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Genetic and metabolic biodiversity of *Trichoderma* from Colombia and adjacent neotropic regions

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ABSTRACT

The genus *Trichoderma* has been studied for production of enzymes and other metabolites, as well as for exploitation as effective biological control agents. The biodiversity of *Trichoderma* has seen relatively limited study over much of the neotropical region. In the current study we assess the biodiversity of 183 isolates from Mexico, Guatemala, Panama, Ecuador, Peru, Brazil and Colombia, using morphological, metabolic and genetic approaches. A comparatively high diversity of species was found, comprising 29 taxa: *Trichoderma asperellum* (60 isolates), *Trichoderma atroviride* (3), *Trichoderma brevicompactum* (5), *Trichoderma crassum* (3), *Trichoderma erinaceum* (3), *Trichoderma gamsii* (2), *Trichoderma hamatum* (2), *Trichoderma harzianum* (49), *Trichoderma koningiopsis* (6), *Trichoderma longibrachiatum* (3), *Trichoderma ovalisporum* (1), *Trichoderma pubescens* (2), *Trichoderma rossicum* (4), *Trichoderma spirale* (1), *Trichoderma tomentosum* (3), *Trichoderma virens* (8), *Trichoderma viridescens* (7) and *Hypocrea jecorina* (3) (anamorph: *Trichoderma reesei*), along with 11 currently undescribed species. *T. asperellum* was the prevalent species and was represented by two distinct genotypes with different metabolic profiles and habitat preferences. The second predominant species, *T. harzianum*, was represented by three distinct genotypes. The addition of 11 currently undescribed species is evidence of the considerable unresolved biodiversity of *Trichoderma* in neotropical regions. Sequencing of the internal transcribed spacer regions (ITS) of the ribosomal repeat could not differentiate some species, and taken alone gave several misidentifications in part due to the presence of nonorthologous copies of the ITS in some isolates.

1. Introduction

Trichoderma species are predominant over wide geographic regions in all climatic zones, where they are significant decomposers of woody and herbaceous materials. They are characterized by rapid growth, an ability to assimilate a diverse array of substrates, and by their production of an array of antimicrobials. Strains have been exploited for production of enzymes and antibiotics, bioremediation of xenobiotic substances, and as biological control agents against plant pathogenic fungi and nematodes. Some species of *Trichoderma* can form intimate associations with plant roots, providing an endemic level of biological control or stimulating plant growth by producing soluble forms of mineral nutrients and growth-promoting metabolites (Altomare et al., 1999; Esposito and Da Silva, 1998; Katayama and Matsumura, 1993; Koenig et al., 1994; Sharon et al., 2001; Sivasithamparam and Ghissalberti, 1998; Yedidia et al., 2000).

The initial approach to understanding the diversity and relationships of species in *Trichoderma*, based on morphological observations, was made by Rifai (1969), and later by Bissett (1984, 1991a,b,c, 1992). *Trichoderma* is monophyletic (Kullnig-Grandiger et al., 2002), with teleomorphs in the genus *Hypocrea* or closely related Hypocreales. Studies on teleomorph–anamorph connections have demonstrated overlapping morphological characteristics among species in the anamorph genus *Trichoderma* (e.g. Chaverri and Samuels, 2003). Consequently, morphological distinctions are not reliable indicators of the degree of genetic divergence between species, and morphological observations alone are insufficient for accurate identification of species. More than 100 species are now recognized in *Trichoderma* (www.isth.info), most described over the last 10 years, and our extensive understanding of species in the genus is possible because of the multidisciplinary approaches and bioinformatics that have been used to understand the variability and diversity in *Trichoderma* and the *Hypocrea* teleomorph. Molecular and metabolic analyses are being used to recognize and characterize new species in *Trichoderma* and to understand the phylogenetic relationships within *Trichoderma* (Bissett et al., 2003; Chaverri et al., 2003a,b; Dodd et al., 2003; Kindermann et al.,

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1998; Kubicek et al., 2002; Kullnig et al., 2000, Lu et al., 2004; Druzhinina et al., 2005, 2006; Jaklitsch et al., 2006; Samuels et al., 2006).

Broad studies on the taxonomy of *Trichoderma* have been carried out in North America and some regions of Europe (e.g. Bissett 1991a,b,c, 1992), where the distribution of species is comparatively well known. Comparatively few comprehensive studies have been undertaken to assess the diversity of *Trichoderma* in areas where the biodiversity is poorly known. Kullnig et al. (2000) examined 76 isolations from Russia, Nepal and northern India, reporting five undescribed taxa. A similar study was carried out by Kubicek et al. (2002) in south-east Asia, in which seven new species were found among 96 strains (Bissett et al., 2003). In the same study isolates of *Trichoderma harzianum* revealed metabolic and genetic variability that may explain the broad distribution of this species aggregate in diverse habitats.

