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ORIGINAL PAPER

Escovopsis trichodermoides sp. nov., isolated from a nest of the lower attine ant *Mycocepurus goeldii*

Virginia E. Masiulionis · Marta N. Cabello · Keith A. Seifert · Andre Rodrigues · Fernando C. Pagnocca

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Abstract Currently, five species are formally described in *Escovopsis,* a specialized mycoparasitic genus of fungus gardens of attine ants (Hymenoptera: Formicidae: tribe Attini). Four species were isolated from leaf-cutting ants in Brazil, including *Escovopsis moelleri* and *Escovopsis microspora* from nests of *Acromyrmex subterraneus molestans*, *Escovopsis weberi* from a nest of *Atta* sp. and *Escovopsis lentecrescens* from a nest of *Acromyrmex subterraneus subterraneus*. The fifth species, *Escovopsis aspergilloides* was isolated from a nest of the higher attine ant *Trachymyrmex ruthae* from Trinidad. Here, we describe a new species, *Escovopsis trichodermoides* isolated from a fungus garden of the lower attine ant *Mycocepurus goeldii*, which differs from the five other species by highly branched, trichoderma-like conidiophores lacking swollen vesicles, with reduced conidiogenous cells and distinctive conidia morphology. Phylogenetic analyses based on partial *tef1* gene sequences support the distinctiveness of this species. A portion of the internal transcribed spacers of the nuclear rDNA was sequenced to serve as a DNA barcode. Future molecular and morphological studies in this group of fungi will certainly unravel the taxonomic diversity of *Escovopsis* associated with fungus-growing ants.

Keywords Attini · Fungus-growing ant · Hypocreales · Mycoparasitism

V. E. Masiulionis · A. Rodrigues · F. C. Pagnocca (✉)
Centro de Estudos de Insetos Sociais, Instituto de Biociências, UNESP – Univ Estadual Paulista, Campus de Rio Claro, Rio Claro, SP 13506-900, Brazil
e-mail: pagnocca@rc.unesp.br

V. E. Masiulionis
e-mail: vemasiulionis@gmail.com

A. Rodrigues
e-mail: andrer@rc.unesp.br

M. N. Cabello
Comisión de Investigaciones Científicas, Facultad de Ciencias Naturales y Museo, Instituto de Botánica Carlos Spegazzini, Universidad Nacional de La Plata, La Plata, Buenos Aires, Argentina
e-mail: mcabello@ymail.com

K. A. Seifert
Biodiversity (Mycology), Eastern Cereal and Oilseed Research Centre, Agriculture & Agri-Food Canada, Ottawa, ON K1A 0C6, Canada
e-mail: Keith.Seifert@agr.gc.ca

A. Rodrigues · F. C. Pagnocca
Departamento de Bioquímica e Microbiologia, Instituto de Biociências, UNESP – Univ Estadual Paulista, Campus de Rio Claro, Rio Claro, SP 13506-900, Brazil

Introduction

Ants in the tribe Attini (Hymenoptera: Formicidae) are known as 'fungus-growing ants' because they cultivate basidiomycetous fungi (Agaricales) for food (Weber 1972; Littledyke and Cherrett 1976; Quinlan and Cherrett 1979; Bass and Cherrett 1995). Schultz and Brady (2008) divided attine fungal cultures into five groups: (i) lower agriculture of the genera *Mycocepurus*, *Myrmicocrypta* and some *Apterostigma* species, in which the cultivated fungus belongs to the tribe Leucocoprineae (group G3); (ii) coral fungus agriculture of the *Apterostigma* 'pilosum group' (cultivating fungi in the Pterulaceae, groups G2 and G4); (iii) yeast agriculture of the lower attine ants in the *Cyphomyrmex* 'rimosus group', in which the cultivated leucocoprineaceous fungi belong to the G3; (iv) generalized higher agriculture of the non-leaf-cutting ants (*Trachymyrmex* and *Sericomyrmex*) that cultivate G1 and, (v) leaf-cutter agriculture (*Atta* and *Acromyrmex*) in which both genera cultivate different fungi belonging to the tribe Leucocoprineae (G1) (Chapela et al. 1994; Villesen et al. 2004). These ancient symbioses possibly originated 50–60 million years ago (Schultz and Brady 2008).

Despite the variety of microorganisms continuously introduced into attine fungus gardens, ants usually successfully maintain their cultivar (Pagnocca et al. 2012). However, in certain circumstances, fungi of the specialized mycoparasitic genus *Escovopsis* (Ascomycota, Hypocreales) threaten the symbiosis (Currie et al. 1999; Reynolds and Currie 2004). The prevalence of these fungi appears to be greater in nests of leaf-cutting ants from Central America (Currie et al. 1999; Gerardo et al. 2006) than in those from South America (Rodrigues et al. 2005, 2008). Unfortunately, despite the molecular diversity presented in these primarily ecological studies, represented only by *tef1-α* sequences, taxonomic and morphological analyses and accompanying ITS barcodes have not been published for most detected species, and few voucher cultures are preserved.

