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Unraveling *Trichoderma* species in the attine ant environment: description of three new taxa

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Abstract Fungus-growing "attine" ants forage diverse substrates to grow fungi for food. In addition to the mutualistic fungal partner, the colonies of these insects harbor a rich microbiome composed of bacteria, filamentous fungi and yeasts. Previous work reported some *Trichoderma* species in the fungus gardens of leafcutter ants. However, no studies systematically addressed the putative association of *Trichoderma* with attine ants, especially in non-leafcutter ants. Here, a total of 62 strains of *Trichoderma* were analyzed using three molecular markers (ITS, *tef1* and *rpb2*). In addition, 30 out of 62 strains were also morphologically examined. The strains studied correspond to the largest sampling carried out so far for *Trichoderma* in the attine ant environment. Our results revealed the richness of *Trichoderma* in this environment, since we found 20 *Trichoderma* species, including three new taxa described in the present work (*Trichoderma attinorum*, *Trichoderma texanum* and *Trichoderma longifialidicum* spp. nov.) as well as a new phylogenetic taxon (LESF 545). Moreover, we show that all 62 strains grouped within different clades across the *Trichoderma* phylogeny, which are identical or closely related to strains derived from several other environments. This evidence supports the transient nature of the genus *Trichoderma* in the attine ant colonies. The discovery of three new species suggests that the dynamic foraging behavior of these insects might be responsible for accumulation of transient fungi into their colonies, which might hold additional fungal taxa still unknown to science.

Keywords Hypocreales · Attini · Richness · Fungus garden · Soil · Transient

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Introduction

The tribe Attini is comprised of approximately 45 ant genera with worldwide distribution (Ward et al. 2015). Most notably in this tribe are the fungus-growing "attine" ants, which cultivate fungi for food (Schultz

and Brady 2008; Ward et al. 2015). These ants are found only in the American continent and comprise 17 genera with more than 250 species (Brandão et al. 2011; Sosa-Calvo et al. 2013). Attine ants maintain a mutualistic interaction with basidiomycetous fungi in an obligatory association that originated about 50 million years ago (Schultz and Brady 2008). In this mutualism, workers collect various types of substrates (e.g. fresh leaves, seeds, and insect carcasses) to nourish the fungal cultivar, besides favoring fungal dispersion during the ants' reproductive stage. In turn, the fungal partner is the primary food source for the brood and minor component in the nutrition of ant workers (Murakami and Higashi 1997; Silva et al. 2003).

Fungus-growing ant colonies represent a complex biological system that harbor a wide range of microorganisms in addition to the fungal cultivar, including bacteria, filamentous fungi and yeasts (Fisher et al. 1996; Rodrigues et al. 2008). Such microbes are found in the soil that composes the nest tunnels and chambers (Rodrigues et al. 2014) as well as in the fungus garden, structure that houses the substrate foraged by the ants and the mycelium of the fungal cultivar (Weber 1972).

Möller (1893) was the first to report the presence of alien fungi such as *Aspergillus*, *Mucor*, *Penicillium* and *Rhizopus* in the gardens of *Acromyrmex disciger*. Later, Kreisel (1972) reported *Cunninghamella*, *Fusarium*, *Rhizopus* and *Trichoderma* in ant-deprived gardens of the leafcutter ant *Atta insularis*. Although several studies have demonstrated the diversity of filamentous fungi found in gardens of attine ants, the origin of these fungi is still uncertain. Subsequent studies suggested such fungal diversity is mostly related to the type of plant substrate that workers use to nourish the fungal cultivar (Fisher et al. 1996; Van Bael et al. 2009).

Filamentous fungi and other microbes that enter the ant colony are derived from the surface of the plant material. Also, due to the complex ant behaviors associated with substrate preparation (scraping and cleaning the surface of leaves to eliminate microorganisms), the origin of some of the alien fungi in leaf-cutting ant colonies is also endophytic (Van Bael et al. 2009). Furthermore, Rodrigues et al. (2005, 2011) suggested that workers that are in direct contact with soil also carry fungi to their colonies. Although most studies focused on leaf-cutting ants, no research investigated the diversity of filamentous fungi in colonies of non-leaf-cutting attine ants. Because these ants use a variety of substrates such as seeds, plant sap, insect frass and parts of dried leaves and flowers to grow the fungal cultivar (Rodrigues et al. 2005, 2008; Mehdiabadi and Schultz 2010), it is possible those substrates contain several fungi that are carried into their colonies, including *Trichoderma* species.

Fungi in the genus *Trichoderma* occur in different parts of the world (Samuels et al. 2006; Druzhinina et al. 2011; Chaverri and Samuels 2013) on various substrates, including: (i) soil; (ii) decaying wood; (iii) on edible mushrooms and (iv) some as endophytes in plants (Samuels et al. 2006; Zhang et al. 2007; Hanada et al. 2008; Bae et al. 2009; Chaverri et al. 2011; Druzhinina et al. 2012; Bailey and Melnick 2013; Chaverri and Samuels 2013). *Trichoderma* species have a high adaptability to different substrates and environments (colonizing diverse ecological niches), probably due to the physicochemical mechanisms (production of enzymes and antibiotics) as tools for obtaining nutrients (Samuels et al. 2006; Jaklitsch 2009; Druzhinina et al. 2011; Druzhinina and Kubicek 2013; Atanasova et al. 2013). Due to the diversity of substrates collected by attine ants, and the cosmopolitan nature of *Trichoderma*, new *Trichoderma* species could enter the colonies of these insects.

Since the first report of *Trichoderma* in colonies of leaf-cutting ants (Kreisel 1972), several studies showed the occurrence of these fungi in colonies of other attine ant genera (Barbosa 2004; Poulsen and Currie 2006; Rodrigues et al. 2008, 2009; Augustin et al. 2011; Rodrigues et al. 2014). However, none of these studies used a detailed taxonomic approach to identify *Trichoderma* species that occur in this environment. Also, some of these studies sequenced the internal transcribed spacer 1 and 2 (ITS 1 and 2) of the rDNA region, which does not resolve species identification in several *Trichoderma* clades, especially for the *Trichoderma harzianum* species complex (Atanasova et al. 2013; Chaverri et al. 2015).

Systematic studies involving other microorganisms associated with leaf-cutting ants showed a high diversity in the fungus gardens or on the integument of ants, including many new yeast species (Middelhoven et al. 2003; Carreiro et al. 2004; Pagnocca et al. 2010; Attili-Angelis et al. 2014; Melo et al. 2014). However, studies focused on the taxonomy and

systematics of filamentous fungi associated with these insects have only considered the genus *Escovopsis*, the specialized parasite of the mutualistic fungus (Muchovej and Della Lucia 1990; Seifert et al. 1995; Currie et al. 1999, 2003; Augustin et al. 2013; Masiulionis et al. 2015; Meirelles et al. 2015a, b). These studies revealed the presence of several new *Escovopsis* species, which suggests that attine ant colonies might also harbor a high diversity of filamentous fungi, including potential new species of the genus *Trichoderma*.

Currently, no study has focused on the taxonomic evaluation of *Trichoderma* species found in the microbial consortium of attine ant colonies. In this sense, this study systematically assessed *Trichoderma* species in colonies of 12 attine ant species (nine species of leaf-cutting and three of non-leaf-cutting ants) belonging to four genera (*Atta*, *Acromyrmex*, *Trachymyrmex*, and *Cyphomyrmex*). We used morphological and molecular markers to identify 62 strains isolated from fungus gardens, soil adjacent to the garden and soil distant from the ant colonies. Here, we report a high number of *Trichoderma* species found in the attine ant environment, including three new *Trichoderma* species as well as a new phylogenetic taxon. Such findings suggest that colonies of these insects may harbor more fungal species still unknown to science.

Materials and methods

Strains examined

The strains examined were obtained in several studies conducted over 10 years (2004–2013) by the research group of the Laboratory of Ecology and Fungal Systematics (LESF) in combination with other laboratory (Section of Integrative Biology, University of Texas at Austin, USA). Ant colonies were excavated to find the fungus gardens. Fragments from the top (new garden) and the bottom (old garden) sections of the fungus gardens were collected in sterile containers. Pieces (3 mm^2) from both sections of this substrate were plated in different culture media supplemented with antibiotics (see Rodrigues et al. 2005, 2008, 2011). In addition, *Trichoderma* strains from soil samples were isolated by the spread plating technique (Rodrigues et al. 2014). Monosporic cultures were obtained for all isolates. All strains are preserved in 10 % glycerol at −80 °C at the UNESP—Microbial Resources Center (CRM-UNESP), Rio Claro, Brazil.

A total of 62 *Trichoderma* strains were revived and examined. From this total, 56 are derived from *Atta* and *Acromyrmex* leaf-cutting ants, comprehending 47 from fungus gardens, two from soil adjacent to garden and seven from soil 10 m away from ant colonies. In addition, we examined six strains derived from fungus gardens of non-leaf-cutting ants in the genera *Trachymyrmex* and *Cyphomyrmex* (Table S1).

Trichoderma strains here studied are derived from a total of 37 ant colonies, corresponding to 12 attine ant species (Table S1). Samples were collected in ten sites from four states in Brazil and two sites in Texas-USA (Fig. S1; Table S1), comprehending so far, the largest sampling of this taxon from attine ant colonies.

