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**UNRAVELING *TRICHODERMA* SPECIES IN ATTINE ANT
ENVIRONMENT: DESCRIPTION OF THREE NEW TAXA**
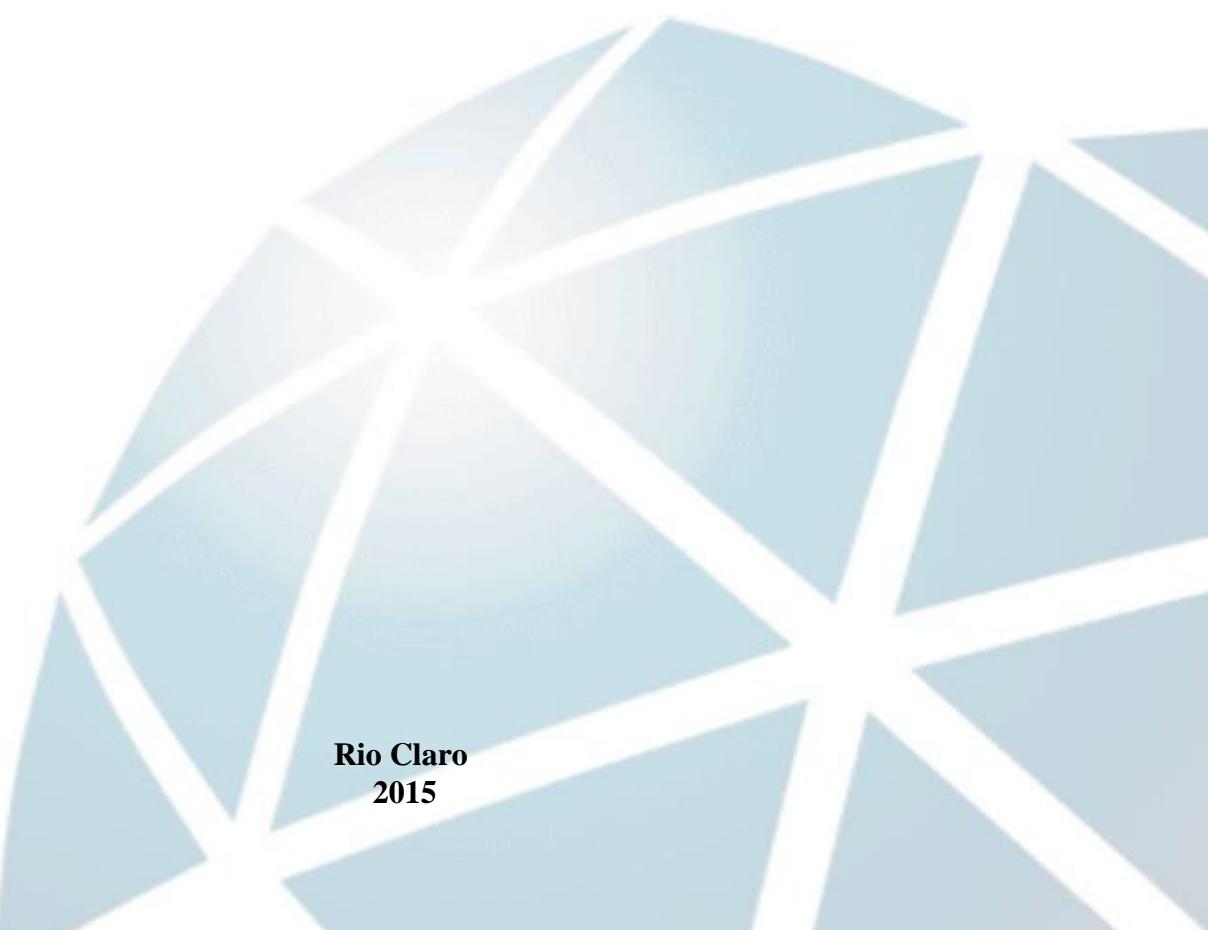
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Orientador: Prof. Dr. André Rodrigues

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TÍTULO: ATTINE ANT COLONIES: A RICH ENVIRONMENT FOR TRICHODERMA SPECIES

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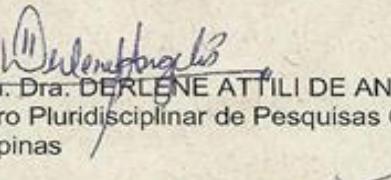
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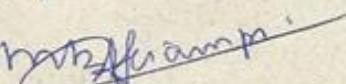
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À Maria Jesus Sutta Martiarena e a minha mãe,
pelos seus ensinamentos e apoio incondicional.

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Summary

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

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Abstract

Fungus-growing "attine" ants forage diverse substrates to grow mutualistic fungi for food. In addition to the fungal partner, colonies of these insects harbor a rich microbiome composed of bacteria, filamentous fungi and yeasts. Previous studies investigated ecological aspects of filamentous fungi in gardens of leaf-cutter ants and reported some *Trichoderma* species; however, no studies systematically addressed the putative association of *Trichoderma* species with attine ants, especially in non-leafcutter ants. Here, a total of 68 strains of *Trichoderma* preserved in our collection were analyzed using three molecular markers (ITS, *tef1* and *rpb2*). In addition, 30 out of 68 strains were also morphologically examined. The strains correspond to samples collected from leaf-cutter and non-leafcutter ants in 12 points from four states in Brazil and two points in Texas (USA), comprising the largest sampling carried out so far for *Trichoderma* in attine ant environment. Our results revealed the richness of *Trichoderma* in this environment, since we found 19 *Trichoderma* species, including three new species described in the present work (*T. attinis*, *T. texana* and *T. longifialidicum*). Moreover, we show that all 68 strains grouped within different clades across the *Trichoderma* phylogeny, which are identical or closely related to strains derived from several other environments, supporting for a transient nature of the genus *Trichoderma* in 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 several fungal taxa still unknown for science.

Keywords: Hypocreales, Attini, diversity, fungus garden, transient.

Introduction

Fungus-growing ants ("attine ants") are found only in the American continent and comprise 17 genera with about 257 species (Brandão et al. 2011; Sosa-Calvo et al. 2013). Attine ants maintain a mutualistic interaction with basiodiomycetous fungi in an obligatory association originated about 50 million years ago (Schultz and Brady 2008). In this mutualism, workers collect various types of substrates (fresh leaves, seeds or 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 workers (Weber 1972).

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 mycelium of the fungal cultivar (Weber 1972).

Möller (1893) was the first to study fungi cultivated by attine ants and also showed the presence of *Aspergillus*, *Mucor*, *Penicillium* and *Rhizopus* in gardens of *Acromyrmex disciger*. Later, Kreisel (1972) reported *Cunninghamella*, *Fusarium*, *Rhizopus* and *Trichoderma* in ant-deprived gardens of *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 diversity is mostly related to the type of plant substrate that workers use to nourish the fungal cultivar (Fisher et al. 1996). Such origin is plausible for endophytic fungi found within the leaf tissue

collected by leaf-cutting workers (Van Bael et al. 2009). Rodrigues et al. (2005, 2011) suggested that fungi are carried to colonies by workers that are in direct contact with soil. The authors also suggested *Cunninghamella elegans*, *Syncephalastrum racemosum* and *Trichoderma harzianum* as common representatives (Rodrigues et al. 2005, 2011). On the other hand, no studies investigated the diversity of filamentous fungi I colonies of non-leaf-cutting attine ants. Because these ants use seeds, plant sap, insect frass and parts of dried leaves and flowers to grow the fungal cultivar (Mehdiabadi and Schultz, 2010), such substrates may also contain and transfer *Trichoderma* to their colonies.

Since the first report of *Trichoderma* in colonies of leaf-cutting ants (Kreisel 1972), several studies reported the occurrence of this genus in colonies of these insects (Barbosa 2004; Poulsen and Currie 2006; Rodrigues et al. 2008, 2009; Augustin 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, these studies sequenced the internal transcribed spacer (ITS) marker, which does not resolve species identification in several *Trichoderma* clades, especially for *T. harzianum* species complex (Atanasova et al. 2013).

Systematic studies involving microorganisms associated with leaf-cutting ants showed a high diversity in 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 (Currie et al. 1999; Currie et al. 2003; Muchovej and Della Lucia 1990; Seifert et al. 1995; Augustin et al. 2013; Masiulionis et al. 2015; Meirelles et al. 2015). These studies showed the presence of

several new *Escovopsis* species, which suggests that attine ants colonies might also harbor a high diversity of filamentous fungi, including potential new species of the genus *Trichoderma*.

To date 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 that occur in the attine ants colonies (considering both leaf-cutting and non-leaf-cutting species). Therefore, we use polyphasic taxonomy (morphological and molecular markers) to identify 68 strains isolated from fungus gardens, soil adjacent to the garden and soil distant from the colonies. Here, we show a high number of *Trichoderma* species found in attine ant colonies, including three new *Trichoderma* species. Such findings suggest that colonies of these insects may harbor more species still unknown for science.

Material and Methods

Strains examined

The strains examined were obtained in several studies conducted 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). All examined strains are preserved in 10% glycerol at -80 °C at the UNESP - Microbial Resources Center (CRM-UNESP). A total of 68 *Trichoderma* strains were revived and examined. From this total, 62 are derived from leaf-cutting ant colonies (*Atta* and *Acromyrmex*) and six from gardens of non-leaf-cutting ants (*Cyphomyrmex* and *Trachymyrmex*). Samples comprised fungi found in the fungus gardens (n = 51 strains from

leaf-cutting ants and n=6 strains from non-leaf-cutting ants), soil adjacent to gardens of leafcutters ants (n = 4) and soil 10 m distant from leaf-cutting ant colonies (n = 7, Table 1).

Trichoderma strains comprised a total of 38 ant colonies corresponding to 13 attine ant species (Table 1). Samples were collected over the past ten years in 12 points from four states in Brazil and two points in Texas-USA (Table 1), so far comprehending 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 five 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 internal transcribed spacer (ITS) and partial sequences of the genes coding for the elongation factor 1 alpha (*tef*1) and the second subunit of RNA polymerase II (*rpb2*). Amplification reactions for the three markers were performed in a final volume of 25 µL (4 µL of dNTPs, [1.25 mM each]; 5 µL of 5X 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 primers and conditions are described in Table 2.

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, Table 2).

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 the strains were compared to homologous sequences deposited in 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) only *tef1* and *rpb2* sequences were used for phylogenetic analyses. Sequences generated in other studies were recovered from the databases following several studies (Table S1). The final dataset comprised 179 partial *tef1* sequences (823 bp) and 146 partial *rpb2* sequences (1058 bp). Alignments were performed separately for each molecular marker using MAFFT v.7 (Katoh and Standley, 2013). Strains LESF 121 and LESF 345 were excluded from the final phylogenetic analysis since they exhibited problems on *tef1* sequencing; however, based on morphological evidence and high-quality *rpb2* sequences, such strains were identified as *T. harzianum*.

The *tef1* and *rpb2* alignments were concatenated using Winclada v.1.00.08 (Nixon, 2002), and the final file contained a total of 1881 bp. We applied Bayesian inference in MrBayes v.3.2.2 to reconstruct the phylogenetic trees (Ronquist et al. 2012). Nucleotide substitution models, HKY+I+G and SYM+I+G, were selected for *tef1* and *rpb2*, respectively, using the Akaike information criterion under 95% of confidence interval in jModelTest 2 (Darriba et al. 2012). Two separate runs were performed, each consisting of three hot chains and one cold chain; each run consisted of a Markov Chain Monte Carlo (MCMC) sampling of 3 million generations, which were sufficient to achieve values for

standard deviation of split frequencies below 0.01. Finally, the first 25% of the generations of MCMC were discarded as burn-in.

Morphological characterization

A total of 30 out of 68 strains were selected for morphological examination based on the results of phylogenetic analysis. We examined (i) macroscopic characteristics of the colony (radius growth, mycelium color, formation of soluble pigment, concentric rings and pustules) and (ii) microscopic features (conidiophore, supporting cells, phialides, conidia and chlamydospores) based on Samuels et al. (2002, 2012), Chaverri et al. (2001, 2003a, 2003b), 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 seven days at 25 °C for 12h (light) and 12 (dark). Growth tests were performed on all three media and incubated at four different temperatures (25, 30, 35 and 40 °C) in the dark. For these tests, the strains were initially grown for seven days in water-agar medium. Thereafter, mycelial 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, the size was sufficient to cover the whole plate. Growth tests were performed in triplicate on three separate time periods for one week. Measurements of growth radius were performed after 72h and 96h.

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 (12h light/12h dark) for a time period of 3 to 5 days. Moreover, we also prepared wet mounts using KOH for

additional observations. All preparations were documented using an optical microscope (Leica DM750, Germany) and measurements of the structures were performed with 30 replicates using the program LAS v4.0 (Leica).

Results

Phylogenetic analyses

The 68 *Trichoderma* strains grouped into 19 different phylogenetic species belonging to 12 clades, following the division proposed by Druzhinina et al. (2005). Among the 19 phylogenetic species, here we present four new phylogenetically strains (LESF 236, LESF 545, LESF 551 and LESF 552, Fig. 1). Moreover, we also identified 15 *Trichoderma* species: *T. afroharzianum* (n = 7 strains), *T. andinense* (n = 2), *T. asperelum* (n = 1), *T. atroviride* (n = 1), *T. deliquescens* (n = 1), *T. eucorticioides* (n = 1), *T. hamatum* (n = 3), *T. harzianum* (n = 7), *T. koningiopsis* (n = 6), *T. longibrachiatum* (n = 1), *T. orientalis* (n = 3), *T. spirale* (n = 15), *T. velutinum* (n = 1), *T. virens* (n = 12) and *T. viride* (n = 1). All these 15 species were originally described in studies not related to attine ants.

Among the four new phylogenetic strains found, three are described here as new species: *T. attinis* (LESF 236), *T. texana* (LESF 551) and *T. longifialidicum* (LESF 552). Strain LESF 545 did not sporulate; therefore, it was not possible to describe its morphological characteristics. 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 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 showed 93% (*tef1*) and 97% (*rpb2*) similarity with *T. velutinum* (AY937415 and JN133569, respectively), 91% (*tef1*) and 96% (*rpb2*) with *T. harzianum* (FJ463318 and FJ442800, respectively). Finally, strain LESF 545 was 89% (*rpb2*) similar to *T. citrinum* (FJ179603) and *T. taxi* (DQ859032).

Growth rate

The 30 strains analyzed (see Material and Methods) grew between 25 and 30 °C. Ten strains grew at 35 °C (LESF 009, 010, 107, 117, 118, 236, 360, 541, 544 and 555) and only three strains grew at 40 °C (LESF 009, 541 and 544). The optimum temperature for growth on PDA was not the same for all strains: while most strains grew better at 25 °C (LESF 006, 010, 107, 108, 115, 117, 118, 119, 120, 121, 132, 330, 360, 551 and 561), ten strains showed better growth at 30 °C (LESF 111, 112, 211, 331, 236, 459, 545, 552, 555 and 556). Two strains showed no difference in growth rate between 25 and 30 °C (LESF 554 and 208) and three strains showed no difference between 25 and 35 °C (LESF 009, 541 and 544). The best mycelium development for all tested strains was observed on PDA, compared to CMD and SNA.

Morphology

The 19 species (corresponding to 11 clades in Fig. 1) had different colony morphological features, but not for all species this difference was significant (e.g. *T. harzianum* and *T. afroharzianum* showed similar morphology). Twelve species formed concentric rings in culture after 72h of growth (*T. afroharzianum*, *T. asperellum*, *T. deliquescens*, *T. harzianum*, *T. hamatum*, *T. koningiopsis*, *T. longibrachiatum*, *T. orientalis*,

T. virens, *T. viride*, *T. attinis* and *T. longifialidicum*). All species, except *T. afroharzianum*, *T. eucorticioides* and *T. deliquescens* formed pustules on CMD and SNA, and less frequently on PDA (Fig. S1-S15). The conidia formation on culture started between the third and fifth day of incubation in all three culture media, but LESF 545 showed no conidia formation on any media up to three months of evaluation at 25 to 30 °C, in the dark, with light alternation (12h light/12h near-UV light) in the three culture media.

The conidia shapes ranged from (i) globose-subglobose (*T. afroharzianum*, *T. pseudoharzianum*, *T. viride*, *T. asperellum*, *T. texana*); (ii) subglobose (*T. atroviride*, *T. eucorticioides*, *T. harzianum*); (iii) subglobose-ovoid (*T. attinis*); (iv) ellipsoidal (*T. andinense*, *T. hamatum*, *T. koningiopsis*, *T. longibrachiatum*, *T. spirale*, *T. velutinum*, *T. deliquescens*, *T. orientalis* and *T. longifialidicum*) to (iv) ellipsoidal-ovoid (*T. virens*). In addition, with the exception of *T. viride*, all strains showed smooth-walled conidia. Green colored conidia was observed for all species, except for *T. eucorticioides* (no pigmentation) (Fig. S1-S15).

Conidiophores formed in aerial mycelia and in pustules (when present). *T. hamatum* and *T. longifialidicum* showed sterile hairs at terminal branches of conidiophores (Fig. S7 and S4). The conidiophores branches were variable depending on the clades; we observed branches with alternate arrangement, in pairs, in whorls and usually with an angle less than 90° with respect to the central axis of the conidiophore. Phialides showed alternate arrangement, regularly paired and in whorls of three or more, which were formed directly from the central hyphae, on branches or supporting cells. The intercalated and terminal chlamydospores formed in the aerial mycelia and in the submerged mycelium in CMD and PDA.

