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LUCAS ANDRADE MEIRELLES

SISTEMÁTICA E FILOGENIA DO FUNGO PARASITA *ESCOVOPSIS* ASSOCIADO ÀS FORMIGAS ATTINI

Dissertação apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas (Área: Microbiologia Aplicada).

Rio Claro
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"We are made of star stuff.
We are a way for the Cosmos to know itself"
Carl Sagan

RESUMO

Ninhos de formigas Attini são conhecidos por abrigar uma diversa e complexa microbiota. Esses insetos possuem uma relação de mutualismo com seu fungo simbionte, utilizado como fonte de alimento. Porém, muitos micro-organismos também são encontrados nos ninho dessas formigas. Dentre eles, destacam-se fungos do gênero *Escovopsis*, considerados parasitas específicos do fungo cultivado por esses insetos. Somente encontrado associado aos ninhos de formigas Attini, *Escovopsis* coevoluiu com as formigas e seus fungos mutualistas em uma relação tripartida. Apesar dos diversos estudos voltados para entender a variedade genética de *Escovopsis* realizados na América Central (especialmente Panamá), não há relatos de grandes amostragens do parasita presente na América do Sul. São também escassos trabalhos taxonômicos para esse grupo de fungo; como resultado, após mais de duas décadas da descrição da primeira espécie do gênero, até o presente trabalho, apenas seis espécies foram formalmente descritas. Nesse contexto, estudamos a filogenia e evolução de *Escovopsis* e separamos nossos resultados em dois capítulos. No primeiro deles, descrevemos uma nova espécie, *E. kreiselii*, primeira a ser descrita pertencente ao clado das formigas Attini basais. Discutimos também a evolução do fungo, indicando que parece haver correlação entre filogenia e características morfológias, com linhagens menos derivadas apresentando ausência de vesícula (célula que suporta as fiálides, produtoras de conídios). Já no segundo capítulo, exploramos a diversidade filogenética de *Escovopsis* associados às formigas Attini derivadas, em diferentes países do continente Americano, especialmente o Brasil. Contrariamente ao que foi previamente proposto, nossos resultados sugerem que as formigas Attini derivadas podem compartilhar linhagens de *Escovopsis*, indicando ausência de co-cladogênese estrita entre formiga-*Escovopsis*-fungo mutualista. A descrição de uma nova espécie e a extensa filogenia de *Escovopsis* aqui apresentadas são um primeiro passo para um melhor entendimento da evolução e taxonomia deste grupo de fungos com potencial biológico para controle de formigas cortadeiras, grupo mais derivado das formigas Attini.

Palavras-chave: formigas cortadeiras, simbiose, parasitas fúngicos, taxonomia

ABSTRACT

Attine ant nests harbor a diverse and complex microbiota. Such insects have a mutualistic relationship with mutualistic fungus, cultured as food source. However, several microorganisms are also present in attine nests. Within this microbiota, fungi in the genus *Escovopsis* are considered specific parasites of the mutualistic fungus. *Escovopsis* is only reported associated with attine ant nests. Furthermore, this parasite coevolved with attine ants and their mutualistic fungi in a tripartite relationship (tripartite coevolution model). Although several studies aimed to understand the genetic diversity of *Escovopsis* in Central America (mostly Panama), no comprehensive reports considering large *Escovopsis* samples are known from South America. There is also few taxonomic work onr this fungus; as a result, after two decades of the description of the first species, up to the present work, only six *Escovopsis* species have been formally described. In this study, we investigated the systematics, phylogeny and evolution of *Escovopsis* and separated our results in two chapters. In the first part, we describe a new species, *E. kreiselii*, first species belonging to basal attine ants. We also discuss the evolution of *Escovopsis*, indicating a correlation between phylogeny and morphological characters, where less derived strains shows absence of vesicles (structures supporting spore bearing-cells). In the second chapter, we exploit the phylogenetic diversity of *Escovopsis* associated with higher-attine ants in different countries of the American continent, especially Brazil. Contrary to previous resports, we show that higher attine ants share *Escovopsis* infections, indicating no strict co-cladogenesis between ant-cultivar-*Escovopsis*. The description of a new species and the extensive *Escovopsis* phylogeny presented here provide a first step towards a better understanding of the evolution and taxonomy of this group of fungi, which has potential for biological control of leaf-cutting ants (the most derived group of attine ants).

Keywords: leaf-cutting ants, symbiosis, fungal parasites, taxonomy**.**

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1 INTRODUÇÃO

As formigas Attini compreendem um grupo de insetos que apresenta como característica principal a capacidade de cultivar fungos como alimento, num processo denominado fungicultura. Estas formigas são também conhecidas como "formigas agricultoras", e estão distribuídas por praticamente todo o continente americano. Contudo, as interações ecológicas presentes dentro dos ninhos destes insetos não são apenas formadas entre as formigas e seu parceiro fúngico mutualista. Diversos outros organismos, incluindo bactérias, leveduras e fungos filamentosos foram encontrados nesse ambiente e são responsáveis por diferentes interações dentro destes ninhos.

Dentre todos os micro-organismos encontrados, este trabalho teve como objetivo estudar a sistemática, filogenia e evolução de um gênero de fungo denominado *Escovopsis*. Esse fungo é considerado um parasita agressivo e específico do fungo mutualista cultivado pelas formigas Attini, sendo somente encontrado associado aos ninhos desses insetos. Diversos estudos exploraram a diversidade de *Escovopsis* em alguns países do continente Americano (especialmente Panamá), contudo, poucos foram os estudos em outras regiões do continente, especialmente no Brasil. Visto que nosso país é detentor da maior diversidade de espécies de formigas Attini, esperávamos encontrar uma grande diversidade do parasita.

Durante os anos de 2003 à 2008, nosso grupo de pesquisa isolou linhagens de *Escovopsis* de diferentes localidades no Brasil e tais linhagens estão mantidas na Central de Recursos Microbianos da UNESP, no Câmpus de Rio Claro. Aqui objetivamos explorar a diversidade desses fungos, bem como analisar um acervo de *Escovopsis* mantido na *University of Texas at Austin* - USA. Os resultados desse estudo estão divididos em dois capítulos, apresentados em formato de manuscritos:

- *Capítulo 1*: Não havia um estudo sistemático sobre *Escovopsis* que infecta ninhos de formigas Attini basais. Desde a descrição do gênero, realizada na década de 90, a carência de um estudo sistemático com linhagens menos derivadas ofuscou nosso conhecimento sobre a evolução de caracteres morfológicos do fungo. Assim, isolamos e estudamos linhagens de *Escovopsis* associadas à formiga Attini basal *Mycetophylax morschi*.

  Nossos resultados permitiram iniciar um novo entendimento das características morfológicas de *Escovopsis*. Concluímos que parece haver uma correlação entre a filogenia e morfologia durante a evolução do fungo, com linhagens localizadas na parte mais basal da filogenia apresentando características plesiomórficas. Além disso, descrevemos uma nova espécie, *Escovopsis kreiselii*, e discutimos aspectos taxonômicos do grupo.

- *Capítulo 2*: Considerando *Escovopsis* associados a formigas Attini derivadas, estudos prévios mostraram a co-evolução entre parasita-formiga-fungo mutualista. Vale salientar que as formigas cortadeiras, um subgrupo das Attini derivadas, compõem a porção mais derivada da filogenia destes insetos. Tal co-evolução teria como resultado co-cladogênese, onde *Escovopsis* infectando formigas cortadeiras (gêneros *Atta* e *Acromyrmex*) não infectariam outras formigas derivadas não-cortadeiras (gêneros *Trachymyrmex* e *Serycomyrmex*) e vice-versa. Contudo, uma ampla amostragem desses fungos não havia sido realizada no Brasil, considerado o centro de diversidade das formigas Attini. Analisamos linhagens de *Escovopsis* isoladas dos quarto gêneros de formigas derivadas no Brasil e em outros países do continente Americano. Nossos resultados mostram várias exceções à hipótese de co-evolução estrita, com compartilhamento de *Escovopsis* entre todos os gêneros de formigas derivadas. Além disso, considerando os resultados obtidos no capítulo I, tentamos correlacionar morfologia e filogenia nas linhagens de *Escovopsis*

de formigas derivadas. Encontramos diferenças na morfologia das vesículas (células que suportam as fiálides, as quais são responsáveis pela produção dos esporos), além de fornecer uma extensa filogenia que poderá ser utilizada como base em próximos estudos taxonômicos, ecológicos e evolutivos de *Escovopsis*.

2 CHAPTER I

New light on the systematics of fungi associated with attine ant gardens and the description of *Escovopsis kreiselii* sp. nov.

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New light on the systematics of fungi associated with attine ant gardens and the description of *Escovopsis kreiselii* sp. nov.

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Abstract

Since the formal description of fungi in the genus *Escovopsis* in 1990, only a few studies have focused on the systematics of this group. For more than two decades, only two *Escovopsis* species were described; however, in 2013, three additional *Escovopsis* species were formally described along with the genus *Escovopsioides*, both found exclusively in attine ant gardens. During a survey for *Escovopsis* species in gardens of the lower attine ant *Mycetophylax morschi* in Brazil, we found four strains belonging to the pink-colored *Escovopsis* clade. Careful examination of these strains revealed significant morphological differences when compared to previously described species of *Escovopsis* and *Escovopsioides*. Based on the type of conidiogenesis (sympodial), as well as morphology of conidiogenous cells (percurrent), non-vesiculated conidiophores, and DNA sequences, we describe the four new strains as a new species, *Escovopsis kreiselii* sp. nov. Phylogenetic analyses using three nuclear markers (Large subunit RNA; translation elongation factor 1-alpha; and internal transcribed spacer) from the new strains as well as available sequences in public databases confirmed that all known fungi infecting attine ant gardens comprise a monophyletic group within the Hypocreaceae family, with very diverse morphological characteristics. Specifically, *Escovopsis kreiselii* is likely associated with gardens of lower-attine ants and its pathogenicity remains uncertain.

Introduction

The diversity of microbial symbionts found within nests of attine ants, as well as the complex relationships among the various participants, makes the attine ant-microbial association among the most complex multipartite interactions known in nature. The tribe Attini comprises 257 ant species within 16 extant genera, including the recently described *Cyatta abscondita*, and one ichnogenus (*Attaichnus* Laza) [1-3]. As has been known for over a century, attine ants cultivate mutualistic basidiomycetous fungi as their primary food source [4]. During the 50 million-year evolutionary history of this tribe, five distinct types of agriculture have arisen: (i) lower attine agriculture (Agaricaceae); (ii) coral fungus agriculture (Pterulaceae); (iii) yeast agriculture (Agaricaceae); and (iv) higher attine ant agriculture, which includes (v) leaf-cutter ant agriculture (Agaricaceae) [1]. These groups differ in the type of substrates used for fungal cultivation, including insect frass, seeds, plant sap, arthropod exoskeletons, fresh leaves, and/or flower parts [5].

The last three decades have revealed that the fungal cultivars are not the only microbial symbionts that have evolved intimate relationships with attine ants. In 1999, Currie et al. [6] demonstrated the existence of a specific parasite that attacks the mutualistic cultivar. Fungi in the genus *Escovopsis* were described as the third member of the attine ant-fungal association. Subsequent studies have focused on evolutionary aspects of *Escovopsis* parasitism [7-11] and it was also demonstrated that the parasite attacks and consumes the cultivar mycelia [12]. Much of this work has focused on leaf-cutting ants, the most derived group within higher attine agriculture [7, 13-16].

Despite becoming a model system for the study of co-evolution and host-parasite dynamics, until recently little attention has been paid to the taxonomy of these fungi. In the 1990s, when the genus *Escovopsis* was proposed, only two species were known: *Escovopsis*

weberi [17] and *E. aspergilloides* [18]. In 2013, three additional *Escovopsis* species -- *E. microspora*, *E. moelleri* and *E. lentecrescen*s -- were described, and a new genus, *Escovopsioides*, was proposed [19].

The five ex-type strains of *Escovopsis* species and *Escovopsioides nivea* (the only described species in the genus), were isolated from gardens of ants that engage in higher attine (including leafcutter) agriculture (i.e. groups (iv) and (v), respectively), which includes the ant genera *Sericomyrmex*, *Trachymyrmex*, *Acromyrmex* and *Atta* [1]. Numerous studies have confirmed that leaf-cutting ants (*Acromyrmex* and *Atta*) cultivate closely related, or even identical, strains of the mutualistic fungus [20-23], which are derived from the fungi cultivated by the higher attine genera *Trachymyrmex* and *Sericomyrmex* [24-26]. Nevertheless, no taxonomic study has been carried out on the parasites that infect gardens of attine ants that perform lower attine agriculture (i.e. group (i)); these fungi were separated into groups depending on their macroscopic characteristics, especially their colony (spore) color (brown, yellow, white and pink) [8-9], but no distinct species has been described in these groups.

We sampled fungus gardens of the lower attine ant *Mycetophylax morschi* and found four strains of a fungus species that are morphologically and phylogenetically distinct from previously described species of *Escovopsis* and *Escovopsioides*. The strains are described as a new species, *Escovopsis kreiselii*, that exhibits percurrent conidiogenous cells with sympodial conidiogenesis; such morphological characteristics may be considered less derived when compared to morphological aspects exhibited by other *Escovopsis* species described to date (including the presence of phialides in vesiculated conidiophores). Phylogenetic analyses of three nuclear loci confirmed the close relationship between *E. kreiselii* and the previously described species in a clade of Hypocreaceae fungi associated with attine ants.

Materials and methods

Ethics statement

Collections made in the first expedition were conducted under collecting permit number 14789-1 issued by the "Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis" (IBAMA) to SES. Collections made in the second expedition were carried out under collecting permit number 31534 issued by the "Instituto Chico Mendes de Conservação da Biodiversidade" (ICMBio) to AR. LAM and QVM joined the second expedition and their names were included in permit 31534. No endangered or protected species were involved in this study.