DNA extraction, PCR and sequencing

DNA extraction was performed directly from the mycelium grown in PDA medium for 5 days in the dark. Genomic DNA extraction followed the method adapted from Möller et al. (1992) and Gerardo et al. (2004). Three molecular markers were amplified: the ITS region (ca. 586–622 bp) with the primer pair ITS4 (5′TCCTCCGCTTATTGATATGC3′) and ITS5 (5′GGAAGTAAAAGTCGTAACAAGG3′ Schoch et al. 2012); the partial sequences (ca. 563–615 bp) of the gene coding for the elongation factor 1 alpha (*tef*1) (EF1-728F: 5′CATCGAGAAGTTCGAGAAGG3′and TEF1R: 5′ GCCATCCTTGGAGATACCAGC3′, Carbone and Kohn 1999; Samuels et al. 2002); and the gene coding for the second subunit (ca. 900–1130 bp) of the RNA polymerase II (*rpb2*) (fRPB2-5F: 5′GA(T/C)GA(T/C)(A/C)G(A/T)GATCA(T/C)TT(T/C)GG-3′ and fRPB2-7cR: 5′CCCAT(A/G)GCTTG(T/C)TT(A/G)CCCAT-3′, Liu et al. 1999). PCRs for the three markers were performed in a final volume of 25 μL (4 μL of dNTPs [1.25 mm each]; 5 μL of 5× buffer; 1 μL of BSA [1 mg mL^{-1}]; 2 μL of $MgCl_2$ [25 mm]; 1 μL of each primer [10 μm]; 0.2 μl of Taq polymerase [5 U μL^{-1}], 2 μL of diluted genomic DNA [1:100] and 8.8 μL of sterile ultrapure water). PCR conditions for ITS were 94 °C/3 min followed by 35 cycles at 94 °C/1 min, 55 °C/1 min and 72 °C/2 min; for *tef*1 and *rpb2*, conditions were 94 °C/2 min followed by 15

cycles at 94 °C/30 s, 65 °C/30 s and 72 °C/1 min; followed by 35 cycles at 94 °C/30 s, 48 °C/30 s and 72 °C/1 min.

Amplicons were purified using Wizard® SV Gel and PCR Clean-Up System Kit (Promega) following the manufacturer's protocol. Then, samples were quantified in NanoDrop® (Thermo Scientific) and subjected to cycle sequencing reaction using BigDye Terminator® v.3.1 Kit (Life Technologies), following the manufacturer's instructions (primers used for sequencing were the same used in the amplification step). The sequencing conditions for all molecular markers were 95 °C/min followed by 28 cycles at 95 °C/15 s, 50 °C/45 s, and 60 °C/4 min. Bidirecional sequences (forward and reverse) were generated in ABI 3330xl sequencer (Life Technologies) and assembled into contigs in BioEdit v.7.0.5.3 (Hall 1999).

Phylogenetic analyses

ITS, *tef1* and *rpb2* contigs of all strains were compared to homologous sequences deposited in NCBI-GenBank. Due to the low intraspecific ITS resolution for *Trichoderma* (Druzhinina and Kubicek 2005; Druzhinina et al. 2006; Atanasova et al. 2013; Chaverri et al. 2015) and also detected in our study, only *tef1* and *rpb2* sequences were used for phylogenetic analyses. However, due to the importance of ITS as a barcode marker for fungi, such sequences are also provided for all strains. Sequences generated in the present study were deposited in the NCBI-GenBank database (see Table S2 for accessions).

Sequences generated in other studies (Table S2) were used in our phylogenetic analyses and were retrieved from the NCBI-GenBank database. These sequences were selected by inferring initial phylogenetic trees (not shown) for each *Trichoderma* clade in attempt to avoid sequences that were too divergent and could hinder the global analysis. After sequences selection, the final dataset comprised 195 partial *tef1* sequences (833 bp) and 155 partial *rpb2* sequences (1058 bp). Alignments were performed separately for each molecular marker using MAFFT v.7 (Katoh and Standley 2013). The *tef1* and *rpb2* alignments were concatenated using Winclada v.1.00.08 (Nixon, 2002), and the final file contained a total of 1891 bp.

We applied Bayesian inference in MrBayes v.3.2.2 to reconstruct our global phylogenetic tree (Ronquist et al. 2012) with the concatenated dataset (*tef1* and *rpb2*).

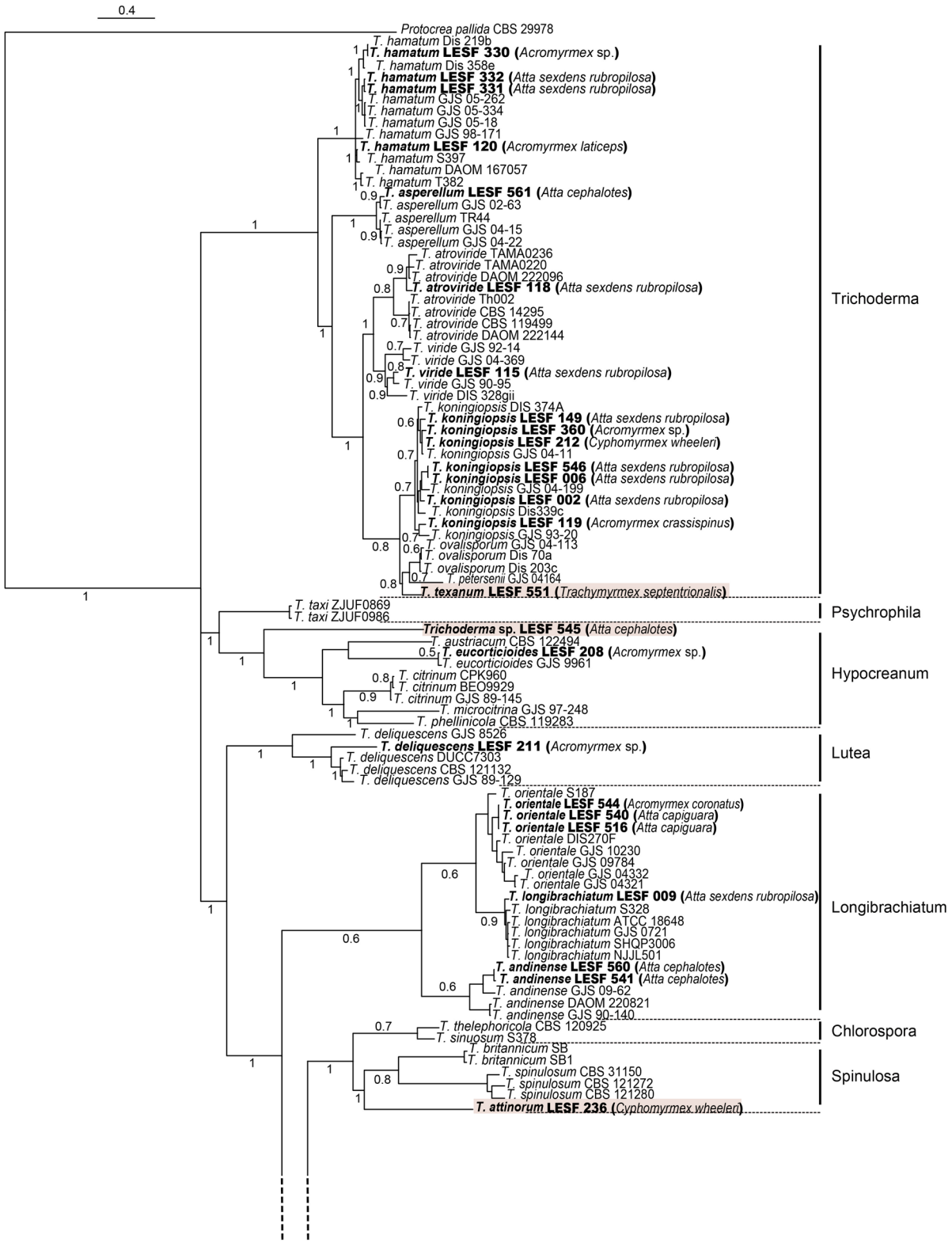
Fig. 1 Phylogenetic tree displaying the position of different *Trichoderma* species isolated from the attine ant environment (fungus gardens, soil next to fungus gardens and soil 10 m away from ant colonies). The analysis was based on concatenated sequences of *tef1* and *rpb2* markers. The tree was built using the Bayesian inference algorithm and the numbers on branches indicate the posterior probabilities. The tree includes a total of 195 sequences of *tef1* and 155 sequences of *rpb2*. The final alignment had 1891 base pairs. We also included *Trichoderma* sequences derived from previous studies obtained from GenBank. Species names are followed by the strains codes. The species identified in this study with its code (LESF) at the end of each name are highlighted in *bold*. For each *Trichoderma* obtained in this study, we show in parentheses the ant species from which fungi strains were isolated. The four new phylogenetic taxa found here (three of them described as new species) are highlighted in *red*. *Dashed lines* indicate the different *Trichoderma* clades sensu Atanasova et al. (2013). *Protodrea pallida* CBS 29978 was used as outgroup. *Bars* indicate nucleotide substitution per site

Nucleotide substitution models, GTR+I+G and SYM+I+G, were selected for *tef1* and *rpb2*, respectively, using the Bayesian information criterion under 95 % of confidence interval in jModelTest 2 (Darriba et al. 2012). We set up two separate runs, each consisting of three hot chains and one cold chain, and a Markov Chain Monte Carlo (MCMC) sampling of seven million generations, which were sufficient to achieve values for standard deviation of split frequencies below 0.01. Finally, the initial 25 % of the generations of MCMC sampling were discarded as burn-in. The refinement of the phylogenetic tree was carried out in FigTree v.1.4 and Adobe Illustrator CC v.17.1.

