min) for 10 d in the dark and 3 d under near-UV light. To induce zoospore formation, each plate was flooded with 10 ml cold (4 °C) sterile distilled water and incubated in a refrigerator for 15 min followed by 30 min at room temperature. The zoospores were collected and the suspension was adjusted to 2×10^5 zoospores ml^{-1}.

In the first experiment, 20 cacao pods (genotype 'comum') from plantations established at Almirante farm, Itajuipe, BA, Brazil were sprayed with the *Trichoderma* conidial suspension. Four days after application of *Trichoderma*, the *Phytophthora* zoospore suspension was also sprayed onto the pods. Control pods were sprayed with sterile distilled water and 4 d later with the *Phytophthora* zoospore suspension. Pods were covered with a humid chamber prepared with plastic bags sprayed with sterile water 24 h before and 24 h after the application of *Trichoderma* and *Phytophthora*. The pods remained attached to the trees until the evaluation, which was done 7 d after the application of *Phytophthora*. The severity of black pod caused by *P. palmivora* was determined on a 1–5 scale, where 1 indicated no symptoms on the pod; 2 indicated localized lesions up to 2 mm diam; 3 indicated lesions in expansion with diameters varying from 2–20 mm; 4 indicated coalesced lesions with an area corresponding to up to 25 % of the pod surface; 5 when more than 25 % of the pod surface was covered with lesions (Hanada 2006).

The second experiment was conducted as described for the first experiment, except that 30 pods were used and an additional treatment with 10.8 g a.i. l^{-1} copper hydroxide (Dupont do Brasil, Barueri) was included.

Statistical analysis

The data of individual experiments were analysed using the one-way non-parametric Kruskal–Wallis test and comparison of the means was done using Wilcoxon's two-sample test as implemented in the program SAS version 9.1 (SAS Institute, Cary, NC).

Recovery of Trichoderma matiale from cacao trees and pods

Five cacao trees (genotype 'comum') from approximately 15-year-old field plantations established at Almirante farm were sprayed with 250 ml per tree of a suspension containing 10^7 conidia ml^{-1} of ALF 247. Recovery was estimated by plating 20 pieces of sapwood of approximately 25 mm^2 from the trunks of each tree, following the protocol of Evans *et al.* (2003). Similarly, 20 pieces from the surface and 20 pieces from the interior of surface-sterilized, two three- to five-month-old pods were evaluated. Isolations were done before spraying and after 5, 20, 35, 55, 80, and 110 d of spraying. The number of pieces colonized by ALF 247 was confirmed under the microscope after its sporulation on the plated fragments.

Results

Phylogenetics

In an earlier work (Jaklitsch *et al.* 2006), based on sequences of *tef1*, ALF 247 (as Vb 2 = G.J.S. 04-40), was shown to form a well-supported, unresolved sister group to *Trichoderma viride*, where it joined an Ecuadorian trunk endophyte of *Theobroma gileri* DIS 328gi (Vb 1), and a clade (Vb 3) that comprised a *Hypocrea* (G.J.S. 90-95) and a soil *Trichoderma* (Tr 21). To confirm this relationship, we sequenced ITS, *tef1* and *rpb2* for ALF 247 and its closest relatives, in addition to reference strains for most of the recognized species in the VIRIDE clade (Samuels 2006); new sequences are deposited in GenBank (Table 1). We used the combined alignment sequences of *tef1* and *rpb2* in the analysis based on evidence that combining gene sequences can improve the accuracy of phylogenies relative to individual gene phylogenes (Darlu & Lecointre 2002). The combined