Nomenclature

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS ONE article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MB/. The online version of this work is archived and available from the following digital repositories: [PubMed Central, LOCKSS].

Sampling site and fungal isolation

Sampling was conducted during two different expeditions to Florianópolis, Santa Catarina, Brazil. In March of 2009, we isolated *E. kreiselii* (strain LESF53) from a nest of *M. morschi* found at Praia da Joaquina (nest AR090306-01, GPS: 27°37'50.01"S 48°27'3.64"W, elev. 1 m). In February 2014, we isolated three additional strains (LESF303, LESF304 and LESF305) of *E. kreiselii*, all associated with *M. morschi*. Strain LESF305 was isolated from a nest located at Praia da Joaquina (nest AR140226-01, GPS: 27°37'49.62"S 48º27'3.6"W, elev. 1.7 m) and the strains LESF303 and LESF304 were isolated from a single nest in Praia de Moçambique, on the northeast shore of the island of Florianópolis (nest AR140227-05, GPS: 27º31'24.96"S 48º25'3.78"W, elev. 1m). All nests were located in a coastal sand dune environment, a typical habitat of *M. morschi* [2, 27]. The whole fungus garden was sampled along with tending workers using a sterile spatula. Fungus gardens were kept in UV-sterilized plastic containers until they reached the laboratory at UNESP in Rio Claro, São Paulo, Brazil. Worker ants were subsequently identified by Dr. Rodrigo Feitosa and deposited in the entomological collection "Padre Jesus Santiago Moure" at Universidade Federal do Paraná, Curitiba, Brazil.

Small (0.5-1 mm in diameter) fragments of the fungus garden were plated on potato dextrose agar (PDA) supplemented with chloramphenicol [150 mg L^{-1}, Sigma] and incubated at 25 °C in the dark for seven days. Plates were monitored daily for fungal growth. Once filamentous fungi emerged from garden fragments, mycelia were subcultured on new PDA plates. Monosporic cultures were obtained for each strain and cultures were stored in 10% glycerol at -80 °C at UNESP - Microbial Resource Center.

Morphological characterization

Four different culture media were used for measuring the radial growth and to determine colony morphology: potato-dextrose agar (PDA), malt extract agar 2% (MA2%),

cornmeal agar (CMD) and synthetic nutrient agar (SNA). Strains were cultivated in triplicates at four different temperatures for each medium (10, 20, 25, 30 and 35 °C). Microscopic morphological characters were examined on slide culture preparations. For this, we placed a 5 mm^2 block of PDA on a microscopic slide and inoculated the fungus; after that, the material was covered with a cover slip and incubated at 25 ºC for seven days. Then, slides were examined under a light microscope. Conidia, conidiophores and conidiogenous cells were measured using the software LAS EZ (Leica Application Suite) using measurements of 30 replicates for each structure.

To visualize these same structures using scanning electron microscopy, fungus samples were fixed in osmium tetroxide vapor for 72 h, dehydrated sequentially through a series of acetone concentrations (50, 75, 90, 95 and 100%) and then dried until the critical point using liquid CO_2 (Balzers CPD030). The dried material was mounted in stubs, sputtered with gold (Balzers SCD050) and examined with a scanning electron microscope (TM3000, Hitachi).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from mycelia grown on PDA for 7 days following a modified version of the CTAB method [8, 28]. Three different nuclear DNA markers were amplified for phylogenetic analysis: (i) large subunit RNA – LSU; (ii) translation elongation factor 1-alpha – *tef1*; and (iii) internal transcribed spacer – ITS. The primers and conditions used for amplification are described in Table 1. PCR products were cleaned up with *Kit Wizard SV Gel and PCR Clean-up System* (Promega) following the manufacturer's protocol. Cycle sequencing reactions were carried out with 20 ng of template using *BigDye Terminator v. 3.1 Cycle Sequencing Kit* (Life Technologies) following the manufacturer instructions. Sequences were generated using ABI3500 (Life Technologies). Contigs were assembled in

Bioedit v. 7.1.3 [29] and queried using the NCBI – GenBank to find the closest known relatives. Sequences of strain LESF53 were deposited in GenBank under accessions KJ808765 - KJ808767. We also sequenced the partial *tef1* gene for *Escovopsis microspora* CBS 135751^{T}, which was deposited in GenBank under accession KJ935030.

Phylogenetic analyses

To determine the phylogenetic position of the new strains, three different phylogenetic reconstructions were performed: (i) a phylogeny within the Hypocreaceae family using LSU partial sequences; (ii) a phylogeny within the *Escovopsis* clade using *tef1* partial sequences (previously examined strains of these fungi have been sequenced for the gene *tef1* and their sequences are available in GenBank); and finally (iii), a phylogeny including only the formally described species of *Escovopsis* and *Escovopsioides* using concatenated sequences of ITS and *tef1*.

Alignments were obtained independently for each molecular marker using MAFFT v.7 [30] and nucleotide substitution models were selected using the Akaike information criterion (AIC) with a confidence interval of 95% in jModelTest 2 [31]. Independent runs in jModelTest 2 were performed for each molecular marker of the concatenated analysis (analysis number iii). Phylogenies were reconstructed using maximum likelihood (ML) in RAxML v.8 [32] and Bayesian Inference (BI) in MrBayes v.3.2.2 [33].

For positioning our strain within the Hypocreaceae, we first reconstructed the phylogeny of several species and genera belonging to this family using the LSU partial sequences. We selected 23 sequences used by Augustin et al. [19] and rooted using two members of Clavicipitaceae. We reconstructed 1000 independent ML trees under the GTR+I+G model in RAxML, and kept the tree with the best score. We evaluated the reliability of the tree topology by performing 2000 bootstrap replicates (bootstrap values

converged at 1150 replicates [34]). Bayesian inference also used the GTR+I+G model of nucleotide substitution in two independent runs in MrBayes, each with three heated chains and one cold chain; each run consisted of Markov Chain Monte Carlo (MCMC) sampling for 2 million generations. Convergence occurred when the standard deviation (SD) of split frequencies fell below 0.01; the first 25% of MCMC generations were discarded as burn-in.

To further refine the position of the new strains within the *Escovopsis* clade, we used phylogenetic inference for *tef1* partial sequences using the same conditions described above for both ML and BI analyses. In this case, bootstrap values converged after 850 replicates. Two million MCMC generations were again sufficient for achieving SD of split frequencies of less than 0.01. This tree was rooted using three sequences of other Hypocreaceae (*Hypomyces polyporinus*, *Hypocrea lutea* and *Trichoderma* sp.) used in previous studies [7, 9].

A third analysis used concatenated alignments of ITS and *tef1* sequences generated using Winclada v.1.00.08 [35]. A partitioned ML analysis was conducted in RAxML using GTR+G for each partition. The alpha shape parameter of the gamma model of rate heterogeneity, empirical base frequencies, and evolutionary rates in the GTR matrix were estimated independently for each partition [32]. As before, 1000 ML trees were reconstructed and 2000 bootstraps replicates were performed (bootstraps values converged at 1950 replicates). For BI, a partitioned analysis was performed in MrBayes under GTR+G for each partition. The number of independent runs, number of chains, and the burn-in were the same as described above, but each run consisted of 1 million MCMC generations, which was sufficient to reach a SD of split frequencies of less than 0.01. We selected three other Hypocreaceae species (*Trichoderma hamatum*, *T. pubens* and *Hypocrea rufa*) that had ITS and *tef1* sequences available in GenBank (NCBI) as the outgroup. The final trees were edited

in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) and Adobe Illustrator CS6 (Adobe Systems).

Results

Morphology

Morphological differences were observed between the *E. kreiselii* strains (LESF53, LESF303, LESF304 and LESF305) and all previously described species of *Escovopsis* and *Escovopsioides* associated with attine ants [19]. The most distinguishing feature of the new species compared with others is the type of conidiogenesis: *E. kreiselii* has holoblastic sympodial conidiogenesis with percurrent conidiogenous cells (Figs. 1 and 2). The colony color also differs from previously described species: *E. kreiselii* spores are pink while other described species of *Escovopsis* are brown, and *Escovopsioides* is white (Fig. 1). Such color differences appear to be important to distinguish fungi within the *Escovopsis* clade [6, 9, 19]. *Escovopsis kreiselii* grows better at 25 °C (but also grows well at 20 ºC, see Fig. S1) which is consistent with thermal preferences described for some attine ants [36]. Moreover, no growth was observed at 30 °C, indicating that *E. kreiselii* is sensitive to high temperatures. *E. kreiselii* also has chlamydospores (Fig. 1M and 2D) similar to *Escovopsioides nivea.*

Phylogenetic analyses

The tree topologies reconstructed using both ML and BI in three different phylogenetic analyses were very similar; the final Bayesian trees are displayed in Figs. 3, 4, and S2 with posterior probabilities and ML bootstrap values to indicate support for individual nodes. The LSU analysis grouped *E. kreiselii* as closely related to *Escovopsis* and *Escovopsioides* species within the phylogeny including other Hypocreaceae members. These

fungi are grouped within an "attine-ants associated clade" (Fig. S2). To compare the new strain with all *Escovopsis* diversity previously explored in other studies, we also performed a phylogenetic analysis using *tef1* (Fig. 3). In this phylogeny, *E. kreiselii* grouped within the clade of pink-colored *Escovopsis* described in Gerardo et al. [9]. Unfortunately, it is not possible to include more genes within this phylogenetic analysis because most studies on the parasite phylogeny used only *tef1* sequences and the strains are not available in public culture collections. Therefore, aiming to increase the reliability of our analysis, we also reconstructed a tree using two markers (ITS and *tef1*) of all described species of *Escovopsis* and *Escovopsioides*, similar to the analysis conducted by Augustin et al. [19]. Overall, our results suggest that *E. kreiselii* is a sister group to the clade that contains all previously described species of *Escovopsis* (Fig. 4), but the phylogenetic position of *Escovopsioides nivea* remains uncertain, varying depending on the molecular marker used (see Figs. 3, 4 and S2). These results along with the morphological data support *E. kreiselii* as a new species within the hypocrealean fungi associated with attine ants.

Taxonomy

Emendation of the genus *Escovopsis* Muchovej & Della Lucia

Augustin et al. [19] added information regarding the conidia morphology to the genus originally described by Muchovej and Della Lucia as *Escovopsis* [17]. However, until now, the genus is described as "*vesiculate; vesicles cylindrical to globose, evanescent; phialides hyaline, swollen at base, extending to a narrow neck; conidia in short basipetal chains, aseptate, hyaline at first becoming pigmented with an ornamented or mucilaginous brown outer coat or sheath, phoretic*" [19]. To accommodate morphological variations observed in *E. kreiselii*, we propose an emendation on the description of the genus *Escovopsis* as follows:

non-vesiculated conidiophores are present in less derived strains, with non-phialidic conidigenous cells producing solitary conidia (as indicated in Figs. 1 and 2).

Escovopsis kreiselii L.A. Meirelles, Q.V. Montoya, S.E. Solomon & A. Rodrigues sp. nov.
Mycobank: MB809176

Colonies on PDA, MA2%, CMD and SNA growing slowly, up to 5.3-6.0 cm, 6.6 – 8.1 cm, 3.9-4.5 cm and 4.3-5.2 cm in diameter after 14 days at 25 °C. Mycelium reaching the edge of a 9 cm Petri dish in 24 and 22 days on PDA and MA2%, respectively, and after 30 days on CMD and SNA. Inconspicuous growth observed at 10 °C; no growth at 30 °C. Concentric growth observed in colonies cultured on PDA and MA2% (Fig. S1). Hyaline hypha present in the aerial mycelia, stolon-like structures not observed. Colonies at first white, later attaining pink to brownish colors depending on the media (Fig. 1A-D). No soluble pigments observed in all media. Colony reverse is uncolored. Intercalary concatenated chlamydospores found in the submerged and in the aerial mycelia, 10.8-21.4 µm in length x 8.3-16.6 µm in width. *Conidiophores* arising from fertile aerial hypha in opposite and intercalary patterns (Fig. 1E and Fig. 2A-B), at first white and quickly becoming light to dark pink. A septum is often observed at the base of each conidiophore. Irregular conidiophore branching pattern, some have verticils (of 3 to 4 branches), some have 2 opposite branches and some are solitary; smooth walled, hyaline, 29.0-229.00 µm in length and 2.5-8.6 µm in diameter. *Conidiogenous cells* are holoblastic, formed at the apex of each branch, intercalary or solitary on the fertile hypha. Each branch of the conidiophore has up to 4-6 conidiogenous cells measuring 5.3-13.4 µm in length and 2.5-7.7 cm in width. Conidiogenous cells are percurrent, hyaline, arising in verticils often with numerous denticle-like structures (Figs 1K, 2E-I). Conidia are globose to subglobose, brown, 2.4-3.2 µm in diameter, thick walled with

smooth or rough surface (Figs. 1N, 2C, and 2E-M). Conidia often bear denticle-like structures (Fig. 2J).

Specimen examined: BRAZIL. Santa Catarina, Florianópolis, Praia da Joaquina, GPS: 27°37'50.01"S; 48°27'3.64"W, elev. 1 m, Fungus garden, 03, 2009. *A. Rodrigues*. Ex-type strain LESF53 (= CBS 139320, = CBMAI 1691). Holotype: CBS H-22062 (dried culture on PDA).

Additional specimens examined: BRAZIL. Santa Catarina, Florianópolis, Praia de Moçambique, GPS: 27°31'24.96"S; 48°25'3.78"W, elev. 1 m, Fungus garden, 02, 2014. *A. Rodrigues*. LESF303 (= CBS 139321, = CBMAI 1692) and LESF304. BRAZIL. Santa Catarina, Florianópolis, Praia da Joaquina, GPS: 27°37'49.62"S; 48°27'3.6"W, elev. 1.7 m, Fungus garden, 02, 2014. *A. Rodrigues*. LESF305 (= CBS 139322, = CBMAI 1693).