Morphological characterization

A total of 30 out of 62 strains were selected for morphological examination based on the results of the phylogenetic analysis. We examined (i) macroscopic characteristics of the colony (radial growth, mycelium color, presence of diffusible pigment, concentric rings and pustules) as well as (ii) microscopic features (shape and size of conidiophores, supporting cells, phialides, conidia and chlamydospores) based on Samuels et al. (2002, 2012a), Chaverri et al. (2001, 2003a, b), Bissett et al. (2003), Druzhinina et al. (2005) and Jaklitsch et al. (2006).

Macroscopic characteristics were evaluated in PDA (Potato Dextrose Agar), CMD (Corn-meal Agar) and SNA (Synthetic Nutrient Deficient Agar) grown for 7 days at 25 °C for 12 h (light) and 12 h (dark).


0.4
Protocrea pallida CBS 29978
T. hamatum Dis 219b
T. hamatum LESF 330 (Acromyrmex sp.)
T. hamatum Dis 358e
T. hamatum LESF 332 (Atta sexdens rubropilosa)
T. hamatum LESF 331 (Atta sexdens rubropilosa)
T. hamatum GJS 05-262
T. hamatum GJS 05-334
T. hamatum GJS 05-18
T. hamatum GJS 98-171
T. hamatum LESF 120 (Acromyrmex laticeps)
T. hamatum S397
T. hamatum DAOM 167057
T. hamatum T382
T. asperellum LESF 561 (Atta cephalotes)
T. asperellum GJS 02-63
T. asperellum TR44
T. asperellum GJS 04-15
T. asperellum GJS 04-22
T. atroviride TAMA0236
T. atroviride TAMA0220
T. atroviride DAOM 222096
T. atroviride LESF 118 (Atta sexdens rubropilosa)
T. atroviride Th002
T. atroviride CBS 14295
T. atroviride CBS 119499
T. atroviride DAOM 222144
T. viride GJS 92-14
T. viride GJS 04-369
T. viride LESF 115 (Atta sexdens rubropilosa)
T. viride GJS 90-95
T. viride DIS 328gii
T. koningiopsis DIS 374A
T. koningiopsis LESF 149 (Atta sexdens rubropilosa)
T. koningiopsis LESF 360 (Acromyrmex sp.)
T. koningiopsis LESF 212 (Cyphomyrmex wheeleri)
T. koningiopsis GJS 04-11
T. koningiopsis LESF 546 (Atta sexdens rubropilosa)
T. koningiopsis LESF 006 (Atta sexdens rubropilosa)
T. koningiopsis GJS 04-199
T. koningiopsis LESF 002 (Atta sexdens rubropilosa)
T. koningiopsis Dis339c
T. koningiopsis LESF 119 (Acromyrmex crassispinus)
T. koningiopsis GJS 93-20
T. ovalisporum GJS 04-113
T. ovalisporum Dis 70a
T. ovalisporum Dis 203c
T. petersenii GJS 04164
T. texanum LESF 551 (Trachymyrmex septentrionalis)
Trichoderma
T. taxi ZJUF0869
T. taxi ZJUF0986
Psychrophila
Trichoderma sp. LESF 545 (Atta cephalotes)
T. austriacum CBS 122494
T. eucorticioides LESF 208 (Acromyrmex sp.)
T. eucorticioides GJS 9961
T. citrinum CPK960
T. citrinum BEO9929
T. citrinum GJS 89-145
T. microcitrina GJS 97-248
T. phellinicola CBS 119283
Hypocreanum
T. deliquescens GJS 8526
T. deliquescens LESF 211 (Acromyrmex sp.)
T. deliquescens DUCC7303
T. deliquescens CBS 121132
T. deliquescens GJS 89-129
Lutea
T. orientale S187
T. orientale LESF 544 (Acromyrmex coronatus)
T. orientale LESF 540 (Atta capiguara)
T. orientale LESF 516 (Atta capiguara)
T. orientale DIS270F
T. orientale GJS 10230
T. orientale GJS 09784
T. orientale GJS 04332
T. orientale GJS 04321
T. longibrachiatum LESF 009 (Atta sexdens rubropilosa)
T. longibrachiatum S328
T. longibrachiatum ATCC 18648
T. longibrachiatum GJS 0721
T. longibrachiatum SHQP3006
T. longibrachiatum NJJL501
T. andinense LESF 560 (Atta cephalotes)
T. andinense LESF 541 (Atta cephalotes)
T. andinense GJS 09-62
T. andinense DAOM 220821
T. andinense GJS 90-140
Longibrachiatum
T. thelephoricola CBS 120925
T. sinuosum S378
Chlorospora
T. britannicum SB
T. britannicum SB1
T. spinulosum CBS 31150
T. spinulosum CBS 121272
T. spinulosum CBS 121280
T. attinorum LESF 236 (Cyphomyrmex wheeleri)
Spinulosa

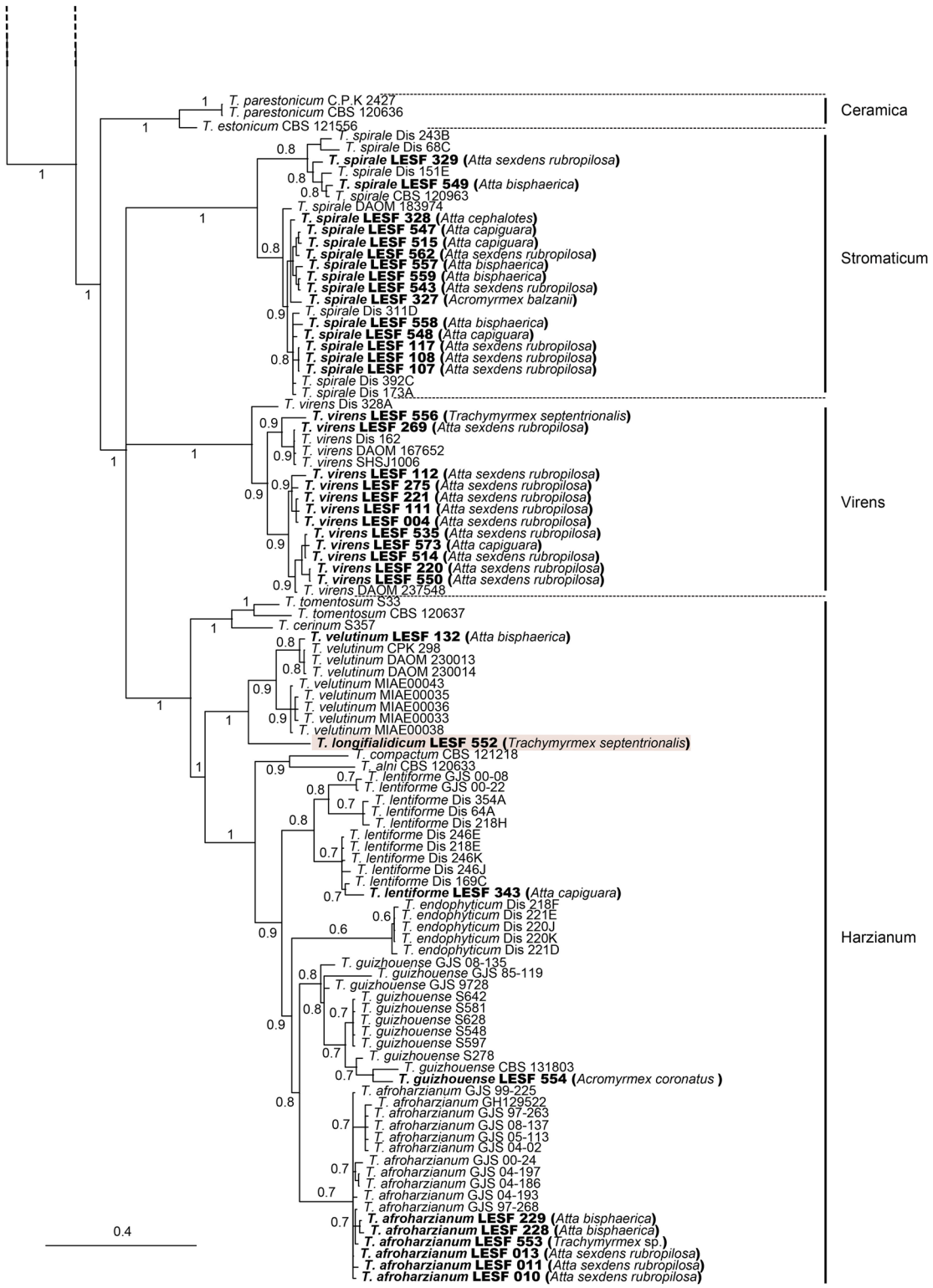


Fig. 1 continued

Growth tests were performed on these three media and incubated at four different temperatures (25, 30, 35 and 40 °C) in darkness. The strains were initially grown for 7 days in water-agar medium. Thereafter, mycelium fragments of 0.5 cm diameter were inoculated on the three culture media (one cm from the edge of the plate). When the mycelium reached 7.5 cm radius, this size was sufficient to cover the entire Petri dish. Growth tests were performed in triplicate on three separate time periods for one week. Measurements of growth radius were performed after 72 h and 96 h.