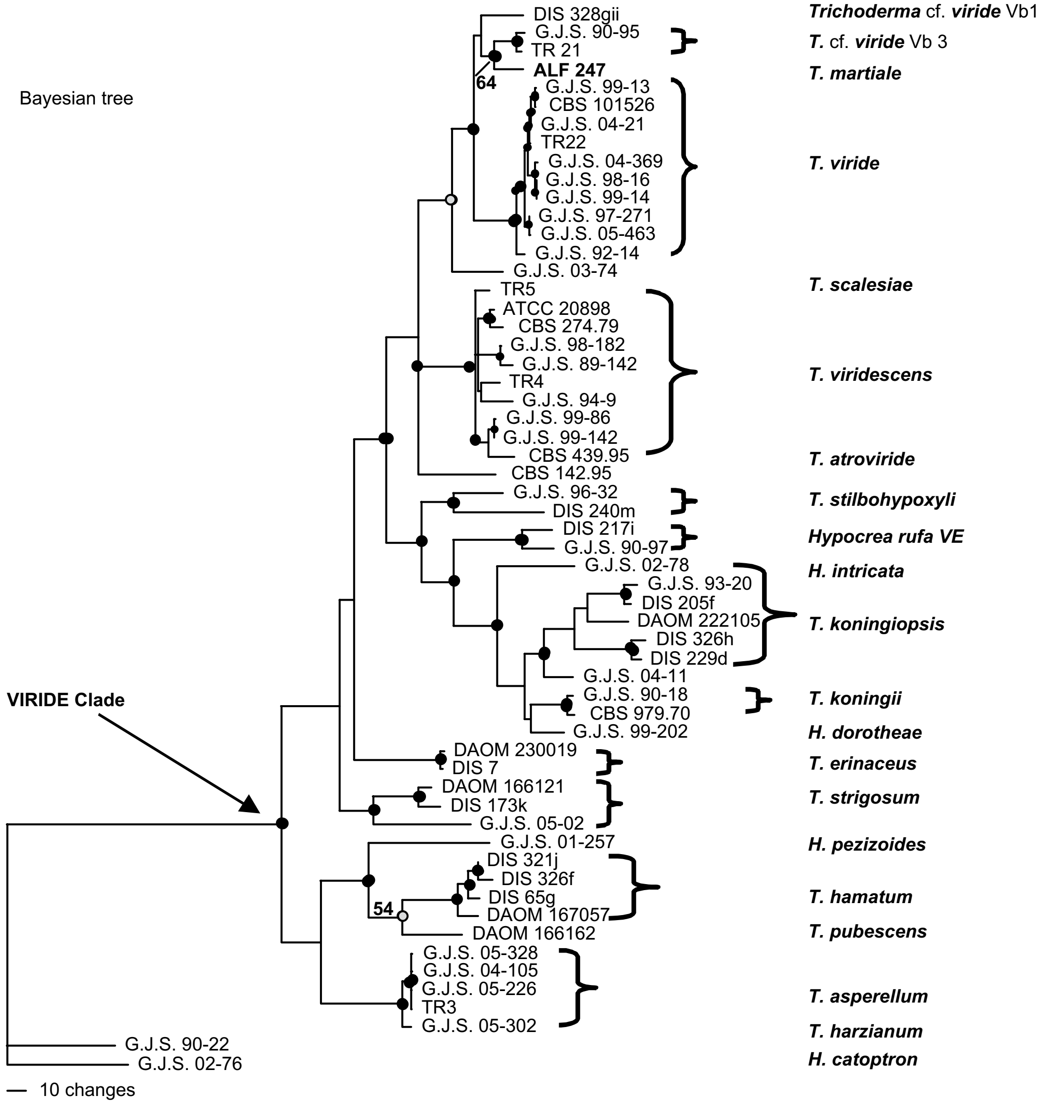

Fig 1 – Fifty percent majority-rule consensus tree of the Bayesian likelihood analysis of the combined *tef1* and *rpb2* data. Except where noted, nodes with filled black dots have a Bayesian PP ≥0.95 and a BS value ≥65 %; light grey nodes have Bayesian PPs ≥0.90 and a BS value ≥65 %.