The three strains described as new species (*T. attinis* LESF 236, *T. texana* LESF 551 and *T. longifialidicum* LESF 552) had distinct morphological characteristics when compared to their phylogenetic closest relatives. *T. attinis* grouped close to *T. sinuosum* (Chaverri et al. 2003a), *T. thelephoricola* (Chaverri and Samuels 2003) and *T. spinulosa* (Chaverri et al. 2003a) on the phylogenetic analysis (Fig. 1). *T. spinulosa* does not form an asexual phase, different from *T. attinis* that only formed the asexual phase (Fig. 2). *T. sinuosum* growth rate is relatively higher than *T. attinis* (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. attinis*). The growth rate of *T. thelephoricola* was lower than *T. attinis* (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 the agar, while *T. attinis* showed soluble pigmentation on all three culture media (Fig. 2A, B, C). Conidiophores of *T. attinis* showed significant differences when compared to *T. sinuosum* and *T. thelephoricola*. In *T. sinuosum* they are branched and sinuous, in *T. attinis* they are usually straight and have short branches (Fig. 2F, G). When compared to *T. thelephoricola*, the difference is even greater since this species has conidiophores similar to *Gliocladium* (*Gliogladum*-like). Regarding the phialides, *T. thelephoricola* presents shape and measures similar to *T. attinis* (lageniform and cylindrical); however, *T. sinuosum* presents ampulliform phialides distinct from those of *T. attinis* (Fig. 2G). The conidia of *T. thelephoricola* are longer than the others. Finally, *T. attinis* presents chlamydospores (Fig. 2H, I) more elongated than *T. sinuosum* (they are globular-subglobose in *T. sinuosum* and subglobose-ovoid in *T. attinis*); *T. thelephoricola* has no chlamydospores formation.

T. texana grouped close to *T. ovalisporum*, *T. koningiopsis* and *T. petersenii* (Fig. 1). The three latter species have concentric rings on PDA, while *T. texana* does not (Fig. 3A). The optimum growth temperature was between 25 and 30 °C for all four species (*T. texana*, *T. ovalisporum*, *T. koningiopsis* and *T. petersenii*). Pustules formation in *T. texana* occurred in less extent on CMD, but always on the edge of the plate (Fig. 3B), unlike *T. ovalisporum*, *T. koningiopsis* and *T. petersenii*, which form abundant pustules in concentric rings. Conidia pigmentation was visible after seven days *T. texana*, differently from *T. ovalisporum* (after 72h), *T. koningiopsis* (after 48h) and *T. petersenii* (after 96h). Only *T. texana* produces soluble pigment in the culture. Conidiophores in *T. texana* are similar to *T. ovalisporum*, *T. koningiopsis* and *T. petersenii* regarding the branches arrangement, but a few differences were also observed: in *T. ovalisporum* and *T. koningiopsis* conidiophores are branched in the base and show long and unbranched (or slightly branched) ends, where the phialides are formed directly from the central conidiophore hyphae; however, in *T. petersenii* and *T. texana* 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, F, G). Additionally, *T. ovalisporum* and *T. koningiopsis* have a central axis of the conidiophore at an angle close to 90°; however, in *T. texana* and *T. petersenii* this angle is smaller than 90° (Fig. 3G). The phialides arrangement is similar in all four species, but *T. ovalisporum*, *T. koningiopsis* and *T. petersenii* show lageniform phialides while in *T. texana* they are cylindrical (Fig. 3G). *T. texana* has longer and thinner phialides when compared to *T. ovalisporum*, *T. koningiopsis* and *T. petersenii*. There are also differences on the conidia shapes: *T. ovalisporum*, *T. koningiopsis* and *T. petersenii* have ellipsoidal conidia, while *T. texana* presents globose-subglobose conidia (Fig 3H). Chlamydospores of

T. texana (Fig. 3I, J) are longer than in the other three species (in which chlamydospores has globose-subglobose shape).

T. longifialidicum grouped close to *T. velutinum* (Bissett et al. 2003) and *T. harzianum* (Rifai 1969) on our phylogenetic analysis (Fig. 1). The growth rate for *T. velutinum* at 30 °C was relatively smaller than *T. longifialidicum* (54 - 62 mm for the first and 65 - 68 mm for the second, both on PDA). Also, when compared to *T. harzianum*, *T. longifialidicum* growth at 25 °C is relatively higher (51 - 57 mm on PDA, 43.5 - 50 mm on SNA for *T. harzianum*; 67 mm on PDA, 60 - 63 mm on SNA for *T. longifialidicum*). Unlike *T. velutinum* and *T. longifialidicum*, *T. harzianum* has no growth at 35 °C. *T. longifialidicum* has pustules with sterile ends in white and green color, similar to *T. velutinum* (Fig. 4E). On the other hand, in *T. harzianum* is not observed the pustules formation according to literature. None of these species including *T. longifialidicum* exhibited production of soluble pigment in agar. The conidiophores of *T. longifialidicum* are scarce, usually straight, little branched and usually they form at the edge of pustules (Fig. 4E.). However, unlike *T. velutinum*, there is no formation of sterile terminations in conidiophore apex. The conidiophores of *T. harzianum* are significantly different when compared to *T. longifialidicum* since in the first they are well branched and the branches are long. The phialide shape is a mark of *T. longifialidicum* when compared to other closest relatives species because *T. longifialidicum* features elongated and cylindrical phialides (7 - 21 μm in length x 1.4 - 2.8 μm wide at the widest point and 1.1 - 2.3 μm wide at the base); the phialides are paired or three whorls (Fig. 4F, G, H). *T. velutinum* presents ampulliform and lageniform phialides (bottle shape), short and thick. On the other hand, *T. harzianum* exhibits smaller phialides with lageniform shape (6.5 - 6.7 μm in length x 2.5 - 3.5 μm in width at the widest point and 1.6 - 2.5 μm in width in basis). Regarding the conidia, *T.*

longifialidicum (Fig. 4K) and *T. velutinum* have similarity in both shape (ellipsoidal) and size; however, they are different in shape when compared to *T. harzianum*, which has subglobose-ellipsoidal conidia. Chlamydospores for the three species are scarce in all culture media and the sizes are similar.

Distribution of Trichoderma strains

We found 16 *Trichoderma* species in leaf-cutting ants colonies and six species present in non-leaf-cutting ants colonies. The two ant groups (leafcutters and non-leaf cutters) shared a total of three species (*T. afroharzianum*, *T. koningiopsis* and *T. virens*, Table 1). Moreover, three species were identified from soil samples adjacent to fungus garden (*T. afroharzianum*, *T. harzianum* and *T. spirale*) and three species from soil samples 10 m distant to colonies (*T. afroharzianum*, *T. spirale* and *T. virens*). All *Trichoderma* species found in soil samples were also found in the fungus garden (Table 1). Finally, the 68 strains analyzed were randomly grouped into clades studied in several works unrelated with the attine ant environment (Fig. 1). The four new phylogenetic species (*T. longifialidicum*, *T. attinis*, *T. texana* and LESF545) clustered into four different clades (Harzianum, Spinulosa, Hypocrearum and Viride, Fig. 1).

Discussion

Attine ant colonies harbor a rich diversity of microorganisms in addition to the mutualistic fungal cultivar. Due to the recent demonstration that such environment houses unknown microbial species stimulated our systematic survey for *Trichoderma* in leaf-cutting ant colonies. In addition, since taxonomic studies investigating filamentous fungi species associated from non-leaf-cutting attine ants are scarce (recent examples are:

Masiulionis et al. [2015] and Meirelles et al. [2015] which focused on *Escovopsis* taxonomy), we also included *Trichoderma* strains isolated from gardens of non-leaf-cutting ants (*Trachymyrmex* and *Cyphomyrmex*) in the present study. Even considering a limited number of isolates, this is the first systematic study to investigate *Trichoderma* species found in colonies of non-leaf-cutting attine ants.

Trichoderma is constantly found in colonies of leaf-cutting ants (Rodrigues et al. 2005, 2008; Augustin et al. 2011); however, there is no systematic study for *Trichoderma* in colonies of these ants. Previous work demonstrated the occurrence of eight *Trichoderma* species: *T. aureoviride, T. harzianum, T. koningii, T. longibrachiatum, T. pseudokoningii, T. hamatum, T. virens* and *T. spirale* (Fisher et al. 1996; Barbosa, 2004; Rodrigues et al. 2005, 2008; Augustin et al. 2011; Rodrigues et al. 2011, 2014). Our results showed that attine ant colonies harbor more *Trichoderma* species than previously reported, since the 68 isolated correspond to 19 species, including three new species described in this study, along with a new phylogenetic strain.

Among the eight *Trichoderma* species previously reported in colonies of leaf-cutting ants, five of them were also found in our study (*T. hamatum, T. harzianum, T. longibrachiatum, T. virens* and *T. spirale*), all isolated from leafcutter ant gardens. The remaining three previously reported species (*T. aureoviride, T. koningii* and *T. pseudokoningii*) were not found in our sample set. However, it is important to note that previous studies used only classical taxonomy methods (morphological markers) or sequenced the ITS region (Fisher et al. 1996; Barbosa, 2004; Rodrigues et al. 2005, 2008, 2011, 2014). Identification of *Trichoderma* species based solely on morphological characterization has proven very difficult due to morphological plasticity among species (Chaverri et al 2003a; Druzhinina and Kubicek 2005; Atanasova et al. 2013; Chaverri et al.

2015). Although considered the barcode marker for filamentous fungi (Schoch et al. 2012), ITS does not exhibit sufficient intraspecific variability in *Trichoderma*, for which is indicated to the use of *tef1* and *rpb2* for species distinction (Druzhinina and Kubicek 2005; Atanasova et al. 2013). Therefore, it is necessary to sequence these markers for the previously identified species such as *T. aureoviride*, *T. koningii* and *T. pseudokoningii* to confirm the presence of these species in attine ant colonies.

Trichoderma is cosmopolitan (Samuels et al. 2006), occurring on various substrates in different parts of the world, including: (i) soil; (ii) decaying wood; (iii) on edible mushrooms and (iv) 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). Due to the diversity of substrates collected by attine ants, their colonies represent potential resources for the discovery of new *Trichoderma* species. Leaf-cutting ants use fresh leaves as substrate for the mutualistic fungus, thus, the endophytic origin of the several fungi found in their colonies (Fisher et al. 1996; Barbosa 2004; Rodrigues et al. 2008; Silva et al. 2010) seems to be the most plausible to explain the diversity of *Trichoderma* species in this environment. Accordingly, Fisher et al. (1996) showed the endophytic origin of some *Trichoderma* species when isolating *T. hamatum* and *T. harzianum* from leaves offered to *Atta cephalotes* colonies. Several other studies with leaf-cutting ants (*Atta* and *Acromyrmex*) 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. harzianum*, *T. longibrachiatum*, *T. orientalis*, *T. viride* and *T. spirale* found on our 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). Regarding *T. viride*, this species was considered to be restricted to the north hemisphere and found in soil, trunks and decaying wood, with only two strains reported in the tropics, isolated as endophytes of *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, Fig. S15) 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.

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 fungal species frequently isolated from the fungus gardens, standing out *T. spirale* (Rodrigues et al. 2014). The authors suggested these fungi would be transported by foragers that are directly in contact with soil. Here, we identified *T. afroharzianum* (LESF 542), *T. harzianum* (LESF 121) and *T. spirale* (LESF 549) isolated from soil adjacent to the chamber holding the fungus garden of *Atta sexdens rubropilosa*. *T. spirale* (LESF 543) was also found in the soil adjacent to the fungus chamber of *Atta bisphaerica* (Table 1). Similarly, we identified *T. afroharzianum* (LESF 228, LESF 229) and *T. spirale* (LESF 557, LESF 558, LESF 559) isolated from soil 10m far from *Atta bisphaerica*; *T. virens* (LESF 550) and *T. spirale* (LESF 562) was also isolated from soil 10m distant from a colony of *A. sexdens rubropilosa* (Table 1). These

four species (*T. afroharzianum, T. harzianum, T. spirale* and *T. virens*) were also found in our fungus gardens samples (Table 1), reinforcing the hypothesis of soil as a second source for diversity of filamentous fungi in leaf-cutting ants 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 1). The strains were identified as *T. koningiopsis, T. afroharzianum, T. virens, T. attinis, T. texana* 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 the colony of such insects, less exploited when compared to leaf-cutting ants, might harbor a richness of *Trichoderma* species, specially due to the wide range of substrates they collect.

Phylogenetic analysis also revealed that none of the 68 strains evaluated forms a specific group of *Trichoderma* associated exclusively with attine ant colonies, since they grouped in clades comprehending *Trichoderma* strains isolated from various sources (see Fig. 1 and Table S1). 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*, Fig. 1 and 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 colony, similarly to other *Trichoderma* species. In our phylogenetic analysis, the three new species grouped in different parts of the tree; if these species are exclusively associated with attine ants, such association would have originated multiples times over evolutionary time, which does not seems to be the case.

Therefore, we believe that the chance of existing *Trichoderma* species exclusively associated with attine ant gardens is unlikely, which supports the hypothesis of these fungi as transient in this environment (Poulsen and Currie 2006). However, future studies considering further sampling in non-leaf-cutting ant gardens and in the substrates collected by these insects may prove this hypothesis. Finally, the presence of three new species (*T. attinis*, *T. texana*, and *T. longifialidicum*) and a new phylogenetic species (LESF 545) in attine ants' gardens corroborates recent results indicating the potential of this environment to harbor a high microbial diversity. Apparently, the dynamic foraging behavior of these insects (gathering several types of substrates) make their colonies a unique environment, which results in a microbiota formed by several sources of biological diversity. Future exploratory studies in this environment will certainly reveal even more unknown species for science, not only within *Trichoderma* genus but for other fungi as well.

Taxonomy

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

Mycobank: XXXXXX

Colonies showed radius of 39 - 41 mm; 25 - 30 mm and 5 - 10 mm on PDA, CMD and SNA, respectively, after 72h at 25 °C in the dark; at 30 °C colonies exhibited radius of 30 - 31 mm, 29 - 38 mm and 6 - 11 mm on PDA, CMD and SNA, respectively. At 35 °C, the radius reached 10 - 15 mm, 2 - 7 mm and 3 - 10 mm on PDA, CMD and SNA, respectively. The colony fills the plate after seven days of incubation at 25 and 30 °C on PDA. After 72h at 25 °C and 30 °C were observed concentric rings alternating in colors green and white for cultures maintained on PDA. The conidia pigmentation starts from the inoculum region and

maintain to the edge of the colony after 48h on PDA. It was observed the presence of yellow soluble pigmentation in CMD and SNA after 48 hours of incubation at all three temperatures. *Hyphae* are hyaline and smooth, usually forming pustules on PDA and CMD, with close growth to agar on SNA. *Pustules* are globose - subglobose and cottony, connecting together in 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, lageniformes 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 forming from supporting cells measuring 4.5 - 14.6 µm in length x 1.7 - 3.4 µm wide; phialides rarely forms from the central conidiophore hyphae. *Conidia* subglobose-ovoid, dark gren-colored and smooth, measuring 3.2 - 4.3 µm x 2.5 - 3.4 µm. *Chlamydospores* hyaline and intercalated, measuring 5.3 - 11.4 µm x 4.5 - 6.8 µm, observed after 72h at 25 °C on CMD and PDA.

Specimen examined: 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, = CBMAI1826). Holotype: CBS H-22180 (dried culture on PDA).

Sequences: XXXXX

Etymology: "*attinis*" in relation to the name of the tribe Attini, the first source from where these species were found.

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

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

Mycobank: XXXXX

Characteristics of colony after 72h at 25 °C in the dark, colonies showed radius of 55 - 56 mm; 45 - 46 mm and 8 - 10 mm on PDA, CMD and SNA, respectively. At 30 °C colonies exhibited radius of 37 - 42mm, 48 - 59 mm and 10 - 23 mm on PDA, CMD and SNA, respectively. No concentric rings or soluble pigmentation was observed. The colony fills the plate after 96h on PDA and CMD and after seven days on SNA. The conidia pigmentation starts from the inoculum region to the edge of the colony after seven 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, 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 x 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 x 1.8 - 3.1 μm in width. *Chlamydospores* hyaline, intercalated, measuring 5.3 - 15.4 μm in length x 3.4 - 7.9 μm in width; chlamydospores were observed on CMD and PDA, after five days of growth at 25 °C.

Specimen examined: 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: XXXX

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

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

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

Mycobank: XXXXXX

Characteristics of colony after 72h at 25 °C in the dark, colonies showed radius of 67 mm; 60 - 63 mm and 39 - 43 mm on PDA, CMD and SNA, respectively; at 30 °C colonies exhibited radius of 65 - 68 mm, 58 - 67 mm 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 96h on all culture media. The conidia pigmentation starts from the center of the colony, after seven 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 x 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 x 1.6 - 2.4 μm in width. *Chlamydospores* are globose, hyaline and intercalated, measuring 7 - 8 x 6 - 6.2 μm; chlamydospores were observed on CMD and PDA, after five days of growth at 25 °C.

Specimen examined: 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, = CBMAI1827). Holotype: CBS H-22181 (dried culture on PDA).

Sequences: XXXXXX.

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

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

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Legends and figures

Fig. 1 Phylogenetic tree displaying the position of different *Trichoderma* species reported in the present studied (LESF ids) 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 Monte Carlo posterior probabilities. The tree includes a total of 179 sequences of *tef1* and 146 sequences of *rpb2*. The final alignment had 1881 base pairs. We also included *Trichoderma* sequences derived from previous studies obtained from GenBank. Species names are followed by the strains codes. *Protodrea pallida* CBS 29978 was used as outgroup. Bars indicate nucleotide substitution per site.

Fig. 2 Morphological characteristics of *Trichoderma attinis*. 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: Chlamidospores.

Fig. 3 Morphological characteristics of *Trichoderma texana*. 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: Chlamidospores. 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.

Table 1. Strains examined in this study.