To assess the microscopic characteristics we carried out slide culture preparations on PDA and CMD. For this purpose, we placed a 5-mm^2 block of each media on a microscopic slide and then inoculated *Trichoderma* spores. Finally, the preparations were covered with a cover slip and incubated at 25 °C with alternating light (12 h light/12 h dark) for a time period of 3–5 days. Moreover, we also prepared wet mounts using 3 % KOH for additional observations of conidia and chlamydospores. All preparations were documented using an optical microscope (DM750, Leica, Germany) and measurements of the structures were performed with 30 replicates of each structure using the program LAS v.4.0 (Leica, Germany).

Results

Phylogenetic analyses

The 62 *Trichoderma* strains grouped into 20 different phylogenetic species. Among these species, we found four new phylogenetic taxa (LESF 236, LESF 545, LESF 551 and LESF 552, Fig. 1). The other 16 *Trichoderma* species identified are species that have been described in previous studies not related to attine ants. The 20 *Trichoderma* phylogenetic species belong to eight clades according to the division proposed by Atanasova et al. (2013): i) Clade Harzianum [*T. afroharzianum* (n = 6 strains), *T. guizhouense* (n = 1), *T. lentiforme* (n = 1), *Trichoderma* sp. LESF 552 (n = 1) and *T. velutinum* (n = 1)]; ii) Clade Hypocreanum [*T. eucorticioides* (n = 1), and *Trichoderma* sp. LESF 545 (n = 1)]; iii) Clade Longibrachiatum [*T. andinense* (n = 2), *T. longibrachiatum* (n = 1), *T. orientalis* (n = 3)]; vi) Clade Lutea [*T. deliquescens* (n = 1)], v) Clade Spinulosa [*Trichoderma* sp. LESF 236, (n = 1)]; vi) Clade Stromaticum [*T. spirale* (n = 15)]; vii) Clade Trichoderma [*T. asperellum* (n = 1), *T. atroviride* (n = 1), *T. hamatum* (n = 3), *T. koningiopsis* (n = 6), *Trichoderma* sp. LESF 551 (n = 1, Fig. 3), *T. viride* (n = 1) and viii) Clade Virens [*T. virens* (n = 12)].

Among the four new strains found, three are described here as new species: *Trichoderma attinorum* (LESF 236), *Trichoderma texanum* (LESF 551) and *Trichoderma longifialidicum* (LESF 552). The strain LESF 545 did not sporulate; therefore, it was not possible to describe this fungus using this key morphological characteristic. This strain showed 88 and 89 % (*rpb2*) similarity with *T. austriacum* and *T. taxii* (FJ860525 and DQ859030, respectively). Strain LESF 236 was found to form a separate branch into the Spinulosa clade with high posterior probability support (100 %, Fig. 1). Regarding the molecular markers used, LESF 236 (Fig. 2) showed 83 % (*tef1*) and 91 % (*rpb2*) similarity with *T. sinuosum* (KJ665729 and KJ665342, respectively), 87 % (*tef1*) and 92 % (*rpb2*) with *T. thelephoricola* (FJ860711 and FJ860600, respectively) and 81 % (*tef1*) and 91 % (*rpb2*) with *T. spinulosum* (FJ860699 and FJ860589, respectively). Strain LESF 551 clustered in the Trichoderma clade but was found to form a separate branch from the other species with a moderate posterior probability support (80 %, Fig. 1). This strain showed 94 % (tef1) and 98 % (rpb2) similarity with *T. koningiopsis* (FJ463288 and FJ442730, respectively) and *T. ovalisporum* (AY376037 and FJ442742, respectively); and 97 % (rpb2) similarity with *T. petersenii* (FJ442783). Regarding strain LESF 552, it clustered in the Trichoderma clade and was found to form a separate branch from the other species with a high posterior probability support (100 %, Fig. 1). This strain showed 93 % (*tef1*) and 97 % (*rpb2*) similarity with *T. velutinum* (AY937415 and JN133569, respectively), 90 % (*tef1*) and 95 % (*rpb2*) with *T. guizhouense* (KF134799 and KF134791, respectively). Finally, strain LESF 545 was 89 % (*rpb2*) similar to *T. citrinum* (FJ179603) and *T. taxi* (DQ859032).

Morphological analyses

When we compared morphological aspects of the three new strains with their phylogenetic closest relatives, we observed distinct characteristics. For instance, *T. attinorum* (LESF 236) grouped close to *T. sinuosum*, *T. thelephoricola* and *T. spinulosum* on our

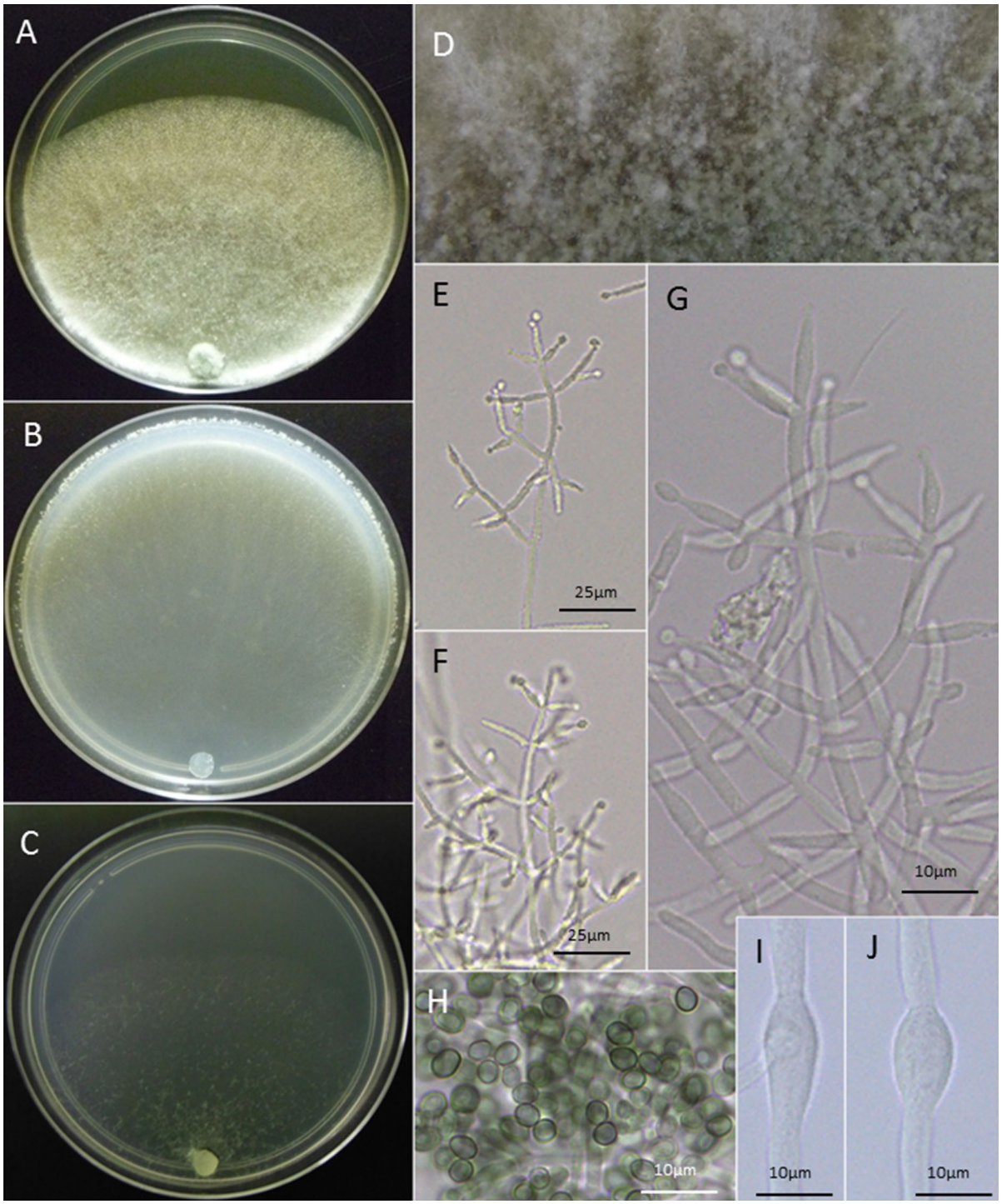


Fig. 2 Morphological characteristics of *Trichoderma attinorum*. **a–c** Cultures on PDA, CMD and SNA, respectively, after 7 days of growth at 25 °C. **d** Dense mycelium with small pustules. **e**, **f** Conidiophore branching pattern. **g** Phialides pattern. **h** Conidia: **i**, **j** Chlamydospores