Möller (1893) first observed these peculiar fungi associated with the fungi cultivated by the ants *Acromyrmex disciger* and *Apterostigma* in Blumenau, Santa Catarina state, Brazil. Kreisel (1972) later found them in nests of *Atta insularis* in Cuba and formally described *Phialocladus zsoltii*, but unfortunately did not designate a type specimen, making the name nomenclaturally invalid. Muchovej and Della Lucia (1990) renamed the genus *Escovopsis* and the species *Escovopsis weberi,* in honor of Neil Weber, an American entomologist. The second fungus observed by Möller (1893) in nests of *Apterostigma* species (*Ap. wasmannii*, *Ap. pilosa* and *Ap. moelleri*) had a similar morphology to *Aspergillus* but was not formally described and named. Seifert et al. (1995) rediscovered this fungus in nests of *Trachymyrmex ruthae* from Trinidad and described it as *Escovopsis aspergilloides,* the epithet indicating its morphological similarity with *Aspergillus.* Augustin et al. (2013) added three new species to *Escovopsis*, namely, *E. lentecrescens*, *E. microspora*, and *E. moelleri* isolated from nests of three ant species in the genus *Acromyrmex.* In addition to the three new species, Augustin et al. (2013) described the third species observed by Möller (1893) as a new genus and species, *Escovopsioides nivea,* morphologically distinguished by the absence of conidiophore swellings, and the production of conidia on clusters of acicular conidiogenous cells. It is clear from molecular phylogenies of fungi isolated from ant nests that many other still undescribed species exist (Currie 2001; Currie et al. 2003; Gerardo et al. 2006; Augustin et al. 2013).

The sexual states of *Escovopsis* species is unknown, whereas the asexual states has only been isolated from fungus gardens and garden waste of attine ants (Currie 2001; Augustin et al. 2013) and the precise nature of the antagonism towards the ants' cultivar is unknown, although it seems clear that they are parasites of the symbiosis (Currie 2001; Reynolds and Currie 2004).

This paper addresses the description of a new species of *Escovopsis* based on morphological and molecular data. The name *Escovopsis trichodermoides* is proposed for the new species isolated from a nest of *Mycocepurus goeldii*, a lower fungus-growing ant.

Materials and methods

Sampling site

Fieldwork was carried out on 13 Aug 2011 on the campus of UNESP—São Paulo State University, Rio Claro, São Paulo state, Brazil. The *M. goeldii* nest was located near the experimental orchard at the university campus (22°23′46.93″S, 47°32′40.12″W). After excavation, samples were collected from the fungus garden of this ant nest.

Fungal isolation

Fragments were removed from the fungus garden of *M. goeldii* with sterile forceps and plated on potato dextrose agar (PDA, Acumedia®) supplemented with 200 μg mL^{-1} of chloramphenicol (Sigma) in 9-cm-diameter Petri dishes. Plates were incubated at 25 °C for 10 days in the dark. After incubation, two morphologically distinct isolates were recovered and further subcultured in additional plates in order to obtain axenic cultures.

Cultural characters

Radial growth, conidia and chlamydospore formation and pigment production were determined on three different culture media without antibiotics: oatmeal agar (OA), 2 % malt extract agar (MEA), and PDA on temperatures ranging from 15 to 35 at 5 °C intervals. Assays were performed in triplicate and lasted 2 weeks. For micromorphological descriptions, the strain was grown on 2 % MEA for 7 days at 25 °C.

To measure the microscopic structures, conidiophores, conidiogenous cells and conidia were prepared for scanning electron microscopy by rinsing in distilled water, removal from the colonies with a fine needle, and air-drying on a metal stub (Allegrucci et al. 2011). Dried spores were sputter-coated with gold/palladium and observed with a Jeol JSM-6360 LV microscope. For SEM micrographs of conidiophores, material was fixed with osmium tetroxide vapor for 24 h, dehydrated and critical point dried using a Balcers CPD030, sputter coated with gold using a SCD050 and imaged using a Tabletop-Hitachi Electron microscope TM3000. Micrographs were taken with a Leica DM750 bright-field microscope.