Strain ID	Species	Colony code	Isolation source	City	Local	Substrate
LESF010	*T. afroharzianum*	Nest 3	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF011	*T. afroharzianum*	Nest 3	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF013	*T. afroharzianum*	Nest 3	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF228	*T. afroharzianum*	AR090429-01	*Atta bisphaerica*	Botucatu-SP	Fazenda Santana	Soil 10m distant
LESF229	*T. afroharzianum*	AR090429-01	*Atta bisphaerica*	Botucatu-SP	Fazenda Santana	Soil 10m distant
LESF542	*T. afroharzianum*	AR090429-01	*Atta bisphaerica*	Botucatu-SP	Fazenda Botucatu	Soil adjacent
LESF553	*T. afroharzianum*	AOMB090904-06	*Trachymyrmex* sp.	Taquara-RS	-	Fungus garden
LESF554	*T. afroharzianum*	AOMB140904-07	*Acromyrmex coronatus*	Timbó-SC	Plantação de mandioca	Fungus garden
LESF541	*T. andinensis*	BMSR120803-02	*Atta cephalotes*	Camacan-BA	Fazenda Paris	Fungus garden
LESF560	*T. andinensis*	BMSR120803-02	*Atta cephalotes*	Camacan-BA	Fazenda Paris	Fungus garden
LESF561	*T. asperellum*	BMSR120803-02	*Atta cephalotes*	Camacan-BA	Fazenda Paris	Fungus garden
LESF118	*T. atroviride*	Nest 39	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF236	*T. attinis*	AR061001-01	*Cyphomyrmex wheeleri*	Austin-Texas, USA	Bull Creek Park	Fungus garden
LESF211	*T. deliquescens*	AR110515-01	*Acromyrmex* sp.	Camacan-BA	Serra Bonita	Fungus garden
LESF208	*T. eucorticioides*	AR110515-01	*Acromyrmex* sp.	Camacan-BA	Serra Bonita	Fungus garden
LESF120	*T. hamatum*	AOMB130904-07	*Acromyrmex laticeps*	Capão Alto-SC	Bosque	Fungus garden
LESF330	*T. hamatum*	ARFVG110517-01	*Acromyrmex* sp.	Camacan-BA	RPPN Serra Bonita	Fungus garden
LESF331	*T. hamatum*	JSP120410-07	*Atta sexdens rubropilosa*	Botucatu-SP	Fazenda Particular	Fungus garden
LESF332	*T. hamatum*	JSP130130-01	*Atta sexdens rubropilosa*	Botucatu-SP	Fazenda Particular	Fungus garden
LESF121	*T. harzianum*	AR090429-01	*Atta bisphaerica*	Botucatu-SP	Fazenda Santana	Fungus garden
LESF343	*T. harzianum*	JSP130307-03	*Atta capiguara*	Botucatu-SP	Fazenda Particular	Fungus garden
LESF345	*T. harzianum*	BMSR120803-02	*Atta cephalotes*	Camacan-BA	Fazenda Paris	Fungus garden
LESF346	*T. harzianum*	AR110511-01	*Acromyrmex balzanii*	Ilhéus-BA	UESC	Fungus garden
LESF517	*T. harzianum*	JSP130307-03	*Atta capiguara*	Botucatu-SP	Fazenda Particular	Fungus garden
LESF555	*T. harzianum*	AOMB100904-03	*Acromyrmex heyeri*	Sentinela do Sul-RS	Pastagem	Fungus garden

| Strain ID	Species	Colony code	Isolation source	City	Local	Substrate
LESF002	*T. koningiopsis*	Nest 16	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF006	*T. koningiopsis*	Nest 1	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF119	*T. koningiopsis*	AOMB060904-01	*Acromyrmex crassispinus*	Nova Petrópolis-RS	Reserva Particular	Fungus garden
LESF149	*T. koningiopsis*	Nest 7	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF212	*T. koningiopsis*	AR061001-01	*Cyphomyrmex wheeleri*	Austin-Texas, USA	Bull Creek Park	Fungus garden
LESF360	*T. koningiopsis*	ARFVG110517-01	*Acromyrmex* sp.	Camacan-BA	RPPN Serra Bonita	Fungus garden
LESF546	*T. koningiopsis*	JSP120411-01	*Atta sexdens rubropilosa*	Botucatu-SP	Fazenda Particular	Fungus garden
LESF009	*T. longibrachiatum*	Nest 2	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF552	*T. longifialidicum*	AR061015-01	*Trachymyrmex septentrionalis*	Smithville-Texas - USA	Stengl "Lost Pines" Biology Station	Fungus garden
LESF516	*T. orientalis*	JSP130305-02	*Atta capiguara*	Botucatu-SP	Fazenda Particular	Fungus garden
LESF540	*T. orientalis*	JSP130307-03	*Atta capiguara*	Botucatu-SP	Fazenda Particular	Fungus garden
LESF544	*T. orientalis*	JSP130307-03	*Atta capiguara*	Botucatu-SP	Fazenda Particular	Fungus garden
LESF545	*Trichoderma* sp.	BMSR120704-01	*Atta cephalotes*	Camacan-BA	RPPN Serra Bonita	Fungus garden
LESF107	*T. spirale*	Nest 6	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF108	*T. spirale*	Nest 6	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF117	*T. spirale*	Nest 5	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF327	*T. spirale*	AR110511-01	*Acromyrmex balzanii*	Ilhéus-BA	UESC	Fungus garden
LESF328	*T. spirale*	BMSR120702-01	*Atta cephalotes*	Camacan-BA	RPPN Serra Bonita	Fungus garden
LESF329	*T. spirale*	JSP120411-01	*Atta sexdens rubropilosa*	Botucatu-SP	Fazenda Particular	Fungus garden
LESF515	*T. spirale*	JSP130307-01	*Atta capiguara*	Botucatu-SP	Fazenda Particular	Fungus garden
LESF543	*T. spirale*	AR090402-01	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Soil adjacent
LESF547	*T. spirale*	JSP130307-01	*Atta capiguara*	Botucatu-SP	Fazenda Particular	Fungus garden
LESF548	*T. spirale*	JSP130307-01	*Atta capiguara*	Botucatu-SP	Fazenda Particular	Fungus garden
LESF549	*T. spirale*	AR090429-01	*Atta bisphaerica*	Botucatu-SP	Fazenda Santana	Soil adjacent
LESF557	*T. spirale*	AR090429-01	*Atta bisphaerica*	Corumbataí-SP	Fazenda Corumbataí	Soil 10m distant
LESF558	*T. spirale*	AR090429-01	*Atta bisphaerica*	Corumbataí-SP	Fazenda Corumbataí	Soil 10m distant

Strain ID	Species	Colony code	Isolation source	City	Local	Substrate
LESF559	*T. spirale*	AR090429-01	*Atta bisphaerica*	Corumbataí-SP	Fazenda Corumbataí	Soil 10m distant
LESF562	*T. spirale*	AR090402-01	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Soil 10m distant
LESF551	*T. texana*	AR061015-01	*Trachymyrmex septentrionalis*	Smithville-Texas – USA	Stengl "Lost Pines" Biology Station	Fungus garden
LESF132	*T. velutinum*	AR090429-01	*Atta bisphaerica*	Botucatu-SP	Fazenda Santana	Fungus garden
LESF004	*T. virens*	Nest 17	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF111	*T. virens*	Nest 15	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF112	*T. virens*	Nest 13	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF220	*T. virens*	Nest 8	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF221	*T. virens*	Nest 15	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF269	*T. virens*	Nest 3	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF275	*T. virens*	Nest 2	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden
LESF514	*T. virens*	JSP130130-02	*Atta sexdens rubropilosa*	Botucatu-SP	Fazenda Particular	Fungus garden
LESF535	*T. virens*	JSP120410-05	*Atta sexdens rubropilosa*	Botucatu-SP	Fazenda Particular	Fungus garden
LESF550	*T. virens*	AR090402-01	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Soil 10m distant
LESF556	*T. virens*	UGM061015-03	*Trachymyrmex septentrionalis*	Smithville-Texas - USA	Stengl "Lost Pines" Biology Station	Fungus garden
LESF573	*T. virens*	JSP130307-01	*Atta capiguara*	Botucatu-SP	Fazenda Particular	Fungus garden
LESF115	*T. viride*	Nest 29	*Atta sexdens rubropilosa*	Corumbataí-SP	Fazenda Corumbataí	Fungus garden

Table 2. Phylogenetic markers amplified, primers and PCR conditions

Marker	Primers	Size (bp)	PCR conditions	Reference
ITS	ITS4 (5'TCCTCCGCTTATTGATATGC3') ITS5 (5'GGAAGTAAAAGTCGTAACAAGG3')	586-622	94 °C/3 min followed by 35 cycles at 94°C /1 min, 55 °C /1 min and 72 °C/2 min.	Schoch et al. (2012)
tef1	EF1-728F (5'CAT CG AG AAGTTCGAGAAGG3') TEF1R (5' GCCATCCTTGGAGATACCAGC3')	563-615	94 °C/2 min followed by 15 cycles at 94°C/30s, 65 °C /30s and 72 °C/1 min; followed by 35 cycles at 94°C /30s, 48°C /30s and 72 °C /1 min.	Carbone e Kohn (1999); Samuels et al. (2002)
rpb2	fRPB2-5F (5'GA(T/C)GA(T/C)(A/C)G(A/T)GATCA(T/C)TT(T/C)G G-3') fRPB2-7cR (5'CCCAT(A/G)GCTTG(T/C)TT (A/G)CCCAT-3')	900-1130	94 °C /2 min followed by 15 cycles at 94°C/30s, 65 °C /30s and 72 °C/1 min; followed by 35 cycles at 94 °C/30s, 48 °C/30s and 72 °C /1 min.	Liu et al. (1999)

Fig 1
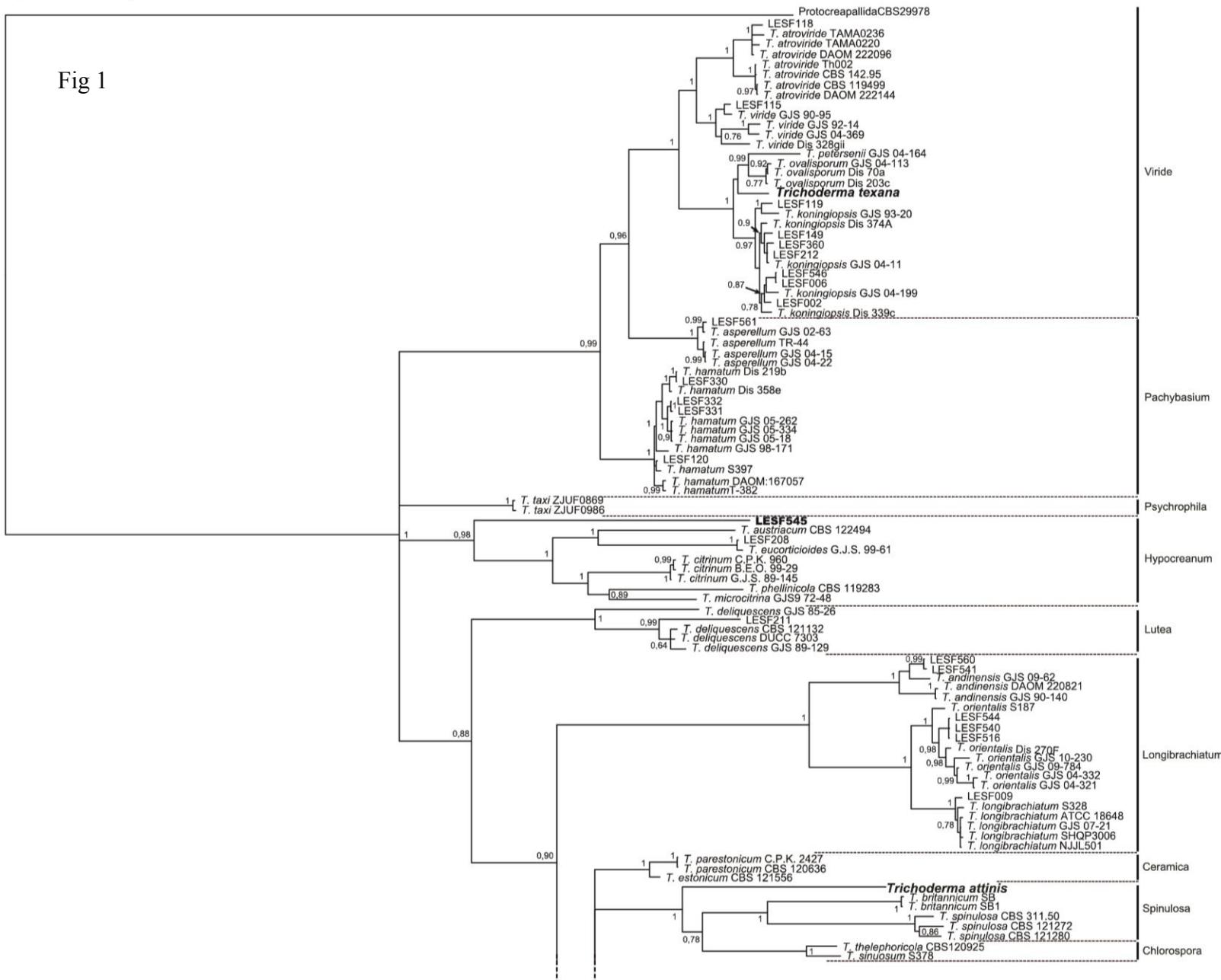
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Fig. 2