phylogenetic analysis (Fig. 1). *T. spinulosum* is only known by the sexual phase (Chaverri et al. 2003a), different from *T. attinorum* that only formed the asexual phase (Fig. 2). *T. sinuosum* growth rate is relatively higher than *T. attinorum* (41–58 mm on PDA and 29–36 mm on SNA at 25 °C; 45–60 mm on PDA and 29–39 mm on SNA at 30 °C for *T. sinuosum*; 39–41 mm on PDA and 5–10 mm on SNA at 25 °C, 30–31 mm on PDA and 6–11 mm on SNA at 30 °C for *T. attinorum*). The growth rate of *T. thelephoricola* is lower than the growth rate of *T. attinorum* (6–16 mm on PDA and 4–10 mm on SNA at 25 °C, 13–15 mm on PDA and 4–7 mm on SNA 30 °C for *T. thelephoricola*). Moreover, *T. sinuosum* and *T. thelephoricola* did not form soluble pigment in artificial culture, while *T. attinorum* does on all three culture media (Fig. 2a–c). Conidiophores of *T. attinorum* showed significant differences when compared to *T. sinuosum* and *T. thelephoricola*. In *T. sinuosum* they are branched and sinuous, in *T. attinorum* they are usually straight and with short branches (Fig. 2e–g). When compared to *T. thelephoricola*, the difference is evident since this species has *Gliocladium*-like conidiophores. The phialides of *T. thelephoricola* have shape and size similar to *T. attinorum* (lageniform and cylindrical); however, *T. sinuosum* presents ampulliform phialides distinct from those of *T. attinorum* (Fig. 2g). The conidia of *T. thelephoricola* are longer than the other two species. Finally, *T. attinorum* presents more elongated chlamydospores (Fig. 2i, j) than the one found in *T. sinuosum* (they are globular-subglobose in *T. sinuosum* and subglobose-ovoid in *T. attinorum*); chlamydospores are not reported for *T. thelephoricola*.

Trichoderma texanum grouped close to *T. koningiopsis*, *T. ovalisporum*, and *T. petersenii* (Fig. 1). The three latter species have concentric rings on PDA, while *T. texanum* does not (Fig. 3a). The optimum growth temperature was between 25 and 30 °C for all four species (*T. koningiopsis*, *T. ovalisporum*, *T. petersenii* and *T. texanum*). Pustule formation in *T. texanum* occurred to a lower extent on CMD, but always on the edge of the plate (Fig. 3b), unlike *T. koningiopsis*, *T. ovalisporum*, and *T. petersenii*, which form abundant pustules in concentric rings. Conidia pigmentation was visible after 7 days in *T. texanum*, differently from *T. koningiopsis* (after 48 h), *T. ovalisporum* (after 72 h), and *T. petersenii* (after 96 h). Only *T. texanum* produces soluble pigment in culture. Conidiophores in *T. texanum* are similar to *T. koningiopsis*, *T. ovalisporum*, and *T. petersenii* regarding the branches arrangement, but with a few differences: the conidiophores in *T. koningiopsis* and *T. ovalisporum* are branched in the base with long and unbranched (or slightly branched) ends, where the phialides are formed directly from the central conidiophore hyphae; however, in *T. petersenii* and *T. texanum* the conidiophores do not have such terminations and phialides are formed from supporting cells and, with less frequency, directly from the conidiophore axis (Fig. 3e–g). Additionally, *T. koningiopsis* and *T. ovalisporum* have a central axis of the conidiophore at an angle close to 90°; however, in *T. petersenii* and *T. texanum* this angle is smaller than 90° (Fig. 3g). The phialide arrangement is similar in all four species, but *T. koningiopsis*, *T. ovalisporum*, and *T. petersenii* have lageniform phialides, while in *T. texanum* they are cylindrical (Fig. 3g). *T. texanum* has longer and thinner phialides when compared to *T. koningiopsis*, *T. ovalisporum*, and *T. petersenii*. There are also differences on the conidia shape: *T. koningiopsis*, *T. ovalisporum*, and *T. petersenii* have ellipsoidal conidia, while *T. texanum* presents globose-subglobose conidia (Fig. 3j). Chlamydospores of *T. texanum* (Fig. 3h, i) are longer than the other three species (in which chlamydospores are globose to subglobose).

Trichoderma longifialidicum grouped close to *T. guizhouense* (Chaverri et al. 2015) and *T. velutinum* (Bissett et al. 2003) on our phylogenetic analysis (Fig. 1). The growth rate of *T. velutinum* is smaller than *T. longifialidicum* at 30 °C on PDA (54–62 mm for the former and 65–68 mm for the latter). In comparison with *T. guizhouense*, *T. longifialidicum* growth was similar at 25 °C (45–70 mm on PDA and SNA for *T. guizhouense*; 67 mm on PDA, 60–63 mm on SNA for *T. longifialidicum*). *T. longifialidicum* often shows white or green-colored globose pustules with sterile ends, similar to those of *T. velutinum* (Fig. 4e) but distinct from *T. guizhouense* that presents irregular pustules without sterile ends. Moreover, *T. longifialidicum* and *T. velutinum* do not exhibit production of soluble pigment in artificial medium, different from *T. guizhouense* that exhibits yellowish to orange pigment at 30 and 35 °C. The conidiophores of *T. longifialidicum* are scarce, usually straight, little or not branched; also, formed mainly at the edge of pustules, rarely forming in the aerial mycelia and with

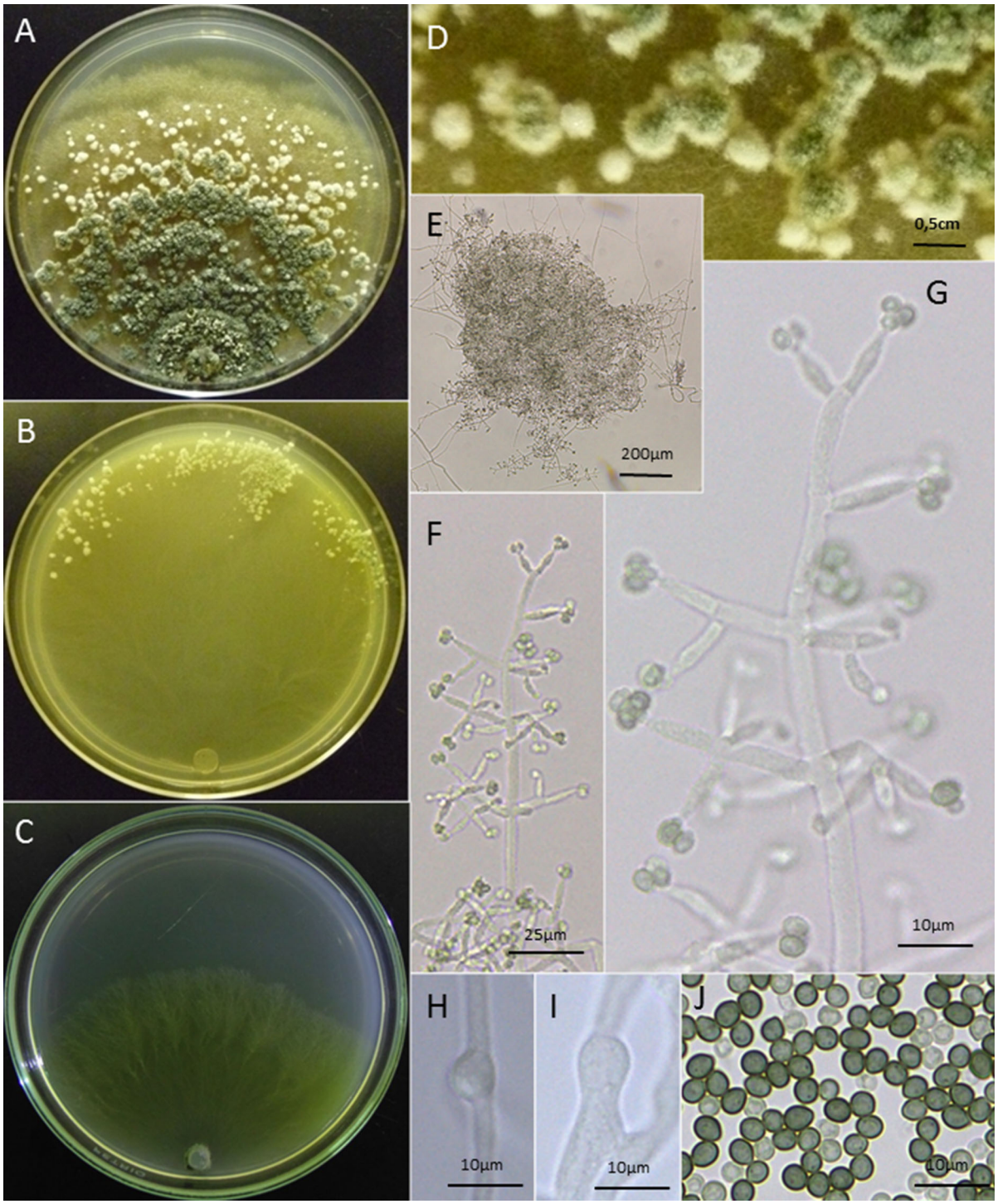


Fig. 3 Morphological characteristics of *Trichoderma texanum*. **a–c** Cultures on PDA, CMD and SNA, respectively, after 7 days of growth at 25 °C. **d**, **e** Pustules formed on CMD. **f** Conidiophore branching pattern. **g** Phialides pattern. **h**, **i** Chlamydospores. **j** Conidia

