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Biodiversity of *Trichoderma* in Neotropics

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1. Introduction

Trichoderma species frequently 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 range 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. The main use of *Trichoderma* in global trade is derived from its high production of enzymes. *Trichoderma reesei* (teleomorph: *Hypocrea jecorina*) is the most widely employed cellulolytic organism in the world, although high levels of cellulase production are also seen in other species of this genus (Baig et al., 2003, Watanabe et al., 2006). Worldwide sales of enzymes had reached the figure of $ 1.6 billion by the year 2000 (Demain 2000, cited by Karmakar and Ray, 2011), with an annual growth of 6.5 to 10% not including pharmaceutical enzymes (Stagehands, 2008). Of these, cellulases comprise approximately 20% of the enzymes marketed worldwide (Tramoy et al., 2009). Cellulases of microbial origin are used to process food and animal feed, biofuel production, baking, textiles, detergents, paper pulp, agriculture and research areas at all levels (Karmakar and Ray, 2011). Most cellulases are derived from *Trichoderma* (section Longibrachiatum in particular) and *Aspergillus* (Begum et al., 2009). *Trichoderma* is also an efficient degrader of heteropolysaccharides such as xylan, and xylanases and mannanases are of importance in the production of fine paper (Watanabe et al., 2006). In addition, some strains of *Trichoderma* are agents of bioremediation, capable of assimilating heavy metals (Akhtar et al., 2009; Guillermina et al., 2002) and of degrading cyanide (Ezzi and Lynch, 2005) and pesticides with high persistence in the environment (Cross, 1999, Tang et al., 2009). The genus *Trichoderma* includes strains altogether producing an extremely wide range of metabolites, including compounds with antifungal activities (phenolic compounds, 6-α-pentyl-pyrone, viridofunginas, harzianopiridona), antibiotics (anthraquinone, harzianodiona, gliotoxin), plant growth regulators (ciclonerodiol, α-harzianopiridona-pentyl-pyrone), antimicrobial peptides including more than 200 peptaibols, and even viridiol phytotoxic compounds with potential pharmaceutical uses as anti-tumor and immunomodulatory compounds (harzianodiona and gliotoxin). These and other metabolites that are unclassified inhibitors and anti-virus agents expand the prospects of industrial, pharmaceutical or other commercial uses of this organism (Sivasithanparam and Ghisalberti, 1998, Supothina et al., 2007, Vinal et al., 2006, Xiao-Yan et al., 2006).

Many species of *Trichoderma* are closely associated with plant roots and specific strains may form endophytic associations with their plant host (Bailey et al., 2006, Evans et al., 2003, Hoyos-Carvajal et al., 2009b; Manesh et al., 2006, Sette et al., 2006, Viterbo & Chet 2006, Yedidia et al., 2000). As endophytes they are particularly effective biological control agents of fungi in the rhizosphere, producing antimicrobials, activating plant defence mechanisms, and stimulating plant growth and vigour by solubilizing minerals and providing other nutrients and growth regulating compounds (Alfano et al., 2007; Altomare et al. 1999; Sharon et al., 2001; Vinale et al., 2006, Woo et al., 2006, Yedidia et al., 2000). The multiple roles of *Trichoderma* in biotrophic decomposition, parasitism and endophytic associations are of particular importance to the sustainability of agricultural and natural ecosystems (Harman et al. 2004). However, one of the great impediments to the study of *Trichoderma* has been the incorrect and confusing application of species names, making comparisons and generalizations from many published studies unreliable (Kopchinskiy et al., 2005). In addition, different isolates of *Trichoderma* species may exhibit as much variation in metabolic activity as observed among species, making careful study of their biodiversity essential to fully exploit the potential of these fungi.

2. *Trichoderma* taxonomy, a tool to assess diversity

There remain many difficulties in the morphological identification of *Trichoderma* due to the homoplasy of morphological and phenetic characters, particularly among the *Trichoderma* anamorph forms (Chaverri & Samuels, 2003; Druzhinina *et al.,* 2006). For very many years since the genus was first described by Persoon in 1794, and connected to its sexual state by Tulasne and Tulasne in 1865, the genus continued without additions and was commonly assumed to comprise a single species, *T. viride.* This concept resulted in misleading species identifications which are still evident today. The type species, *T. viride sensu stricto,* is a relatively rare species more or less restricted in its distribution to regions in Europe and North America, and yet it is frequently cited as a native biological control agent in other regions (Jacklist *et al.*, 2006). Similarly, the widely reported *T. aureoviride*, for example, appears to have a limited distribution in northern Europe (Lieckfeldt *et al*., 2001).