Studies on *Trichoderma* in neotropical regions have been performed for biocontrol of plant pathogens with economical importance in cacao plantations, orchards, coffee, flowers and rubber tree plantations (Castro, 1996; Carsolio et al., 1994; Esposito and Da Silva, 1998; Hebbar et al., 1999; Samuels et al., 2000, 2006), as well as to control the symbiotic fungus of the leaf-cutting ant *Atta cephalotes* (Ortiz and Orduz, 2001, Lopez and Orduz, 2003). A study by Samuels et al. (1998) on *Trichoderma* section *Longibrachiatum* revealed diversity in neotropical areas resulting in the description of new species in this section. Recent work undertaken to discover biocontrol agents in specific crops such as cocoa has resulted in identification of other new species in neotropical regions (Jaklitsch et al., 2006; Samuels et al., 2000, 2006; Holmes et al., 2004). However, methodical studies to identify prevalent species of *Trichoderma* in neotropical areas have not been undertaken. The objective of the current study is to understand the biodiversity and distribution of *Trichoderma* in Colombia and adjacent neotropical regions through a multidisciplinary approach using genetic, metabolic and morphological characters. Metabolic characters can validate genetic differences by demonstrating significant variation in the expression of phenetic characteristics. The combined study of genetic and metabolic characters is important in evaluating biodiversity, accounting for the diversity of both species and their genes, and has particular relevance when the organism has potential for economic impact. This is especially true in neotropical regions where the genetic diversity of *Trichoderma* is not well documented and the species have significant potential as effective bioinoculants and biological control agents.

2. Materials and methods

2.1. Collections

Soils from different locations in Colombia, Ecuador, Peru and Brazil were collected in sterile polyethylene bags and stored at 4 °C until isolation. Pure cultures of *Trichoderma* were isolated as described by Elad et al. (1981). One hundred nineteen isolates (116 from Colombia) were obtained and preserved in the Microorganism Culture Collection of Unidad de Biotecnología y Control Biológico (Corporación para Investigaciones Biológicas, Medellín). Sixty seven additional isolates from Guatemala, Mexico, Panama and Peru provided by Martha Christensen (University of Wyoming, Laramie) and John Bissett (Agriculture and Agri-Food Canada, Ottawa) also were included in this study. Collection information for all isolates is given in Table 1.

2.2. Morphological examination

Cultures were grown on 2% Oxoid™ malt extract agar (MA) and Oxoid™ potato dextrose agar (PDA) at 20 °C under ambient daylight conditions, or in a 12 h:12 h light:dark cycle under fluorescent and near-UV light. Linear growth rates were determined by placing fresh mycelial plugs near the edge of 9 cm diameter PDA plates, incubating at 20 °C, and measuring the colony radius at 24 h intervals. Microscopic observations and measurements were made from preparations mounted in lactic acid. Conidiophore structure and morphology were observed from macronematous conidiophores taken from the edge of conidiogenous pustules or fascicles when conidia were maturing, usually after 4–7 days of incubation. Conidial morphology and measurements were recorded after 14 days. Preliminary species identifications based on morphological observations were made by comparison with descriptions and keys in the recent taxonomic literature (Bissett 1984, 1991a,b,c, 1992; Gams and Bissett, 1998).

2.3. Metabolic analyses

Carbon assimilation and mitochondrial activity were investigated using Biolog FF MicroPlates™. The FF MicroPlate test panel comprises 95 wells with different carbon-containing compounds and a control well. Iodonitrotetrazolium violet (INT) is used as a redox dye to colorimetrically measure mitochondrial activity resulting from oxidation of metabolizable carbon sources. The oxidation of succinate to fumarate in the citric acid cycle, mediated by succinate dehydrogenase and FAD, causes INT to be reduced to a red-colored formazan dye with peak absorbance at 490 nm. In the current study, absorbance readings were taken at 490 and 750 nm. The 750 nm reading measures turbidity resulting from assimilation of the test substrate and mycelial production. Since the absorbance spectrum of hyaline mycelium is essentially level over the range from 490 to 750 nm, a corrected "redox" value for the production of formazan was obtained by subtracting the 750 nm reading (490–750 nm). Metabolic studies using the FF MicroPlates were performed using the protocol prescribed by the manufacturer (Biolog Inc., Hayward, CA). Conidial suspensions were prepared from ~7 days old cultures on 2% malt extract media and adjusted to 70% transmission at 490 nm (~1×10^5 conidia/ml). Microplates were inoculated with 100 µl/well of the conidial suspension, incubated at 26 °C in darkness, and read after 24, 48, 72 and 96 h incubation. Preliminary species identifications based on morphological observations were confirmed by comparison with a metabolic database for more than 1000 strains of *Trichoderma* (ECORC, Agriculture and Agri-Food Canada, Ottawa), including all *Trichoderma* species currently described. Statistical analyses were performed to study metabolic variation within species, and to compare closely related species groups for metabolic differences. Analyses of variance (ANOVAS) and canonical variate analyses were performed using SAS using data at 96 h (SAS Institute Inc., 1989).

2.4. DNA sequencing and phylogenetic analyses

DNA was isolated from mycelium of cultures on PDA using the UltraClean™ DNA isolation kit following the manufacturer's protocol (BioCan Scientific, Mississauga, ON). A region of nuclear rDNA, containing the internal transcribed spacer regions 1 and 2 and the 5.8S rRNA gene, was amplified for all isolates by polymerase chain reaction (PCR) using the primer combinations NS5-fw (5′-TGGAAGTAAAAGTCGTAACAAGG-3′) and NS4-rev (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990), in an automated temperature-cycling device (GeneAmp® 2700, Applied Biosystems, Foster City, CA), using the following parameters: 3 min initial denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 75 s primer annealing at 58 °C, 105 s extension at 72 °C, and a final extension period of 10 min at 72 °C. A 0.9-kb fragment of the 5′ end of the translation elongation factor-1α (*tef*) gene (eEF1a1) containing three introns was amplified using the primer pair *tef*71f (C