DNA extraction, PCR and sequencing

Fungal mycelia were grown on PDA at 25 °C for 8 days. Genomic DNA extractions were performed after breaking the mycelia with a mortar and pestle in liquid nitrogen, as described by Almeida (2005). A single exon of the nuclear translation elongation factor 1-α gene (*tef1-α*) gene was amplified with *Escovopsis* primers EF6-20F (5′AAGAACATGATCACTGGTACCT3′) and EF6-1000R (5′CGCATGTCRCGGACGGC3′), following methods used in previous studies on *Escovopsis* phylogeny (Currie et al. 2003; Gerardo et al. 2004; Taerum et al. 2007, 2010). PCR was performed with an initial denaturation at 96 °C for 3 min, followed by 35 cycles at 96 °C for 1 min, 61 °C for 1 min, and 72 °C for 1 min. PCR products were purified with an Illustra GFX DNA and Gel Band Purification Kit (GE Healthcare). Sequencing reactions were performed with Big Dye® Terminator v3.1 Cycle Sequencing Kit using the same primers as in PCR amplification and placed in a 3130 Genetic Analyzer (Applied Biosystems). Sequences were assembled in BioEdit v. 7.0.5.3 (Hall 1999). The ITS fungal barcode was also amplified and sequenced with primers ITS5 (5′GGAAGTAAAAGTCGTAACAAGG3′) and ITS4 (5′TCCTCCGCTTATTGATATGC3′) (White et al. 1990). PCR conditions were an initial denaturation at 96 °C for 3 min, 35 cycles at 96 °C for 30 s, 63 °C for 45 s, and 72 °C for 1 min. The amplicon purification protocol was the same as for *tef1-α*.

Phylogenetic analysis

Partial *tef1-α* gene and ITS sequences of *Escovopsis* species were retrieved from GenBank. Sequence selection was based (i) on the phylogenetic diversity of *Escovopsis* found in the different fungicultures of attine ants, (ii) on the different spore colours described by Gerardo et al. (2006) and (iii) those available for type strains. Sequences were aligned with MAFFT v7 (Katoh and Standley 2013) and trimmed with BioEdit (Hall 1999). Maximum likelihood (ML) and Bayesian inference (BI) phylogenetic analyses were performed on the *tef1-α* and ITS data set. ML analyses were conducted using the MEGA v6 phylogenetic software package (Tamura et al. 2013). A bootstrap analysis was performed to assess the confidence limits of the branching (2,000 replicates) (Felsenstein 1985) and BI analyses was carried out with MrBayes v3.2.1 (Ronquist et al. 2012). The model of sequence evolution was selected based on the Akaike information criterion (AIC) as calculated in MrModeltest v2 (Nylander 2004). The best models selected were GTR+I+G for *tef1-α* and K80+I model for ITS. In BI analyses, two independent Markov chain Monte Carlo (MCMC) runs were performed for each data set for 3 million generations, with sampling every 1,000 generations. The first 25 % of trees was removed as burn-in and the final consensus tree was inferred using the 50 % majority rule with posterior probabilities for each node.

Results

During the isolation procedure, two distinct isolates were recovered on PDA. One was the mutualistic fungus of *M. goeldii* (GenBank accession #KF033129) with an ITS similarity of 99 % (584/587 bp identical) with the cultivar of *Mycetagroicus cerradensis* (GenBank accession #HM245775) and with other mutualistic cultivars from different attine ant species. This finding conforms to the survey by Solomon et al. (2011), who reported that the mutualistic fungus belongs to the group of lower attine cultivars (G3-clade2). The other isolate recovered from the fungus garden of *M. goeldii*, initially labeled as VEM001 (GenBank accession #KF033128 for *tef1* sequence) recovered from the fungus garden of *M. goeldii* had white colonies that later turned yellow and then brown, with stolon-like structures and rapid growth, which we considered to be a hitherto unknown species of *Escovopsis*, described below as *E. trichodermoides.*

Phylogenetic analysis

A comparison of partial (784 bp) *tef1-α* sequences of *E. trichodermoides* and those retrieved from GenBank showed that the closest relatives were *Escovopsis* sequences deposited by Gerardo et al. (2006). The first was strain nmg011101-03 (GenBank accession #DQ848209), isolated from the fungus garden of *Cyphomyrmex longiscapus* in Panama, which had 96 % similarity (33 substitutions, 15 transversions and 18 transitions). The second was strain agh020629-02 esc6 (GenBank accession #DQ848167) with 95 % similarity (43 substitutions, 17 transversions and 23 transitions), which was found in the fungus garden of *Apterostigma dentigerum* in Costa Rica. The length of the alignment used for the phylogenetic analysis after trimming and excluding gaps was 395 bp.

Our phylogenetic results (Fig. 1) show that *E. trichodermoides* is related to the Hypocreales (Ascomycota), in agreement with the results of Currie et al. (2003) and Augustin et al. (2013). *Escovopsis trichodermoides* is associated with fungus gardens of lower attine ants and occupies a somewhat remote position, although with lower bootstrap support, in the clade that includes other *Escovopsis* species associated with lower attine ants (Fig. 1). A portion of the ITS was sequenced but because most known *Escovopsis* species have not been barcoded, and no reference vouchers or DNA are available, no phylogenetic analysis was possible. The ITS sequence of *E. trichodermoides* (GenBank accession #KJ485699) showed 84 % similarity with *Hypomyces* and *Cladobotryum* species.

Growth

The strain VEM001 grew well on all agar media from 15 to 30 °C; the optimum range is 25–30 °C and no growth was observed at 35 °C. Radial growth diameter (in mm) after 72 h of incubation at 15–30 °C on OA, MEA and PDA are shown in Table 1. Conidia were observed after 3 days at all temperatures tested on PDA and after 4 and 12 days at 20–30 °C on OA and MEA, respectively.