Sequences: LSU: KJ808765, *tef1*: KJ808766, ITS: KJ808767

Etymology: Named in honor of Dr. Hanns Kreisel, who first described the fungal parasite *Phialocladus* currently known as *Escovopsis*. Kreisel did not designate a type strain for *Phialocladus* [37], thus this name was considered a *nomen invalidum* [17].

Habitat: isolated from fungus gardens of the attine ant *Mycetophylax morschi*.

Discussion

The intricacy of symbioses between animals and microbes is emerging as a major theme in 21st century biology (e.g. the human microbiome project). Despite a new appreciation of the ubiquity and importance of such symbioses, the attine ants and their fungal symbionts have long been recognized as an example of the complex interactions between animals and microbes. Although the fungal cultivar used as a food source for ants was first

recognized in 1874 [38], it was not until the 1990s, and especially the 2000s, that the high fungal diversity associated with attine ants began to be recognized [6, 13, 17-19, 39-46].

The genus *Escovopsis* was likewise first observed in the 19th century [47]. During the 20th century, some early studies documented the presence of these fungi in attine ant nests [37, 48], but the genus and the first two species were not formally described until the 1990s [17, 18]. Recently, Augustin et al. [19] described three additional species within *Escovopsis* and the new genus *Escovopsioides* (originally studied by Möller in 1893 [47]; however he interpreted this genus to be a "weak form" of the fungus cultivated by leaf-cutting ants). Moreover, no taxonomic study was performed for *Escovopsis* associated with lower attine ants and the morphological aspects of these fungi are still poorly known.

Gerardo et al. [9] examined different *Escovopsis* morphotypes, documenting the existence of at least four different colored groups (pink, white, yellow and brown) associated with *Apterostigma* and *Cyphomyrmex* ants. However, that study aimed to explore the phylogenetic diversity of the fungus, and no detailed morphological examination was carried out. In our phylogenetic analyses, *E. kreiselii* falls within the pink-colored *Escovopsis* clade (Fig. 3), suggesting that species of the pink clade may exhibit significant morphological differences when compared to other described *Escovopsis* species; nevertheless, detailed morphological analyses on these strains are necessary to confirm this hypothesis.

Currie et al. [6, 7] provided evidence that *Escovopsis* is a specific parasite of the mutualistic fungus cultivated by attine ants and that *Escovopsis* has co-evolved with the ants' fungal cultivar, as evidenced by co-cladogenesis between species of both fungi. The morphological analyses conducted by Currie et al. [7] were not sufficient to identify detailed differences in conidiophore structure or conidiogenesis type for the distinct *Escovopsis* clades. Our study extends the findings of Currie et al. [7] and corroborates that *Escovopsis* is a genus that exhibits diverse morphological features. This concept is consistent with the known

diversity of this group of ants [1, 3] and that of their mutualistic fungi [24, 26, 49]. Morphological characteristics of *E. kreiselii* support its distinction from other *Escovopsis* species due to the conidiogenesis type, which is sympodial in *E. kreiselii* and phialidic for the other *Escovopsis* described so far. Moreover, no vesicles were observed in the conidiophores of *E. kreiselii*, which is contrary to the vesiculated conidiophores found in other *Escovopsis* species. In addition, all the described *Escovopsis* species are brown, whereas *E. kreiselii* is pink. The conidiogenous cells of the new species are percurrent, contrary to the phialidic conidiogenous cells observed in *Escovopsis* from higher attines. Furthermore, *E. kreiselii* produces solitary conidia. Due to the inclusion of *E. kreiselii* in the genus *Escovopsis*, it is clear that this group exhibits very diverse morphological aspects and an extension of the genus is necessary to accommodate the new morphological diversity. Future taxonomic work will enhance our understanding of this diversity. Although previous phylogenetic approaches have hinted at the vast genetic diversity of *Escovopsis* [7, 9-11], detailed examination of the morphology of the yellow, white and other strains of the pink-spored *Escovopsis* is essential to better understand the systematics of this group. Such examination will also aid in studies regarding evolutionary and ecology of host-pathogen dynamics in this system.

An important event in the study of this group was the description of *Escovopsioides* by Augustin et al. [19]. However, the phylogenetic position of this taxon remains uncertain. In our LSU phylogeny, *Escovopsioides* was an outgroup to the clade consisting of all formally described *Escovopsis* species (Fig. S2); nevertheless, in the phylogeny reconstructed with *tef1*, *Escovopsioides* is sister to the brown-spored *Escovopsis*, which together with the yellow-spored *Escovopsis* form a clade that is sister to the white-spored and pink-spored *Escovopsis* (Fig. 3). A similar topology was suggested by the concatenated (ITS and *tef1*) phylogeny, but with low statistical support for *Escovopsioides* position (Fig. 4). Under a morphological approach, *Escovopsioides* seems to be more similar to species described within the brown-

spored *Escovopsis* group (both have phialides with conidia produced in chains). The main difference is that the phialides for the *Escovopsioides* are lageniform and produced on terminal and intercalary globose vesicles [19]. *Escovopsioides nivea* has no pigmentation while all *Escovopsis* species exhibiting phialidic conidiogenese are brown in color [19]. Moreover, the percurrent conidiogenous cells of *E. kreiselii* may be considered a less derived character when compared to the phialides of brown *Escovopsis* and *Escovopsioides*. Nevertheless, only one species of *Escovopsioides* is known, represented by a single DNA sequence. Thus, sampling more strains of *Escovopsioides* may resolve these incongruences in the phylogenetic reconstructions using different genes and clarify its position. If this fungus is in fact more related to the brown *Escovopsis* clade than to the other color clades (i.e. if it groups within the *Escovopsis* clade), future studies might reclassify *E. nivea* as an *Escovopsis* species, maintaining the monophyly of the hypocrealean fungi specialized in infecting gardens of attine ants. It seems that, over evolutionary time, this group of fungi developed different conidiogenesis types and such morphological aspects might indicate adaptations related to their interactions with the ants and/or the mutualistic cultivar.

Finally, as a member of the *Escovopsis* group, *E. kreiselii* is presumed to be a fungal parasite of attine fungal gardens; however, the pathogenicity of this species remains unknown. Currie [50] demonstrated that *Escovopsis* is a specific parasite of the mutualistic fungus that decreases garden biomass accumulation. Reynolds and Currie [12] suggested that *Escovopsis* attacks the mutualistic fungus using chemical compounds and Gerardo et al. [8] demonstrated specificity in host-parasite interactions among the pink-spored *Escovopsis*. Moreover, several studies have tested the *in vitro* pathogenicity of brown-spored *Escovopsis* of leaf-cutting ants [51, 52]. However, no study to date has considered this approach for the other *Escovopsis* clades. It is possible that *E. kreiselii* attacks the mutualistic fungus of *M. morschi*, but this hypothesis remains to be supported empirically. It is noteworthy that all the three *M. morschi*

nests we sampled were apparently healthy, suggesting that *E. kreiselii* was either in a dormant state within the fungus gardens at the time of sampling or that *E. kreiselii* is not in fact a virulent pathogen of lower attine fungal gardens.

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Figure legends

Fig. 1. General morphological characteristics of *Escovopsis kreiselii* CBS 139320 (= LESF53). A-D: cultures grown in PDA, MA2%, CMD and SNA after 14 days at 25 °C, respectively; E: Conidiophores pattern formation on the aerial mycelia; F-H: Conidiophore branching pattern; I-L: Percurrent conidiogenous cells; M: Chlamydospores developed in chains and N: Globose to subglobose conidia.

Fig. 2. SEM of *Escovopsis kreiselii* CBS 139320 (= LESF53) showing morphological aspects. Slide cultures grown in PDA and SNA for five days at 25 °C. A-B: Conidiophore growing patterns on the aerial mycelia; C: Conidiophore branching pattern; D: Chlamydospores; E-I: Percurrent conidiogenous cells showing conidia attached to the cells by denticles; J-M: Conidia. J: Conidia with attached denticle; K-L: Conidia without denticles and M: smooth walled conidia.

Fig. 3. Phylogenetic position of *Escovopsis kreiselii* within the *Escovopsis* clade based on *tef1* sequences reconstructed using Bayesian Inference. All *Escovopsis* species described so far are denoted in bold. In addition to the newly described species, forty-three *tef1* sequences representing all *Escovopsis* morphotypes used in previous studies were retrieved from GenBank. Sequences of *tef1* from other Hypocreaceae were used as outgroup. The voucher accession numbers in culture collections follow the taxon names. GenBank accessions and the ant species from which the fungi were isolated are given in parentheses. Different colors indicate the fungal morphotypes found in the *Escovopsis* clade. Bootstrap values from ML analyses are also indicated from a similar topology. Only PP and bootstrap values ≥ 0.5 or 50 are shown. Phylogeny based on Gerardo et al. [9]. T: ex-type strains. Bar: 0.05 substitutions per nucleotide position.

Fig. 4. Phylogeny of described species within the *Escovopsis* clade. This tree was generated from Bayesian analyses of two different markers: ITS and *tef1*. Sequences of other Hypocreaceae species were used as outgroup. The voucher accession numbers in culture collections follow the taxon names, and GenBank accessions and the ant species from which the fungi were isolated are given in parentheses. The new species, *Escovopsis kreiselii* is denoted in bold. ML analyses resulted in an identical topology and ML bootstraps values are shown above nodes. T: ex-type strains. Bar: 0.05 substitutions per nucleotide position.

Table 1. Molecular markers, primers and PCR conditions used in this study.

Marker	Primers	Conditions
LSU	CLA-F (5'- GCATATCAATAAGCGGAGGA); CLA-R (5'- GACTCCTTGGTCCGTGTTTCA) [7]	2 min of denaturation at 95 °C, 40 cycles consisting of 30 s at 95 °C, 60 s at 62 °C, 90 s at 72 °C and 5 min of extension at 72 °C [19]
tef1	EF6-20F (5'-AAGAACATGATCACTGGTACCT-3'); EF6-1000R (5'-CGCATGTCRCGGACGGC-3') [10]	96 °C for 3 min, 35 cycles at 96 °C for 30 s, 61 ºC for 45 s and a final extension step at 72 °C for 1 min [53]
ITS	ITS4 (5'-TCCTCCGCTTATTGATATGC-3'); ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') [54]	96 °C for 3 min, 35 cycles at 94 °C for 1 min, 55 °C for 1 min and a final extension step at 72 °C for 2 min [54]

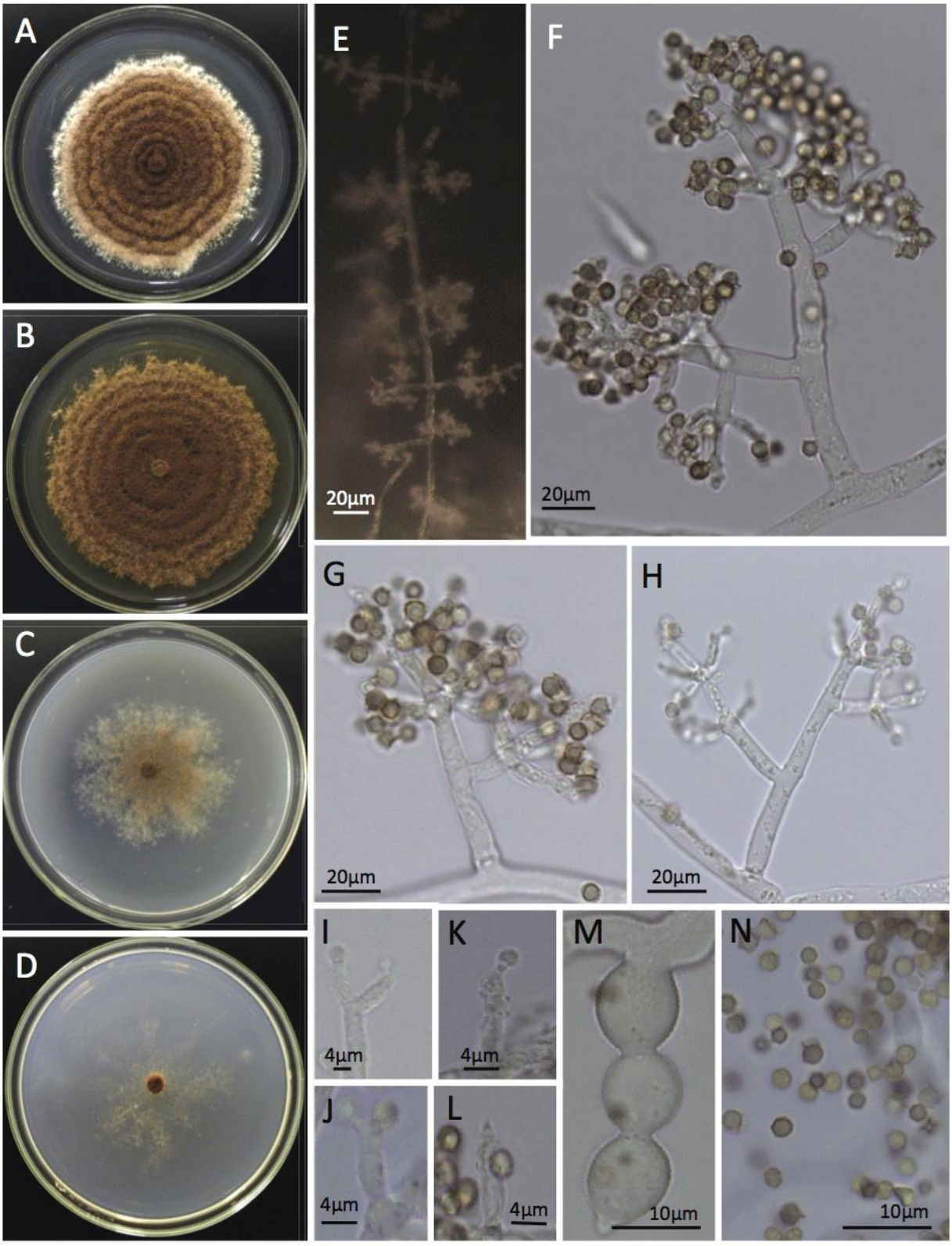
Supporting Information Legends

Fig. S1. *Escovopsis kreiselii* CBS 139320 (= LESF53) mycelial growth after 14 days grown in different culture media at two temperatures. PDA: potato-dextrose agar; MA2%: malt extract agar 2%; CMD: cornmeal agar; SNA: synthetic nutrient agar.