Rifai (1969) made the initial approach to understand the diversity of *Trichoderma*, introducing the concept of "species aggregates" in *Trichoderma* and featuring Nine of them, but clarifying that these aggregate species could include multiple species not distinguishable by morphological characters. Later revisions of Bissett (1984, 1991 a, b, c, 1992) and Gams & Bissett (1998), increased the number of species based on morphological distinctions and made connections between anamorph and teleomorph states to include also some species previously placed in the genus *Gliocladium*. Studies on *Hypocrea* demonstrated the overlapping morphological characteristics among species in the anamorph genus *Trichoderma* (e.g. Chaverri and Samuels, 2003; Jaklitsch, 2009), definitively showing that morphological distinctions were not reliable indicators of the degree of genetic divergence between species, and that morphological observations alone were insufficient for accurate identification of species of *Trichoderma*.

To compensate for the lack of reliable morphological characters, research in *Trichoderma* biodiversity over the past 20 years has concentrated on the development of a variety of molecular markers to differentiate species, including isozymes, RAPDs, RFLP, AFLP and, most recently, the nucleotide sequences of various gene loci. The introduction of molecular tools resulted in greatly expanding the number of species recognized in *Trichoderma*. 104

species of *Trichoderma* are listed on the website of the International Commission on the Taxonomy of Fungi subcommision on *Trichoderma* and *Hypocrea* (www.isth.info, Druzhinina & Kopchinskiy 2008), and 193 named taxa are represented to date by sequences deposited in Genbank (www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=29859).

2.1 Species concepts

In the past, species in *Trichoderma* were defined primarily by the application of the concept Morphological Species Recognition (MSR), sometimes in combination with other phenetic characters. However, morphological identifications are highly prone to error due to the lack of definitive morphological characteristics and variations in culture. Consequently, perhaps 50% or more of the *Trichoderma* isolates deposited in culture collections may be incorrectly named based on morphological identifications. Furthermore, *Trichoderma* strains evidently cannot consistently be crossed to apply the Biological Species Recognition (BSR) concept based on their reproductive behavior. Therefore, Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (Taylor et al., 2000), based on the concordance of multiple gene phylogenies, is an attractive alternative to apply the Phylogenetic Species Concept (PSC) in recognizing species of *Trichoderma*.

2.1.1 Cryptic species or phylogenetic species

Complementary methodologies have been applied to differentiate and characterize *cryptic species* or *phylogenetic species* in a fungi, correlating morphological, biogeographic, biochemical, ecological and, most recently, phylogenetic traits (e.g. refs). Applying the PSC proposed by Taylor et al. (2000), Chaverri et al. (2003) examined the internal transcribed spacer regions of rDNA (ITS1 and ITS2), the large intron of the transcription elongation factor 1-α (*tef1a)*, and short fragments of the actin (*act*1) and calmodulin (*cal1*) exon sequences in *H. lixii/T. harzianum*, to determine seven phylogenetic lineages in *T. harzianum*. However, they declined to recognize the lineages as phylogenetic species since they could not be reliably distinguished morphologically. Similarly applying GCPSR, Samuels et al. (2006) found that the *T. koningii* species aggregate includes three well-separated main lineages defined by phenotypic characters, and further recognized twelve taxonomic species and one variety within the three lineages: (1) T. *koningii, T. ovalisporum* and the new taxa *T. caribbaeum* var. *caribbaeum, T. caribbaeum* var. *aequatoriale, T. dorotheae, T. dingleyae, T. intricatum, T. koningiopsis, T. petersenii and T. taiwanense*; (2) the new species *T. rogersonii* and *T. austrokoningii*, and (3) the new anamorph species *T. stilbohypoxyli*. Druzhinina et al (2010b) recently revisited the genetic diversity in *T. harzianum,* examining three unlinked gene loci for 93 strains isolated worldwide. Their data illustrated clearly the complex history of speciation in the *H. lixii-T. harzianum* species group, rejecting the anamorph/teleomorph combination in favour of separate species status for *H. lixii* and *T. harzianum*, with the phylogenetic position of most isolates not resolved and attributed to a diverse network of recombining strains lacking strict genetic borders. In a similar study employing multiple gene phylogenies and multiple methods of phenotype profiling, Druzhina et al. (2010a) demonstrated that isolates previously identified as *H. jecorina* comprised four phylogenetic species, including *H. jecorina/T. reesei sensu stricto* containing most of the teleomorph isolates and the wild-type strain of *T. reesei* (QM6a) that has subsequently been genetically modified and employed in biofuel production. Conversely, all of the strains isolated as anamorphs from soil were referred to *T. parareesei.* It becomes clear from these studies that

phylogenetic structure within these complex species groupings must be taken into account in selecting potential isolates to use in industrial applications. For example, although the name "*T. harzianum*" has been uniformly applied to the biological control agent in the past, there is now increasing evidence that several, genetically diverse species are used in biocontrol (Druzhinina and Kubicek, 2005).