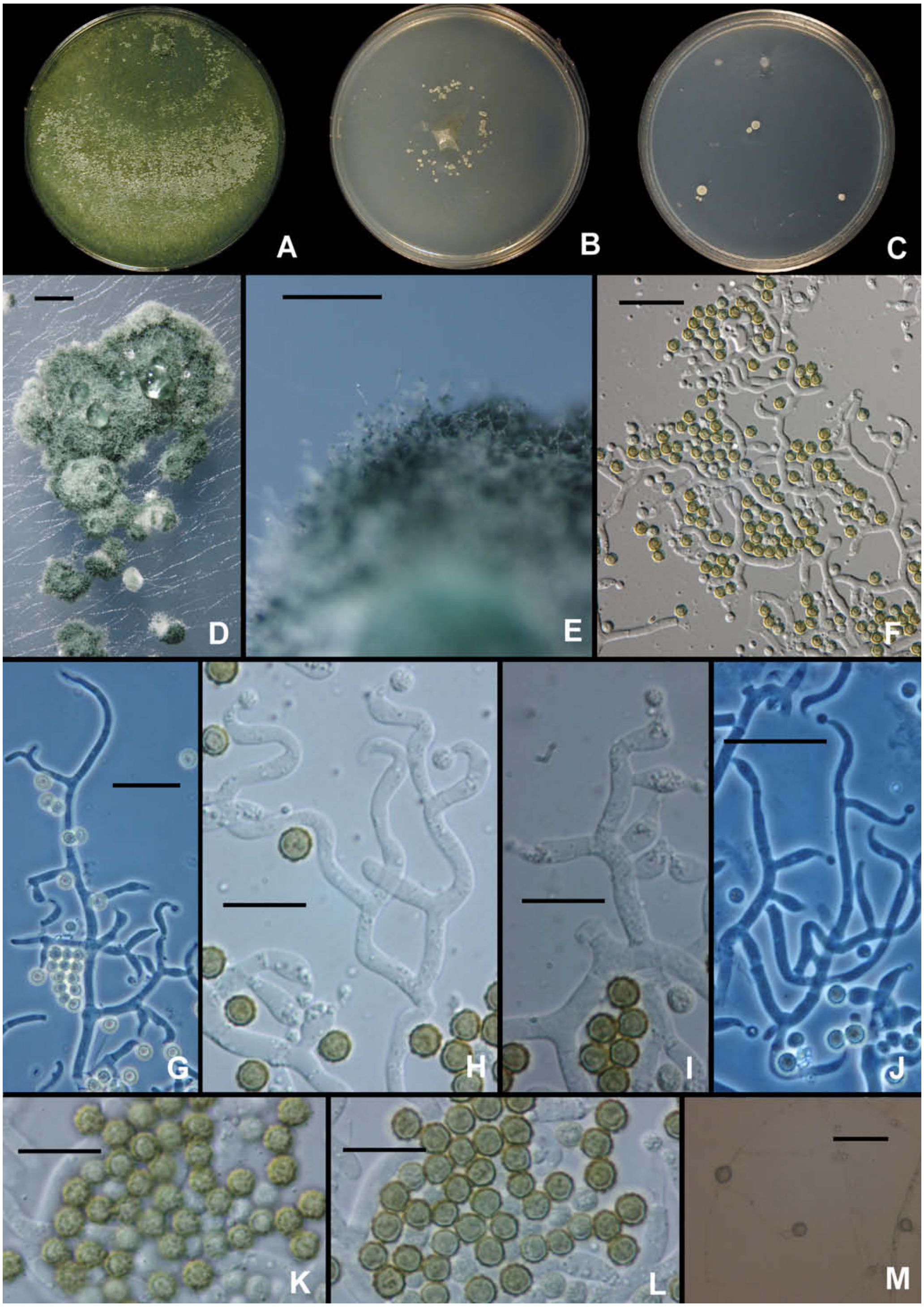

Fig 2 – *Trichoderma martiale.* (A–C) Colony grown at 25 °C, alternating 12 h darkness/12 h cool, white, fluorescent light. (A) PDA. (B) SNA. (C) CMD. (D–E) Conidium pustules on SNA. Projecting conidiophores seen in (E). (F–J) Conidiophores and phialides. (K–L) Conidia in surface view (K) and optical section (L). (M) Chlamydospores. (F–G, J) From SNA; (H–I, K–M) from CMD. Bars = (D) 1 mm, (E) 150 μm; (F–G, J, M) 20 μm; (H–I, K–L) 10 μm.