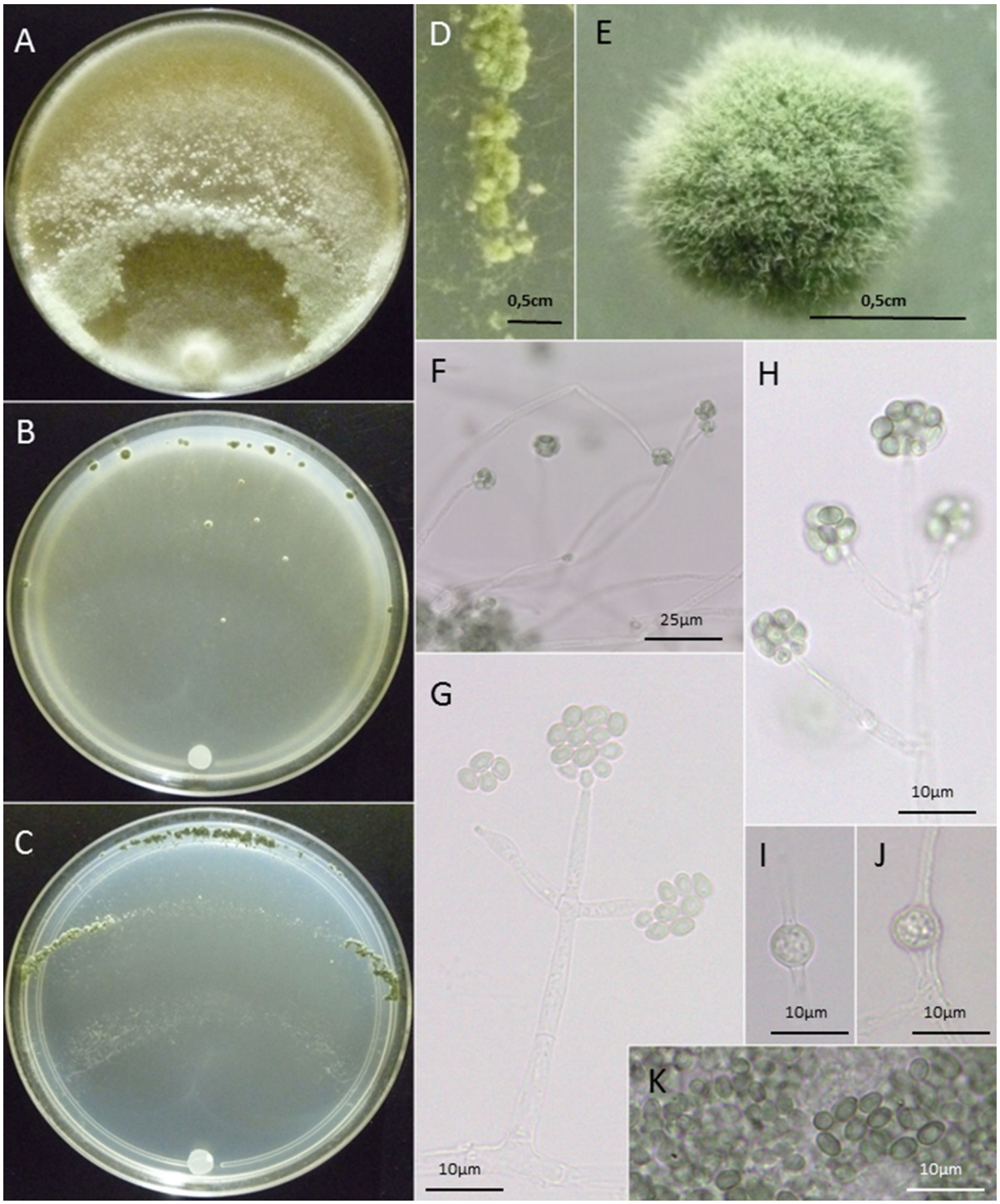


Fig. 4 Morphological characteristics of *Trichoderma longifialidicum*. **a–c** Cultures on PDA, CMD and SNA, respectively, after 7 days of growth at 25 °C. **d**, **e** Pustules with sterile terminations. **f** Long phialides on the edge of the pustule. **g**, **h** Conidiophores. **i**, **j** Chlamydospores **k** Conidia

three phialides in cross at the end of the conidiophore (Fig. 4e.). *T. longifialidicum* has a similar conidiophore when compared to *T. guizhouense*, but only *T. velutinum* has the main axis of the conidiophore ending in a elongate sterile hair. The phialide shape is a distinguishing feature of *T. longifialidicum* in comparison to other closely related species due to the elongated and cylindrical phialides (7–21 µm in length × 1.4–2.8 µm wide at the widest point and 1.1–2.3 µm wide at the base). Phialides in *T. longifialidicum* are found in whorls of two or three phialides (Fig. 4f–h). Nevertheless, *T. guizhouense* and *T. velutinum* present ampulliform and lageniform phialides (bottle shape), short and thick-walled (4.0–7.2 µm in length × 2.8–4.5 µm in width for *T. velutinum* and 4.7–7.5 µm in length × 3–4 µm in width for *T. guizhouense*). The conidia of *T. longifialidicum* (Fig. 4k) and *T. velutinum* are ellipsoidal with similar sizes; however, they differ in shape in comparison to *T. guizhouense*, which has globose to subglobose conidia. Chlamydospores of *T. longifialidicum* and *T. velutinum* are scarce in all culture media and the sizes are similar. Chlamydospores were not observed in *T. guizhouense*.

Phylogenetic distribution of *Trichoderma* strains found in the attine ant environment

We found fourteen *Trichoderma* species in leaf-cutting ant colonies and six species in non-leaf-cutting ant colonies. The two ant groups (leafcutters and non-leafcutters) shared a total of three species (*T. afroharzianum*, *T. koningiopsis* and *T. virens*, Table S1). Regarding the substrate from where we isolated *Trichoderma*, all the 20 species were found in the fungus garden, three species were observed from the soil samples collected 10 m distant to the ant colonies (*T. afroharzianum*, *T. spirale* and *T. virens*) and only *T. spirale* was found in soil samples adjacent to the fungus garden (see Table S1 for substrate sources). All *Trichoderma* species found in soil samples were also found in the fungus garden (for a full comparison of *Trichoderma* species with ant species and geographic regions, see Fig. S1).

Finally, the 62 strains analyzed in the present work grouped into eight clades which include *Trichoderma* species previously found in several habitats unrelated with the attine ant environment. The four new phylogenetic taxa (*T. attinorum*, *T. longifialidicum*, *T. texanum,* and LESF 545) clustered into four different clades (Harzianum, Spinulosa, Hypocreanum and Trichoderma, Fig. 1).

Discussion

Fungus-growing attine ant colonies harbor a rich diversity of microorganisms in addition to the mutualistic fungal cultivar. The recent demonstration that such environments house unknown microbial species stimulated our systematic survey for *Trichoderma* in leaf-cutting ant colonies. In addition, since taxonomic studies investigating filamentous fungi in non-leaf-cutting attine ants are scarce (recent examples are: Masiulionis et al. (2015) and Meirelles et al. (2015a) which focused on *Escovopsis*), we also included *Trichoderma* strains isolated from gardens of non-leaf-cutting ants (genera *Cyphomyrmex* and *Trachymyrmex*) in our study. Even considering a limited number of isolates examined, this is the first systematic study to investigate *Trichoderma* species found in colonies of non-leafcutter attine ants.

Trichoderma species are commonly found in colonies of leaf-cutting ants (Augustin et al. 2011). Previous work demonstrated the occurrence of eight *Trichoderma* species: *T. aureoviride*, *T. hamatum*, *T. harzianum*, *T. koningii*, *T. longibrachiatum*, *T. pseudokoningii*, *T. spirale*, and *T. virens* (Fisher et al. 1996; Barbosa 2004; Rodrigues et al. 2005, 2008; Augustin et al. 2011; Rodrigues et al. 2011, 2014). Our results show that attine ant colonies harbor more *Trichoderma* species than previously reported, since the 62 strains corresponded to 20 species, including three new species described in this study, along with a new phylogenetic taxon.