2.2 Methods to identify species in *Trichoderma*

2.2.1 Morphological analysis

Different laboratories have used a variety of media culture for morphological observations in *Trichoderma*. In general, a relatively simple media, such as malt extract agar 2% (MEA), is useful for the production of conidia and the observation of complex branching conidiophores (macronematous). A rich culture medium such potato dextrose agar (PDA) is useful for observing pigment production and harvesting mycelium to isolate DNA. Conidiophore structure and morphology is observed from conidiophores taken from the edge of conidiogenous pustules or fascicles when conidia were maturing (usually after 4-7 days of incubation). The morphology and size of conidia should be observed at maturity after approximately 14 days of growth (Bissett 1984, 1991a, b, c, 1992; Gams and Bissett, 1998). The preliminary identification of species or species aggregates based on characteristic morphologies can be attempted using keys and descriptions in the available taxonomic literature (e.g. Gams & Bissett, 1998).

2.2.2 Molecular analysis

Initially, it was presumed that sequences of the ITS regions of rDNA were sufficient for identification of most fungal species (e.g. Lieckfeldt & Seifert, 2000). However, the popular application of BLAST (in Genbank for example) to identify species based on ITS sequence homologies can provide misleading identifications. Kopchinskiy et al. (2005) found numerous identification errors among sequences deposited in Genbank, which from time to time also does not include all species of the genus. It is now apparent that ITS alone is not sufficiently informative to resolve closely related species in *Trichoderma*, illustrated by the more recent studies employing GCPSR based on multiple gene phylogenies to resolve cryptic species. In addition, paralogous copies of RNA coding genes have been found in some genera of Hypocreales which can result in misleading identifications based on ITS alone (O'Donnell, 2000; Lieckfeldt & Seifert, 2000, Chaverry et al., 2003b, Hoyos et al., 2009a). Numerous genes have now been investigated for the application of GCPSR to resolve species limits in *Trichoderma,* with genes such as translation elongation factor 1-α (TEF), RNA polymerase II subunit B2 (RPB2), chitinase 18-5 (ECH42), calmodulin 1 (CALM1), actin, β-tubulin2 (TUBB2), LAS1 nuclear protein and ATP citrate lyase subunit A (ACLA) providing informative loci for multi-gene studies.

Other molecular tools have been developed for identification of *Trichoderma*. Druzhinina et al. (2005) presented a unique 'bar-code' system for *Trichoderma,* based on 'hallmark' regions in sequences of ITS 1 and 2, and using several of these oligonucleotide regions as genetic fingerprints. These are stored in a MySQL database and integrated with their TrichOKey program for identification (www.isth.info). This program can be used to supplement traditional identification methods. For other gene loci they have developed the program TrichoBLAST (Kopchinskiy et al., 2005), which allows alignment and comparison of sequences of ITS 1 and 2 and fragments of *tef*1α and RPB2. Complementing these methods

they developed TrichoMARK to detect one or more sequence fragments of these genes as phylogenetic markers. The latter program is capable of distinguishing the five groups of species haplotypes that have identical ITS 1 and 2 sequences, *viz*.: *T. tomentosum / T. cerinum, T. longipile / T. crassum, T. koningii / T. ovalisporum / T. muroiana, H. lutea / H. melanomagna,* and *T. longibrachiatum / H. orientalis / H. cerebriformis.* In the case of *H. lixii / T. harzianum*, the program detects intraspecific differences accurately in this cluster, which contains several putative phylogenetic species. The ISTH website (www.isth.info) also provides the primer sequences and protocols necessary for sequencing the genes used for identification.

2.2.3 Metabolic tests

These may be based on the profiles of particular enzyme classes such as chitinases or cellulases, although other metabolic profiling techniques have been developed to validate new species which can also potentially provide data on the ecological roles of species (e.g. Kubicek et al. 2003, Hoyos-Carvajal et al., 2009a). The latter studies employed Biolog FF ® microplates (Biolog Inc., Hayward CA) comprising 96 cells containing different carbon sources and redox reagents sensitive to the activity of the enzyme succinate dehydrogenase in the citric acid cycle. Photometry at 590 nm and 750 nm provide quantitative measurements of assimilation and growth (measuring mycelial density) and respiratory activity on the different substrates. The metabolic profiles obtained may be characteristic of species, and the assimilation of specific substrates may allow hypotheses on the ecological role of species. For example, the assimilation of polyols such as maltitol and adonitol could indicate activity of dehydrogenases relevant to survive droughty conditions.