sequence alignment of the two loci, *tef1* and *rpb2*, included a total of 1490 bp; *tef1* had 642 characters of which 46 were excluded from analyses due to ambiguity in alignment. The remaining 596 characters had 310 constants, 71 parsimony uninformative, and 215 (36 %) parsimony-informative characters. The sequence of *rpb2* included 848 characters of which 611 were constant, 49 parsimony uninformative and 188 (22 %) were parsimony-informative. Analysis of the data using MP and Bayesian analysis yielded essentially identical trees and thus only the Bayesian phylogram is presented with BS values from the parsimony analysis included on the tree branches (Fig 1). The 50 % majority-rule phylogram based on Bayesian analysis with *T. harzianum* G.J.S. 90-22 and *Hypocrea catoptron* as outgroups showed that ALF 247 is a member of the VIRIDE clade. Along with the endophyte DIS 328gi, and G.J.S. 90-95 and TR21, ALF 247 formed a weakly supported clade (0.86 PP) that had strong sister relationship with a larger clade of *T. viride* (PP 1 and 96 % BS values). Within the weak clade, isolate ALF 247 formed a moderately supported subclade with taxa GJS 90-95 and TR 21 with 0.97 and 64 % PP and BS values, respectively. The present work confirms the phylogenetic distance of ALF 247 from its closest relatives and from *T. viride*.

The rDNA ITS sequences of the four isolates in the weakly supported clade were identical except for ALF 247, which differed from the other three by 1 bp. However, the ITS sequences of the many of species in the VIRIDE clade are identical, rendering this locus inappropriate for recognition of species within the clade (Jaklitsch *et al.* 2006), thus we did not include it in the phylogenetic analyses.

Phenotype

The isolate ALF 247 is typical of a clade that is characterized by rather large (3–4 μm diam), globose to subglobose, warted, green conidia and ± cylindrical phialides that are often solitary and hooked or sinuous and produced from conidiophores that are irregularly branched (Jaklitsch *et al.* 2006). The dominant, most common species in this clade is *Trichoderma viride*, the type species of *Trichoderma*. ALF 247 differs from its closest relatives (G.J.S. 90-95/TR21 and DIS 328gi) and *T. viride*, all of which have slower rates of growth. The phylogenetic species represented by G.J.S. 90-95/TR21 (Vb 3 in Jaklitsch *et al.* 2006) is phenotypically indistinguishable from *T. viride*. ALF 247 and DIS 328gi are phenotypically different from each other and from *T. viride*. Conidia of ALF 247 and DIS 328gi form on SNA and CMD in distinctive, large (2–3 mm diam), flat pustules; pustules in *T. viride* are not conspicuous or are at most cottony aggregates that are highly irregular in shape and smaller. ALF 247 in comparison to DIS 328gi has longer phialides (8.8 ± 1.2 μm vs. 5.8 ± 1.1 μm), and smaller phialide l/w ratio (2.3 ± 0.6 *versus* 2.1 ± 0.5). Conidiophores of ALF 247 and DIS 328gi are irregularly branched; however, the branches are more or less typical of the VIRIDE clade (Samuels *et al.* 2006a; Jaklitsch *et al.* 2006), wherein lateral branches, which increase in length with distance from the tip, are far more common in DIS 328gi than in ALF 247. Also, in DIS 328gi phialides tend to be held in whorls whereas in ALF 247 phialides tend to arise singly from the conidiophore.