Fig. S2. Phylogeny of Hypocreaceae reconstructed from the LSU gene indicating the position of *Escovopsis kreiselii*. The phylogeny of 22 Hypocreaceae species and two Clavicipitaceae species as outgroup was reconstructed using Bayesian Inference. The voucher accession numbers in culture collections follow the taxon names and the GenBank accession numbers are given in parentheses. The clade highlighted in gray represents fungi strictly associated with attine ants. Posterior probabilities of nodes are given along with ML bootstraps values for a similar topology; only PP and bootstrap values ≥ 0.5 or 50 are shown on branches, respectively. Phylogeny based on Augustin et al. [19]. Bar: 0.05 substitutions per nucleotide position. T: ex-type strains.

Figures

Fig. 1.



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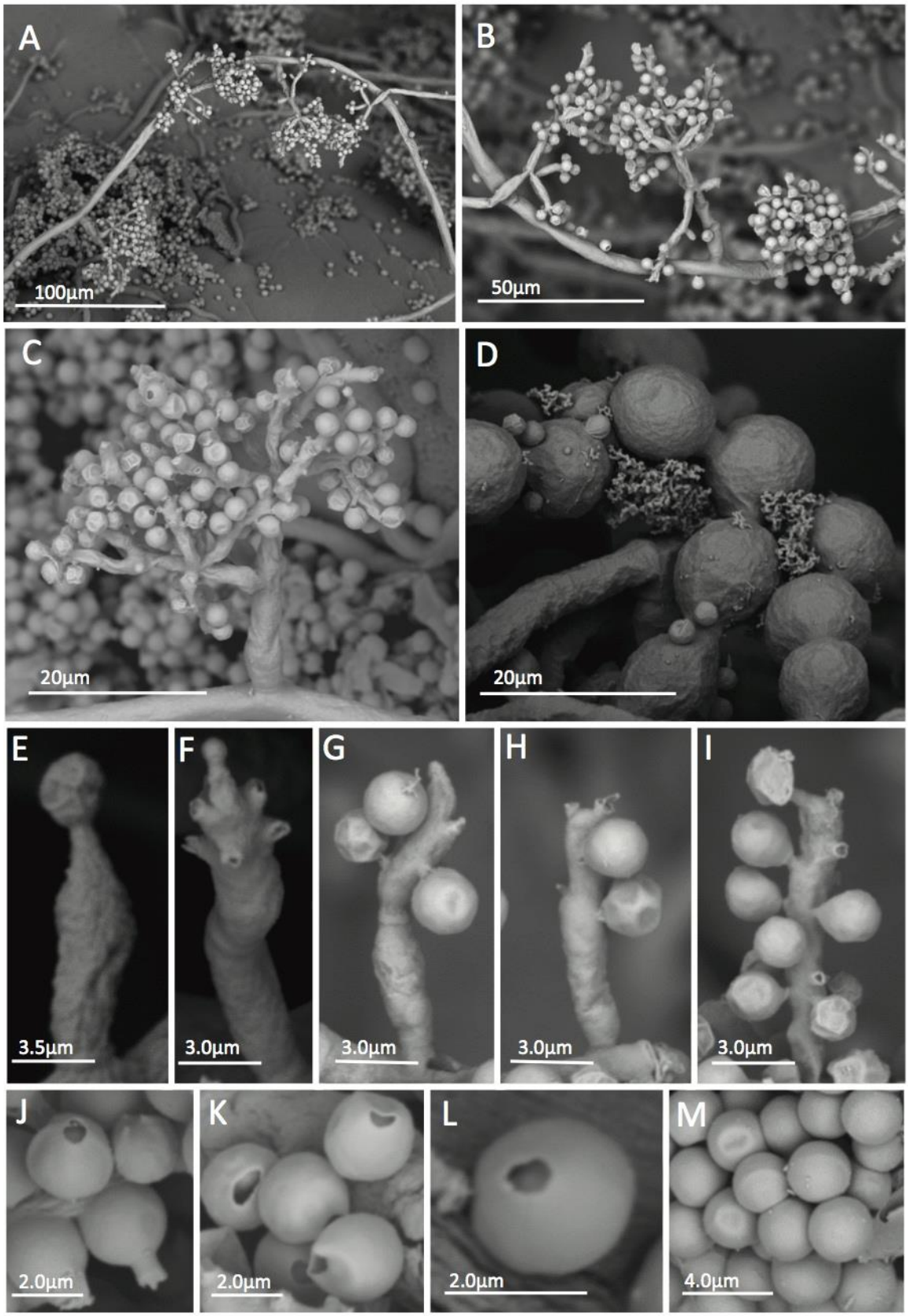

A
100µm
B
50µm
C
20µm
D
20µm
E
3.5µm
F
3.0µm
G
3.0µm
H
3.0µm
I
3.0µm
J
2.0µm
K
2.0µm
L
2.0µm
M
4.0µm

Fig. 3.

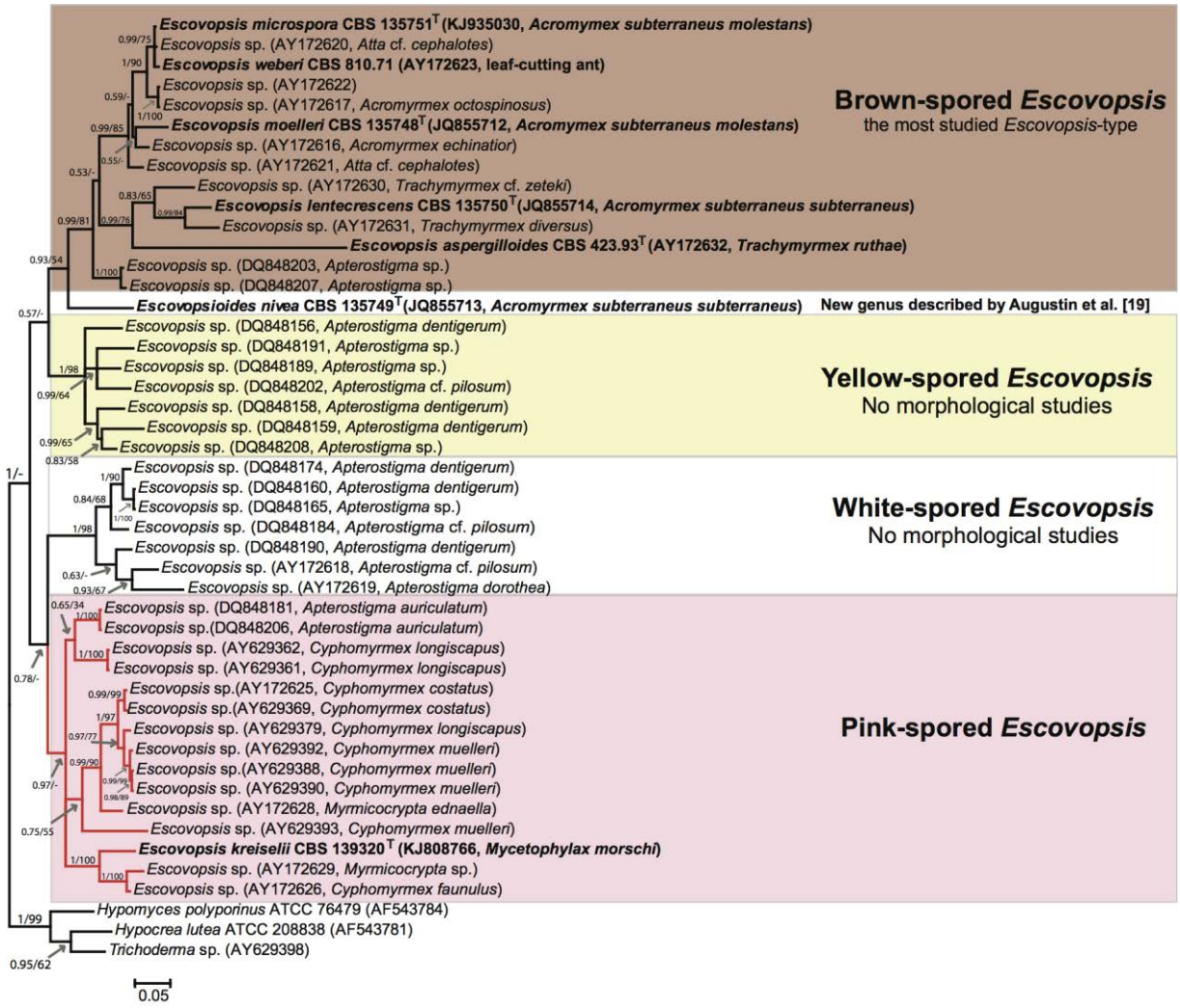

Escovopsis microspora CBS 135751T (KJ935030, Acromymex subterraneus molestans)
Escovopsis sp. (AY172620, Atta cf. cephalotes)
Escovopsis weberi CBS 810.71 (AY172623, leaf-cutting ant)
Escovopsis sp. (AY172622)
Escovopsis sp. (AY172617, Acromyrmex octospinosus)
Escovopsis moelleri CBS 135748T (JQ855712, Acromymex subterraneus molestans)
Escovopsis sp. (AY172616, Acromyrmex echinatior)
Escovopsis sp. (AY172621, Atta cf. cephalotes)
Escovopsis sp. (AY172630, Trachymyrmex cf. zeteki)
Escovopsis lentecrescens CBS 135750T (JQ855714, Acromyrmex subterraneus subterraneus)
Escovopsis sp. (AY172631, Trachymyrmex diversus)
Escovopsis aspergilloides CBS 423.93T (AY172632, Trachymyrmex ruthae)
Escovopsis sp. (DQ848203, Apterostigma sp.)
Escovopsis sp. (DQ848207, Apterostigma sp.)
Escovopsioides nivea CBS 135749T (JQ855713, Acromyrmex subterraneus subterraneus)
Brown-spored Escovopsis
the most studied Escovopsis-type
New genus described by Augustin et al. [19]
Escovopsis sp. (DQ848156, Apterostigma dentigerum)
Escovopsis sp. (DQ848191, Apterostigma sp.)
Escovopsis sp. (DQ848189, Apterostigma sp.)
Escovopsis sp. (DQ848202, Apterostigma cf. pilosum)
Escovopsis sp. (DQ848158, Apterostigma dentigerum)
Escovopsis sp. (DQ848159, Apterostigma dentigerum)
Escovopsis sp. (DQ848208, Apterostigma sp.)
Yellow-spored Escovopsis
No morphological studies
Escovopsis sp. (DQ848174, Apterostigma dentigerum)
Escovopsis sp. (DQ848160, Apterostigma dentigerum)
Escovopsis sp. (DQ848165, Apterostigma sp.)
Escovopsis sp. (DQ848184, Apterostigma cf. pilosum)
Escovopsis sp. (DQ848190, Apterostigma dentigerum)
Escovopsis sp. (AY172618, Apterostigma cf. pilosum)
Escovopsis sp. (AY172619, Apterostigma dorothea)
White-spored Escovopsis
No morphological studies
Escovopsis sp. (DQ848181, Apterostigma auriculatum)
Escovopsis sp.(DQ848206, Apterostigma auriculatum)
Escovopsis sp. (AY629362, Cyphomyrmex longiscapus)
Escovopsis sp. (AY629361, Cyphomyrmex longiscapus)
Escovopsis sp.(AY172625, Cyphomyrmex costatus)
Escovopsis sp.(AY629369, Cyphomyrmex costatus)
Escovopsis sp. (AY629379, Cyphomyrmex longiscapus)
Escovopsis sp. (AY629392, Cyphomyrmex muelleri)
Escovopsis sp.(AY629388, Cyphomyrmex muelleri)
Escovopsis sp. (AY629390, Cyphomyrmex muelleri)
Escovopsis sp. (AY172628, Myrmicocrypta ednaella)
Escovopsis sp. (AY629393, Cyphomyrmex muelleri)
Escovopsis kreiselii CBS 139320T (KJ808766, Mycetophylax morschi)
Escovopsis sp. (AY172629, Myrmicocrypta sp.)
Escovopsis sp. (AY172626, Cyphomyrmex faunulus)
Pink-spored Escovopsis
Hypomyces polyporinus ATCC 76479 (AF543784)
Hypocrea lutea ATCC 208838 (AF543781)
Trichoderma sp. (AY629398)
0.05

Fig. 4.