3. *Trichoderma:* distribution and biogeography

Broad studies on the taxonomy and biodiversity 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 now reasonably well known, particularly for specific taxa or groups (Lieckefeldt et al., 2001). Some regions have been studied in detail, e.g. Wuczkowski et al., 2003, investigated the genetic diversity of a European river-floodplain landscape near Vienna, and Migheli et al., 2009 studied the biodiversity of *Trichoderma* in Sardinia, a Mediterranean hot spot of biodiversity, analyzing the influence of abiotic factors on the distribution of species *Trichoderma.* In the latter study, 482 strains of *Hypocrea/Trichoderma* were identified from undisturbed and disturbed environments (forest, shrub lands and undisturbed or extensively grazed grass steppes), with the finding that most of the strains were pan-European and/or pan-global species. Meinke et al., 2010 described the *Trichoderma* communities in rhizosphere of four varieties and transgenic lines of potato in Germany. They observed a heterogeneous distribution and varying diversity of *Trichoderma* dependent on soil characteristics, climate and management practices, in this case not related to the crop variety.

Studies in previously uninvestigated regions or habitats have frequently led to the discovery of new taxa. Kullning et al. (2000) examined 76 isolates from Russia, Nepal and North India, reporting seven species (*T. asperellum, T.atroviride, T. ghanense, T. hamatum, T. harzianum, T. virens* and *T. oblongisporum*) and five new taxa. They also found T. *harzianum* the most genetically diverse speices, with the *T. harzianum* complex representing the majority of isolates. A similar study was conducted by Kubicek et al. (2003) in Southeast Asia, where they reported *T. asperellum, T.atroviride, T. ghanense, T. hamatum, T. harzianum, T. koningii, T. spirale, T. virens, T. viride* and *H. jecorina* (anam: *T. reesei*), along with seven new species

among 96 isolates tested (Bissett et al., 2003). The *T. harzianum* complex was equally prevalent, exhibiting high metabolic and morphological variability that may explain the wide distribution of this species aggregate over different habitats (Kubicek et al., 2003).

Sadfi-Zouaoui et al., 2009, in a study encompassing four different bioclimatic zones in Tunisia, assessed the genetic diversity of endemic species of *Trichoderma* and their association to bioclimatic zones. *T. harzianum*, divided into six clades, was the prevalent species complex. *T. harzianum* and *T. longibrachiatum* were predominant in forest soils in north Tunisia; *T. harzianum, T. saturnisporum* and *Trichoderma* sp. indet. were isolated from forest soils in central Tunisia; *T. atroviride* and *T. hamatum* were found in cultivated fields in northeast Tunisia; and *T. harzianum* and *T. hamatum* were present in oasis soils in south Tunisia. Zhang et al. (2005) assessed the biodiversity and biogeography of *Trichoderma* in China, sampling four disparate regions: north (Hebei province), south-east (Zhejiang province), west (Himalayan, Tibet) and south-west (Yunnan province). *T. asperellum, T. koningii, T. atroviride, T. viride, T. velutinum, T. cerinum, T. virens, T. harzianum, T. sinensis, T. citrinoviride,* and *T. longibrachiatum* were identified along with two putative new species. This study revealed a north-south gradient in species distribution in eastern Asia. Tsurumi et al. (2010) explored the biodiversity of *Trichoderma* in Mongolia, Japan, Vietnam and Indonesia, isolating 332 strains and finding T. *harzianum, T. hamatum, T. virens* and *T. crassum* in most habitats. *T. koningiopsis, T. atroviridae* and *T. stramineum* also were frequently isolated, except in cool sites where they were replaced by *T. polysporum* and *T. viridescens.* In tropical areas *T. ghanense, T.brevicompactum* and *T. erinaceum* were prevalent. In addition they discovered five unidentifiable isolate groups and 26 singular unidentified strains.

The most comprehensive survey over any one biogeographic region was performed by Jaklitsch (2009, 2011). He employed three genetic markers to identify 620 specimens of *Hypocrea* occurring in 14 countries in temperate regions of Europe, identifying 75 species including 29 previously undiscovered, thus greatly expanding the number of species known in that region. His observations suggest that the biodiversity of *Hypocrea/Trichoderma* above soil exceeds the diversity residing in soil. He also speculated that the majority of species may be nectrotrophic on other fungi colonizing wood and bark. It now appears that the majority of *Trichoderma* species are capable of sexual recombination and form a teleomorph, and a comparatively smaller number may be clonally propagating agamospecies.

As a result of these recent discoveries, generalizations on the distribution of *Trichoderma* have become increasingly problematic. Their occurrence will be modulated by microclimatic components, substrate availability, rhizosphere associations, soil chemistry, complex ecological interactions and many other factors. The introduction of invasive species, biocontrol agents, and agricultural perturbations result in changes in specific patterns of distribution that cannot be clearly identified, as suggested by Migheli et al. (2009) in finding the colonization of pan-European pan-global *Hypocrea/Trichoderma* species on the island of Sardinia, which may or may not involve the displacement of native strains.