Fig. 3

Fig. 4

0,5cm

0,5cm

25µm

10µm

10µm

10µm

10µm

10µm
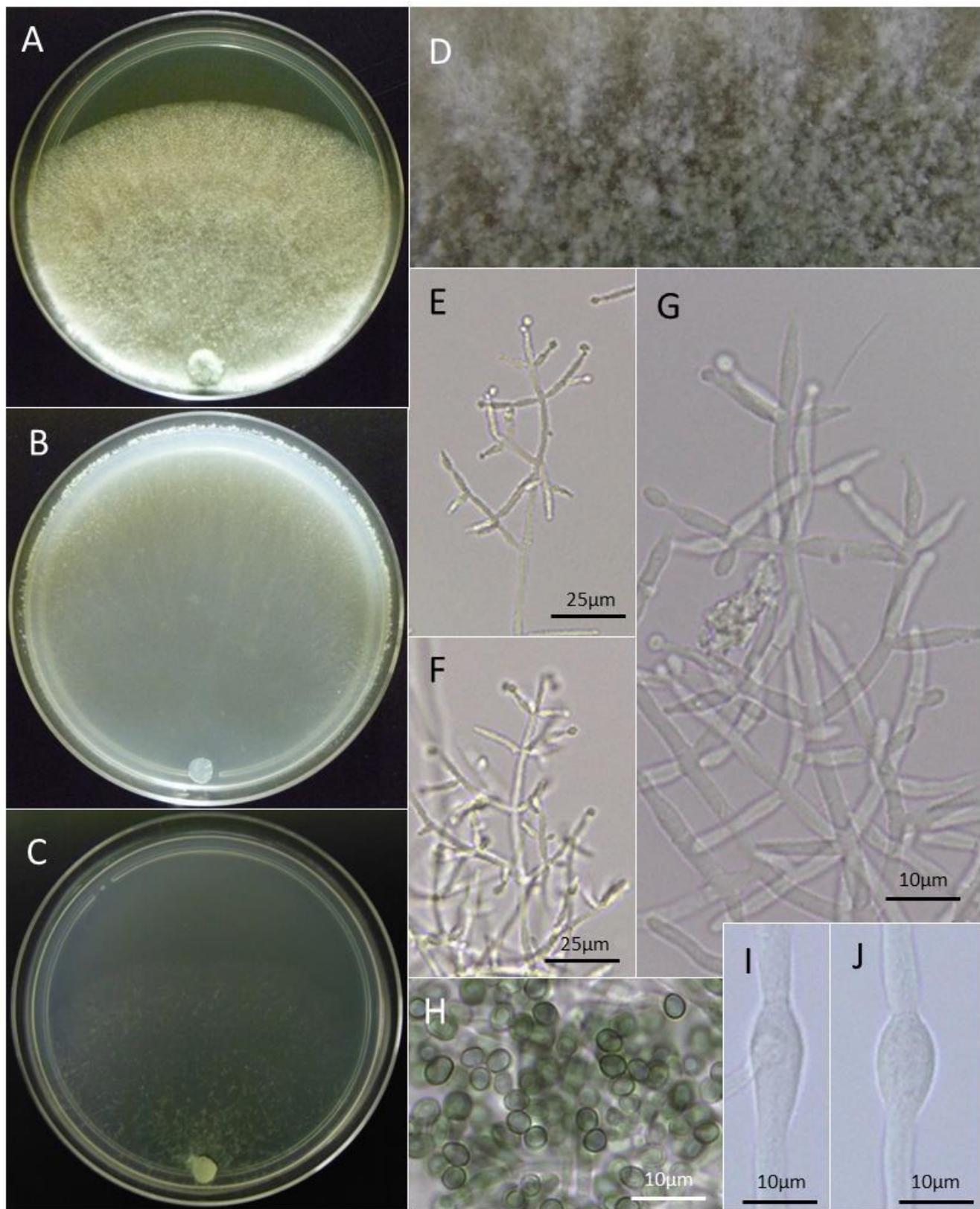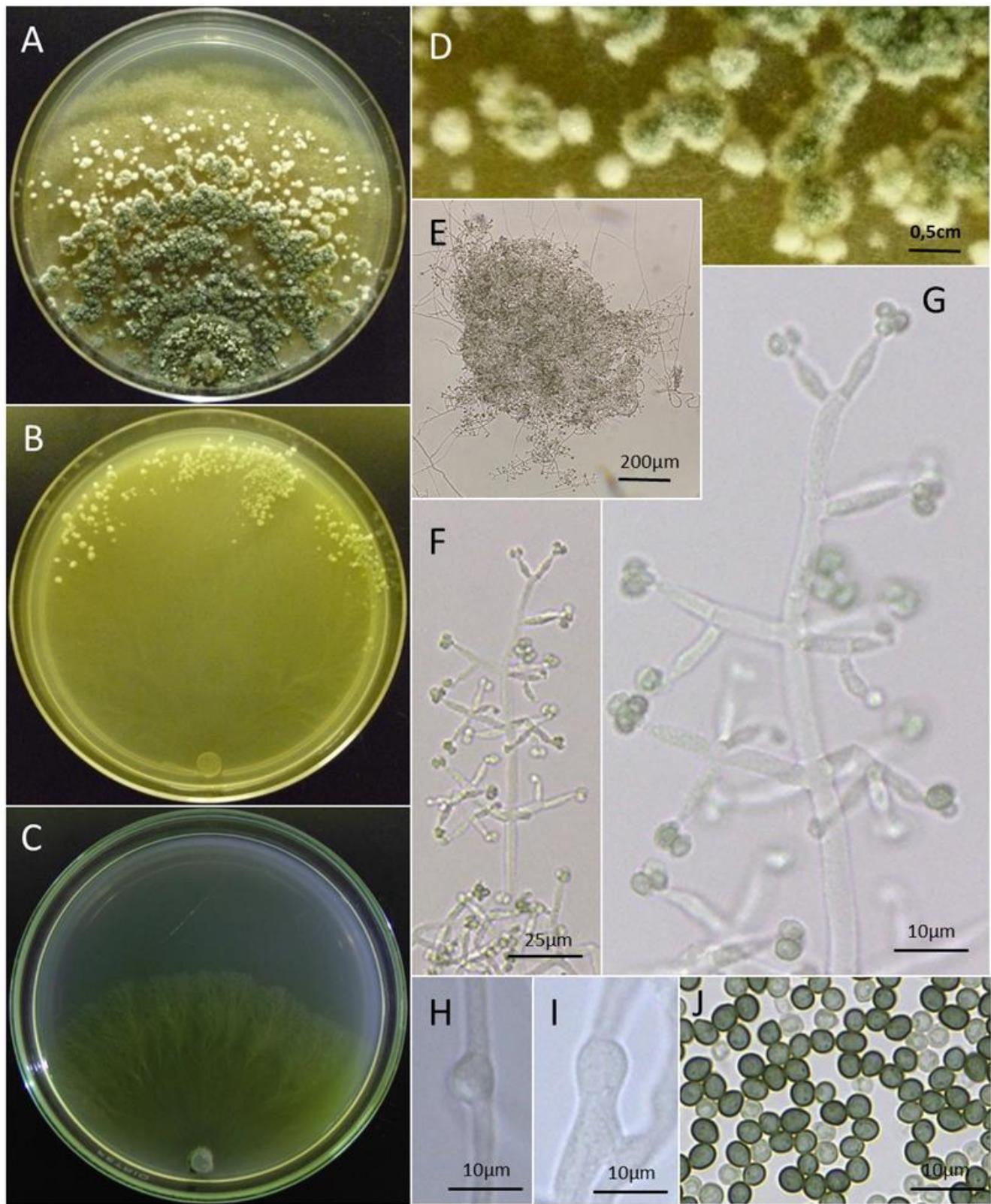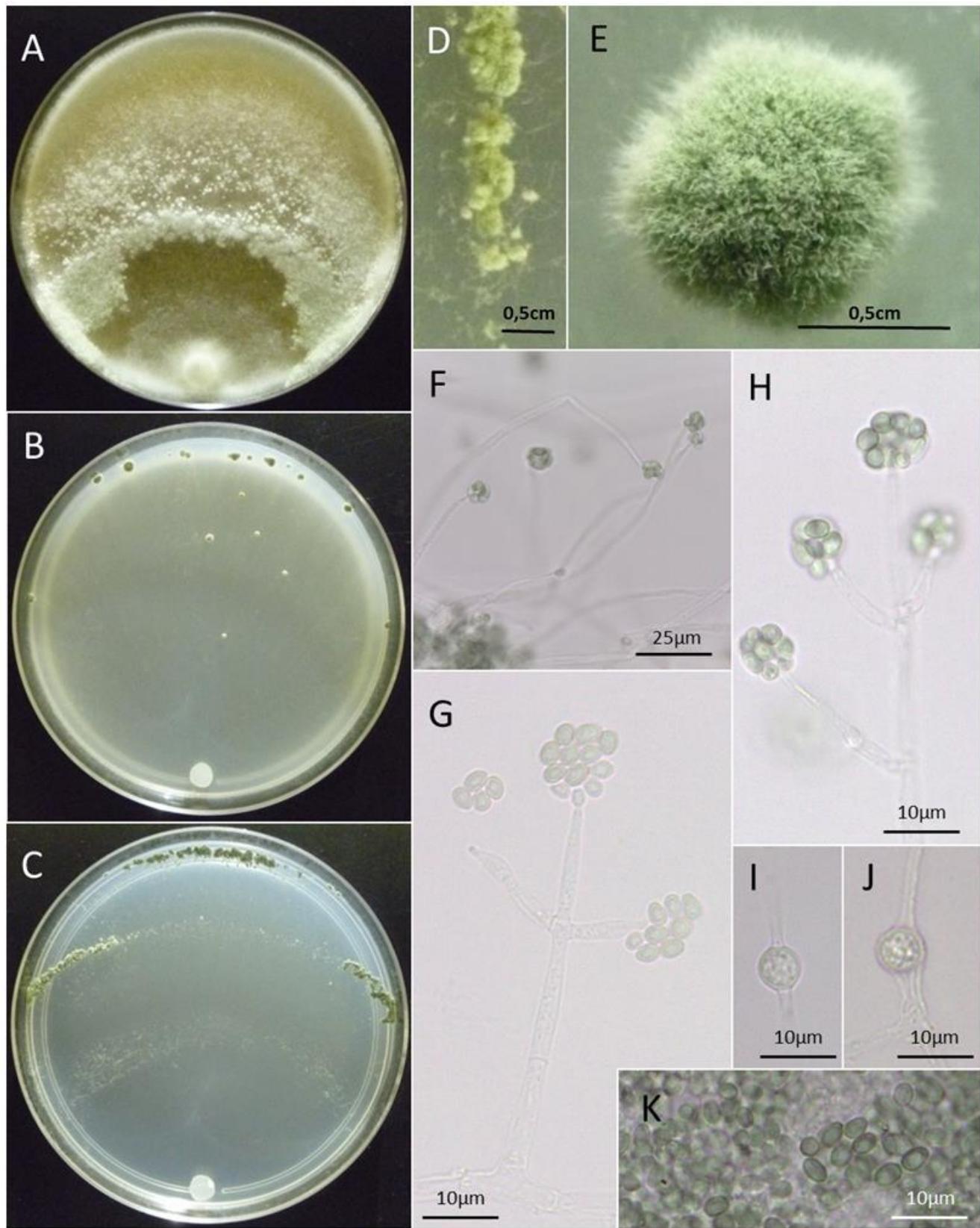
Supplementary material