Discussion

Möller (1893) provided detailed descriptions of the rapid growth of a fungus with wider hyphae than those of the mutualistic fungus, which produced conidia from a lateral, perpendicular branch that extended and gave rise to new lateral, perpendicular branches. The fungus produced stolon-like hyphae and rapidly formed conidia, features characteristic of all known *Escovopsis* species.

The new species *E. trichodermoides* differs morphologically from other described species by the trichoderma-like branching of the conidiophores, the absence of vesicles, monoblastic conidiogenous cells and conidial morphology. *Escovopsis trichodermoides* produces branched conidiophores up to 230 µm long and 4.5 µm wide. From the main axis, side branches develop at more or less right angles. At each turn of every branch, new fertile branches arise. Branches are covered with conidiogenous cells, 10–20 × 7–8.5 µm, with no trace of supporting vesicles. In contrast, *E. weberi* ATCC 64542^{T} has terminal more or less clavate vesicles, 43–58 × 11.5–14 µm, covered with conidiogenous cells that are 3–4.5 × 4.5 µm. In *E. aspergilloides,* the conidiophores are polycephalous on stipes up to 1,350 µm long, terminating in globose vesicles with a uniseriate layer of phialides, similar in overall appearance to species of *Aspergillus*. Conidiophores of *E. moelleri* are 55–90 × 10–16 µm, branched in one or two levels at a more or less right angles, with single or opposite branches, and each branch terminating