4. Diversity of *Trichoderma* in tropical America

Comparatively few comprehensive studies have been undertaken to assess the diversity of *Trichoderma* in neotropical regions. Since agriculture is a vital segment of local economies in the neotropics, most research in this region on *Trichoderma* has been directed to their biological control activities against phytopathogens. Studies have focused on biological control of plant pathogens with economical importance in cacao plantations,

orchards, coffee, beans, cotton, flowers and rubber tree plantations, (Castro, 1996, Carsolio *et al*., 1994, Hebbar *et al*., 1999; Hoyos-Carvajal et al., 2008; Rivas & Pavone, 2010; Samuels *et al*, 2000, Samuels *et al.,* 2006,), to control the symbiotic fungus of the leaf-cutting ant *Atta cephalotes* (López & Orduz, 2003), as well as to study the ability of *Trichoderma* to improve plant vigour and stimulate crop growth (Bae et al., 2009, Hoyos-Carvajal et al., 2009b, Resende et al., 2004).

Our knowledge of the distribution of *Trichoderma* species is constantly evolving in the context of current advances toward resolving the taxonomy of the genus. As a consequence, we can anticipate in future years to better understand the biogeography of *Trichoderma* species as research is pursued in new regions and to resolve complex species aggregates. For example, Samuels et al. (2006) determined that the species commonly cited in literature, *Trichoderma koningii,* in the strict sense is a relatively uncommon species restricted to temperate Europe and North America. From within the *T. koningii* aggregate he erected numerous new species, describing *T. caribbaeum* var. *aequatoriale, T. koningiopsis,* and *T. ovalisporum* as endophytes of *Theobroma* species in tropical America, and *T. ovalisporum* also from the woody liana *Banisteropsis caapi* in Ecuador. *T. koningiopsis* (previously identified as *T. koningii*) was determined to be common in tropical America, occurring also on natural substrata in East Africa, Europe and Canada, from ascospores in eastern North America, and as an endophyte in *Theobroma. T. stilbohypoxyli*, described as a parasite of *Stilbohypoxylon* species in Puerto Rico, was found to be more common in the tropics. Samuels et al. (1998) reported on the diversity of *Trichoderma* section *Longibrachiatum,* revealing diversity in neotropical areas resulting in the description of new species in this section. Jaklitsch et al. (2006), in revising the *T. viride* species complex, reported *T. viridescens* as a species found in Peru at high elevation, and *T. neokoningii* in a tropical region in Peru. He also described, as new species, *T. scalesiae* isolated as an endophyte from the trunk of daisy tree (*Scalesia pedunculata*) in the Galapagos Islands of Ecuador, *T. paucisporum* as a mycoparasite of *Moniliophthora roreri* on pods of *Theobroma cacao* in Ecuador, and *T. gamsii*, an apparently cosmopolitan species that has been found in Italy, Rwanda, South Africa, and Romania as well as Guatemala. Recent studies undertaken to find biocontrol agents in specific crops such as cocoa also has resulted in the determination of other new species in neotropical regions (Jaklitsch et al., 2006, Samuels et al., 2000, Samuels et al., 2006).

4.1 Can we generalize on the soil-inhabiting species of *Trichoderma* occurring in the neotropics?

Hoyos-Carvajal et al. (2009a) carry out a systematic survey of *Trichoderma* species in seven countries: Mexico, Guatemala, Panama, Peru, Ecuador, Brazil and Colombia, isolating primarily from soil and employing complementary observations on morphology, metabolism and sequences of ITS 1 and 2 and the 5′ region of *tef*-1a encompassing four introns. They identified 182 *Trichoderma* isolates finding a wide diversity of species over this region of the neotropics - *T. asperellum* (26 isolates), *T. asperelloides* (34 isolates, as *T. asperellum* 'B') *T. atroviride* (3), *T. brevicompactum* (5), *T. crassum* (3), *T. erinaceum* (3), *T. gamsii* (2), *T. hamatum* (2), *T. harzianum* (49), *T. koningiopsis* (6), *T. longibrachiatum* (3), *T. ovalisporum* (1), *T. pubescens* (2), *T. rossicum* (4), *T. spirale* (1), *T. tomentosum* (3), *T. virens* (8), *T. viridescens* (7), *T. parareesei* (3, as *H. jecorina*), along with 11 presumptive new species that have not yet been described. Analyses of variance were performed on metabolic data for the Colombian isolates. Highly significant differences ($P < 0.0001$) in assimilation were observed for 42 substrates among the 12 species isolated from Colombia (*T. asperellum*, *T. atroviride*, *T.*