Among the eight *Trichoderma* species previously reported in colonies of leaf-cutting ants, four of them were also found in our study (*T. hamatum*, *T. longibrachiatum*, *T. spirale* and *T. virens*), all isolated from leafcutter ant colonies. The remaining four previously reported species (*T. aureoviride*, *T. harzianum*, *T. koningii* and *T. pseudokoningii*) were not found in our sample set. It is important to note that previous studies used only classical taxonomic methods (morphological markers) or sequenced the ITS region to identify these isolates (Fisher et al. 1996; Barbosa 2004; Rodrigues et al. 2005, 2008, 2011, 2014). Several studies showed that the morphological

species concept presents serious problems when used as the only tool for species identification in the genus *Trichoderma* (Chaverri et al. 2003a; Druzhinina and Kubicek 2005; Atanasova et al. 2013; Chaverri et al. 2015). On the other hand, using only the phylogenetic species concept for the identification of *Trichoderma* may eventually fall into the same problem, especially when a molecular marker with insufficient intraspecific variability (such as ITS region) is used alone (Druzhinina and Kubicek 2005; Atanasova et al. 2013; Chaverri et al. 2015). In that sense, it is more plausible to identify a species with a combination of morphological and phylogenetic data, using various molecular markers in a concatenated analysis [Genealogic Concordance Phylogenetic Species Recognition (GCPSR; Taylor et al. 2000; Druzhinina and Kubicek 2005; Jaklitsch 2009; Chaverri and Samuels 2013; Atanasova et al. 2013; Chaverri et al. 2015)]. Following this reasoning, we think it is necessary to sequence additional markers (as *tef1* and *rpb2*) for the previously identified species (*T. atroviride*, *T. harzianum*, *T. koningii* and *T. pseudokoningii*) to indeed confirm their presence in the attine ant colonies.

Moreover, for a description of new taxon, it is desirable to find several isolates to understand the variability of the proposed taxon regarding its geographic range, substrate preferences, morphology and physiology of the sexual (if existent) and the asexual phases (Atanasova et al. 2013). Also, phylogenetic analysis using various molecular markers (minimum *tef1* and *rpb2*) is essential (Atanasova et al. 2013; Chaverri et al. 2015). Despite the efforts to meet these recommendations, several studies showed that it is not always possible to isolate more than one strain, although this issue did not invalidate the description of several species: *Trichoderma taiwanense* (Samuels et al. 2006), *T. neokoningii*, *Trichoderma scalesiae* (Jaklitsch et al. 2006), *Trichoderma aerugineum* (Jaklitsch 2009), *Trichoderma junci*, *Trichoderma valdunense* (Jaklitsch 2011), *Trichoderma caesareum*, *Trichoderma floccosum*, *Trichoderma ivoriense*, *Trichoderma vermipilum* (Samuels et al. 2012b) and *T. lixii* (Chaverri et al. 2015).

The three taxa (LESF 236, 551 and 552) described here as new species have one strain each. Although this issue might hinder the description of the intraspecific variability of these taxa, our results show strong evidence of the distinct morphology and phylogenetic position (based with *tef1* and *rpb2* markers) that support those taxa as true taxonomic novelties. Also, when considering the biology of attine ants, these insects forage random substrates in different places; thus, it is possible that this foraged material (along with the different microorganisms found in that substrate) may not be collected again. Hence, the collection of a particular *Trichoderma* species will be influenced by how it is distributed in the environment and where the ants are foraging. Therefore, future works including more sampling of attine ant colonies and also from different substrates that surrounds the ant colonies (soil, leaves, etc.) might increase the chance to obtain more strains of the new species described here.

Fisher et al. (1996) showed the endophytic origin of some *Trichoderma* species when isolating *T. hamatum* and *T. cf. harzianum* from leaves offered to *Atta cephalotes* colonies. Several other studies with leaf-cutting ants suggested that *Trichoderma* fungi are associated with the plant substrate foraged by workers (Barbosa 2004; Rodrigues et al. 2008, 2009, 2011). Our results support this hypothesis, since 51 out 62 strains derived from leaf-cutting ants were isolated from plant material present in the fungus gardens. Moreover, *T. atroviride*, *T. eucorticioides*, *T. hamatum*, *T. longibrachiatum*, *T. orientalis*, *T. spirale* and *T. viride* found on fungus garden samples, have been reported as endophytes on substrates not related to leaf-cutting ants (Overton et al. 2006; Samuels et al. 2006; Bae et al. 2009; Chaverri et al. 2011; Druzhinina et al. 2012; Bailey and Melnick 2013). However, *Trichoderma* species are also parasites of other fungi (Bailey et al. 2008; John et al. 2010; Chaverri and Samuels 2013); in this sense, they could also enter the fungus garden together with the leaves collected by the ants, possibly as mycoparasites of plant pathogenic fungi. In fact, this route of entry for *Trichoderma* was not considered in previous studies and future efforts might look for evidences for this hypothesis.

Regarding *T. viride*, this species was considered to be restricted to the northern hemisphere and found in soil, trunks and decaying wood, with only two strains reported in the tropics, isolated as endophytes on *Theobroma gileri* (Ecuador) and *Theobroma cacao* (Brazil) (Lieckfeldt et al. 1999; Jaklitsch et al. 2006; Samuels et al. 2006). The present study identified a *T. viride* strain (LESF 115) in the fungus garden of *Atta sexdens rubropilosa*, which indicates this fungus could have been carried by the ants as an endophyte.

Moreover, this result supports the hypothesis proposed by Samuels et al. (2006) suggesting this taxon also occurs in the tropics, but probably in less exploited environments, such as the attine ant colonies.

The non-leaf-cutting attine ants collect several substrates to feed their mutualistic fungus (Mehdiabadi and Schultz, 2010). Given the cosmopolitan characteristic of the genus *Trichoderma*, such substrates are likely sources of *Trichoderma* spores. In our study, we analyzed a total of six *Trichoderma* strains isolated from gardens of non-leaf-cutting ants, including *Trachymyrmex* spp. (n = 4) and *Cyphomyrmex wheeleri* (n = 2, Table S1). The strains were identified as *T. koningiopsis*, *T. afroharzianum*, *T. virens*, *T. attinorum*, *T. texanum* and *T. longifialidicum*; intriguingly, the three latter strains are new species. The fact of finding new species in gardens of non-leaf-cutting attine ants indicates that the colonies of such insects, less exploited when compared to leaf-cutting ants, might harbor a high richness of *Trichoderma* species, especially due to the wide range of substrates they collect.

On the other hand, Augustin et al. (2011) and Rodrigues et al. (2014) suggested that the soil could also be an alternative source for diversity of *Trichoderma* species present in colonies of leaf-cutting ants. For example, Rodrigues et al. (2014) showed that soil samples distant from the ant colonies have several fungal species such as *T. spirale*, which are also frequently isolated from the fungus gardens. The authors suggested that these fungi would be transported by foragers that are directly in contact with soil. Here, we identified *T. spirale* (LESF 549 and LESF 543) isolated from the soil adjacent to the chamber that holds the fungus garden of *Atta sexdens rubropilosa* and *Atta bisphaerica* (Table S1). Similarly, we identified *T. afroharzianum* (LESF 228, LESF 229) and *T. spirale* (LESF 557, LESF 558, LESF 559) isolated from soil 10 m far from *Atta bisphaerica*; *T. virens* (LESF 550) and *T. spirale* (LESF 562) was also isolated from soil 10 m distant from a colony of *A. sexdens rubropilosa* (Table S1). These three species (*T. afroharzianum*, *T. spirale* and *T. virens*) were also found in fungus garden samples (Table S1), reinforcing that the soil is another source for the diversity of *Trichoderma* in leaf-cutting ants colonies.

Our phylogenetic analysis also revealed that none of the 62 strains evaluated form a specific group of *Trichoderma* associated exclusively with attine ant colonies, since they grouped in clades comprehending *Trichoderma* strains isolated from various sources (Fig. 1; Table S2). Furthermore, we did not observe *Trichoderma* strains associated with a specific genus of leaf-cutting ants (i.e. the same *Trichoderma* species can occur in both *Atta* and *Acromyrmex* genera, Fig. 1; Table S1). Regarding the three new species isolated from gardens of non-leaf-cutting ants, the most parsimonious hypothesis is to consider that they may have been indirectly brought to the colonies, similarly to other *Trichoderma* species. In our phylogenetic analysis, the three new phylogenetic species grouped in different parts of the tree; if these species were exclusively associated with attine ants, such association would have originated multiples times over the evolutionary time. However, the fact that only few strains have been isolated indicates that they are not a common occurrence in this environment, which also supports the idea that they were brought in by ants suggesting the transient nature of *Trichoderma* in attine ant colonies (Poulsen and Currie 2006).

The genus *Trichoderma* has several ecological roles (Druzhinina and Kubicek 2005; Samuels et al. 2006; Jaklitsch 2009; Atanasova et al. 2013; Chaverri and Samuels 2013; Druzhinina and Kubicek 2013). Because ants feed their mutualistic fungus with the forage plant material, we can infer that some *Trichoderma* species (those who manage to resist the cleaning process of the ants) could use nutrients found in the fungus garden matrix as well as some species could act as an antagonist of the mutualistic fungus, especially those that are mycotrophic (for example: *T. lixii*, *T. ceramicum*, *T. parestonicum*, *T. viride*). However, so far there is no evidence that *Trichoderma* performs any of these roles in the attine ant colonies.