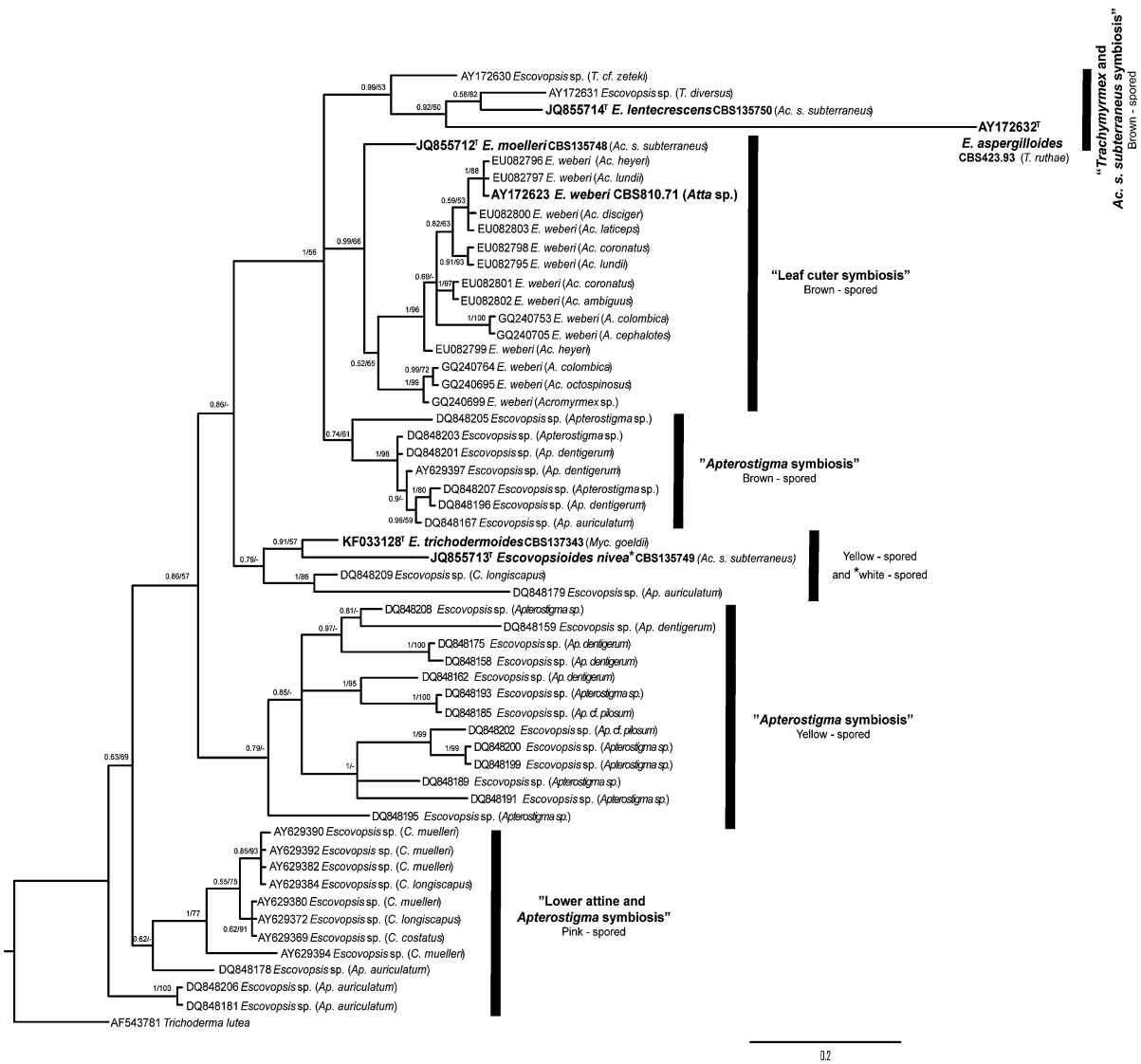

Fig. 1 Phylogenetic tree showing the position of *E. trichodermoides* in the *Escovopsis* clade. The tree was inferred with the Bayesian algorithm using an alignment of the partial *tef1-α* gene sequences (alignment length 395 bp). Numbers on branches are posterior probabilities followed by maximum-likelihood bootstrap support values. Only bootstrap values higher than 50 % are shown. Names in bold denote the currently described *Escovopsis* species that have vouchers deposited in culture collections. The GenBank accessions are represented before each taxon name and the ant species related to each *Escovopsis* strain are in parentheses. *Scale bar* 0.02 substitutions per site. T = ex-type strain

in a more or less cylindrical vesicle covered with phialides, a similar pattern to what is observed in *E. microspora* and *E. lentecrescens.* All three species described by Augustin et al. (2013) seem to have similar branching patterns and none have the overall pyramidal appearance seen in *E. trichodermoides*. The conidia of *E. weberi* are globose to ovoid (2.0–3.5 × 2–3 μm), rough and produced in basipetal chains. In *E. aspergilloides,* conidia are ellipsoidal, 2.5–3.5 × 2 μm and also produced in basipetal chains. The conidia of *E. moelleri* are oblong and 7–10 × 3.0–3.5 μm, whereas they are ovoid in *E. microspora* (2.5 × 1.5 μm) and in *E. lentecrescens* (3.0–4.0 × 2.0–2.5 μm). Finally in *E. trichodermoides,* conidia are ovoid (3.5–4.5 × 2–3 μm), coarsely verrucose, with a distinctly truncate base, and solitary rather

Table 1 Growth diameter (mm) of *E. trichodermoides* (VEM001) after 72 h of incubation at temperature range of 15–30 °C on different culture media

Culture media	Temperature (°C)			
	15	20	25	30
OA	25–40	30–40	80–81	80–81
MEA	30–35	45–50	65–72	65–72
PDA	25–30	45–50	65–72	65–72

No growth was observed at 35 °C

OA Oatmeal agar, *MEA* malt extract agar, *PDA* potato-dextrose agar

than produced in chains. The production of chlamydospores in *E. trichodermoides* is typical and they are smaller than those of *E. microspora*. The growth rate of *E. trichodermoides,* covering a 9-cm-diameter Petri dish after 4 days at 25–30 °C on OA and in 5 days on MEA and PDA at the same temperatures, is similar to that of *E. weberi* and *E. aspergilloides* (Seifert et al. 1995). *Escovopsis moelleri, E. microspora* and *E. lentecrescens* (Augustin et al. 2013) are slower growing.

In the past, the taxonomy of hyphomycetes was dominated by interpretations of conidium ontogeny (Kendrick 1971), but now there are many examples of genera with variable conidiogenesis, such as mixtures of sympodial and percurrent conidiogenous cells, or percurrent conidiogenous cells and phialides among species classified within one genus (Seifert et al. 2011). The inclusion of species with either solitary or catenate conidia within *Escovopsis* is thus consistent with current taxonomic practice. According to Muchovej and Della Lucia (1990), *E. weberi* has holoblastic ontogeny of conidia, as does the new species *E. trichodermoides,* while in *E. aspergilloides* conidiogenesis was reported as enteroblastic (Seifert et al. 1995). In his considerations of the relationships between phialidic hyphomycetes producing multiple conidia versus solitary conidia, Gams (1973) noted that in all phialides, the first formed conidium has a wall continuous with the conidiogenous cell, and could thus be considered holoblastic, and the subsequently formed conidia are initiated from a new, internal wall and are thus enteroblastic. He suggested that species producing solitary conidia from conidiogenous cells that otherwise appear to be phialides may have evolved from those producing serial conidia (Gams 1973). However, even if the conidiogenous denticles of *E. trichodermoides* are considered morphologically reduced phialides, Gams's hypothesis does not apply here, because this fungus produces solitary conidia and is in a basal position in the phylogenetic tree (Fig. 1). Evidently, in this clade, phialides producing basipetal conidial chains are a derived character.