brevicompactum, *T. erinaceum*, *T. hamatum*, *T. harzianum*, *T. koningiopsis*, *T. longibrachiatum*, *T. virens*, *T. viridescens*, *T. parareesei* and *Trichoderma* sp. 210). The highest growth rates were observed on 23 substrates for *T. viridescens* isolated from mostly from rhizosphere of *Impatiens*, for *T. asperellum* obtained from a broad range of substrates on five substrates, and for *T. harzianum* from varied habitats and *T. parareesei* from African palm on three substrates. Seven substrates for which *T. viridescens* had the fastest growth rate were substrates not or scarcely assimilated by any other species (sedoheptulosan, glucuronamide, 2-aminoethanol, D-lactic acid methyl ester, putrescine, L-alaninamide, γ-hydroxyphenylacetic acid), perhaps indicating an ability to grow on recalcitrant substrates, although a similar pattern has been observed in other studies, contrasting isolates from undisturbed forests habitats, capable of growing on recalcitrant substrates, with isolates from agricultural habitats (Bissett unpublished). *Trichoderma viridescens* and *T. harzianum* showed positive growth on the largest number of significant substrates (41 and 34 respectively), indicating possible adaptation to a relatively broad range of habitats or niches and reflected in their wide distibutions. Slowest growth rates were observed for *T. erinaceum* from maize rhizosphere on 15 substrates, and for *Trichoderma* sp. 210 from river sand on 11 substrates. *T. longibrachiatum* and *Trichoderma* sp. 210 in section Longibrachiatum, along with *T. erinaceum* assimilated the fewest substrates (19-25 substrates).

The considerable biodiversity of *Trichoderma* in neotropical regions was evident in the study by Hoyos-Carvajal et al. (2009a). Nineteen species were identified from 182 isolations, and eleven so far undescribed species were discovered from rainforest soils and other specific habitats such as river sand, humus and wood in Peru, Mexico, Guatemala and Colombia. In a study of *Trichoderma* in Venezuelan soils the most abundant species was *T. harzianum*, followed by *T. virens, T. brevicompactum, T. theobromicola, T. koningiopsis, T. ovalisporum, T. asperellum, T. pleurotum* and *T. koningiopsis* (Rivas & Pavone, 2010). These observations are added to new species of *Trichoderma* from neotropics described in recent years, mainly as endophytes in plants (Jaklitsch et al., 2006, Samuels et al., 2006), and are evidence of the significant biodiversity of *Trichoderma* in the tropical region (table 1).

Unlike the studies conducted by Kullning et al. (2000) and Kubicek et al. (2002), assessing *Trichoderma* biodiversity of specific geographic areas, where the most common species was *T. harzianum*, in contrast in neotropical areas studied by Hoyos-Carvajal et al. (2009a) was *T. asperellum* (33% of strains) and *T. harzianum* the second most common (27%). Genetic variation was evident for both species, and two (*T. asperellum*) or three (*T. harzianum*) distinct genotypes were evident in the analysis of *tef* sequences and metabolic profiles. *T. asperellum,* which is often isolated from tropical regions (Druzhinina et al., pers. comm.), could be divided into two groups (A and B), which more recently have been described as separate species, *T. asperellum* and *T. asperelloides* respectively (Samuels et al., 2009, 2010). *T. asperellum* includes isolates from Brazil, Peru and Colombia originating in soils with poorly degraded materials such as fallen leaves or crop residuals in colder climate zones. T. *asperelloides* includes strains collected in Colombia and Ecuador that exhibit a preference for soils and substrates with high organic content, and often adapted to the rhizosphere of crops in Andean zones. These two species could not be differentiated by morphological characters or by growth rates, suggesting the development of ecologically or geographically isolated lineages as has been reported for *T. harzianum* (Chaverri et al., 2003) and *T. koningii* (Samuels et al., 2006). Metabolic differences were apparent between the closely related species, with *T. asperellum* better able to assimilate a wider range of C-substrates including some organic acids, polyols and amino acids, although growth rates on readily assimilated substrates such

as glucose and N-acetyl-D-glucosamine were essentially the same. *T. asperelloides* had faster growth only on the disaccharide gentobiose. All differences between the two species were a matter of rate of growth, rather than growth/no growth. Notably, *T. asperellum* had significantly higher growth on substrates that are usually not at all assimilated by fungi, such as D-psicose, sedoheptulosan and γ-hydroxybutyric acid. *T. asperellum* was isolated from soils with poorly incorporated litter or crop residuals in colder climate zones (15ºC), growing on a wider variety of poorly metabolized substrates, and concomitantly under more difficult nutritional conditions in forest soils or debris. The pattern of growth on recalcitrant substrates for *T. asperellum* may be correlated with occurrence in relatively undisturbed, forested soils or other natural habitats. *T. asperelloides* was associated with agricultural soils crops, and in Colombia displayed a pattern of affinity for readily available substrates such as sugars, from comparatively warmer climates (23–28 ºC), and the rhizosphere soils where this strain was collected are associated with tropical crops such as African palm (*Elaeis guineensis*), coffee (*Coffea arabiga*), black mulberry (*Morus* sp.), avocado (*Persea americana*) and some grasses, in areas with a comparatively high organic matter contents. Despite the apparent different habitat preferences, *T. asperellum* and *T. asperelloides* did not exhibit differences in growth rates over the range 5-40°C, both species with temperature optima near 28˚C.