Finally, the presence of three new species (*T. attinorum*, *T. longifialidicum* and *T. texanum*) and a new phylogenetic taxon (LESF 545) in the attine ants' gardens corroborates recent results indicating the potential of this environment to harbor high microbial diversity. Apparently, the dynamic foraging behavior of these insects (gathering several types of substrates) offers a unique environment for a rich and diverse microbiome. Future exploratory studies in this environment will certainly reveal even more unknown species for science, not only within the genus *Trichoderma* but for other fungal groups as well.

Taxonomy

Trichoderma attinorum Q.V. Montoya, L.A. Meirelles, P. Chaverri & A. Rodrigues sp. nov. (Fig. 2)

Mycobank: MB814486

Etymology: "*attinorum*" in relation to the name of the tribe Attini, the first source from where the species was isolated.

Typification: USA. Austin, Texas, Bull Creek Park, GPS 30°22′16″N; 97°47′08″W, Fungus garden, 10, 2006. *A. Rodrigues.* Ex-type strain LESF 236 (= CBS 139783, = CBMAI 1826). Holotype: CBS H-22180 (dried culture on PDA).

Sequences: ITS (HQ608035), *tef1* (KT279039) and *rpb2* (KT278971).

Teleomorph unknown.

Culture characters: *Colonies* showed a radius of 39–41; 25–30 and 5–10 mm on PDA, CMD and SNA, respectively, after 72 h at 25 °C in the dark; at 30 °C colonies exhibited a radius of 30–31, 29–38 and 6–11 mm on PDA, CMD and SNA, respectively. At 35 °C, the radius reached 10–15, 2–7 and 3–10 mm on PDA, CMD and SNA, respectively. The colony fills the plate after 7 days of incubation at 25 and 30 °C on PDA. After 72 h at 25 and 30 °C, concentric rings of alternating green and white colour were observed in cultures maintained on PDA. The conidia pigmentation starts from the inoculum region and continues instead of maintain to the edge of the colony after 48 h on PDA. The presence of yellow soluble pigmentation was observed in CMD and SNA after 48 h of incubation at all three temperatures. *Hyphae* are hyaline and smooth, usually forming pustules on PDA and CMD, no aerial mycelium on SNA. *Pustules* are globose to subglobose and cottony, connecting together forming dense masses and measuring 1–3 mm in diameter. *Conidiophores* are straight, with short branches, matched and in whorls, forming at the edge of pustules and in aerial mycelium. *Phialides* are formed usually in whorls, paired and rarely isolated, lageniform to cylindrical-shaped, usually straight and rarely curved measuring 5.2–11.2 µm in length, 1.8–2.8 µm in width at the widest point, 1.2–2.3 µm in width at the base. Phialides are formed from supporting cells measuring 4.5–14.6 µm in length × 1.7–3.4 µm wide; phialides rarely form from the central conidiophore hyphae. *Conidia* subglobose to ovoid, dark green-colored and smooth, measuring 3.2–4.3 × 2.5–3.4 µm. *Chlamydospores* hyaline and intercalated, measuring 5.3–11.4 × 4.5–6.8 µm, observed after 72 h at 25 °C on CMD and PDA.

Habitat: isolated from fungus garden of *Cyphomyrmex wheeleri*.

Notes: *T. attinorum* is closely related to *T. sipulosum*, *T. sinuosum* and *T. thelephoricola* and its most distinctive features are the dense pustules and yellow soluble pigment observed in the three culture media and at all temperatures. Moreover, differences in the shape of the conidiophores are present when compared to *T. sipulosum*, *T. sinuosum* and *T. thelephoricola* (the last one has a *Gliocladium*-like conidiophore, an even more remarkably-different conidiophore morphology when compared to *T. attinorum*).

Trichoderma texanum Q.V. Montoya, L.A. Meirelles, P. Chaverri & A. Rodrigues sp. nov. (Fig. 3)

Mycobank: MB814487

Etymology: "*texanum*" in relation to the state of "Texas" (EUA), from where the species was first isolated.

Typification: USA. Smithville, Texas, Stengl "Lost Pines" Biology Station, GPS 30°05′13″N; 97°10′15″W, Fungus garden, 10, 2006. *A. Rodrigues*. Ex-type strain LESF 551 (= CBS 139784, = CBMAI 1828). Holotype: CBS H-22182 (dried culture on PDA).

Sequences: ITS (HQ608136), *tef1* (KT278988) and *rpb2* (KT278920).

Teleomorph unknown.

Culture characters: *Colonies* showed a radius of 55–56; 45–46 and 8–10 mm on PDA, CMD and SNA, respectively after 72 h at 25 °C in the dark. At 30 °C colonies exhibited a radius of 37–42, 48–59 and 10–23 mm on PDA, CMD and SNA, respectively. No concentric rings or soluble pigmentation was observed. The colony fills the plate after 96 h on PDA and CMD and after 7 days on SNA. The conidia pigmentation starts from the inoculum region to the edge of the colony after 7 days on PDA. *Hyphae* are hyaline, smooth and sinuous, but not in a spiral. *Pustules* are small, globoid, cottony in masses, forming from the inoculum center on PDA and from the edge of the plate on CMD. *Conidiophores* usually straight, but rarely curved or sinuous, forming both from on the edge of the pustules and from aerial hyphae. The conidiophores have branches, usually paired, and alternated, but sometimes in whorls. *Phialides* are generally paired and whorls of three,

forming usually from supporting cells, which measures 2.1–15.5 μm in length × 1.6–3.1 μm in width; or from the conidiophore apex. Phialides are cylindrical-shaped, usually arranged in whorls, isolated or paired, measuring 7.1–13 μm in length, 1.2–2.8 μm in width at the widest point, and 1.2–2.3 μm in width at the base. *Conidia* are globose to subglobose, dark green, smooth-walled and measuring 1–4 μm in length × 1.8–3.1 μm in width. *Chlamydospores* hyaline, intercalated, measuring 5.3–15.4 μm in length × 3.4–7.9 μm in width; chlamydospores were observed on CMD and PDA, after 5 days of growth at 25 °C.

Habitat: isolated from fungus garden of *Trachymyrmex septentrionalis*.

Notes: *T. texanum* is closely related to *T. koningiopsis*, *T. petersenii* and *T. ovalisporum. T. texanum* morphologically differs from these three species, due to (i) the pustules formation (rare in *T. texanum* and abundant and dense on the other species); (ii) conidia pigmentation (after seven days in *T. texanum* and from two to four days for the other species); (iii) the conidia shape (globose-subglobose for *T. texanum*, and ellipsoid for the other species); (iv) chlamydospores' size and shape (larger and more elongated on *T. texanum*).

Trichoderma longifialidicum Q.V. Montoya, L.A. Meirelles, P. Chaverri & A. Rodrigues sp. nov. (Fig. 4)

Mycobank: MB814488

Etymology: "*longifialidicum*" relating to the long length of the phialides when compared to phylogenetic close species.

Typification: USA. Smithville, Texas, Stengl "Lost Pines" Biology Station, GPS 30°05′13″N; 97°10′15″W, Fungus garden, 10, 2006. *A. Rodrigues*. Ex-type strain LESF 552 (= CBS 139785, = CBMAI 1827). Holotype: CBS H-22181 (dried culture on PDA).

Sequences: ITS (KT278901), *tef1* (KT279020) and *rpb2* (KT278955).

Teleomorph unknown.

Culture characters: *Colonies* showed a radius of 67; 60–63 and 39–43 mm on PDA, CMD and SNA, respectively after 72 h at 25 °C in the dark. At 30 °C colonies exhibited a radius of 65–68, 58–67 and 40–47 mm on PDA, CMD and SNA, respectively. Concentric rings were observed on PDA and SNA. No soluble pigmentation was present. The colony filled all the plate after 96 h on all culture media. The conidia pigmentation starts from the center of the colony, after 7 days on PDA. *Hyphae* are hyaline, smooth and sinuous. *Pustules* are globoid, cottony, isolated on CMD and in dense masses on PDA and SNA, forming from the center of the plate on PDA and disperse on CMD and SNA. *Conidiophores* are usually straight, forming more often on the edge of pustules and fewer in the aerial hyphae. The conidiophores have few branches, usually alternated. *Phialides* generally paired and in whorls of three, forming usually directly from the central axis of the conidiophore and from supporting cells, which measures 6–16 × 1.7–3.2 μm. Phialides are long, cylindrical, usually paired or isolated, measuring 7–21 μm in length, 1.4–2.8 μm in width at the widest point, and 1.1–2.3 μm in width at the base. *Conidia* are ellipsoidal, green, smooth-walled and measuring 2.5–3.5 μm long × 1.6–2.4 μm in width. *Chlamydospores* are globose, hyaline and intercalated, measuring 7–8 × 6–6.2 μm; chlamydospores were observed on CMD and PDA, after 5 days of growth at 25 °C.

Habitat: isolated from fungus garden of *Trachymyrmex septentrionalis*.

Notes: *T. longifialidicum* is closely related to *T. velutinum* and *T. guizhouense*. *T. longifialidicum* has few morphological differences compared to the other two species; however, its most distinct morphological feature is the shape and size of its phialides (cylindrical and elongated in *T. longifialidicum*; short and ampuliform in the other species).

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