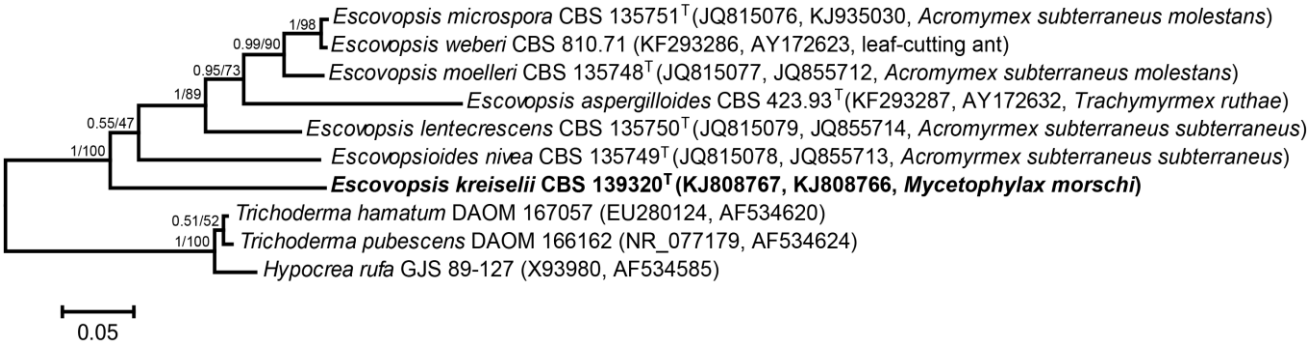

Escovopsis microspora CBS 135751T (JQ815076, KJ935030, Acromymex subterraneus molestans)
Escovopsis weberi CBS 810.71 (KF293286, AY172623, leaf-cutting ant)
Escovopsis moelleri CBS 135748T (JQ815077, JQ855712, Acromymex subterraneus molestans)
Escovopsis aspergilloides CBS 423.93T (KF293287, AY172632, Trachymyrmex ruthae)
Escovopsis lentecrescens CBS 135750T (JQ815079, JQ855714, Acromyrmex subterraneus subterraneus)
Escovopsioides nivea CBS 135749T (JQ815078, JQ855713, Acromyrmex subterraneus subterraneus)
Escovopsis kreiselii CBS 139320T (KJ808767, KJ808766, Mycetophylax morschi)
Trichoderma hamatum DAOM 167057 (EU280124, AF534620)
Trichoderma pubescens DAOM 166162 (NR_077179, AF534624)
Hypocrea rufa GJS 89-127 (X93980, AF534585)
0.05

Fig. S1.

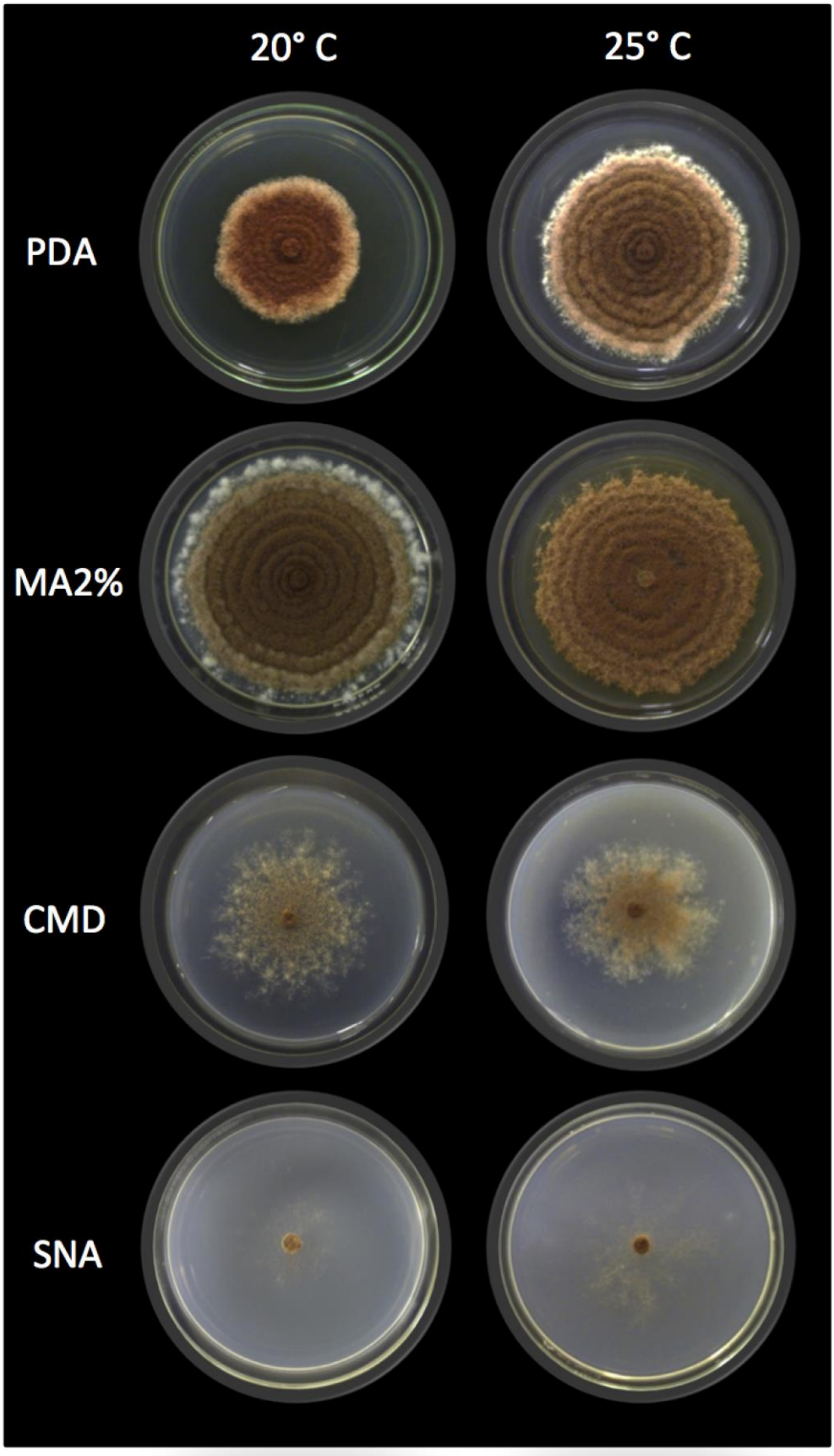
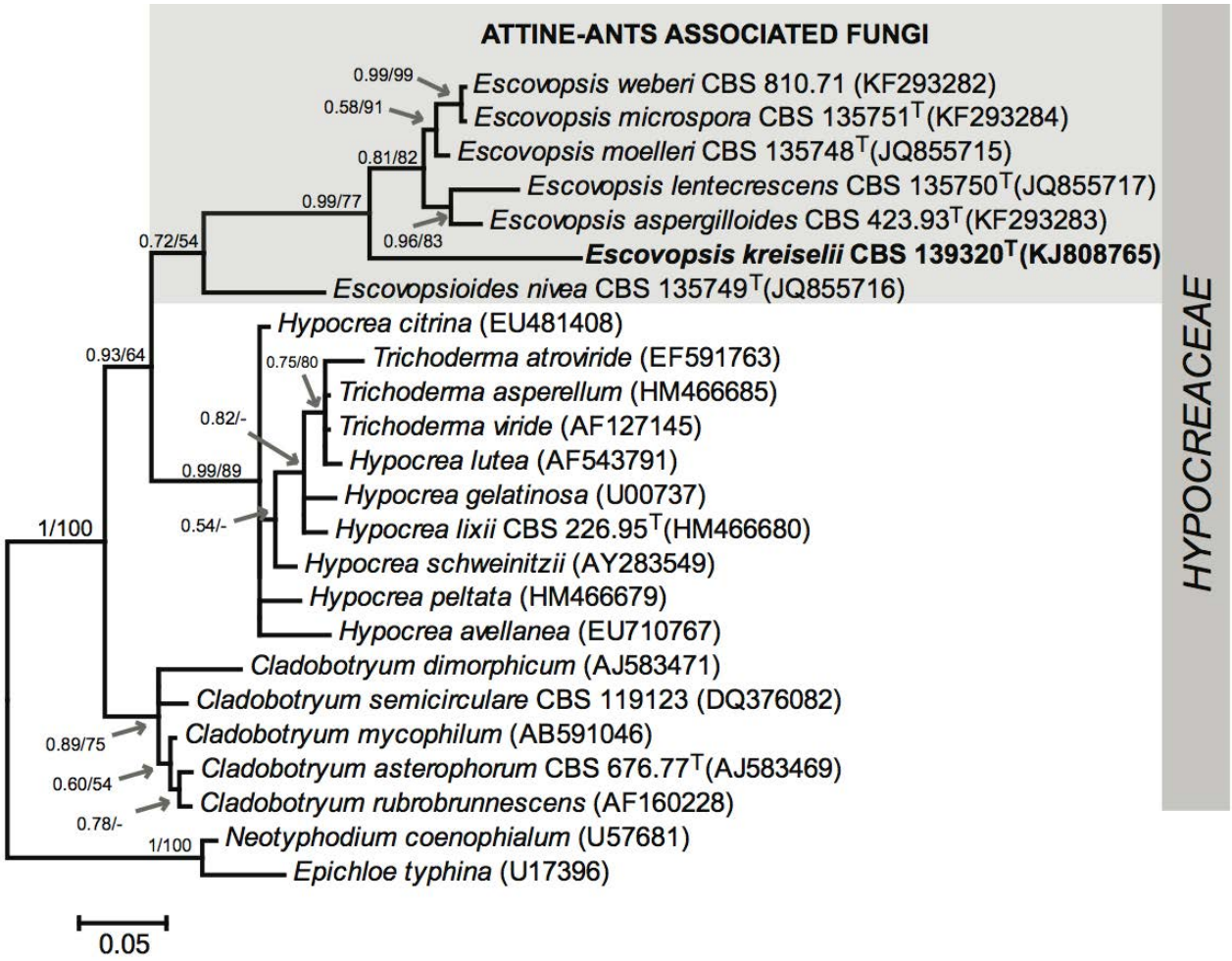


Fig. S2.


ATTINE-ANTS ASSOCIATED FUNGI
HYPOCREACEAE
0.99/99
0.58/91
0.81/82
0.99/77
0.96/83
0.72/54
0.93/64
0.75/80
0.82/-
0.99/89
0.54/-
1/100
0.89/75
0.60/54
0.78/-
1/100
Escovopsis weberi CBS 810.71 (KF293282)
Escovopsis microspora CBS 135751T (KF293284)
Escovopsis moelleri CBS 135748T (JQ855715)
Escovopsis lentecrescens CBS 135750T (JQ855717)
Escovopsis aspergilloides CBS 423.93T (KF293283)
Escovopsis kreiselii CBS 139320T (KJ808765)
Escovopsioides nivea CBS 135749T (JQ855716)
Hypocrea citrina (EU481408)
Trichoderma atroviride (EF591763)
Trichoderma asperellum (HM466685)
Trichoderma viride (AF127145)
Hypocrea lutea (AF543791)
Hypocrea gelatinosa (U00737)
Hypocrea lixii CBS 226.95T (HM466680)
Hypocrea schweinitzii (AY283549)
Hypocrea peltata (HM466679)
Hypocrea avellanea (EU710767)
Cladobotryum dimorphicum (AJ583471)
Cladobotryum semicirculare CBS 119123 (DQ376082)
Cladobotryum mycophilum (AB591046)
Cladobotryum asterophorum CBS 676.77T (AJ583469)
Cladobotryum rubrobrunnescens (AF160228)
Neotyphodium coenophialum (U57681)
Epichloe typhina (U17396)
0.05

3 CHAPTER II

Shared *Escovopsis* infections destabilize the tripartite co-evolution hypothesis in the higher attine fungus-growing ant symbiosis

Status: *In review* – Proc Royal Society B - London

Shared *Escovopsis* infections destabilize the tripartite co-evolution hypothesis in the higher-attine fungus-growing ant symbiosis

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Short title: Higher-attine ants share *Escovopsis*

Keywords: ancestral state reconstruction, attine ants, host-parasite co-evolution, phylogeny

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Abstract

Fungus-gardening (attine) ants grow fungus for food in protected gardens, which contain beneficial, auxiliary microbes, but also microbes harmful to gardens. Among these potentially pathogenic microorganisms, the most consistently isolated are fungi in the genus *Escovopsis*, which are thought to co-evolve with ants and their cultivar in a tripartite model. To test strict clade-to-clade correspondence predicted by this tripartite model for the higher-attine symbiosis, we amassed a geographically comprehensive collection of *Escovopsis* (Mexico to southern Brazil) from higher-attine gardens and reconstructed the *Escovopsis* phylogeny. Contrary to a previous analysis reporting ant-*Escovopsis* clade-to-clade correspondences within the higher-attine symbiosis, we found no evidence for such specialization; rather, gardens from different higher-attine ant genera can be infected by closely-related strains of the parasite. *Escovopsis* appears to switch readily between higher-attine nests, destabilizing the strict tripartite coevolution hypothesis within the higher-attine symbiosis. We also identified rare *Escovopsis* strains that might represent biogeographically restricted endemic species. Phylogenetic patterns correspond to morphological variation of vesicle type (structures supporting spore-bearing cells), separating *Escovopsis* with phylogenetically derived cylindrical vesicles from ancestral globose vesicles. The phylogenetic patterns are inconsistent with the prevailing strict coevolution model and provide an improved basis for future taxonomic and ecological studies of *Escovopsis*.

1. Introduction

Symbiotic relationships are a significant source for evolutionary innovation [1-3], generating diversity in mutualistic and parasitic interactions, particularly among microbe-insect associations. During the last decades, understanding of microbe-insect interactions has significantly improved, revealing more complex interactions among an increasingly diverse set of participants than was previously appreciated. This is especially true for attine ants and their microbial symbionts, which have become among the best-studied examples for parasitic and mutualistic insect-microbe coevolution [4,5].

All known attine ants (257 described species within 16 genera) cultivate basidiomycetous fungi as the primary source of food [6,7]. Since the origin of the symbiosis approximately 67 million years ago, five distinct types of fungiculture evolved [8-10]. We focused here on the so-called higher-attine fungiculture involving four ant genera: *Trachymyrmex*, *Sericomyrmex*, *Acromyrmex* and *Atta* (the last two genera comprise the leaf-cutting ants, the behaviorally most complex group within the entire tribe [9,10]). Higher-attine ants cultivate phylogenetically derived fungi that exhibit hyphal swellings, called gongylidia, which are specialized structures used to feed the queen and the brood [11-13].