Escovopsis is commonly associated with colonies of attine ants and according to Currie (2001) and Augustin et al. (2013), this fungus is prevalent in the exhausted substrate in the bottom of the fungus garden. Interestingly, our strain of *E. trichodermoides* was isolated from young parts of the garden. According to the phylogenetic analyses of Currie et al. (2003), *Escovopsis* is divided into four lineages, each associated with a particular group of fungi cultivated by attine ants: (i) the "lower attine symbiosis" that cultivates leucocoprinaceous fungi; (ii) the "*Apterostigma* symbiosis" that cultivates pterulaceous fungi; (iii) the "*Trachymyrmex* symbiosis" that cultivates derived leucocoprinaceous fungi, and (iv) the "leaf-cutter symbiosis" that cultivates the highly derived leucocoprinaceous fungi. The same clades occur in our phylogenetic analyses (Fig. 1). Because *E. trichodermoides* was isolated from the fungus garden of a lower attine ant that cultivates G3 fungus, we expected that the mycoparasite from this nest would cluster with similar *Escovopsis* found infecting nests of ants cultivating other G3 fungi. As expected, *E. trichodermoides* clustered with *Escovopsis* isolated from gardens of *Ap. auriculatum* and *C. longiscapus*, two other lower attine ants that cultivate fungi of the G3 group (Fig. 1). More interesting, these particular *Escovopsis* strains from such ant species have yellow spores (Fig. 1). According to N. M. Gerardo (personal communication), such yellow *Escovopsis* morphotypes are unusual in gardens of lower attines.

The *Escovopsis* species that infect lower attine cultivars appear to be very diverse in morphological characteristics (Gerardo et al. 2006) and it would be interesting to study the micromorphology of the many unnamed species that have been sequenced to determine whether all *Escovopsis* species isolated from lower attine cultivars lack phialides, which might then constitute a synapomorphy for this group.

Augustin et al. (2013) described the new genus *Escovopsioides* based on morphological differences from the type strains of *Escovopsis*, which then

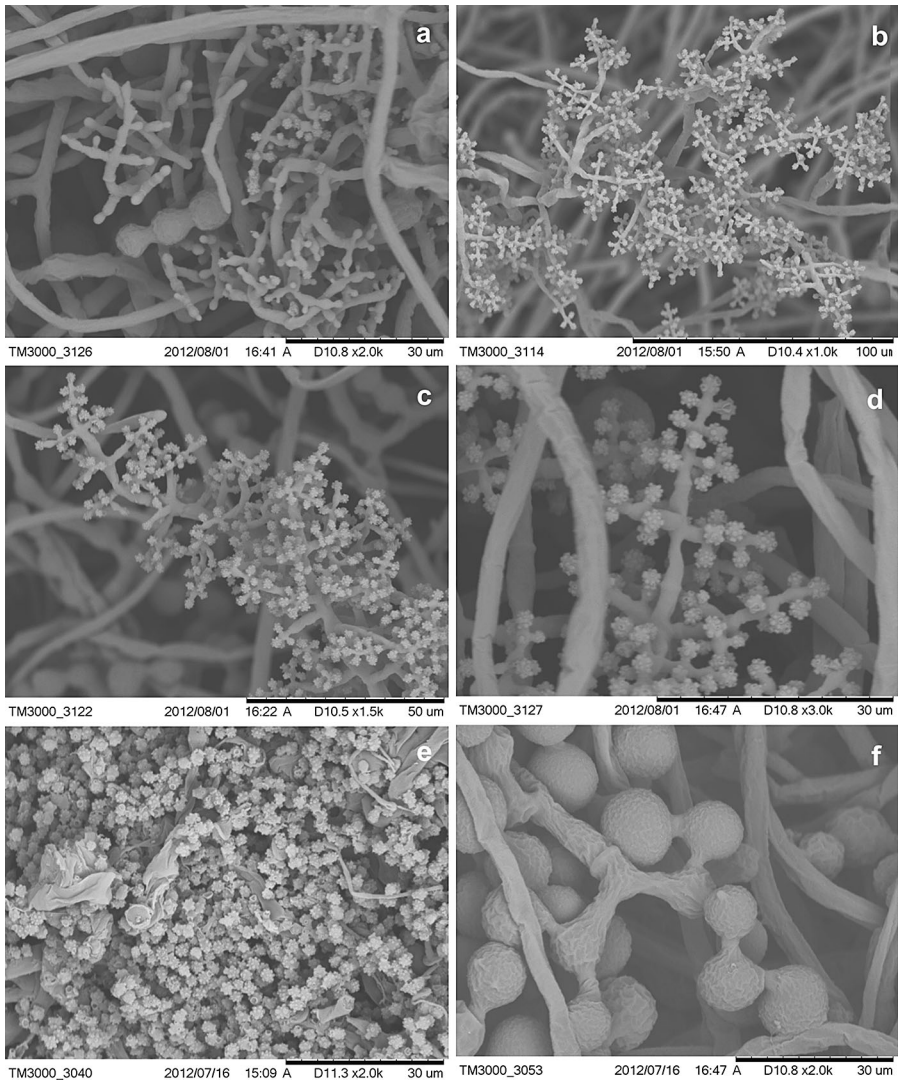

Fig. 2 SEM images of *E. trichodermoides.* **a–e**, Morphology of conidiophores, and conidia, **f** chlamydospores. Growth on PDA at 25 °C after 7 days (Electron microscope TM3000 Tabletop-Hitachi)

included only species with swollen or vesiculate conidiophores and phialide-like conidiogenous cells. Our analysis apparently contradicts the conclusion by Augustin et al. (2013) that *Escovopsioides* is distinct from *Escovopsis*. In our phylogeny, *Escovopsioides* clusters within the broad *Escovopsis* clade. However, additional sampling coupled with a broader, multigene phylogenetic analysis would shed additional light on the taxonomic tenability of *Escovopsioides*.