Fig. S1 Morfological characteristics of *Trichoderma afroharzianum* LESF 010. A-C: cultures in PDA, CMD and SNA, respectively, after seven days at 25 °C. D: Mycelium on CMD. E: General aspect of conidiophores. F-H: Branching pattern of conidiophore and phialides. I: chlamydospores. J: Conidia.
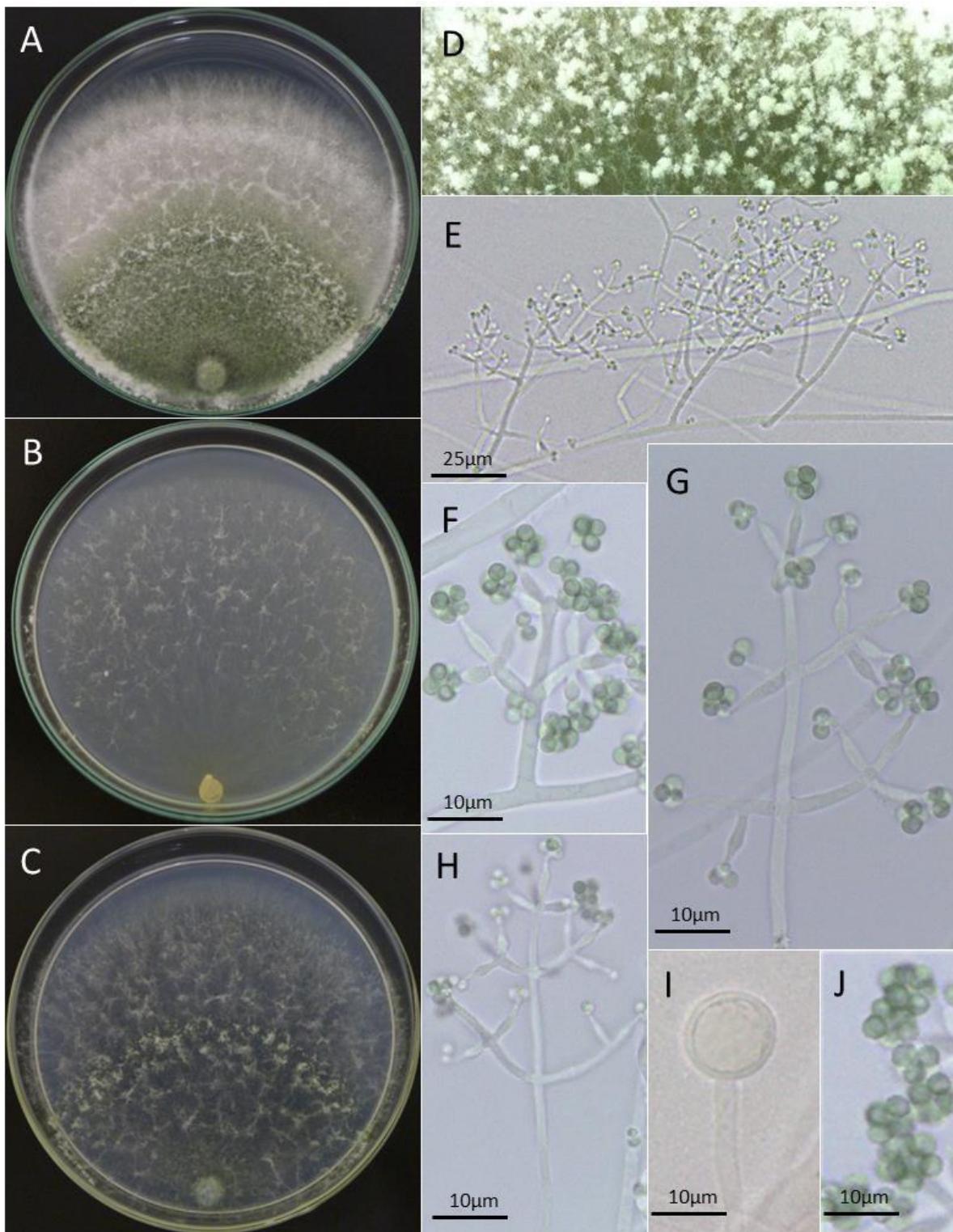
Fig. S2 Morfological characteristics of *Trichoderma andinense* LESF 560. A-B: culture in PDA after seven days at 25 °C and 30 °C, respectively. C-D: cultures on CMD and SNA, respectively, after seven days at 25 °C. E: Mycelium in CMD. E: General aspect of conidiophores. F: Pustules on CMD. H-I: Phialides. J-K: Chlamydospores. L: Conidia.
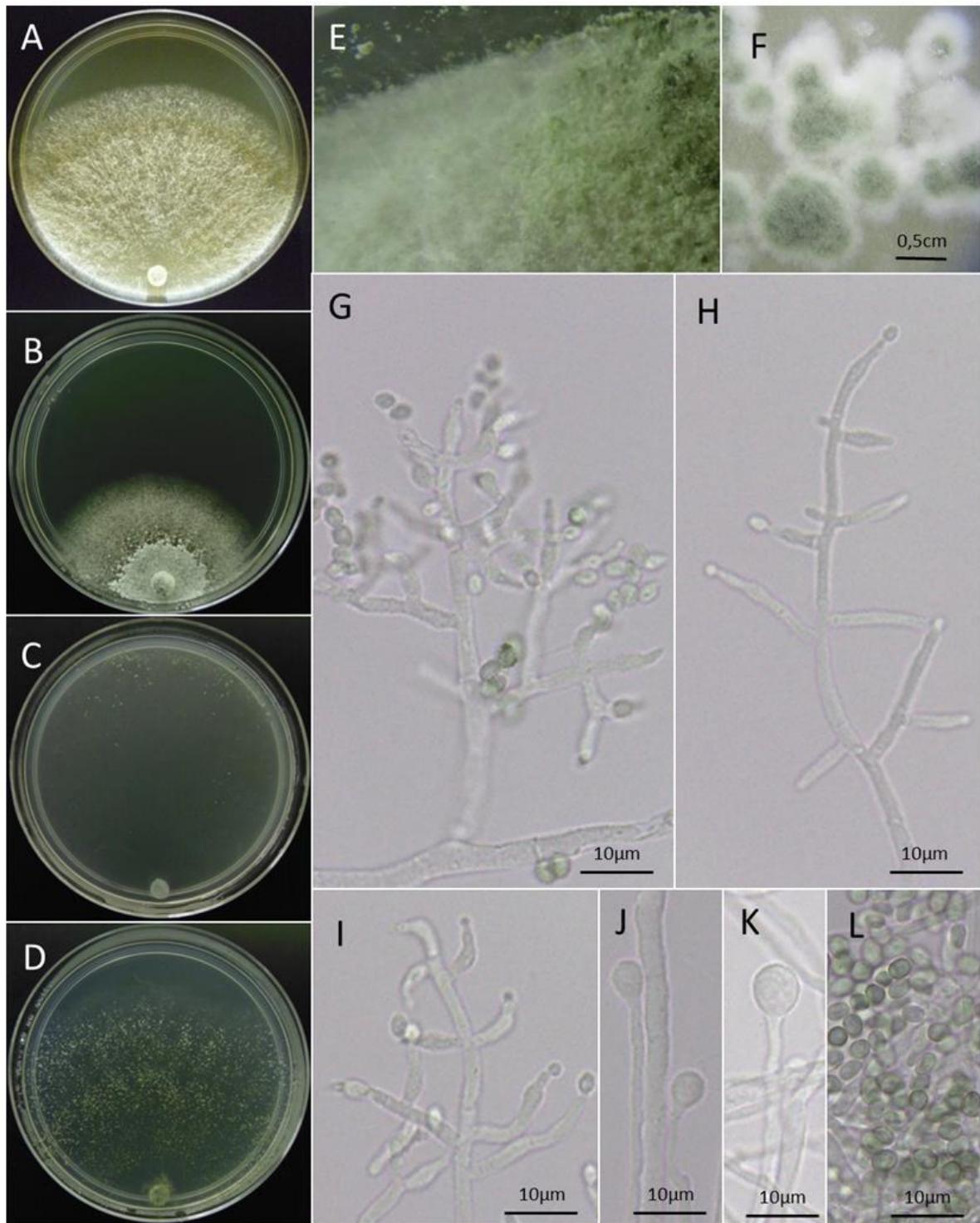
Fig. S3 Morfological characteristics of *Trichoderma asperellum* LESF 561. A-C: culture on PDA, CMD and SNA, respectively, after seven days at 25 °C. D: Mycelium forming pustules on CMD. E-G: General aspect of conidiophores and phialides. H: Chlamydospores. I: Conidia.
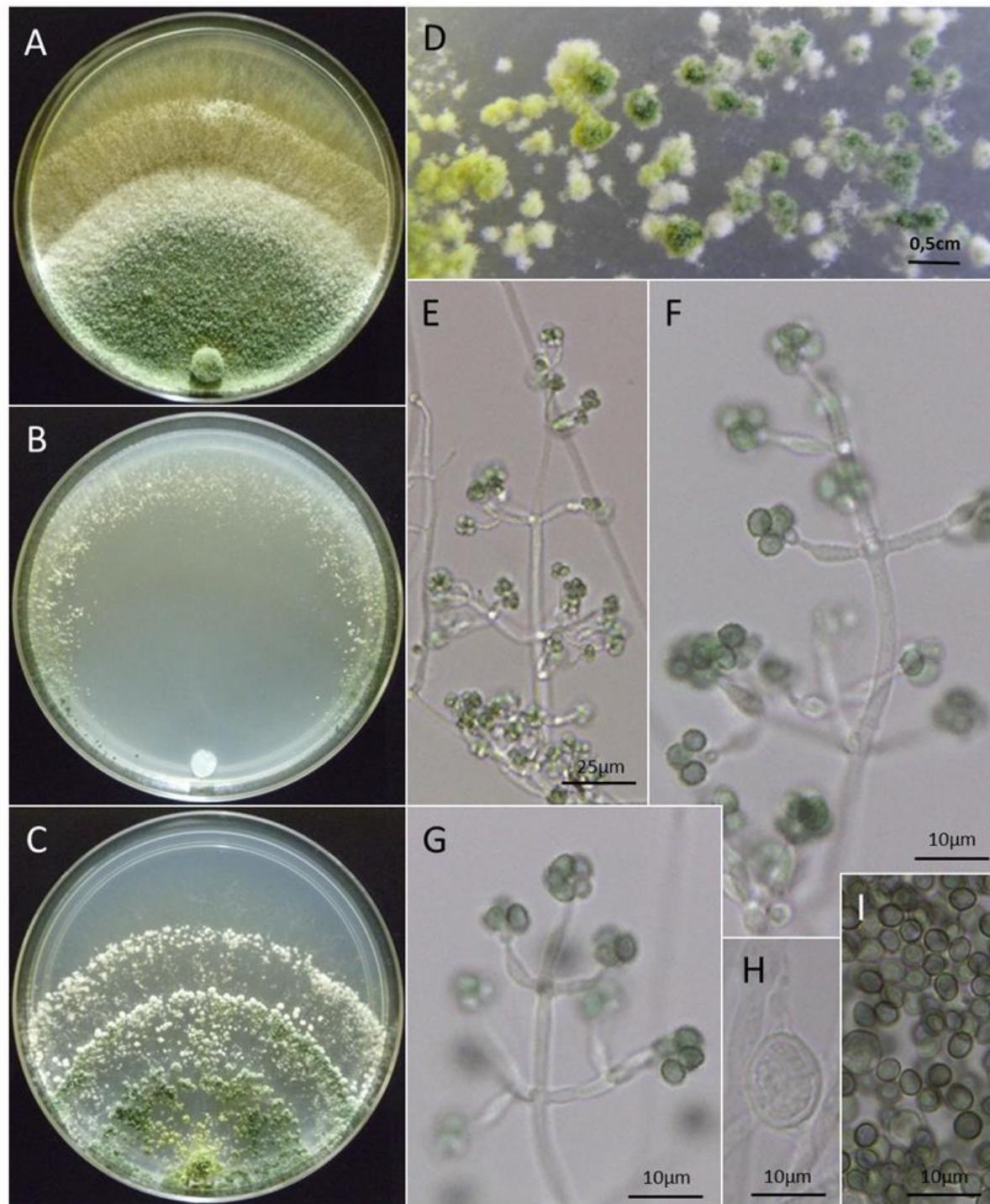
Fig. S4 Morfological characteristics of *Trichoderma atroviride* LESF 118. A-C: culture on PDA, CMD and SNA, respectively, after seven days at 25 °C. D-E: Pustules formed on CMD. F: General aspects of conidiophores. G: Phialides. H: Conidia. I-J: Chlamydospores.
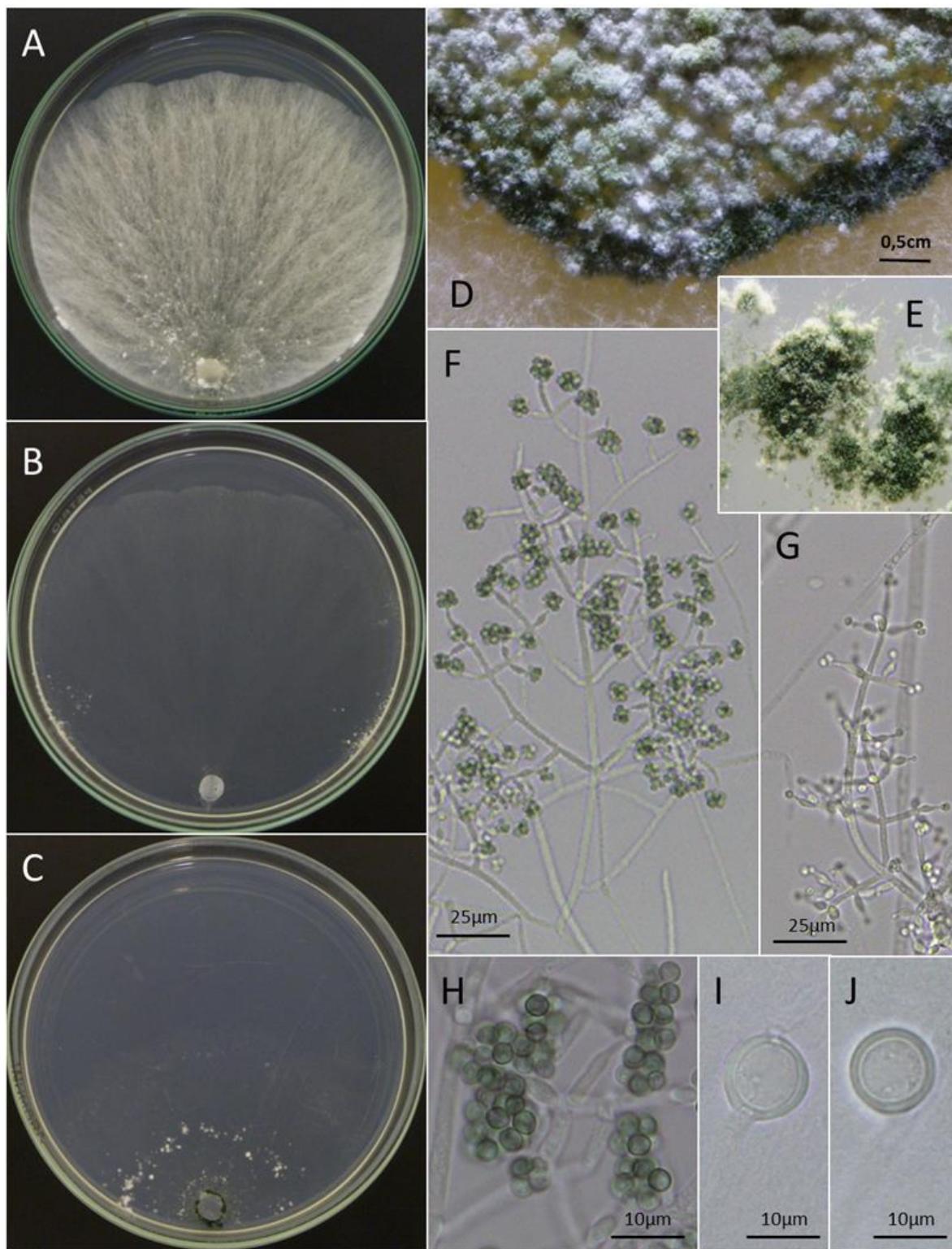
Fig. S5 Morfological characteristics of *Trichoderma deliquescens* LESF 211. A-B: culture on PDA, CMD, respectively, after seven days at 25 °C. C: Disposition of conidiophores formed on aerial mycelium. D-G: General aspect of conidiophores and phialides. H-I: Chlamydospores. J: Conidia.
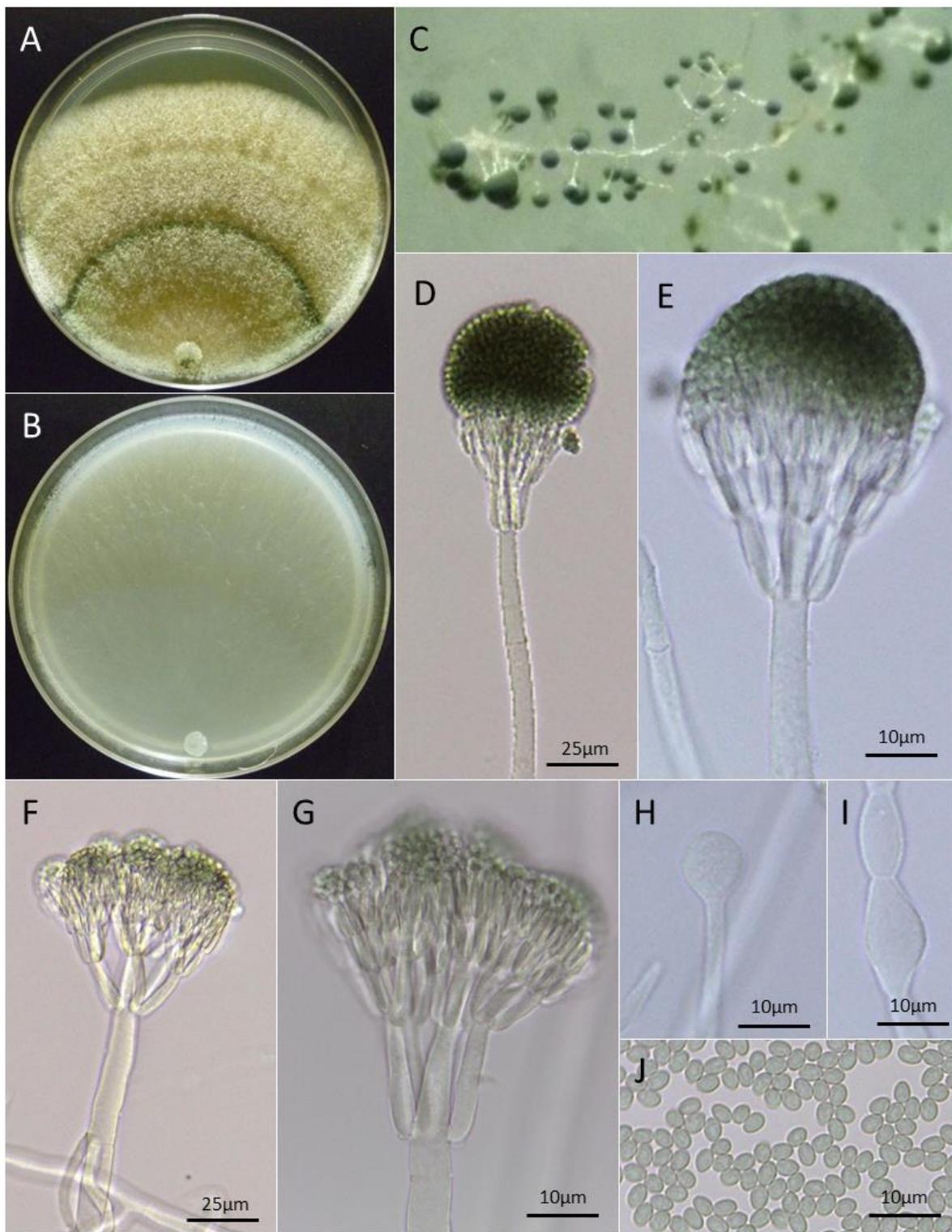
Fig. S6 Morfological characteristics of *Trichoderma eucorticioides* LESF 208. A-B: culture on PDA and CMD, respectively, after seven days at 25 °C. C: Mycelium forming pustules on PDA. D: General aspect of conidiophores. E-G: Branching pattern of conidiophores and phialides. H: Chlamydospores. I-J: Conidia.
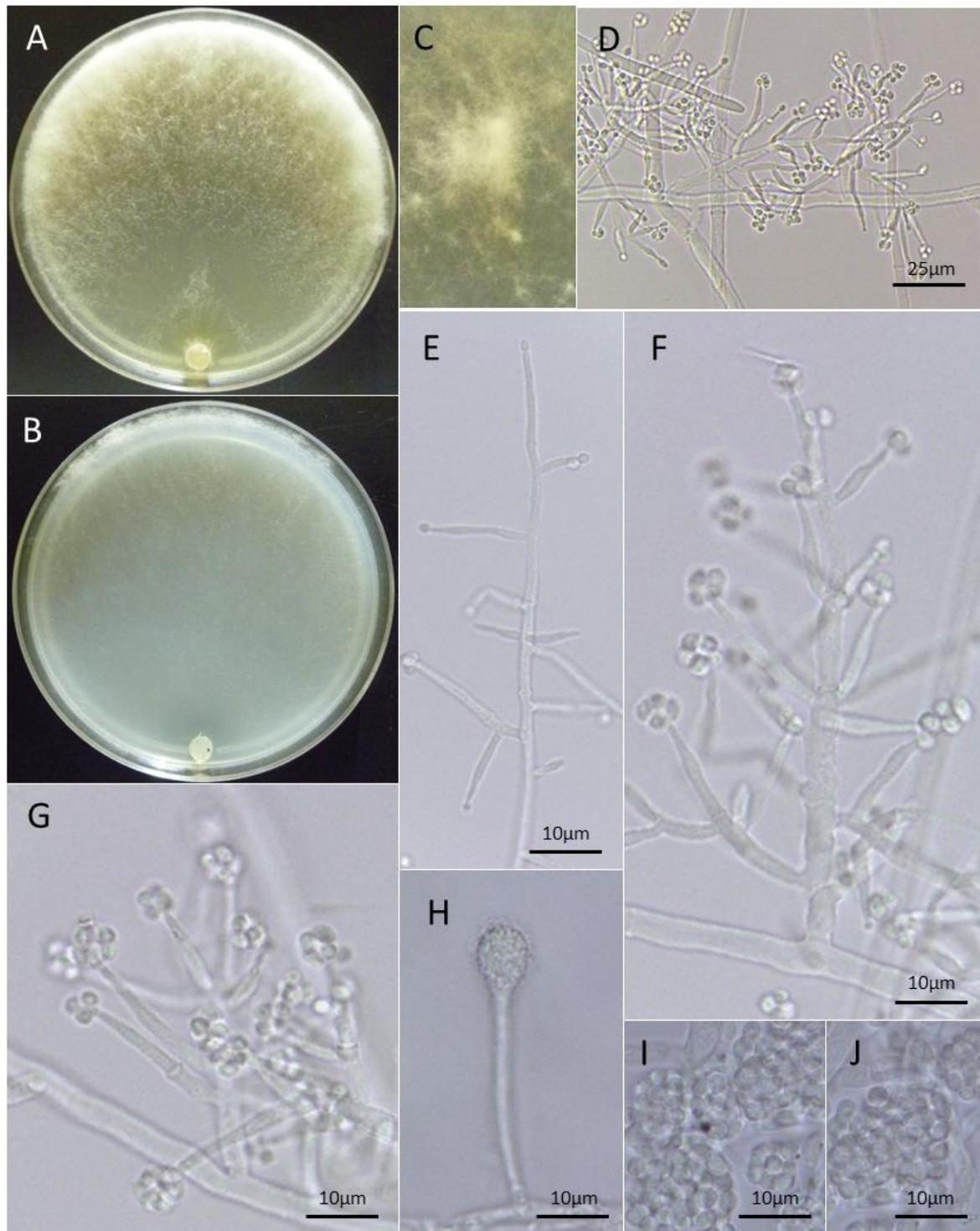
Fig. S7 Morfological characteristics of *Trichoderma hamatum* LESF 120. A-B: culture on PDA after seven days at 25 °C. C-D: Culture in CMD and SNA, respectively, after seven days at 25 °C. E-F: Pustules formed on PDA. G-H: General aspect of conidiophores. I: Sterile hairs. J-K: Phialides. L-M: Chlamydospores. N: Conidia.
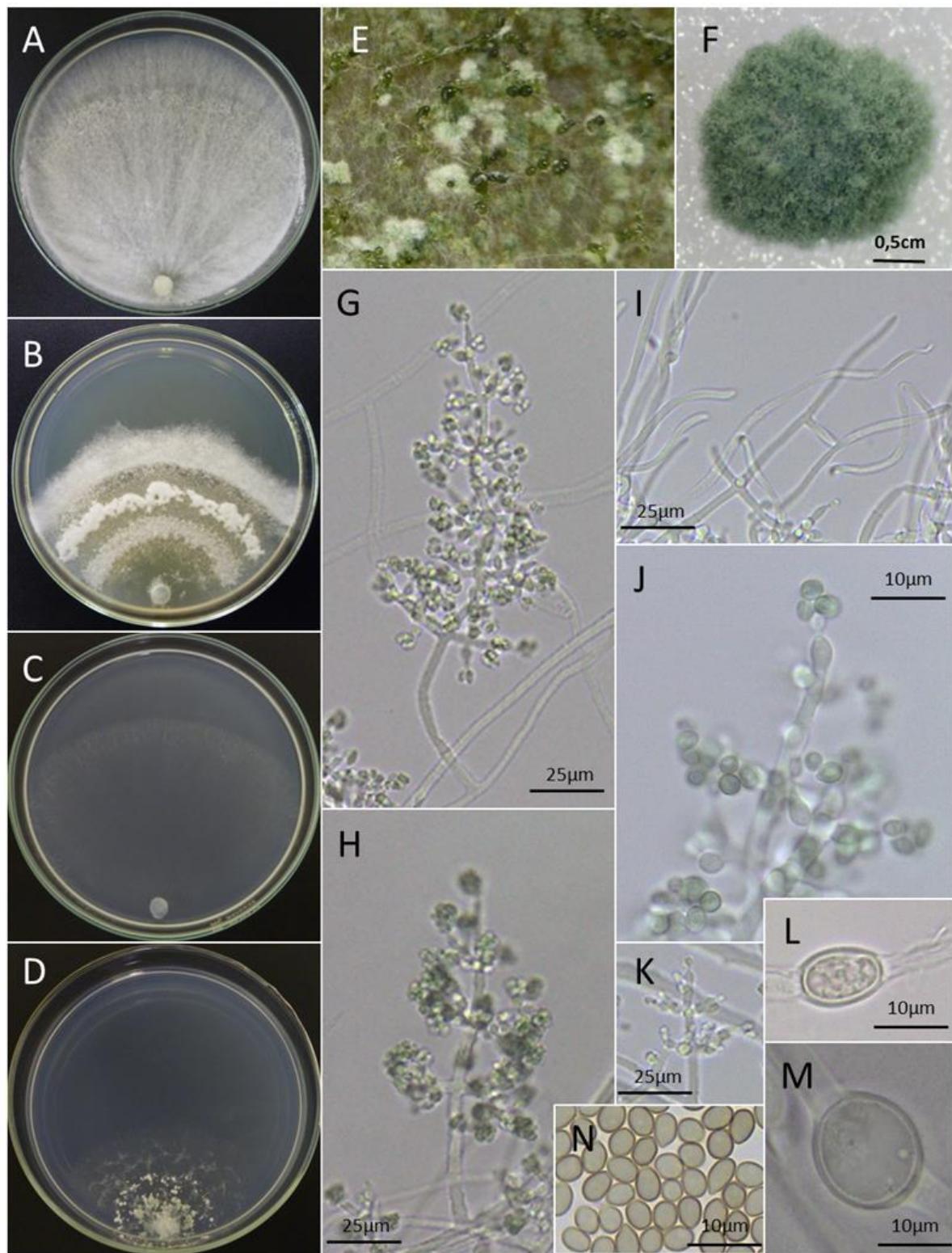
Fig. S8 Morfological characteristics of *Trichoderma harzianum* LESF 555. A-C: culture on PDA, CMD and SNA, respectively, after seven days at 25 °C. D: Mycelium forming pustules on CMD. E-F: Conidiophores. G: Branching pattern and of phialides. H: Chlamydospores. I: Conidia.
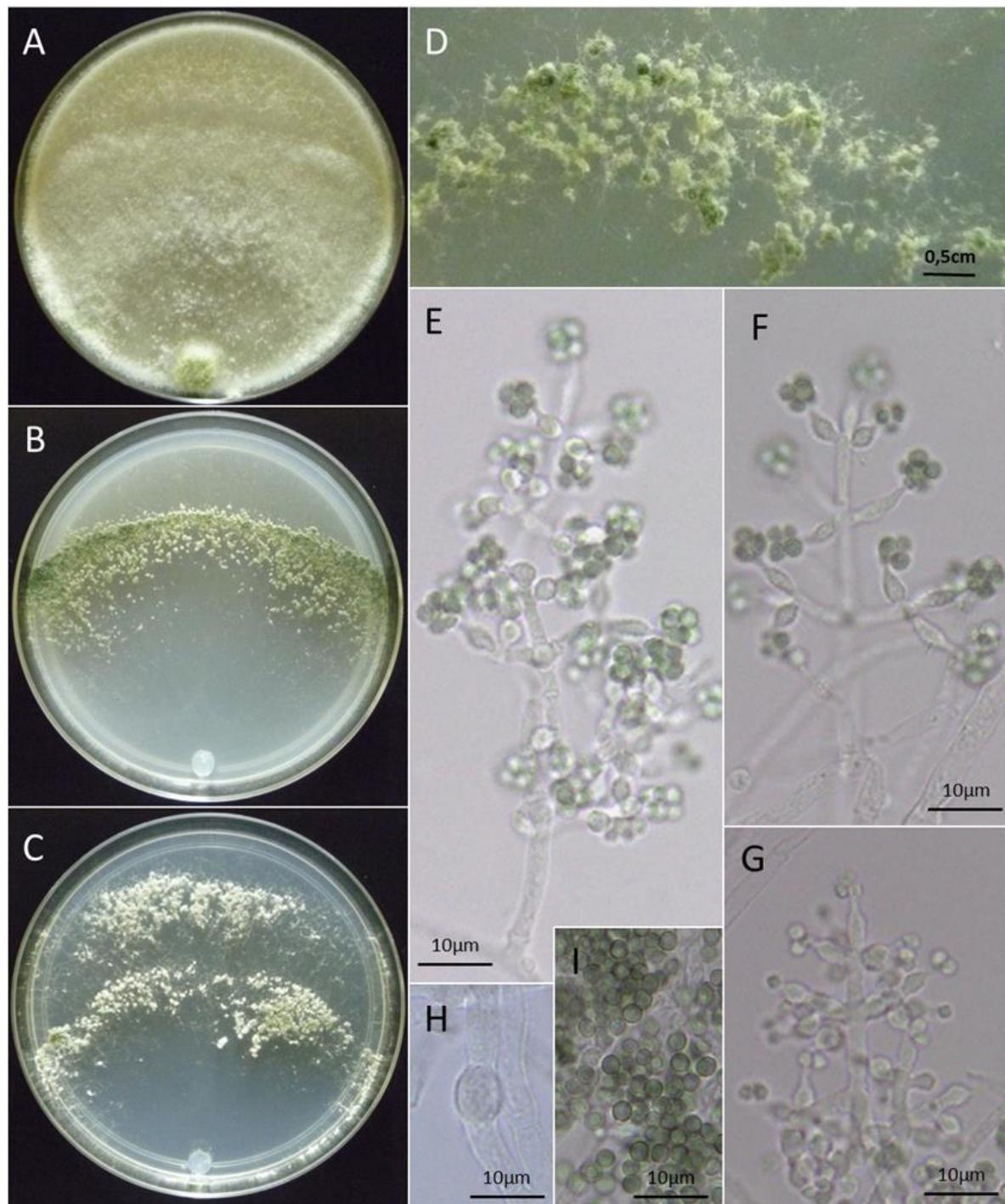
Fig. S9 Morfological characteristics of *Trichoderma koningiopsis* LESF 006. A-C: culture on PDA, CMD and SNA, respectively, after seven days at 25 °C. D: Reverse on PDA after seven days at 25 °C. E-F Pustules on CMD. G: Branching pattern of conidiophore. H-J: Phialides. K-M: Chlamydospores. N: Conidia.
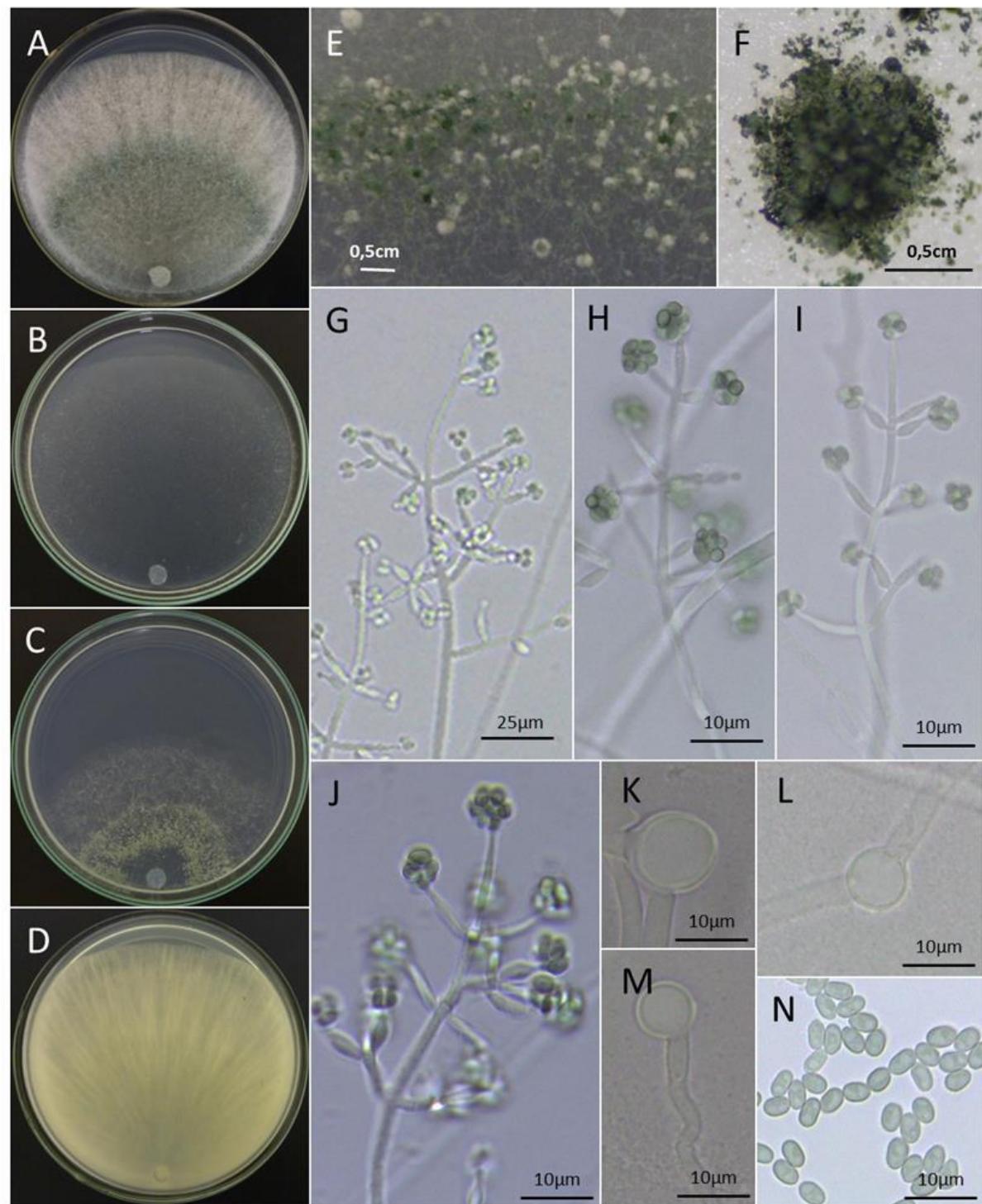
Fig. S10 Morfological characteristics of *Trichoderma longibrachiatum* LESF 009. A-C: culture on PDA, CMD and SNA, respectively, after seven days at 25 °C. D: Diffusion of yellow pigment. E: Pustules formed on CMD. F-G: Hyphae. H-I: Branching pattern of conidiophore. J-K: Phialides. L: Conidia. M: Chlamydospores.
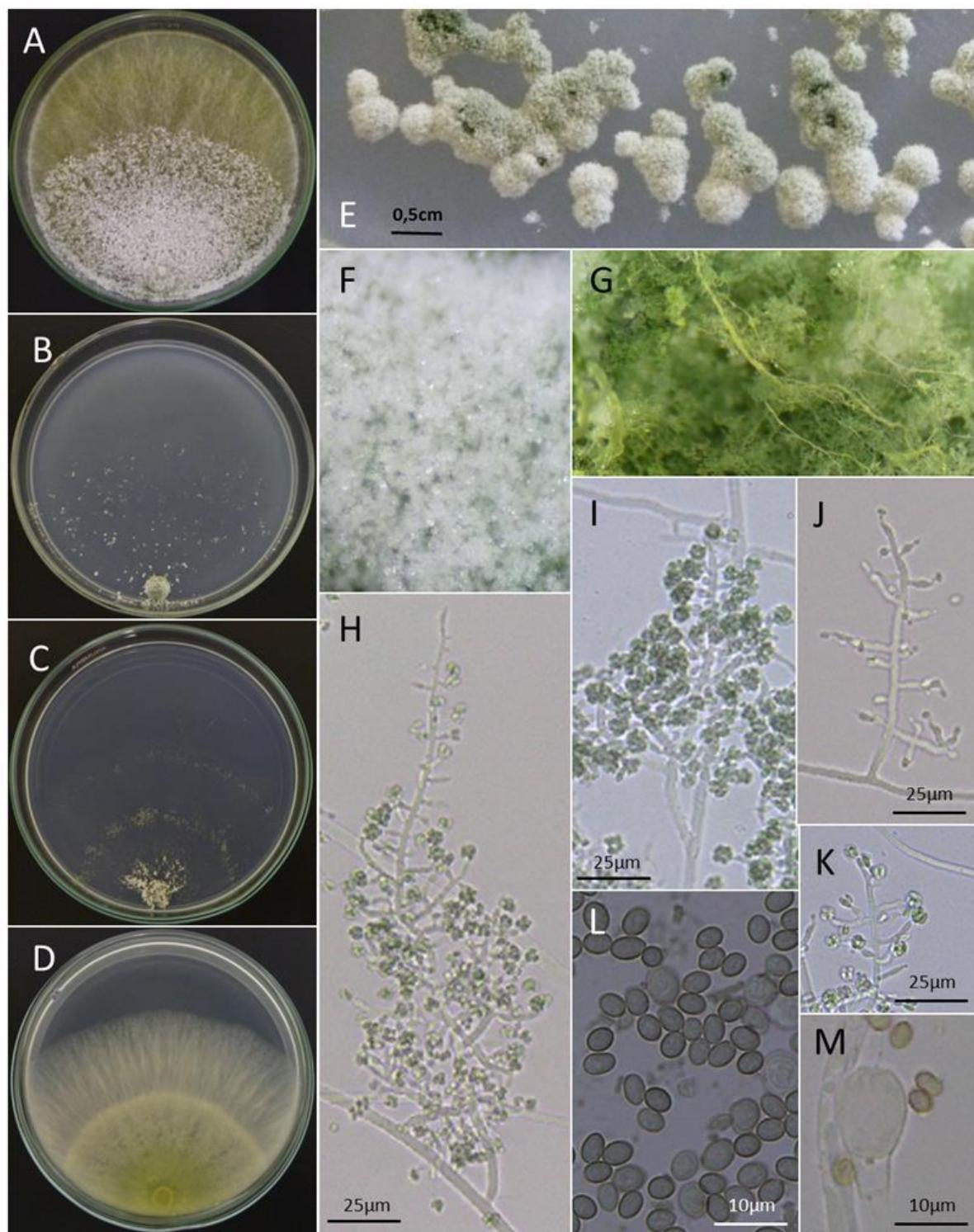
Fig. S11 Morfological characteristics of *Trichoderma orientalis* LESF 544. A-C: culture on PDA, CMD and SNA, respectively, after seven days at 25 °C. D: Pustules formed on CMD. E-G: Branching pattern of conidiophore and phialides. H-I: Chlamydospores. J: Conidia.
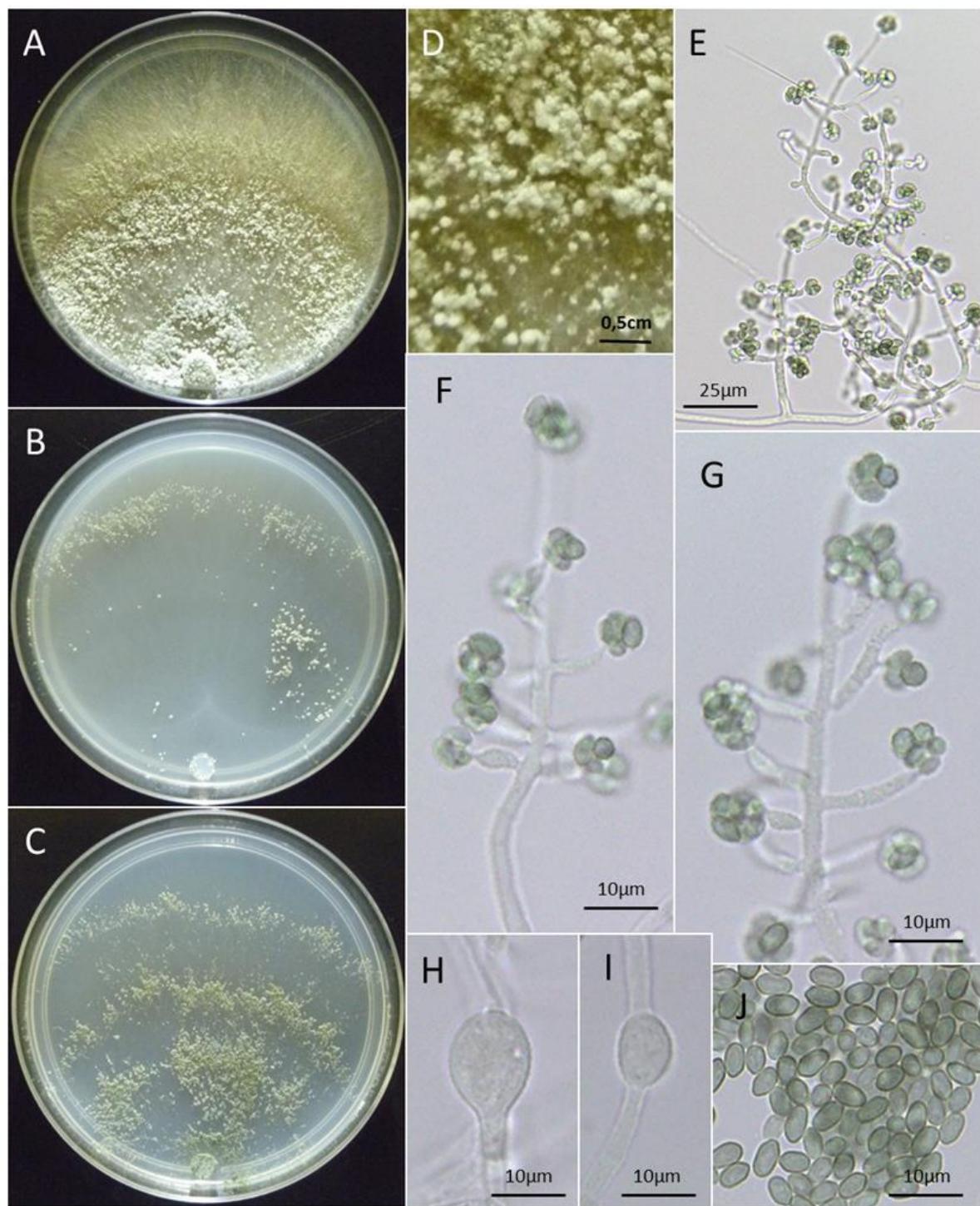
Fig. S12 Morfological characteristics of *Trichoderma spirale* LESF 107. A-B: Cultures on PDA after 14 and seven days at 25 °C, respectively. C-D: Cultures on CMD and SNA after seven days at 25 °C, respectively. E: Pustules formed on CMD. F-G: Branching pattern of conidiophore H-I: Phialides. J-K: Chlamydospores. L: Conidia.
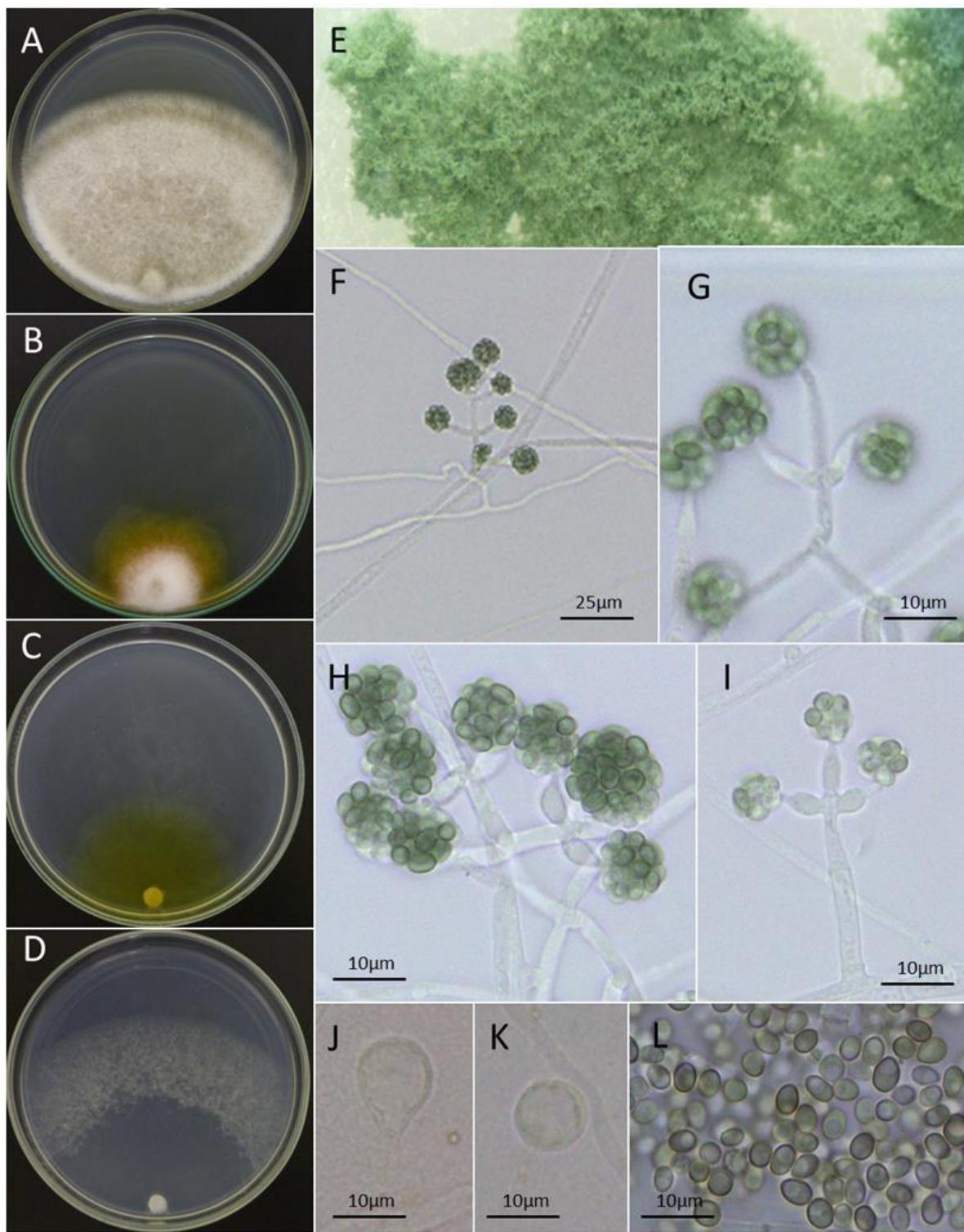
Fig. S13 Morfological characteristics of *Trichoderma velutinum* LESF 132. A-C: Cultures on PDA, CMD and SNA, respectively, after seven days at 25 °C. D-E: Pustules formed on CMD. F: Branching pattern of conidiophore G-H: Phialides. I: Chlamydospores. J: Conidia.
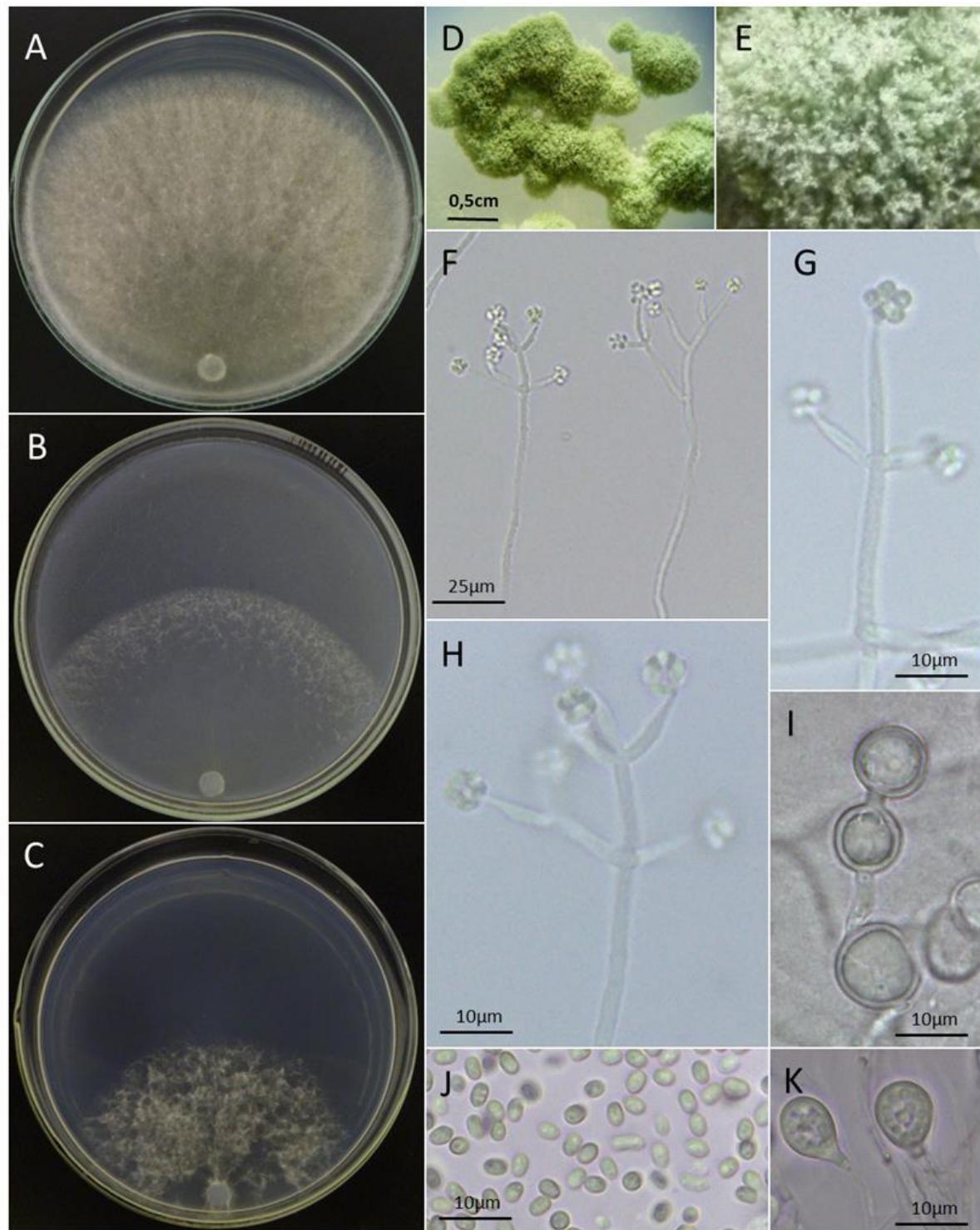
Fig. S14 Morfological characteristics of *Trichoderma virens* LESF 111. A-C: Cultures on PDA, CMD and SNA, respectively, after seven days at 25 °C. D: Pustules formed on CMD. E-F: Phialides. G: Branching pattern of conidiophore. H-I: Chlamydospores. J: Conidia.
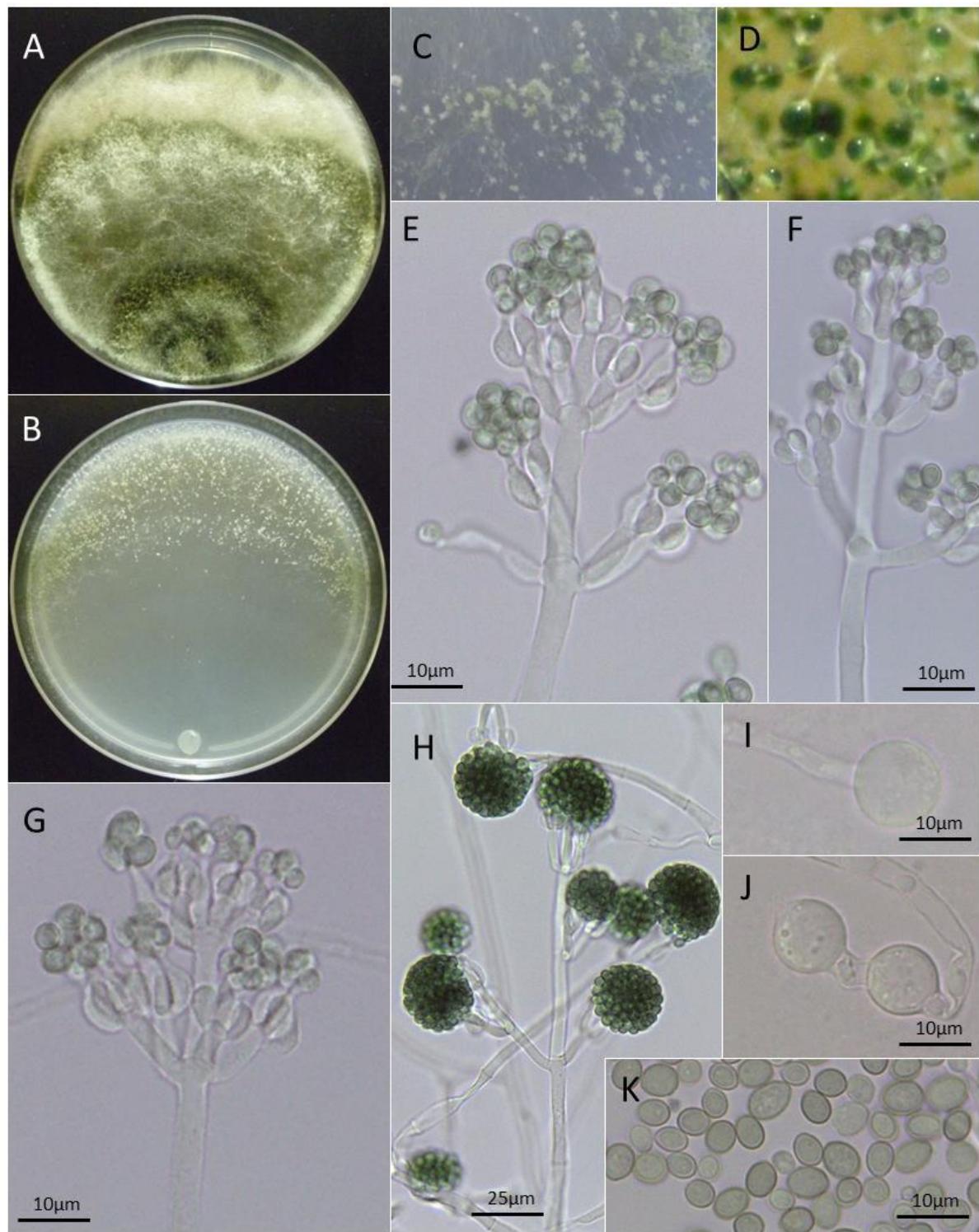
Fig. S15 Morfological characteristics of *Trichoderma viride* LESF 115. A-C: Cultures on PDA, CMD and SNA, respectively, after seven days at 25 °C. D-E: Pustules formed on CMD. F-G: Branching pattern of conidiophore. G: H-I: Phialides. J: Chlamydospores. K: Conidia.
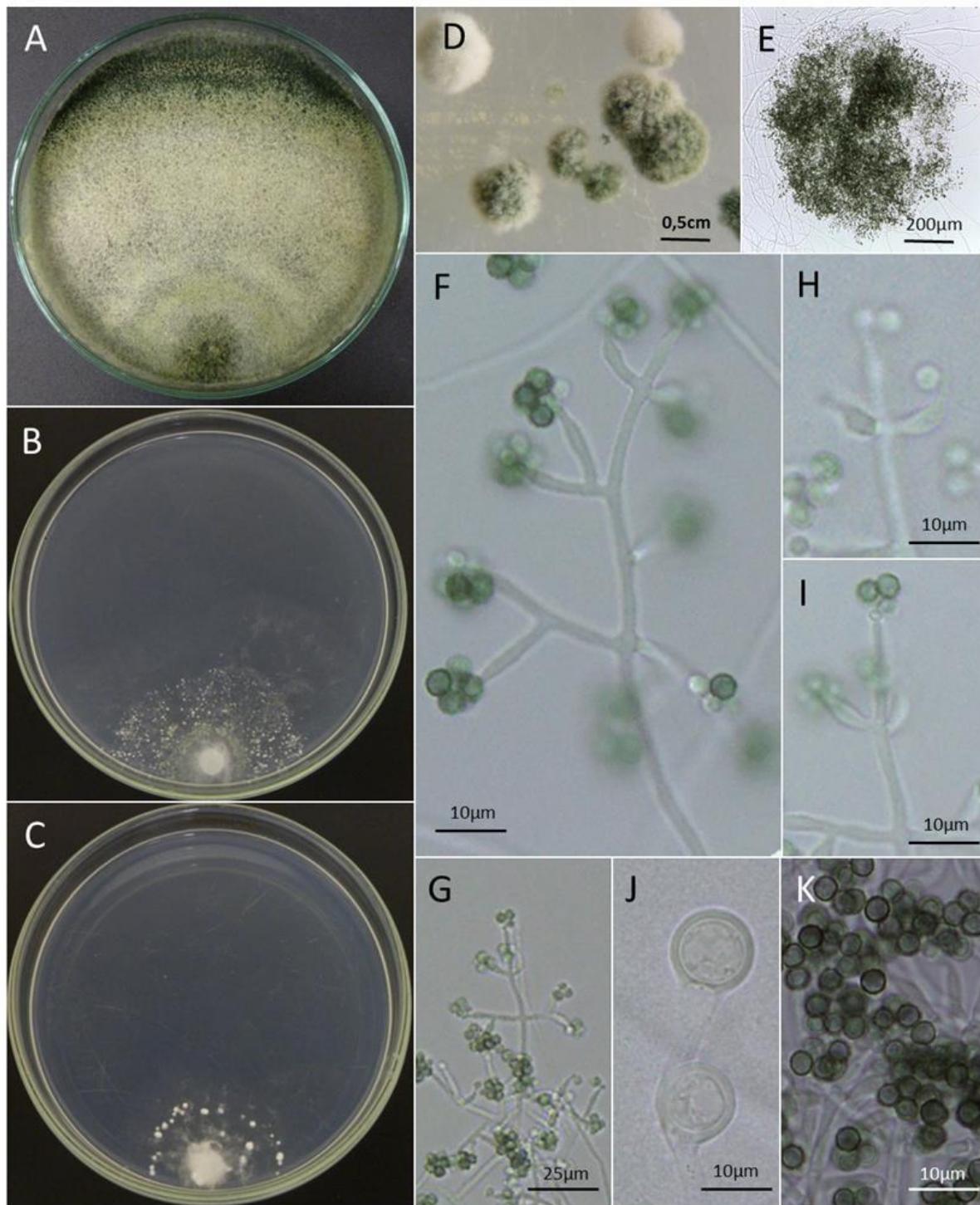
Table S1. *Trichoderma* strains used in phylogenetic analyses and their associated metadata.