In the neotropics, the second most prevalent species from neotropical soils studied by Hoyos-Carvajal et al. (2009a) was *T. harzianum*, commonly associated with the rhizosphere of cultivated plants and frequently employed as a biocontrol agent against soil-borne phytopathogens. The predominance of *T. harzianum* in many different environments might be explained by its ability to assimilate a comparatively wide array of carbon substrates. The concept of *T. harzianum* as a genetically variable complex, comprising reproductively isolated biological species, recent agamospecies and phylogenetically unresolved relict lineages as determined by Druzhinina et al. (2010) is coherent with the adaptive range of this taxon. In the study of neotropical *Trichoderma* by Hoyos-Carvajal et al. (2009a), *T. harzianum* A was characterized by growth on poorly metabolized substrates, and strains from Colombia were isolated from a variety of environments, but commonly Andean soils associated with *Impatiens* sp. Group A also included strains from Mexico, consistent with the distribution found by Chaverri et al. (2003) for *H. lixii* lineage 1, and also includes isolates from Panama and Peru. The second clade, *T. harzianum* B, comprised mostly strains from the rhizosphere of tobacco, and sequences are coincident with lineage 5 of Chaverri et al. (2003) that included strains from Japan, Mexico and Cameroon, and with lineage 6 from Europe. *T. harzianum* C comprised nine strains from Colombia together with lineage 3 identified by Chaverri et al. (2003) from the United States, lineage 7 from Japan and Mexico, lineage 2 from Europe, and lineage 4 from Cameroon. *T. harzianum* A had fastest growth on L serine, i-erythriol, glycerol, D-sorbitol, D-ribose, α-D-lactose, L-threonine, L-proline, D-mannitol, and L-sorbose; however, there was no significant difference among the three genotypes on glucose, and all three genotypes had similar linear growth rates in culture on PDA over the temperatures range 5-40ºC. Significantly higher growth rates for *T. harzianum* A on the monosaccharide polyols i-erythritol, D-sorbitol, and D-mannitol, and on the fatty acid glycerol could indicate the presence of dehydrogenases allowing an adaptation to relatively dry habitats. *T. harzianum* B was the only genotype able to assimilate the disaccharide sucrose, although it exhibited relatively poor growth on the disaccharide lactose which was readily assimilated by genotypes A and C.

In the study carried out by Hoyos-Carvajal et al. (2009a), Colombia becomes the most intensively surveyed neotropic region for *Trichoderma* biodiversity to date, comprising 116 isolates, representing 11 described species and one new taxon. As was mentioned, the most commonly isolated species from Colombia were *T. asperellum* (inclusive of more recently distinguished *T. asperelloides)* and *T. harzianum*. The prevalence of these species in Colombia may be explained by their genetic variability, seen in the several distinct genotypes found for each species, and their corresponding ability to grow on a wide variety of carbon substrates. However, the isolation methods used in this study, which are commonly used to isolate from the soil and rhizosphere, would be selective for soil-inhabiting species such as *T. asperellum* and *T. harzianum,* which are fast growing and sporulate early, allowing them to be recognized ahead of slower developing species. The majority of new species in this study

Species	**Reference**
T. asperellum	Samuels *et al*., 1999
T. asperelloides	Samuels *et al*., 2010
T. atroviride	Bissett, 1992.
T. brevicompactum	Krauss *et al*., 2004.
T. caribbaeum	Samuels *et al*. 2006
T. caribbaeum var. aequatoriale	Samuels *et al*. 2006
T. crassum	Bissett, 1991 a
T. erinaceum	Bissett *et al*., 2003
T. evansii	Samuels & Ismaiel, 2009
T. gamsii	Jaklitsch *et al*., 2006
T. hamatum	Gams y Bissett, 1998
T. harzianum	Chaverry *et al*., 2003; Gams & Bissett, 1998
T. koningiopsis	Samuels *et al*., 2006
T. lieckfeldtiae	Samuels & Ismaiel, 2009
T. longibrachiatum	Gams y Bissett
T. neokoningii	Jaklitsch *et al*., 2006
T. ovalisporum	Holmes *et al*., 2004
T. parareesei	Atanasova *et al*., 2010
T. paucisporum	Samuels *et al*. 2006b
T. pleurotum	Komoń-Zelazowska et al., 2007
T. pubescens	Bissett 1991a
T. reesei (H. jecorina)	Gams and Bissett, 1998
T. rossicum	Bissett *et al*., 2003.
T. scalesiae	Jaklitsch *et al*., 2006
T. spirale	Bissett 1991a
T. stilbohypoxyli	Samuels *et al*. 2006a
T. theobromicola	Samuels *et al*. 2006b
T. tomentosum	Bissett 1991a
T. virens	Bissett 1991[a]
T. viridescens	Jaklitsch *et al*., 2006

