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

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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.

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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*,

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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 $\mu g\, ml^{-1}$ 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′-GCCATCCTTGGGAGA TACCAGC (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

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