Species	Strain ID	Origin	Habitat	GenBank accession numbers			Reference
				ITS	*tef*1	*rpb*2	
T. afroharzianum	G.J.S. 00-24	Mexico	Soil	AF443922	FJ442726	AF443940	Chaverri et al. (2015)
T. afroharzianum	G.J.S. 04-197	Peru	On basidioma of *Moniliophthora roreri* on fruit of *Theobroma*	FJ442214	FJ463302	FJ442740	Chaverri et al. (2015)
T. afroharzianum	G.J.S. 04-186	Peru	On basidioma of *Moniliophthora roreri* on fruit of *Theobroma*	FJ442265	FJ463301	FJ442691	Chaverri et al. (2015)
T. afroharzianum	G.J.S. 04-193	Peru	On basidioma of *Moniliophthora roreri* on fruit of *Theobroma*	FJ442233	FJ463298	FJ442709	Chaverri et al. (2015)
T. afroharzianum	PPRC RW20	Harerge, Ethiopia	coffee rhizosphere		FJ716621		Druzhinina et al. (2010)
T. afroharzianum	PPRC RW14	Holleta, Ethiopia	coffee rhizosphere		FJ577788		Druzhinina et al. (2010)
T. afroharzianum	DAOM 231421	Kigali, Rwanda	clay Soil		AY605770		Druzhinina et al. (2010)
T. afroharzianum	LESF010	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. afroharzianum	LESF011	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. afroharzianum	LESF013	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. afroharzianum	LESF228	Botucatu-SP-Brasil	Soil 10m distant from *Atta bisphaerica* colony				
T. afroharzianum	LESF229	Botucatu-SP-Brasil	Soil 10m distant from *Atta bisphaerica* colony				
T. afroharzianum	LESF542	Botucatu-SP-Brasil	Soil adjacent of *Atta bisphaerica* colony				
T. afroharzianum	LESF553	Taquara-RS-Brasil	Fungus garden of *Trachymyrmex* sp.				
T. afroharzianum	LESF554	Timbó-SC-Brasil	Fungus garden of *Acromyrmex coronatus*				
T. alni	CBS 120633	Europe	Wood		EU498312	EU498349	Jaklitsch et al. (2014)
T. andinensis	GJS 90-140	Venesuela	cut end of tree		AY956321	JN175531	Unpublished
T. andinensis	DAOM 220821	Venezuela	…….		EU280042	KJ842208	Druzhinina et al. (2012)
T. andinensis	GJS 09-62	Peru	soil		JN133570	JN175533	Chaverri and Samuels (2013)
T. andinensis	LESF541	Camacan-BA-Brasil	Fungus garden of *Atta cephalotes*				
T. andinensis	LESF560	Camacan-BA-Brasil	Fungus garden of *Atta cephalotes*				
T. asperellum	GJS 02-63	Cameroon	soil		GU198230	GU198260	Samuels et