Escovopsis is little-studied and there is still little information about DNA sequence diversity. It seems appropriate to broaden the diagnostic morphological characters for the genus to allow for various conidiophore branching patterns, presence or absence of vesicles, and solitary or catenate conidia. This results in an inclusive, ecologically meaningful generic concept that correlates with our present state of phylogenetic knowledge.

Description of *Escovopsis trichodermoides* sp. nov.

Escovopsis trichodermoides Cabello, Masiulionis, Seifert, Rodrigues and Pagnocca (Fig. 2) MycoBank MB804146.

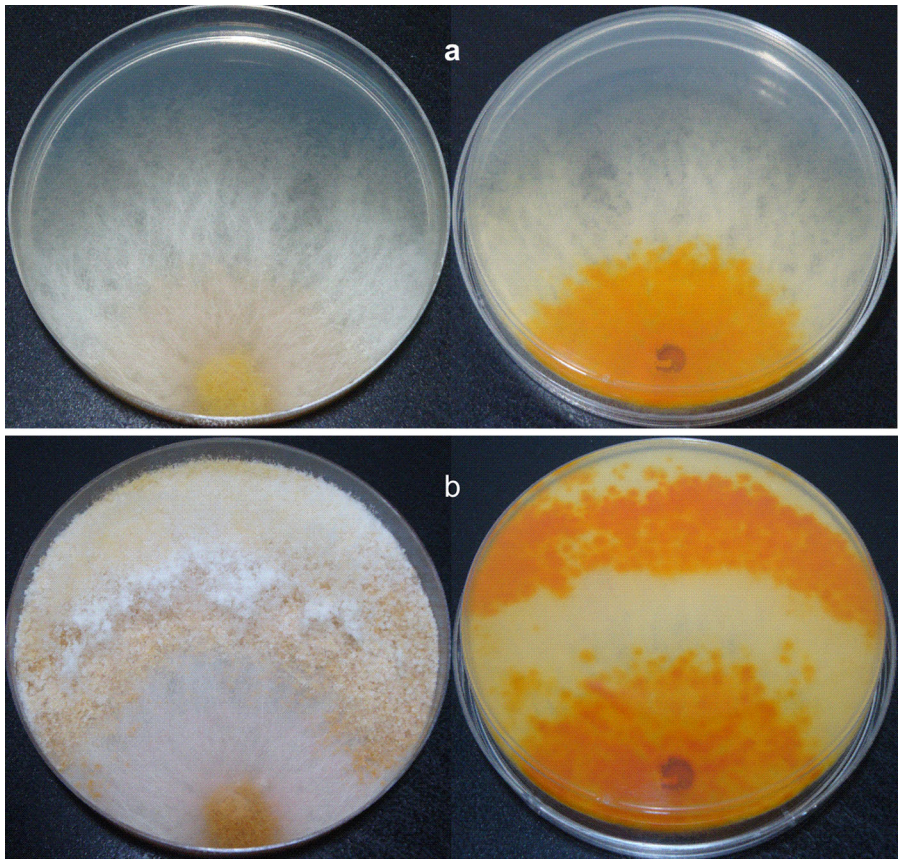

Fig. 3 Growth of *E. trichodermoides* on PDA at 25 °C after 5 days (**a**), and 9 days (**b**). Front and reverse of Petri dishes

Etymology: *Trichodermoides*, referring to the branching pattern, which resembles that of species of *Trichoderma*.

Colonies on 2 % MEA after 7 days at 25 °C filling a 9-cm Petri dish, margin diffuse, uneven; mycelium submerged but forming aerial stolon-like structures; colony surface smooth, conidial masses at first white, becoming yellow in the center and eventually turning brown (Figs. 3, 4). Aerial mycelium sparse. Colony reverse colourless on MEA but yellow on OA and PDA after 2–3 days at 25–30 °C (Figs. 3, 4). Exudates or odors not produced. Yellow soluble pigment observed on OA and PDA after 2 days at 10, 15, 20, 25 and 30 °C (Fig. 3). Conidiophores trichoderma-like, pyramidal in outline, with a main axis 230 µm long, often with 5–6 levels of primary branches arising at right angles from the axis, longest near the base of the axis, and becoming shorter towards the top, with the lower branches sometimes with one or two additional levels of right angled branches; the terminal branches at each level 4.5–5 µm wide, terminal branches fertile, conidiogenous cells ampulliform, producing one conidium, but otherwise phialide-like, hyaline 10–20 × 7–8.5 µm. Conidia ovate with a truncate base, apex rounded, 3.5–4.5 × 2.5–3 µm, clearly verrucose, warts ≤1 µm (0.650–1.12 µm, as measured from SEM images), crowded, slightly ochraceous, solitary, dry. Chlamydospores observed after 4 days at temperatures of 15–25 °C in MEA and PDA and at 30 °C in OA medium. Chlamydospore diameters similar on the three media and at different temperatures, 12–16.5 µm diameter (Fig. 4f), hyaline, smooth walls that seem to be thin. Aerial mycelia and stolon-like hyphae observed regularly.