Despite the morphological and phylogenetic similarity of ALF 247 and DIS 328gi to *T. viride*, we conclude that they are not *T. viride*. Even though the two endophytic *Trichoderma* cultures, ALF 247 and DIS 328gi, are phylogenetically and phenotypically very similar, their small differences indicate that they are not the same species. Accordingly, we describe ALF 247 as a new species, *T. martiale*. The taxonomy of DIS 328gi and the phylogenetic species represented by G.J.S. 90-95/TR21 will be dealt with in a future publication.

Reduction in black pod rot severity by Trichoderma martiale

Field assays were conducted to assess the severity of disease symptoms 7 d after the pods were sprayed with *Trichoderma martiale* strain ALF 247. In the first field assay, the effect of the application of ALF 247 was compared with water alone control and in the second assay a copper fungicide control was used in addition to the water control. ALF 247 significantly reduced the severity of black pod symptoms in both the assays (Fig 3). In the first assay, where 20 replicate pods were used, the disease index was 2.65 in the control treatment and was only 2.05 when treated with *T. martiale*. In the second experiment, where 30 pods were inoculated, and where copper fungicide was used as a fungicide control, the disease indices were 2.13, 1.4, and 1 for the control, *T. martiale,* and copper treatments, respectively. ALF 247 was significantly better than the control but not as good as the copper hydroxide fungicide.

Recovery of ALF 247 from cacao trees

The survival of ALF 247 on the various parts of the tree was estimated by conducting isolations from the surface and from the interior of pod tissue and also from the tree trunks over a period of approximately four months. Although *Trichoderma martiale* was recovered from 80–100 % of the surface of pod-piece samples during the first 55 days of the assay, the recovery was less than 5–10 % from within the pod tissue. The isolations from within the trunks of cacao trees indicated a steady initial rise (35 d) in the recovery rates followed by a steady decline. After being sprayed on cacao trees, *T. martiale* could be recovered for at least 80 d from the surface of pods and up to 110 d from within the tree trunks (Fig 4). There was no recovery of ALF 247 before it was sprayed, or from non-sprayed trees.

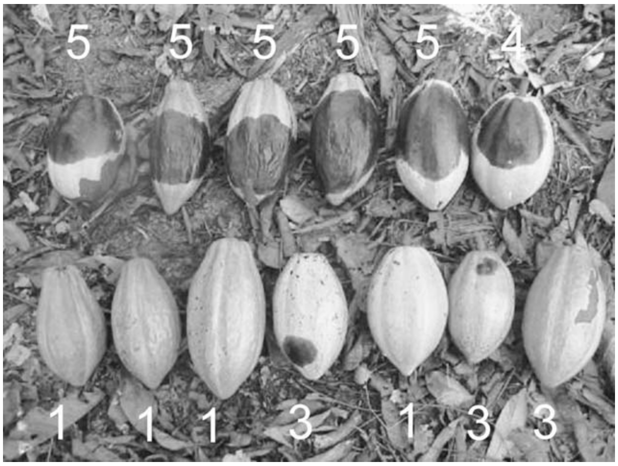

Fig 3 – Reduction in black pod disease severity by treatment with *Trichoderma martiale* under field conditions. Cacao pods were sprayed with water (upper row) or with a suspension containing 10^7 conidia ml^{-1} of *T. martiale* (lower row). For details see the text. Pods were detached from the trees just before being photographed.