The gardens grown by attine ants are susceptible to invasion by fungi; some of these are only known from associations with attine ant nests and are considered specific parasites [14]. All these parasitic fungi are grouped within the genus *Escovopsis* [14-21]. Recently, a new closely-related genus, *Escovopsioides*, was described, but the parasitic nature of this fungus is unknown [20].

Previous research showed that *Escovopsis* coevolved with attine ants and their cultivars [17]; in this association, a clade-to-clade correspondence was demonstrated, in which different clades of the parasite would be specifically associated with distinct clades of ants and their corresponding fungi. For example, a monophyletic clade of three *Escovopsis*

strains was found infecting *Trachymyrmex* gardens from Panama, Ecuador, and Trinidad and Tobago, suggesting that a specific clade of *Escovopsis* infects the *Trachymyrmex* symbiosis [17]. Based on the three strains examined (each from a single nest from each of these locations), the *Trachymyrmex*-associated *Escovopsis* was thought to be distinct from *Escovopsis* infecting leaf-cutting ants (*Acromyrmex* and *Atta*). However, to date no study has extensively explored the geographic diversity of *Escovopsis* associated with *Trachymyrmex*, especially in South America, the center of diversity for all genera of higher attines. Studying *Escovopsis* strains from leaf-cutting ants, Taerum *et al*. [22,23] identified three main, monophyletic groups among leafcutter-associated *Escovopsis*, all of which were found in South and Central America. However, Taerum *et al*. [22,23] studied mostly *Escovopsis* collected in Panama (some samples from Argentina, Ecuador and one sample from Guiana), but few representatives from across South America. Augustin *et al*. [20] studied *Escovopsis* strains associated with *Acromyrmex* species within a small geographic area in Brazil (Viçosa – Minas Gerais state) and showed that, even within a restricted area, three new species of *Escovopsis* were found infecting gardens of *Acromyrmex* ants. This previously unknown diversity uncovered in the small sample of Augustin *et al*. [20] suggests that South America might hold a significant unexplored diversity of *Escovopsis*.

Using a greatly expanded collection of *Escovopsis* spanning almost the entire range of higher-attine ants, including Brazil (n=55 strains), Argentina (n=1), Panama (n=13), Lesser Antilles (n=2), Trinidad and Tobago (n=1), Mexico (n=3), we here reevaluate the prevailing strict tripartite coevolution hypothesis, specifically testing whether *Escovopsis* isolated from leaf-cutting nests group separately from those isolated from *Trachymyrmex* nests. Analyzing *Escovopsis* strains isolated from 70 higher-attine colonies, from at least 20 ant species in 31 locations, we show that leaf-cutter ants and other higher-attine ants can share similar *Escovopsis* infections, indicating no ant-parasite strict coevolution within this group of ants.

We also identify several new *Escovopsis* strains forming previously unknown clades, which may correspond to new species. Correlations between morphological traits and phylogenetic patterns suggest the need for taxonomic and ecological studies on these newly identified fungal pathogens.

2. Material and methods

(a) Collection, isolation and morphological aspects

From 2001 to 2013, we sampled *Escovopsis* from Brazil (22 sites), Panama (4 sites), the Caribbean island of Guadeloupe (1 site), Argentina (1 site) and Mexico (1 site). To these collections, we added DNA sequence data from published studies of *Escovopsis* collected in Trinidad and Tobago by Seifert *et al*. [16] and in Viçosa, Brazil by Augustin *et al*. [20]. The combined data set contains samples from 31 collection sites spanning the known geographic range of *Escovopsis* (see table S1 for complete information).

To isolate *Escovopsis*, pieces of attine fungus garden (1-5 pieces with 3-5 cm^3 each, depending on the size of the chamber) along with some worker ants were collected *in situ* from live ant colonies using a sterile spatula. Fungus gardens were maintained in UV-sterilized plastic containers until they reached the laboratory. Small fragments of the fungus garden (0.5-1 mm in diameter) were plated on potato dextrose agar (PDA) supplemented with chloramphenicol [150 mg L^{-1}, Sigma] and incubated at 25 °C in the dark for the following 14 days. Plates were checked twice daily for fungal growth and were subcultured on new PDA plates when *Escovopsis* growth became visible. To confirm the purity of *Escovopsis* cultures, monosporic cultures were obtained for each strain after isolation. Some strains were isolated from middens, but the isolation process was the same described above (see Table S1). We obtained 61 new *Escovopsis* strains from our survey and also analyzed nine *Escovopsis* strains isolated by our research group and superficially characterized in previous studies [24,25].

Moreover, we included morphological data derived from five strains for brown-spored *Escovopsis* species previously identified [16,20]. Therefore, our morphological analysis comprised 75 *Escovopsis* strains associated with higher-attine ants: 55 from Brazil, 13 from Panama, three from Mexico, two from the Caribbean island of Guadeloupe, one from Argentina and one from Trinidad and Tobago. *Escovopsis* strains RS019, RS020, RS030, RS053, RS055, RS061 and RS076 were superficially analyzed in Rodrigues *et al*. [24], while strains NL007 and SES005 were used in *in vitro* bioassays in Meirelles *et al*. [25].

In addition to the 75 strains, we also analyzed the morphology of five brown-spored *Escovopsis* strains isolated from *Apterostigma* by Gerardo *et al*. [19]; these strains were included mainly for comparison with our strains associated with higher attines. *Escovopsis* sampled from Brazil are stored in 10% glycerol at -80 °C at UNESP – Microbial Resource Center in Rio Claro, SP, Brazil, while *Escovopsis* obtained from other countries are stored under the same conditions in the Section of Integrative Biology – University of Texas at Austin, Austin, TX, USA.

We performed morphological examinations on all new strains. For this purpose, the strains were grown in PDA at 25 °C in the dark and, after 7 days of growth, microscopic slides were prepared to analyze the vesicle shape (globose or cylindrical).

(b) DNA extraction, PCR and sequencing

Using a modified CTAB method [18,26], we extracted genomic DNA directly from colony mycelia of all strains after 7 days of growth in PDA. Previous studies used elongation factor 1-alpha (*tef1*) to reconstruct *Escovopsis* phylogenies [17-19, 22,23]. Augustin *et al*. [20] showed the potential of using the internal transcriber spacer (ITS) as a phylogenetic marker for this fungus; this region is now considered the “barcoding region” for fungi [27]. To increase our phylogenetic signal, we used both *tef1* and ITS for phylogenetic analyses.

For *tef1* amplification, we used primers derived from Taerum *et al*. [22]. The forward primer was the same EF6-20F (5'-AAGAACATGATCACTGGTACCT-3'); however, we modified one base on the reverse primer ("R" was replaced by "A") and the final primer was EF6**A**-1000R (5'-CGCATGTC**A**CGGACGGC-3'). This change increased the melting temperature of the primer, minimized co-amplification and improved the sequencing results. DNA was diluted 1000 – 10000x (final concentration ≅ 0.5-5ng/μL) and the PCR conditions were: 96 °C for 3 min, 35 cycles at 96 °C for 30 s, 61 ºC for 45 s and a final extension step at 72 °C for 1 min following Meirelles *et al*. [25].

ITS amplification was performed using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). DNA was diluted 100x (final concentration ≅ 50-100ng/μL) and the PCR conditions were: 96 °C for 3 min, 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 55 °C for 1 min and a final extension step at 72 °C for 2 min [28]. Due to the high CG content in ITS for *Escovopsis* (> 65% in some strains), we prepared reactions with 5% Dimethyl sulfoxide (DMSO) to enhance primer annealing.

PCR products were cleaned up with *Kit Wizard® SV Gel and PCR Clean-up System* (Promega) following the manufacturer's protocol. Cycle sequencing reactions were prepared with 15-20 ng of template using *BigDye® Terminator v. 3.1 Cycle Sequencing Kit* (Life Technologies) following the manufacturer instructions. Sequences were generated using ABI3500 (Life Technologies). Contigs were assembled using Bioedit v.7.1.3 [29]. Sequences of all strains were deposited in GenBank under accessions KM817043 – KM817173 (see Table S1).

(c) Phylogenetic analysis

We included in our analysis all the 61 new strains derived from this study as well as nine *Escovopsis* strains from Brazil used in previous studies [24,25], all associated with higher-attine ants (see Table S1). Moreover, sequences of five brown-spored *Escovopsis* strains associated with *Apterostigma* ants [19] were included along with sequences from described species for *Escovopsis* and *Escovopsioides* [16,20,21]. Finally, three other Hypocreaceae fungi used as outgroup by Meirelles *et al*. [21] were maintained here. The final dataset comprised two files, one for *tef1* with 85 sequences and other for ITS with 80 sequences (for the five *Escovopsis* strains derived from Gerardo *et al*. [19], only *tef1* sequences were available). Alignments were obtained independently for each file using MAFFT v.7 [30]. Nucleotide substitution models were selected under separated runs for each file using the Akaike information criterion (AIC) with a confidence interval of 95% in jModelTest 2 [31]. The phylogeny was reconstructed by two different methods: maximum likelihood (ML) using RAxML v.8 [32] and Bayesian Inference (BI) using MrBayes v.3.2.2 [33].

The two different alignments were concatenated using Winclada v.1.00.08 [34]. ML analysis was conducted in RAxML using GTR+I+G for each partition; 100 independent ML trees were reconstructed and the one with the best score was kept. To evaluate the tree reliability, 2000 bootstraps replicates were performed using the same model (bootstraps values converged at 1100 replicates [35]). For BI, GTR+I+G was also used as initial model for each partition. Two independent runs were performed; for both, three heated chains and one cold chain was used; each run consisted of Markov Chain Monte Carlo (MCMC) sampling for two million generations. Convergence occurred when the standard deviation (SD) of split frequencies fell below 0.01; the first 25% of MCMC generations were discarded

as "burn-in". The final tree was edited in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) and Adobe Illustrator CS6 (Adobe Systems).

(d) Ancestral state reconstruction

Realizing that *Escovopsis* with globose vesicles grouped in less derived clades when compared to strains with cylindrical vesicles, the first character could be considered the ancestral state. To test this hypothesis, we performed ancestral state reconstruction. Ancestral state inference using a phylogenetic tree assumes a single tree that is known without error. However, heuristic searches for phylogenetic tree estimation do not guarantee the discovery of the true tree topology and branch lengths. Therefore, we performed ancestral state reconstruction using the Ape package in R [36,37] over the maximum likelihood topology estimated in RAxML, the Bayesian consensus topology estimated in MrBayes, a sample of trees from the Bayesian posterior sample and a sample from the RAxML bootstrap replicates to assess the robustness of our conclusions to slight variations in topology and/or branch lengths.

Ape incorporates multiple models that can be used for ancestral state reconstruction. We performed model fitting using likelihood ratio tests to determine if a simple equal-rates model could be rejected in favor of a more complex model, in which the rate of changing from globose to cylindrical vesicles differed from the rate of changing from cylindrical to globose vesicles. With an average p-value of 0.749, we could not reject the simpler model in favor of the more complex model for any tree in our sample; therefore, we performed the analysis under the equal rates model.

3. Results

Escovopsis strains derived from higher-attine ants grouped within nine different clades, and four of them (I, II, V and VII) contain both *Escovopsis* from leaf-cutting ants and *Trachymyrmex* ants (Fig. 1). Representative strains from these four clades were isolated from multiple points in Brazil, Panama, Argentina, Mexico, Guadeloupe and Trinidad and Tobago (Fig. 1). Clades I and II consist mainly of *Escovopsis* isolated from leaf-cutting ants, while clades V and VII consist mostly of *Escovopsis* derived from *Trachymyrmex*, but we also found multiple exceptions to this clade-to-clade correspondence. Specifically, 12 of the 75 *Escovopsis* strains (16%) isolated from higher attine ants did not follow this pattern (Fig. 1). These results show that *Escovopsis* infecting leaf-cutting ants and those infecting other higher-attine ants are not reciprocally monophyletic, suggesting that these insects can share infections caused by closely related strains of the parasite.

This is the first study to report the occurrence of clades IV, V, VI and VIII. These results indicate that the phylogenetic diversity of *Escovopsis* is much greater than what was known. This is likely due to the inclusion of samples from a geographic range unexplored before, especially from Brazil. Our results suggest that clades IV, VI and VIII may consist of rare *Escovopsis* types, as strains from these clades were each found in only one locality (Fig. 2). Clade IV consists of three phylogenetic identical strains isolated from two different *Atta cephalotes* colonies in Camacan, Bahia – BRA (site #9); despite possible abundance at this site, representatives of this clade have not been isolated at any other collection site (Figs. 1 & 2) suggesting the possibility of an endemic *Escovopsis* strain from this region.

As demonstrated by Taerum *et al*. [22], there was no correlation between leaf-cutting ant genera and particular *Escovopsis* strains. *Atta cephalotes* populations from Camacan were infected by *Escovopsis* from clades I, II and IV. In *Atta sexdens*, we found *Escovopsis* from clades I, V and also one strain (*Escovopsis* UT010) that grouped with brown-spored

Escovopsis associated with *Apterostigma* [19], called here as clade IX. The phylogenetic position of *Escovopsis* UT010 is intriguing, because it was thought that this group of *Escovopsis* was not able to infect higher-attine ants [19]. Finally, the type strains of *E. microspora* and *E. moelleri*, both isolated from the same ant species *Acromyrmex subterraneus molestans* [20], grouped in clades I and V, respectively (Fig. 1). These results support the low host-specificity between *Escovopsis* and its leaf-cutting ant hosts, demonstrated by Taerum *et al*. [22].