The ex-type strain was isolated from the upper part of a fungus garden of *M. goeldii* (Hymenoptera: Formicidae: tribe Attini) in Rio Claro, São Paulo, Brazil, isolation number VEM001, ex-type CBS 137343 and CBMAI 1620 and LPSC 1176. The GenBank accession for CBS 137343 is #KF033128

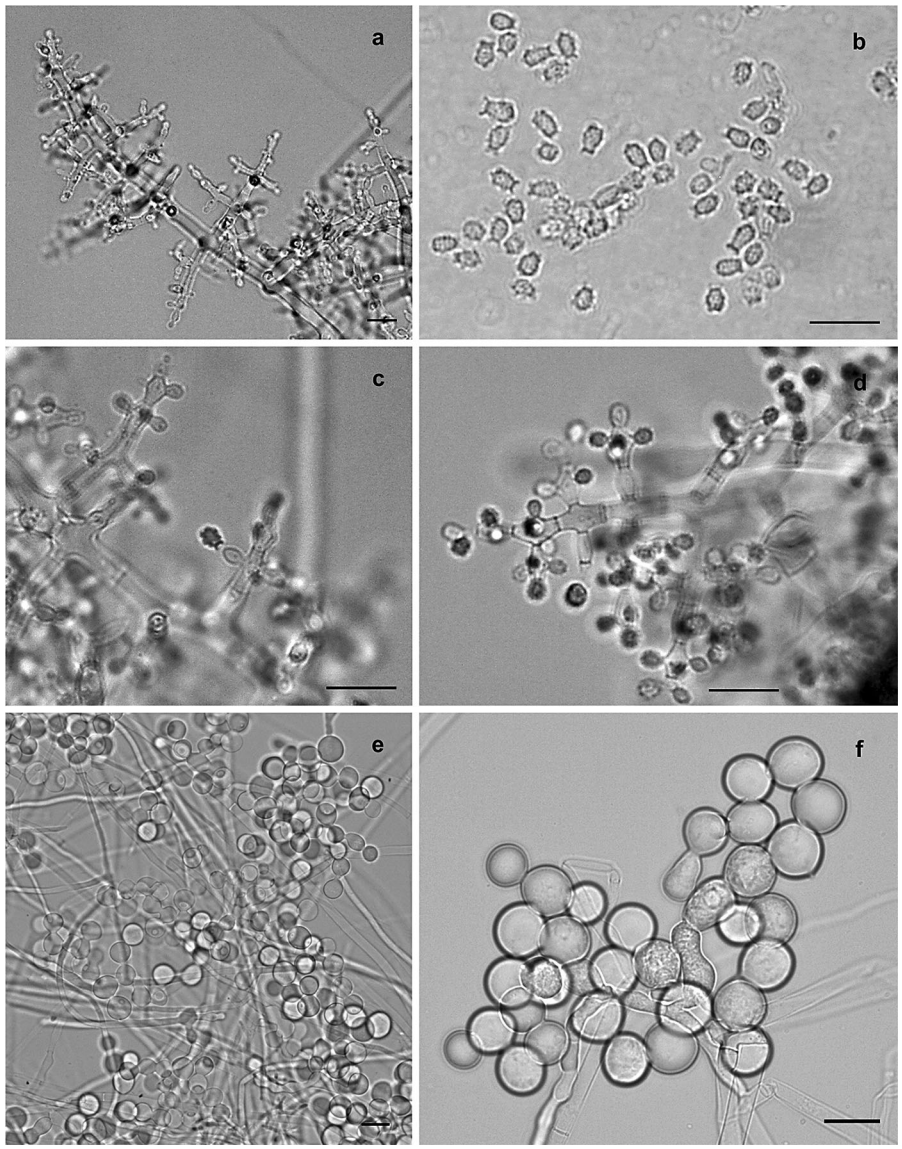

Fig. 4 *Escovopsis trichodermoides*. **a** Conidiophores (×400, *scale bar* 20 μm), **b** Conidia (×1,000, *scale bar* 50 μm), **c**, **d**) Conidiophores (×1,000, *scale bar* 20 μm), **e**, **f** Chlamydospores (×200, *scale bar* 100 μm and ×1,000, *scale bar* 50 μm, respectively)

(*tef1*-α) and #KJ485699 (ITS). The holotype is preserved as a freeze-dried sample at CBS with the number 14.0662.

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