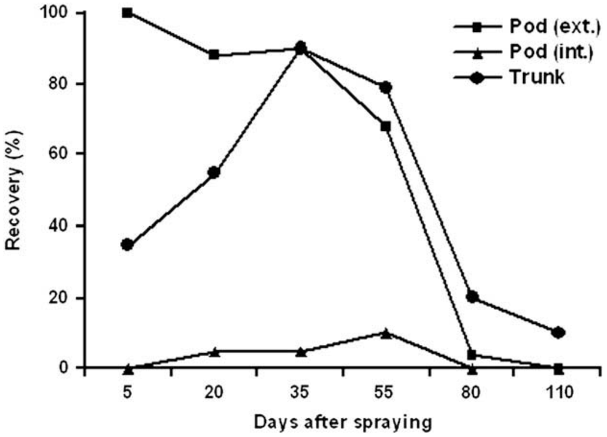

Fig 4 – Recovery of *Trichoderma martiale* 247 from cacao trees established in the field. Cacao trees were sprayed with a spore suspension and isolations were made before spraying (0) and after 5, 20, 35, 55, 80, and 110 d of spraying. The number of fragments from the trunk, pod surface (ext.) and interior of pods (int.) colonized by *T. martiale* were determined by examining its sporulation on the fragments.

Taxonomy

Trichoderma martiale Samuels, sp. nov. (Fig 2)
MycoBank no.: MB 5118877
Etym: Latin '*martiale*' = bellicose, referring to the apparent ability of this species to protect cacao pods from the parasite *Phytophthora palmivora*.

Trichodermae viridae Pers simile sed in agaro dicto PDA et SNA magis celeriter crescens; in regione tropicali inventum.

Typus: Brazil: *Bahia*, Inema, isolated from sapwood of trunk of a tree of *Theobroma cacao,* 2004, *A. Pomella* ALF 247 (dried colony on SNA, BPI 878377—holotypus; live culture G.J.S. 04-40 = CBS 123052).

Optimum temperature for growth on PDA and SNA 25–30 °C; after 72 h on PDA colony radius 45 mm, on SNA 30–35 mm; not growing at 35 °C. Colonies grown on PDA not forming conidia within 96 h; colonies grown on SNA forming conidia within 96 h at (25–)30 °C. Colonies on CMD after one week: conidia in scattered, 2–3 mm diam, grey–green pustules. Colonies on PDA after one week: forming in three rings of yellow–green pustules, pustules more green (K&W 28D) toward the colony margin and more yellow (K&W 1A7) toward the colony centre; pustules 1–2 mm diam, forming in the aerial mycelium, densely disposed in the concentric rings; no pigment forming; no distinctive odour noted. Colonies on CMD after one week: forming few, large (4–6 mm diam), flat pustules; pustules with a white fringe, conidia K&W 27E6. On SNA after one week at 25 °C: colonies similar to CMD but pustules more abundant and disposed in a ring, and smaller (1–2 mm diam); conidia darker green (K&W 27F8). No diffusing pigment or distinctive odour on CMD or SNA. Pustules on CMD and SNA compact, easily removed from agar surface, uniformly woolly or cottony, lacking projecting conidiophores or sterile hairs. Pustules formed of intertwined, 2.5–3 μm wide conidiophores; conidiophores highly irregularly branched; phialides arising singly from conidiophores or in pairs and terminating fertile, unicellular branches; occasionally more 'typical' *Trichoderma* conidiophores seen with two or three levels of lateral branches, the lateral branches increasing in length with distance from the tip. Phialides cylindrical or lageniform and slightly swollen in the middle, often hooked at the tip or sinuous, (6–)7–10.5(–12) μm long, (1.5–)2.5–3(–3.5) μm at the widest point, L/W = (1.5–)2–4(–6), (1.5–)2–2.5(–3.5) μm at the base; arising from a cell (1.5–)2.5–3 μm wide. Conidia globose to subglobose, (3–)3.5–4 (–6) × (3–)3.5(–4.5) μm, L:B = (0.7–)0.9–1.2(–1.3). Chlamydospores forming abundantly on CMD after one week at 25 °C.

Habitat. Isolated as an endophyte from sapwood of *Theobroma cacao* in Brazil (Bahia). Known only from the holotype.