The results of our phylogenetic groupings based on two nuclear markers are further supported by the presence of two morphological characters that distinguish clades I-IV and VIII from clades VI, VII and IX (Fig. 1). Based on our samples, all *Escovopsis* strains from clades I – IV and VIII have cylindrical vesicles, while all strains from clades VI, VII have globose vesicles (Fig. 1). All the *Escovopsis* strains from the clade mainly associated with *Apterostigma* (clade IX) analyzed here also have globose vesicles. Both vesicle morphotypes were previously reported by studies that described the five known brown-spored *Escovopsis* species [15,16,20]. However, we show here that this morphological character correlates with phylogeny (i.e. we did not find strains within the same clade exhibiting different types of vesicles), suggesting that this trait is important for taxonomic studies.

Moreover, we also performed ancestral state reconstruction to test which of the two morphotypes could be considered the ancestor state using "Ape" package in R [36,37] (see Material and methods for details). Under the equal rates model, the average support for globose vesicles as the ancestral state average is 96.99% (pie-graphs on Fig. 1) and in none of the trees were cylindrical vesicles supported as the ancestral state. When estimated over a sample of trees from a RAxML bootstrap sample, the average support for globose vesicles as the ancestral state was 69.04%. This is lower than the support values obtained from the Bayesian sample (96.99%). One possible cause for this discrepancy is that the RAxML trees

were, on average, shorter in total branch lengths than the Bayesian trees. Shorter branch lengths decrease the possibility of change along a given branch, and the trees estimated by RAxML tend to have short branch lengths near the root of the tree. These branch lengths could inflate the probability of observing cylindrical vesicles at the root.

Additional macroscopic differences (e.g. growth, time for sporulation and intensity of sporulation) were noted between strains from different clades that warrant more careful examination by taxonomists (Fig. S1).

4. Discussion

(a) Escovopsis *occurrence in higher-attine ants*

Despite the efforts of attine workers to maintain their fungal gardens as monocultures, alien fungi are commonly associated with attine nests [24,40]. Some are cosmopolitan fungi within the genera *Trichoderma*, *Fusarium* or *Syncephalastrum* that also exist outside the attine symbiosis [24]. However, *Escovopsis* represents a group of fungi that are so far only known from attine colonies and therefore are thought to be obligately dependent on attine nests for survival [14,17]. These parasites have stimulated broad interest due to their potential as biological control agents of leaf-cutting ants, which are major agricultural pests throughout the tropical New World [41]. To date, the largest studies of *Escovopsis* diversity associated with higher attine ants are those of Taerum *et al*. [22,23], however, these authors did not deeply explore geographic areas in South America (the origin of higher-attine ants) as well as the diversity of the parasite associated with non-leafcutter higher-attine ants (they focused mostly on *Escovopsis* from the leafcutter ants *Acromyrmex* and *Atta*, but not from the other higher-attine genera *Trachymyrmex* and *Sericomyrmex*).

Our results do not support the prevailing model of co-cladogenesis between higher-attine ants and their respective *Escovopsis* parasites [17,22]. Under this model, *Escovopsis* associated with higher-attine ants was thought to have undergone cladogenesis in parallel with the corresponding ant lineages. This original model was supported by the apparent presence of two reciprocally monophyletic *Escovopsis* clades, each associated with its own clade of higher-attine ants: (i) *Escovopsis* from the leaf-cutting ants *Acromyrmex* and *Atta*, and (ii) *Escovopsis* from *Trachymyrmex*, a non-leaf-cutter higher-attine genus [17,22]. A similar pattern of co-cladogenesis had been suggested for the mutualistic cultivar, which likewise consists of a higher-attine clade, within which "Attamyces" is cultivated by leaf-cutting ants, and so-called "Trachymyces" by *Trachymyrmex* [42]. Our phylogenetic reconstruction (Fig. 1) is inconsistent with this view, as we found 12 examples of *Escovopsis* (16% of our total sampling) infecting ants other than the clade with which they were thought to be exclusively associated (Fig. 1). Our data suggest that, rather than tight correspondence between ant genera and particular *Escovopsis* strains, higher-attine ants from any genus can share infections by close related *Escovopsis* strains. However, these patterns are not inconsistent with broad congruence between *Escovopsis* and attine ants at higher taxonomic levels [17], for example between higher and lower attines other than *Apterostigma* (i.e. we did not find pink, white or yellow-spored *Escovopsis* infecting higher-attine ants; these morphotypes seem to be less derived and restrict to lower-attine ants as indicated by previous studies [17,19,21,43]).

Cultivar switching, which is known to occur among some attine species, could be one of the mechanisms for *Escovopsis* sharing between leaf-cutting and other higher-attine ants; nevertheless, this does not seem to be the only mechanism for this infection sharing. Augustin *et al*. [20] found three different *Escovopsis* species infecting the same garden within a nest of *Acromyrmex subterraneus molestans* (see Table S2, nest #4 in Augustin *et al*. [20]). Strains of *E. moelleri* and *E. microspora* were found in top-portions of the garden, while strains of *E.*

moelleri and *E. lentecrescens* were found on the bottom of the garden. Therefore, *Escovopsis* strains infecting the same garden grouped here in three different clades (*E. microspora* - Clade I; *E. moelleri* - Clade V and *E. lentecrescens* - Clade VII). These clades comprise *Escovopsis* from leaf-cutting ants as well as *Escovopsis* thought to be specialized on *Trachymyrmex* ants, indicating no specificity in *Escovopsis* infections within the higher-attine group (i.e., it is possible to isolate from a single ant garden *Escovopsis* strains that span a broad diversity of higher-attine-associated *Escovopsis*).

One possible interpretation of the new phylogenetic pattern is that some *Escovopsis* clades have generalist pathogenic potential (Clades I, II, V and VII), infecting different higher-attine ant genera, while other clades (IV, VI, VIII) may be more restricted to specific ant populations or geographic regions (corresponding to endemic *Escovopsis* species). For example, although the mechanisms of *Escovopsis* transmission between ant nests is still unknown, it is possible a generalist vector might be able to transmit *Escovopsis* from Clades I, II, V, and VII between nests from the majority of higher-attine ants, whereas other *Escovopsis* from clades IV, VI and VIII may be transmitted by less generalized vectors. An alternative hypothesis would be that these fungi might be more adapted to the specific ecological conditions in the regions where they are found.

The presence of *Escovopsis* in the Caribbean island of Guadeloupe (strains UT001 and UT002, Fig. 1) is particularly interesting. *Acromyrmex octospinosus* has been recently introduced to Guadeloupe and is an invasive species there [44], which makes the presence of *Escovopsis* in these colonies somewhat of a curiosity. We propose three potential hypotheses for how this relationship came to be: (i) *Escovopsis* was present in the nest that was initially introduced, suggesting that *Escovopsis* sometimes do not represent a deadly disease, since this nest survived; (ii) *Escovopsis* was present on the queen(s) that was/were introduced, implying that *Escovopsis* can sometimes be vertically transmitted; or (iii) *Escovopsis* can be dispersed

or vectored widely across the Neotropics, including remote islands, indicating that sometimes *Escovopsis* is able to disperse more broadly than nest-founding queens.

Gerardo *et al*. [19] were the first to document exceptions to the co-cladogenesis model when they found brown-spored *Escovopsis* (close related to *Escovopsis* associated with higher attines) infecting *Apterostigma* ants that cultivate "coral fungi" in the family Pterulaceae, which are distantly related to the higher-attine cultivars. We also found one strain (*Escovopsis* UT010) infecting *Atta sexdens* that grouped with those brown-spored *Escovopsis* types that Gerardo *et al*. [19] showed were prevalent in *Apterostigma* gardens, indicating that these fungi, albeit rarely, could infect leaf-cutting gardens. Recently, *Apterostigma* ants cultivating "Attamyces" fungi were found in Brazil [45]. These combined findings suggest that the hypothesized strict co-cladogenesis between *Escovopsis*, the attine ants, and their fungal cultivars breaks down across much of South America. Additional sampling of lower-attine ants, especially in Brazil, may reveal further exceptions to the co-cladogenesis model of ant-cultivar-parasite symbiosis.

(b) Phylogenetic and morphological diversity of higher attine Escovopsis

Escovopsis diversity is likely affected by environmental and climate conditions, and may follow a latitudinal diversity gradient. Four colonies of *Atta cephalotes* from Camacan, Bahia, Brazil (Table S1 and Fig. 2) contained *Escovopsis* from three different clades (Fig. 1), including clade IV, currently known only from that site. Augustin *et al*. [20] also found *Escovopsis* from three different clades (I, V and VII) associated with a single colony of *Acromyrmex* in Viçosa (site #23), Brazil. In contrast, our sampling of *Acromyrmex* species from southern Brazil only revealed *Escovopsis* from clade I.

Until recently, *Escovopsis* was superficially characterized by "*weberi* morphology" and "*aspergilloides* morphology", corresponding to cylindrical and globose vesicles,

respectively [15,16]. However, Augustin *et al*. [20] were the first to recognize new species within each of these two morpho-groups when describing *E. microspora*, *E. moelleri* and *E. lentecrescens*. Our analyses (Fig. 1) expand Augustin *et al*. [20] work by identifying new clades, as well as several new strains within the two previously known morpho-groups. Therefore, aiming to avoid name confusion, future studies should consider using either "globose" or "cylindrical" vesicles for a morphological classification for this group (instead of using "*weberi* morphology" and "*aspergilloides* morphology"). Moreover, we found that globose vesicle appears to be the ancestral state when compared to cylindrical vesicles, being present in *Escovopsis* that commonly infect *Apterostigma* gardens and in *Escovopsis* UT010 (clade IX) (Figs. 1 & S2, prob = 0.97). Therefore, two main scenarios are possible: (i) cylindrical vesicles arose on the ancestor between clade VIII and all other clades and after that, independent reversions to globose vesicles occurred for clades VI and VII, while all other clades maintained the derived character; (ii) cylindrical vesicles arose independently two times, one in clade VIII and then on the ancestral of all derived clades I, II, III, IV and V. Our data support the first scenario less strongly (see pie-graphs at Figs. 1 & S2), however in any case, future sampling in *Apterostigma* ants from Brazil (especially searching for brown-spored *Escovopsis*) will be necessary to better understand the morphological evolution of these fungi and verify if these ants can also be infected by *Escovopsis* exhibiting cylindrical vesicles or not. Further, with such sampling, researchers will be able to confirm if additional switches between *Escovopsis* infecting higher-attine ants and the lower-attine *Apterostigma* indeed occur.

In addition to these morphological differences, we found that strains from different clades can exhibit very distinct growth, time of sporulation and sporulation intensity when cultivated under the same conditions (Fig. S1). Such differences, as well as more detailed microscopic morphology (e.g. conidia structure and size or conidiophore morphology) will be

necessary for future taxonomic work. The phylogenetic patterns documented here (Fig. 1) hint at an underappreciated diversity of *Escovopsis* and the complexity of its evolutionary history, but provide a basis for future taxonomic and ecological studies of *Escovopsis* fungi associated with higher-attine ants.

Data accessibility

DNA sequences: GenBank accessions KM817043-KM817173. For details, see Table S1.

Phylogenetic data, including alignments: TreeBASE accession number S16556 (http://purl.org/phylo/treebase/phylows/study/TB2:S16556)

Competing interests

We have no competing interests.

Author's contributions

LAM carried out the molecular lab work, carried out the phylogenetic and morphological analysis, participated in the design of the study and drafted the manuscript; SES participated in field collections and helped draft the manuscript; MBJr. participated in the design of the study and helped draft the manuscript; AMW carried out the ancestral state analyses and helped draft the manuscript; UGM participated in field collections, helped to coordinate the study and to draft the manuscript; AR conceived the study, designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

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Figures legends

Fig. 1. Phylogenetic tree of *Escovopsis* associated with higher attine ants based on elongation factor 1-alpha (*tef1)* and internal transcribed spacer (ITS) markers analyzed under Bayesian criteria. Pie-graphs at key nodes show probabilities of ancestral character states for globose (red) and cylindrical (blue) vesicles. Colored bars on the right side indicate vesicle morphology observed and the black arrows point each vesicle structure (400x). The main clades found are highlighted in different colors and were consistent in both ML and Bayesian analysis. We also included strains from brown-spored *Escovopsis* associated with *Apterostigma* ants [19], *Escovopsioides nivea* and *Escovopsis kreiselii* (the only species described for the pink-spored *Escovopsis*, a less derived group that infects lower attine ant gardens, Meirelles *et al*. [21]). Three other species of Hypocreaceae fungi were used as outgroup. *Escovopsis* strains in bold indicate exceptions of the co-cladogenesis model (see text). The ant species from which an *Escovopsis* strain was isolated, its corresponding collection sites and ID numbers are given in parentheses. GenBank accession number for *tef1* and ITS of each strain are available on Table S1. Only posterior probabilities and bootstrap values $\geq$ 0.5 or 50 are shown. The letter "T" indicates ex-type strains.

Abbreviations: Ser. = *Sericomyrmex*; *sub.* = *subterraneus*.

Fig. 2. Geographic distribution of *Escovopsis* sampling sites. Colored circles correspond to clade colors in Fig. 1. Location number corresponds to the listing in Table S1. For sites from which different clades of *Escovopsis* were sampled, more than one colored circle is showed, which does not indicate that different *Escovopsis* strains derived from the same colony (see Table S1 for nests IDs). The green shading indicates the approximate distribution of higher

attine ants [6, 38]. Cartographic data (original map) was obtained using a free package for the software DIVA-GIS [39].