							al. (2010)
T. asperellum	GJS 04-22	Maryland	soil under cacao seedling		KF274022	KF27402	Samuels et al. (2010)
T. asperellum	TR-44	USA: Georgia	Soil		GU198231	GU198261	Samuels et al. (2010)
T. asperellum	GJS 04-15	USA: Maryland	coffee seedling	GU198311	GU198290	GU198276	Samuels et al. (2010)
T. asperellum	LESF561	Camacan-BA-Brasil	Fungus garden of *Atta cephalotes*				
T. atroviride	DAOM 222096	Canada	mushroom compost		AF456903	AF456920	Dodd et al. (2003)
T. atroviride	TAMA0220	Japan: Okinawa	….	AB853783	AB853815	AB853846	Unpublished
T. atroviride	TAMA0236	Japan: Tokyo	….	AB853792	AB853824	AB853855	Unpublished
T. atroviride	CBS 119499	Austria	bark and wood	FJ860726	FJ860611	FJ860518	Jaklitsch (2011)
T. atroviride	DAOM 222144	Canada	mushroom compost	AF456916	AF456889	FJ442754	Dodd et al. (2003)
T. atroviride	CBS 142.95	Slovenia	gallery of beetle	AF456917	AY376051	EU341801	Samuels et al. (2002)
T. atroviride	Th002	Colombia	Soil		AB558906	AB558915	Samuels et al. (2002)
T. atroviride	LESF118	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. attinis	LESF236	Austin - Texas, USA	Fungus garden of *Cyphomyrmex wheeleri*				
T. austriacum	CBS 122494	Vienna	wood and effete	FJ860735	FJ860619	FJ860525	Jaklitsch et al. (2011)
T. britanicum	SB	Germany	on rotten wood of *Quercus*		KF134795	KF134786	Jaklitsch et al. (2009)
T. britanicum	SB1	England	on rotten wood of *Quercus*		KF134796	KF134787	Jaklitsch et al. (2009)
T. cerinum	S357	France	forest soil		KF134797	KF134788	Jaklitsch et al. (2014)
T. citrinum	G.J.S. 89-145	Devon, Budleigh, Saltaton, U.K.	.....	DQ835414	DQ835483	DQ835457	Overton et al. (2006)
T. citrinum	B.E.O. 99-29	Oswego County, New York, U.S.A.		DQ835412	DQ835482	DQ835464	Overton et al. (2006)
T. citrinum	C.P.K. 960	Finland	on material freshly		FJ860631	FJ179603	Jaklitsch et al. (2009)
T. compactum	CBS 121218	China	Rhizosphere of tobacco		KF134798	KF134789	Jaklitsch et al. (2014)
T. deliquescens	GJS 89-129	New York	….	AY737773	AF534581	AF545517	Jaklitsch et al. (2011)
T. deliquescens	CBS 121132	United Kingdom	on well-decomposed, crumbly wood	FJ860772	FJ860644		Jaklitsch et al. (2011)
T. deliquescens	DUCC 7303	Korea	Wood		KC291140		Suh et al. (2012)
T. deliquescens	GJS 85-26	Ecuador	*Theobroma gileri*	EU330951	EU338288	EU338328	Degenkolb et al. (2008)
T. deliquescens	LESF211	Camacan-BA-Brasil	Fungus garden of *Acromyrmex* sp.				
T. estonicum	CBS 121556	Europa	on and around		FJ860637	FJ860536	Jaklitsch et