Discussion

We originally identified the endophytic culture ALF 247 as *Trichoderma viride* based on its morphology. However, on closer inspection, we found biogeographic and phenotypic differences. *T. viride* is primarily found in temperate parts of the northern hemisphere, rarely found outside of North America and Europe (Jaklitsch *et al.* 2006), despite much past literature that reported a cosmopolitan distribution for the species, whereas *T. martiale* is found near the Equator as an endophyte. Combined sequences of the *tef1* and *rpb2* datasets confirmed that ALF 247 is distinct from the *T. viride*. Facets of its biology (endophyte in sapwood), biogeography (tropical), morphology (conidia arising from discrete pustules), and the faster growth rate reinforce this distinction. Although describing a species based on a single individual is not ideal, the combined molecular and phenotypic data justify recognizing ALF 247 as a new species.

Phytophthora species are the greatest cause of loss in cacao, ranging from 30–90 % of the crop depending on local conditions (Brasier & Griffin 1979; Bowers *et al.* 2001, Nyasse *et al.* 2007). In West and Central Africa, *P. megakarya* is in an invasive phase and can cause losses of up to 100 % on a cacao farm. Various fungi have been investigated as potential biological control agents of black pod disease of cacao. In Cameroon, a soil isolate of *T. asperellum* reduces the incidence of pod rot caused by *P. megakarya*, most probably by its mycoparasitic activity while enhancing fruit set (Tondje *et al.* 2007). The cacao leaf endophytes *Colletotrichum gloeosporioides, Clonostachys rosea,* and *Botryosphaeria ribes* limit foliar damage in cacao caused by *P. palmivora* in Panama (Arnold & Herre 2003; Herre *et al.* 2007; Mejía *et al.* 2008). Krauss & Soberanis (2002) cited *C. rosea* and an unidentified *Trichoderma* species as reducing black pod disease in Peru. However, apart from *T. asperellum,* none of these isolates has been able to protect the pods as efficiently as chemical fungicides. This may be due to their inability to colonize the pod tissue and offer long-term protection.

T. martiale survives on the pod surface for as long as 80 d and can establish an endophytic association that can be detected as much as 3.5 months after its inoculation into trees. Moreover, *T. martiale* can reduce the severity of symptoms caused by *P. megakarya* on pods in the field. With these two abilities, *T. martiale* joins the growing list of *Trichoderma* species that have potential for incorporation into IPM schemes for the control of diseases of cacao. Next steps include large-scale field trials that will determine the ability of *T. martiale* to protect pods over time, and studies of the mode of action. Although the path from successful field trials to a product that can be distributed to farmers is long and difficult, the potential benefits to the crop, including adding value and diminishing environmental damage caused by chemical fungicides, are well worth the effort.

Acknowledgements

We appreciate the support of Mars in parts of this research. An anonymous reviewer corrected the Latin description.

REFERENCES

Arnold AE, Herre EA, 2003. Canopy cover and leaf age affect colonization by tropical fungal endophytes: ecological pattern and process in *Theobroma cacao* (*Malvaceae*). *Mycologia* **95**: 388–398.

Bartley BGD, 2005. *The Genetic Diversity of Cacao and its Utilization*. CABI Publishing, Wallingford.

Bowers JH, Bailey BA, Hebbar PK, Sanogo S, Lumsden RD, 2001. The impact of plant disease on world chocolate production. *Plant Health Progress* doi:10.1094/PHP-2001-0709-01-RV. http://www.plantmanagementnetwork.org/sub/php/review/cacao/Bowers.pdf.

Brasier CM, Griffin MJ, 1979. Taxonomy of '*Phytophthora palmivora*' on cocoa. *Transactions of the British Mycological Society* **72**: 111–143.

Carbone I, Kohn LM, 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* **91**: 553–556.

Darlu P, Lecointre G, 2002. When does the incongruence length difference test fail? *Molecular Biology and Evolution* **19**: 432–437.

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS, 1991. Touchdown PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research* **19**: 4008.