Table 1. *Trichoderma* species currently identified from the tropics with references to morphological descriptions.

were isolated from other neotropical countries, notably Peru (6 new species) and Guatemala (3 new species) from which there were far fewer isolates. However, sampling in these countries was selective for unusual substrates above ground, resulting in the high proportion of novel strains. Therefore, the study by Hojos-Carvajal et al. (2009) would not account for the above ground biodiversity of species in Colombia on account of a relatively selective (but typical) sampling regime, although it is indicative of the wide distribution of *T. asperellum* and *T. harzianun* in soils, as reported in previous studies for other regions (Kullnig et al., 2000, Kubicek et al., 2003, Chaverry et al., 2003).

The various studies of *Trichoderma* in the neotropics have expanded the known biogeographical and ecological distribution of many *Trichoderma* species. For example, *T. virens* (rain forest in Perú; rotten wood, rhizosphere of rice, tobacco and grassland in Colombia), *T. pubescens* (rain forest soil in Perú*), T. strigosum* (Perú rain forest soil), and *T. tomentosum* (cloud forest soil, Guatemala), were originally described from North America and Europe where they are relatively uncommon (Bissett, 1991 b). *T. ovalisporum*, previously reported from Ecuador as an endophyte in *Banisteriopsis caapi* and *Theobroma* sp. (Samuels et al., 2006), was isolated as an apparent saprophyte from soil in Panama. The infrequent isolation of these species also from neotropical soils suggests that these species may be restricted to specific ecozones, habitats or niches (Hoyos-Carvajal et al., 2009a). Samuels et al. (1998) reported *H. jecorina* (anam.: *T. reesei*) to be common in the pantropical region, and it is an important producer of cellulase enzymes. Hoyos-Carvajal et al., 2009a reported the species in typically warm soils cultivated with African palm in Colombia, but these strains did not assimilate sucrose, which had been reported for isolations of *H. jecorina* from the eastern Pacific (Samuels et al., 1998). We now know that the species reported by Hoyos-Carvajal et al. (2009) was in fact *T. parareesei,* recently differentiated from the sympatric species *H. jecorina* by Druzhinina et al. (2010a).

Eleven neotropical clades were differentiated from known *Trichoderma* species by Hoyos-Carvajal et al. (2009a) based on morphologic, metabolic and molecular differences and these remained undescribed. These are presumed to represent new taxa in *Trichoderma* and are the subject of ongoing investigations. The high proportion of apparently new species in this study is an indication that we have only begun to explore the biodiversity of *Trichoderma* in the neotropic regions.

5. Conclusions

Trichoderma species represent a major component of soil biodiversity with an important role in maintaining soil and plant health. The numbers, diversity, roles, and interactions of *Trichoderma* species in the environment are only now being discovered as tools are developed to distinguish the anamorph forms most commonly encountered. Significant and novel biodiversity of *Trichoderma* in the neotropics has been demonstrated, although we have only begun to explore the diversity of regions, habitats and substrates that exist in the region. We are now able to account taxonomically for a significant component of their biological diversity, to begin to predict biological activities, and to communicate results through the use of accurately determined names. The identification of *Trichoderma* species, as for species in other economically important and species-rich genera, is increasingly reliant on molecular data as the limits of phenotypic characters to distinguish species are reached. Many new species of *Trichoderma* will undoubtedly be distinguished as molecular tools are developed for ecological and metagenomic studies. Agriculture is the main economic

activity in neotropical regions, and *Trichoderma* is the most important biocontrol agent against soil-borne phytopathogens. Consequently neotropical investigations have concentrated on the application of *Trichoderma* to control crop diseases, and discovering new metabolites and mechanisms of action. We can now appreciate the importance in preserving the biodiversity of delicate ecosystems such as rain forest and Andean forest, as reservoirs of metabolites and diverse and unique ecological niches for habitation by animals, plants and microorganisms. Conservation is facilitated as we increase our knowledge of the fundamental role of *Trichoderma* in nutrient cycling and in the complex interactions within the soil biota.

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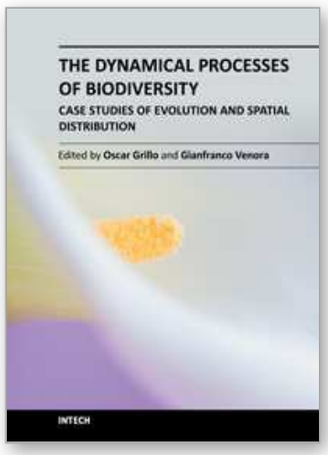

The Dynamical Processes of Biodiversity - Case Studies of Evolution and Spatial Distribution

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