			basidiomes of *Hymenochaete tabacina*				al. (2009)
T. eucorticioides	G.J.S. 99-61	Argentina	Typically found on bark of decaying wood	DQ835467	DQ835474	DQ835518	Overton et al. (2006)
T. eucorticioides	LESF208	Camacan-BA-Brasil	Fungus garden of *Acromyrmex* sp.				
T. hamatum	T-382	USA:Ohio	composted hardwood bark	DQ151583	DQ151582		Samuels (2006)
T. hamatum	DAOM: 167057	Canada	….	EU280124	AF456911	HM466686	Hoyos - Carvajal et al. (2009)
T. hamatum	GJS 05-18	Cameroon	Soil	EU856290	EU856315	FJ150780	Samuels and Ismaiel (2009)
T. hamatum	GJS 05-334	Cameroon	Soil	EU856291	EU856316	FJ150781	Samuels and Ismaiel (2009)
T. hamatum	Dis 219b	Ecuador	*Theobroma gileri*, surface sterilized pod	EU856287	EU856312	EU856342	Samuels and Ismaiel (2009)
T. hamatum	Dis 358e	Ecuador	*Theobroma gileri*, trunk endophyte	EU856283	EU856307	EU856335	Samuels and Ismaiel (2009)
T. hamatum	GJS 05-262	Cameroon	Soil	EU856292	EU856317	EU856348	Samuels and Ismaiel (2009)
T. hamatum	GJS 98-171	New Zealand	Soil	EU856282	EU856306	EU856333	Samuels and Ismaiel (2009)
T. hamatum	S397	Spain	….		JN715614	JN715601	Jaklitsch and Voglmayr (2012)
T. hamatum	LESF120	Capao Alto-SC-Brasil	Fungus garden of *Acromyrmex laticeps*				
T. hamatum	LESF330	Camacan-BA-Brasil	Fungus garden of *Acromyrmex* sp.				
T. hamatum	LESF331	Botucatu-SP-Brasil	Fungus garden of ant colony of *Atta sexdens rubropilosa*				
T. hamatum	LESF332	Botucatu-SP-Brasil	Fungus garden of ant colony of *Atta sexdens rubropilosa*				
T. harzianum	C.P.K. 1934	Lower Austria	*Fagus sylvatica*	EF392746	FJ179573	FJ179608	Druzhinina et al.(2010)
T. harzianum	Dis 253B	Ecuador	*Theobroma cacao*	FJ442619	FJ851875	FJ442756	Unpublished
T. harzianum	Dis 167C	Brasil	*Theobroma cacao*	FJ442269	FJ463309	FJ442689	Unpublished
T. harzianum	GJS 06-94	Cameroon	Soil	FJ442277	FJ463318	FJ442800	Unpublished
T. harzianum	GJS 04-212	Italy	*Vitis vinifera*	FJ442672	FJ463385	FJ442767	Unpublished
T. harzianum	DAOM 222137	Leamington, ON, Canada	commercial mushroom bed	AY605752	AY605796		Druzhinina et al.(2010)
T. harzianum	DAOM 222151	Temple, PA, USA	commercial mushroom bed	AY605753	AY605797		Druzhinina et al.(2010)
T. harzianum	O. Yarden 3907	Mediterraneo	*Psammocinia sp.* sponges		JF421266		Gal-Hemed et al. (2011)
T. harzianum(T)	CBS 226.95	England	botanical garden soil	AF057606	AF348101	AF348101	Samuels et al. (2002)
T. harzianum	LESF343	Botucatu-	Fungus garden of				

		SP-Brasil	*Atta capiguara*				
T. harzianum	LESF344	Ilhéus - BA-Brasil	Fungus garden of *Acromyrmex balzanii*				
T. harzianum	LESF346	Botucatu-SP-Brasil	Fungus garden of *Atta capiguara*				
T. harzianum	LESF517	Sentinela do Sul-RS-Brasil	Fungus garden of *Acromyrmex heyeri*				
T. harzianum	LESF542	Botucatu-SP-Brasil	Fungus garden of *Atta capiguara*				
T. harzianum	LESF554	Ilhéus-BA-Brasil	Fungus garden of *Acromyrmex balzanii*				
T. harzianum	LESF555		*Atta capiguara*				
T. koningiopsis	GJS 04-199	Peru	soil	FJ442654	FJ463268	FJ442789	Unpublished
T. koningiopsis	Dis 374A	Cameroon	stem endophyte	FJ442213	FJ463288	FJ442730	Unpublished
T. koningiopsis	Dis 339c	Ecuador	*Theobroma gileri*	DQ31314	DQ284968	FJ442784	Unpublished
T. koningiopsis	GJS 04-11	USA: Texas	Soil	DQ323421	DQ289009	FJ442693	Samuels et al. (2006)
T. koningiopsis	GJS 93-20	Cuba	branch	DQ313140	DQ284966	EU241506	Samuels et al. (2006)
T. koningiopsis	LESF002	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. koningiopsis	LESF002	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. koningiopsis	LESF119	Nova Petrópolis - RS-Brasil	Fungus garden of *Acromyrmex crassispinus*				
T. koningiopsis	LESF149	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. koningiopsis	LESF212	Austin - Texas, USA	Fungus garden of *Cyphomyrmex wheeleri*				
T. koningiopsis	LESF360	Camacan - BA-Brasil	Fungus garden of *Acromyrmex* sp.				
T. koningiopsis	LESF546	Botucatu-SP-Brasil	Fungus garden *Atta sexdens rubropilosa*				
T. longibrachiatum	NJJL501	China	…..	JQ040373	JQ040434		Unpublished
T. longibrachiatum	SHQP3006	China	…..	JQ040374	JQ040433		Unpublished
T. longibrachiatum	ATCC 18648	Switzerland	Wood of *Aquilaria crassna*	KM279989	AY937412	HM466682	Unpublished
T. longibrachiatum	GJS 07-21	Ghana	….		JN175569	JN175513	Druzhinina et al. (2012)
T. longibrachiatum	S328	Europe	wood	JQ685875	JQ685867	KJ665291	Jaklitsch and Voglmayr (2012)
T. longibrachiatum	LESF009	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. longifialidicum	LESF552	Smithville-Texas - USA	Fungus garden of *Trachymyrmex septentrionalis*				
T. microcitrina	G.J.S. 97-248	Georgia	….	DQ835424	DQ835479	DQ835462	Overton et al. (2006)
T. orientalis	S187	Europe	wood	JQ685873	JQ685868	JQ685884	Jaklitsch and Voglmayr (2012)

T. orientalis	Dis 270F	Ecuador	*Herrania* sp		JN175577	JN175521	Druzhinina et al. (2012)
T. orientalis	GJS 10-230	Brasil	*Theobroma cacao*		JN175579	JN175523	Druzhinina et al. (2012)
T. orientalis	GJS 09-784	Peru	soil under *Theobroma cacao*		JN175578	JN175522	Druzhinina et al. (2012)
T. orientalis	GJS 04-321	Peru	*Theobroma cacao* pot		JN175573	JN175517	Druzhinina et al. (2012)
T. orientalis	GJS 04-332	Peru	*Manihot esculenta*		JN175574	JN175518	Druzhinina et al. (2012)
T. orientalis	LESF516	Botucatu-SP-Brasil	Fungus garden *Atta capiguara*				
T. orientalis	LESF540	Botucatu-SP-Brasil	Fungus garden *Atta capiguara*				
T. orientalis	LESF544	Botucatu-SP-Brasil	Fungus garden of ant colony of *Atta capiguara*				
T. ovalisporum	Dis 203c	Brasil	*Theobroma speciosum*	DQ315458	DQ307540	FJ442796	Unpublished
T. ovalisporum	Dis 70a	Ecuador	*Banisteriopsis caapi*		AY376037	FJ442742	Unpublished
T. ovalisporum	GJS 04-113	Viet Nam	peanut	FJ442614	FJ463281	FJ442781	Unpublished
T. parestonicum	CBS 120636	Europe	on and around basidiomes of *Hymenochaete tabacina*	FJ860803	FJ860667	FJ860565	Jaklitsch et al. (2009)
T. parestonicum	C.P.K 2427	Europe	on and around basidiomes of *Hymenochaete tabacina*	FJ860802	FJ860666	FJ860564	Jaklitsch et al. (2009)
T. petersenii	GJS 04-164	USA	decorticated wood	DQ323442	DQ289004	FJ442783	Unpublished
T. phellinicola	CBS 119283	Austria	on *Phellinus ferruginosus/Fagus sylvatica*	FJ860808	FJ860672	FJ860569	Jaklitsch et al. (2011)
Protocrea pallida	CBS 299.78	....	....	EU703925	EU703900	EU703948	Jaklitsch et al. (2009)
T. sinuosum	S378	Spain	On decorticated wood	JN198099	KJ66572	KJ665342	Chaverri and Samuels, (2003)
T. spinulosa	CBS 121280	Denmark	an apple plantation	FJ86084	FJ860699	FJ860589	Jaklitsch et al. (2009)
T. spinulosa	CBS 121272	Austria	on stems of *Juncus effuses* and *Festuca* sp.	FJ860843	FJ860700	FJ860590	Jaklitsch et al. (2009)
T. spinulosa	CBS 311.50	France	on piled culms of *Molinia coerulea*	FJ860844	FJ860701	FJ860591	Jaklitsch et al. (2009)
Trichoerma sp.		Camacan-BA-Brasil	Fungus garden *Atta cephalotes*				
T. spirale	CBS 120963	Turkey	Soil *Solanum tuberosum*	FJ442608	FJ463291		Unpublished
T. spirale	Dis 151E	Costa Rica	stem endophyte *Theobroma grandiflorum*	FJ442230	FJ463374	FJ442766	Unpublished
T. spirale	Dis 68C	Ecuador	stem endophyte *Theobroma sp.*	FJ442222	FJ463372	FJ442698	Unpublished
T. spirale	Dis 243B	Ecuador	stem endophyte *Theobroma bicolor*	FJ442231	FJ463373	FJ442785	Unpublished
T. spirale	Dis 311D	Cameroom	stem endophyte *Irvingia gabonensis*	FJ442232	FJ463369	FJ442694	Unpublished
T. spirale	DAOM183974	Thailand	……	EU280068	EU280049	AF545553	Unpublished

T. spirale	Dis 173A	Brazil	stem endophyte *Theobroma* sp.	FJ442217	FJ463371	FJ442705	Unpublished
T. spirale	Dis 392C	Cameroom	stem endophyte *Irvingia* sp.	FJ442622	FJ463370		Unpublished
T. spirale	LESF107	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. spirale	LESF108	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. spirale	LESF117	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. spirale	LESF327	Ilhéus-BA-Brasil	Fungus garden of *Acromyrmex balzanii*				
T. spirale	LESF328	Camacan-BA-Brasil	Fungus garden of *Atta cephalotes*				
T. spirale	LESF515	Botucatu-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. spirale	LESF543	Botucatu-SP-Brasil	Fungus garden of *Atta capiguara*				
T. spirale	LESF547	Corumbataí-SP-Brasil	Soil adjacent *Atta sexdens rubropilosa* colony				
T. spirale	LESF548	Botucatu-SP-Brasil	Fungus garden of *Atta capiguara*				
T. spirale	LESF557	Botucatu-SP-Brasil	Fungus garden of *Atta capiguara*				
T. spirale	LESF559	Botucatu-SP-Brasil	Soil adjacent of ant *Atta bisphaerica*				
T. spirale	LESF562	Corumbataí-SP-Brasil	Soil 10m distant from *Atta bisphaerica* colony				
T. taxi	ZJUF0986	China	from old trunk of *Taxus mairei*	DQ470074	DQ859029	DQ859032	Zhang et al. (2007)
T. taxi	ZJUF0869	China	from old trunk of *Taxus mairei*	DQ859025	DQ859027	DQ859030	Chaverri and Samuels, (2003)
T. texana	LESF551	Smithville-Texas-USA	Fungus garden of *Trachymyrmex septentrionalis*				
T. thelephoricola	CBS 120925	Austria	on hymenium of *Steccherinum ochraceum*	FJ860858	FJ860711	FJ860600	Jaklitsch et al. (2009)
T. tomentosum	CBS 120637	Europe	……	FJ860744	FJ860629	FJ860532	Jaklitsch et al. (2011)
T. tomentosum	S33	France	forest soil		KF134801	KF134793	Jaklitsch et al. (2014)
T. velutinum	MIAE00038	France	Soil	HM176568	HM176586		Anees et al. (2010)
T. velutinum	MIAE00033	France	Soil	HM176563	HM176581		Anees et al. (2010)
T. velutinum	MIAE00035	France	Soil	HM176565	HM176583		Anees et al. (2010)
T. velutinum	MIAE00036	France	Soil	HM176566	HM176584		Anees et al. (2010)
T. velutinum	MIAE00043	France	Soil	HM176573	HM176591		Anees et al. (2010)
T. velutinum	DAOM	Nepal	forest soil		AY937415	JN133569	Jaklitsch et

Species	Code	Location	Substrate				Reference
	230013						al. (2014)
T. velutinum	DAOM 230014	Nepal	forest soil		AY937446		Samuels (2006)
T. velutinum	C.P.K. 298	Nepal	forest soil		KJ665769	KF134794	Jaklitsch et al. (2014)
T. velutinum	LESF132	Botucatu-SP-Brasil	Fungus garden of *Atta bisphaerica*				
T. virens	SHSJ1006	China	….	JQ040399	JQ040415		Unpublished
T. virens	DAOM 237548	Peru	Soil		EU280065		Hoyos-Carvajal et al. (2009)
T. virens	DAOM 167652	Georgia	forest soil		AY750891		Jaklitsch et al. (2014)
T. virens	Dis 328A	Ecuador	stem endophyte	FJ442670	FJ463363	FJ442738	Unpublished
T. virens	Dis 162	Costa Rica	stem endophyte	FJ442669	FJ463367	FJ442696	Unpublished
T. virens	LESF004	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. virens	LESF111	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. virens	LESF112	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. virens	LESF220	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. virens	LESF221	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. virens	LESF269	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. virens	LESF275	Corumbataí-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. virens	LESF514	Botucatu-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. virens	LESF535	Botucatu-SP-Brasil	Fungus garden of *Atta sexdens rubropilosa*				
T. virens	LESF550	Corumbataí-SP-Brasil	Soil 10m distant from *Atta sexdens rubropilosa* colony				
T. virens	LESF556	Smithville-Texas - USA	Fungus garden of *Trachymyrmex septentrionalis*				
T. virens	LESF573	Botucatu-SP-Brasil	Fungus garden of *Atta capiguara*				
T. viride	Dis 238gii	Ecuador	*Theobroma gileri*	DQ841739	DQ841724		Jaklitsch et al. (2006)
T. viride	GJS 90-95	USA: North Carolina	decorticated wood	DQ315455	DQ307535	EU248596	Samuels et al. (2006)
T. viride	GJS 04-369	Austria	sapwood of *Theobroma cacao*	DQ323430	DQ307553	EU252003	Hanada et al. (2008)
T. viride	GJS 92-14	New Zealand	sapwood of *Theobroma cacao*	DQ313155	DQ288988	EU252006	Hanada et al. (2008)
T. viride	LESF115	Corumbataí-SP-Brasil	Soil 10m distant from *Atta sexdens rubropilosa* colony				

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