Evans HC, Holmes KA, Thomas SE, 2003. Endophytes and mycoparasites associated with an indigenous forest tree, *Theobroma gileri*, in Ecuador and a preliminary assessment of their potential as biocontrol agents of cocoa diseases. *Mycological Progress* **2**: 149–160.

Hanada RE, 2006. *Controle de Phytophthora palmivora, agente causal da podridão-parda dos frutos de cacaueiro com fungos endofíticos*. PhD thesis, Universidade Federal do Amazonas, Manaus.

Herre EA, Mejia LC, Kyllo D, Rojas EI, Maynard Z, Butler A, Van Bael S, 2007. Ecological implications of anti-pathogen effects of tropical fungal endophytes and mycorrhizae. *Ecology* **88**: 550–558.

Huelsenbeck JP, Ronquist F, 2001. Mr Bayes: Bayesian inference of phylogeny. *Bioinformatics* **17**: 754–755.

Jaklitsch WM, Samuels GJ, Dodd S, Lu BS, Druzhinina IS, 2006. *Hypocrea rufa/Trichoderma viride*: a reassessment, and description of five closely related species with and without warted conidia. *Studies in Mycology* **56**: 135–177.

Kornerup A, Wanscher JH, 1978. *Methuen Handbook of Colour*, 3rd edn. Eyre Methuen, London.

Krauss U, Soberanis W, 2002. Effect of fertilization and biocontrol application frequency on cocoa pod diseases. *Biological Control* **24**: 82–89.

Leache AD, Reeder TW, 2002. Molecular systematics of Eastern Fence lizard (*Sceloporus* undulates): a comparison of parsimony, likelihood and Bayesian approaches. *Systematic Biology* **51**: 44–68.

Liu YJ, Whelen S, Hall BD, 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. *Molecular Biology and Evolution* **16**: 1799–1808.

Maddison DR, Maddison WM, 2001. *MacClade 4: Analysis of Phylogeny and Character Evolution (version 4.06)*. Sinauer Associates, Sunderland MA.

Mejía LC, Rojas EI, Maynard Z, Van Bael S, Arnold EA, Hebbar P, Samuels GJ, Nancy Robbins N, Edward Allen Herre EA, 2008. Endophytic fungi as biocontrol agents of *Theobroma cacao*. *Biological Control* **46**: 4–14.

Nirenberg HI, 1976. Untersuchungen über die morphologische und biologische Differenzierung in der *Fusarium*-Sektion *Liseola*. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin-Dahlem* **169**: I–v, 1–117.

Nyasse S, Efombagn MIB, Kébe BI, Tahi M, Despréaux D, Cilas C, 2007. Integrated management of *Phytophthora* diseases on cocoa (*Theobroma cacao* L): impact of plant breeding on pod rot incidence. *Crop Protection* **26**: 40–45.

Posada D, Crandall KA, 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 917–918.

Samuels GJ, 2006. *Trichoderma*: systematics, the sexual state, and ecology. *Phytopathology* **96**: 195–206.

Samuels GJ, Dodd SL, Gams W, Castlebury LA, Petrini O, 2002. *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. *Mycologia* **94**: 146–170.

Samuels GJ, Dodd S, Lu BS, Petrini O, Schroers H-J, Druzhinina I, 2006a. The *Trichoderma koningi* aggregate species. *Studies in Mycology* **56**: 67–133.

Swofford DL, 2002. *PAUP*: phylogenetic analysis using parsimony (*and other methods)*, version 4.06b10. Sinauer Associates, Sunderland, MA.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, 1997. The ClustalX windows interface; flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**: 4876–4882.

Tondje PR, Roberts DP, Bon M-C, Widmer T, Samuels GJ, Nyemb-Tschombe E, Ndoumbe-Nkeng M, Bateman RP, Fontem D, Hebbar KP, 2007. Isolation and identification of mycoparasitic isolates of *Trichoderma asperellum* with potential for suppression of black pod disease of cacao in Cameroon. *Biological Control* **43**: 202–212.