Figures

Fig. 1

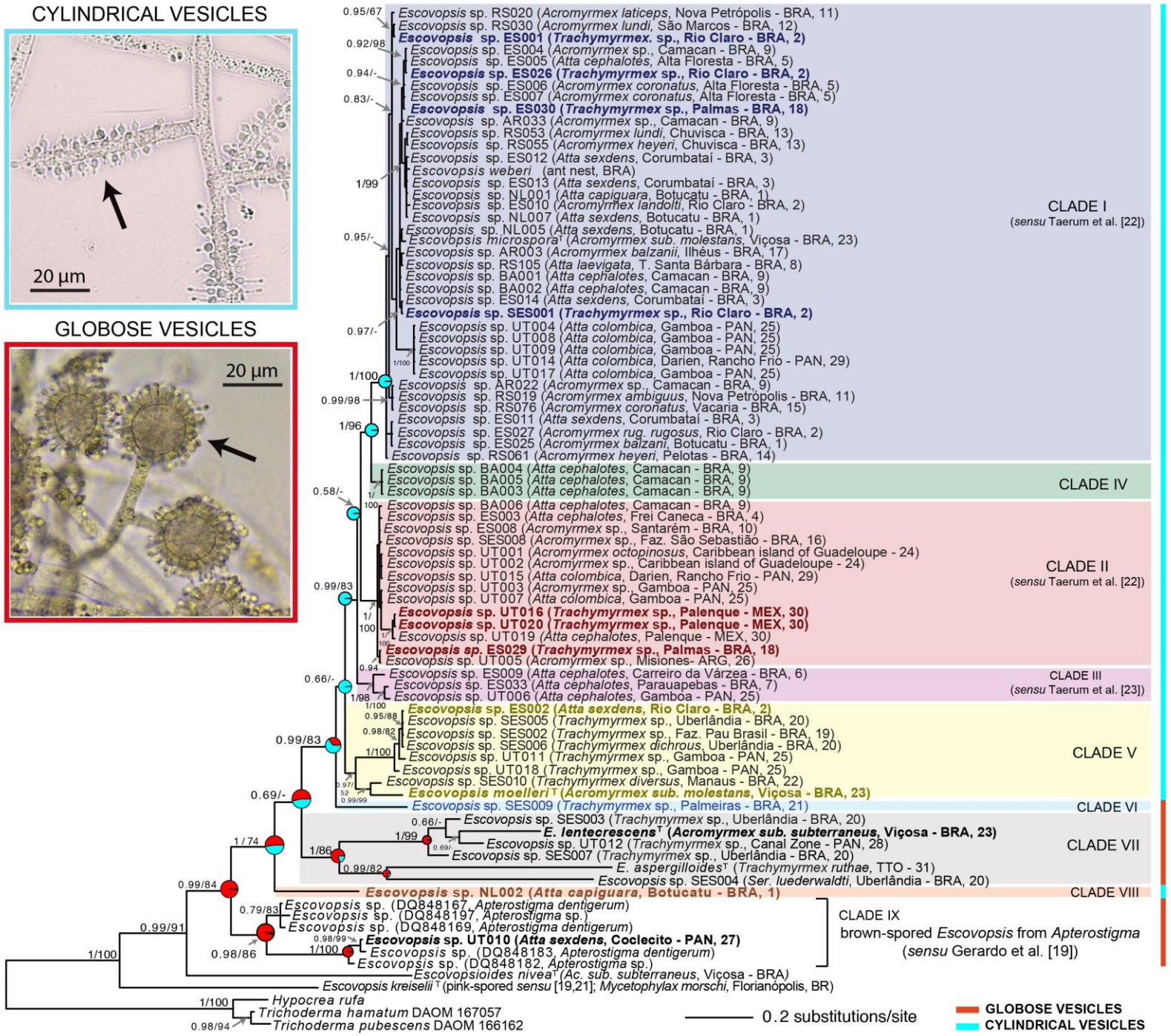
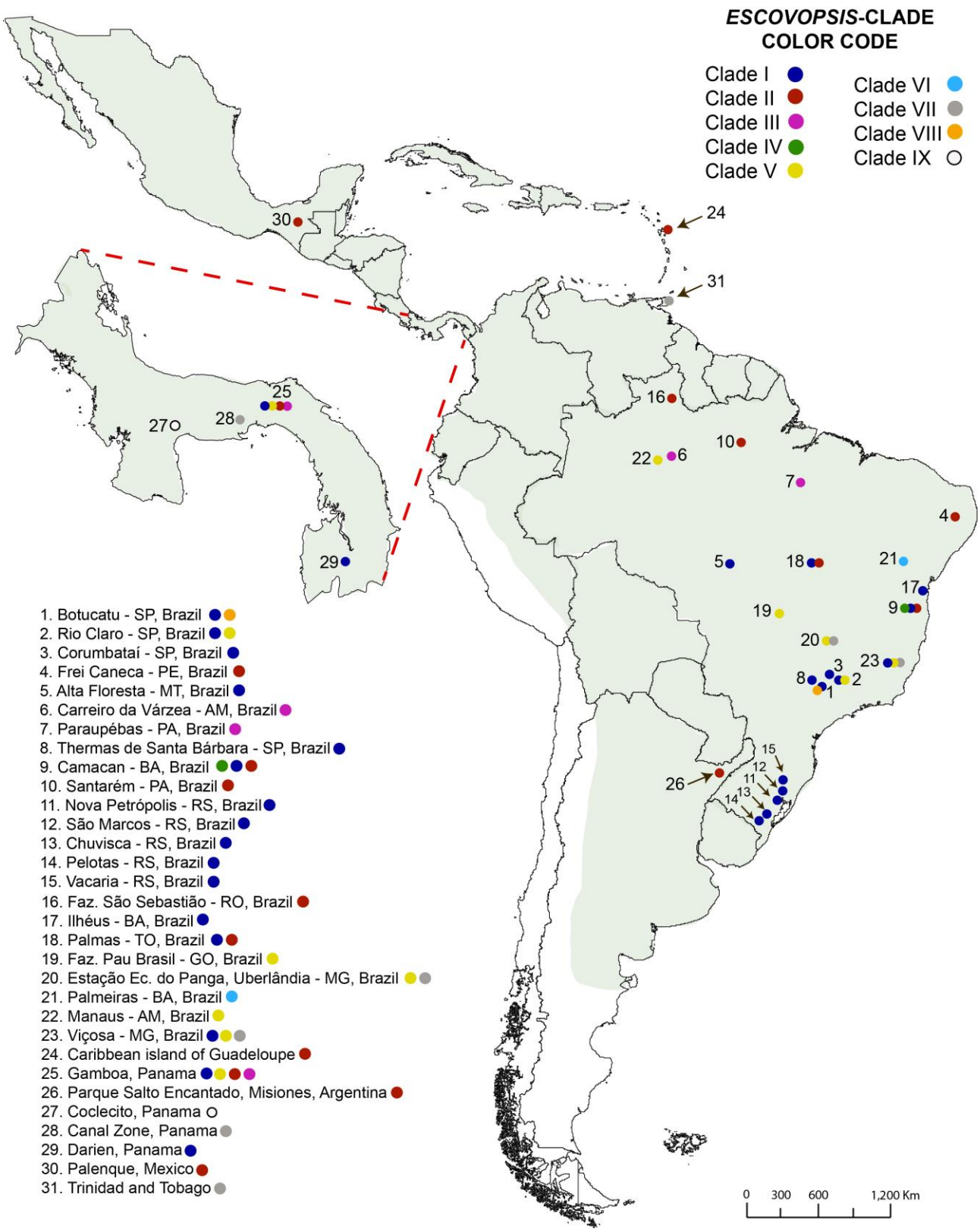


Fig. 2


ESCOVOPSIS-CLADE COLOR CODE
Clade I
Clade II
Clade III
Clade IV
Clade V
Clade VI
Clade VII
Clade VIII
Clade IX
1. Botucatu - SP, Brazil
2. Rio Claro - SP, Brazil
3. Corumbataí - SP, Brazil
4. Frei Caneca - PE, Brazil
5. Alta Floresta - MT, Brazil
6. Carreiro da Várzea - AM, Brazil
7. Paraupébas - PA, Brazil
8. Thermas de Santa Bárbara - SP, Brazil
9. Camacan - BA, Brazil
10. Santarém - PA, Brazil
11. Nova Petrópolis - RS, Brazil
12. São Marcos - RS, Brazil
13. Chuvisca - RS, Brazil
14. Pelotas - RS, Brazil
15. Vacaria - RS, Brazil
16. Faz. São Sebastião - RO, Brazil
17. Ilhéus - BA, Brazil
18. Palmas - TO, Brazil
19. Faz. Pau Brasil - GO, Brazil
20. Estação Ec. do Panga, Uberlândia - MG, Brazil
21. Palmeiras - BA, Brazil
22. Manaus - AM, Brazil
23. Viçosa - MG, Brazil
24. Caribbean island of Guadeloupe
25. Gamboa, Panama
26. Parque Salto Encantado, Misiones, Argentina
27. Coclecito, Panama
28. Canal Zone, Panama
29. Darien, Panama
30. Palenque, Mexico
31. Trinidad and Tobago
0 300 600 1,200 Km

Supplementary material

Shared *Escovopsis* infections destabilize the tripartite co-evolution hypothesis in the higher attine fungus-growing ant symbiosis

Lucas A. Meirelles, Scott E. Solomon, Mauricio Bacci Jr., April M. Wright, Ulrich G. Mueller, Andre Rodrigues

Fig. S1. Morphological aspects of *Escovopsis*. Conditions were 7 seven days of growth in PDA medium at 25 º C. I-IX represents colony aspects for one strain of each clade displayed in Fig. 1, indicated by the colors used in the edge. In the figure, it can be noted differences on growth, sporulation time and conidia maturation (i.e. sporulation for brown-spored *Escovopsis* is characterized by the white young conidia, and becoming brown when mature).

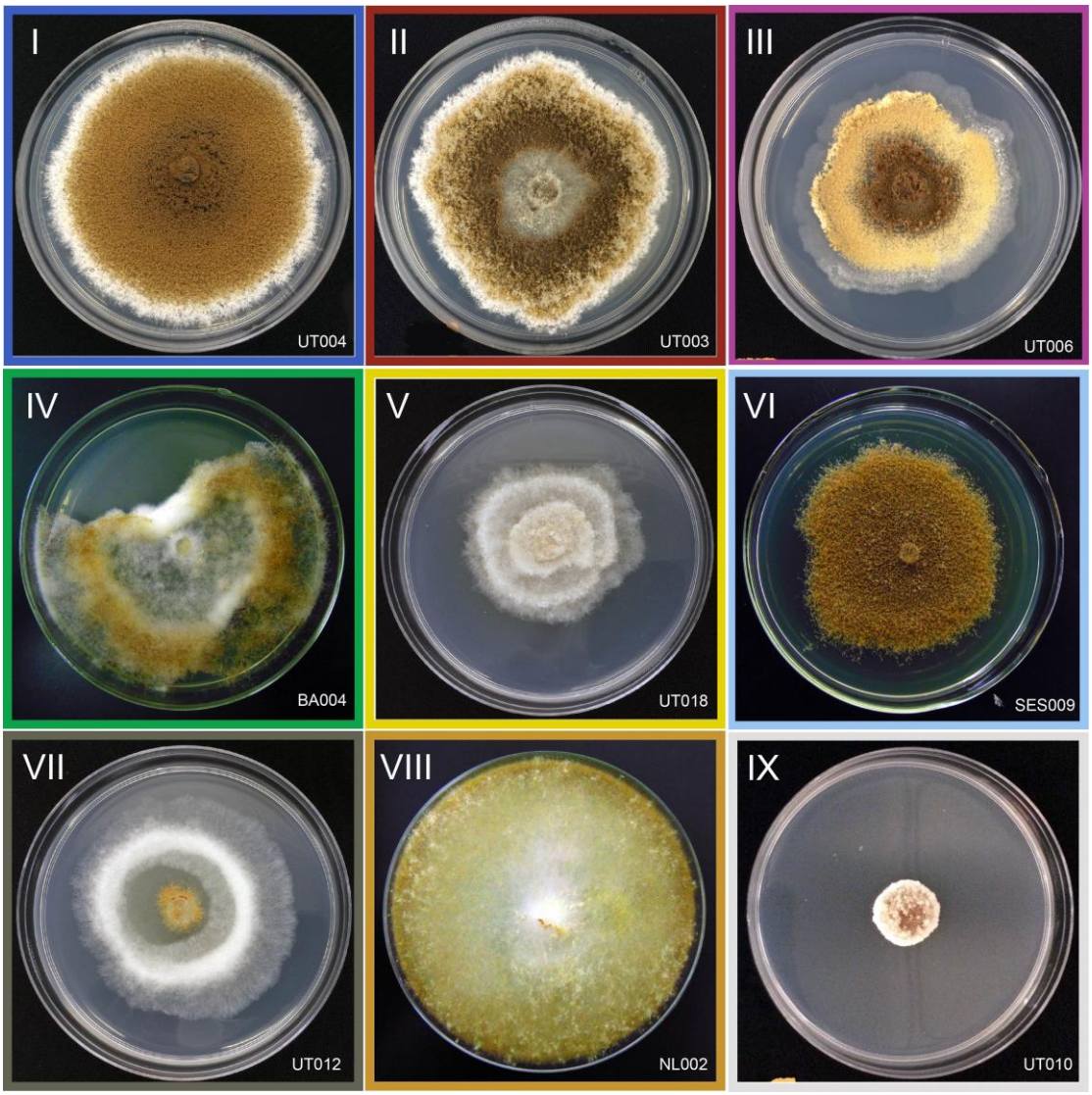


Fig. S2. Ancestral state reconstruction for vesicle type of *Escovopsis* displayed on the Bayesian consensus tree. Probabilities are shown in pie-graph format where color red indicated globose vesicles and color blue indicates cylindrical vesicles. The tree is the same displayed on Fig. 1 on the manuscript; however, here results are shown for detailed clades.

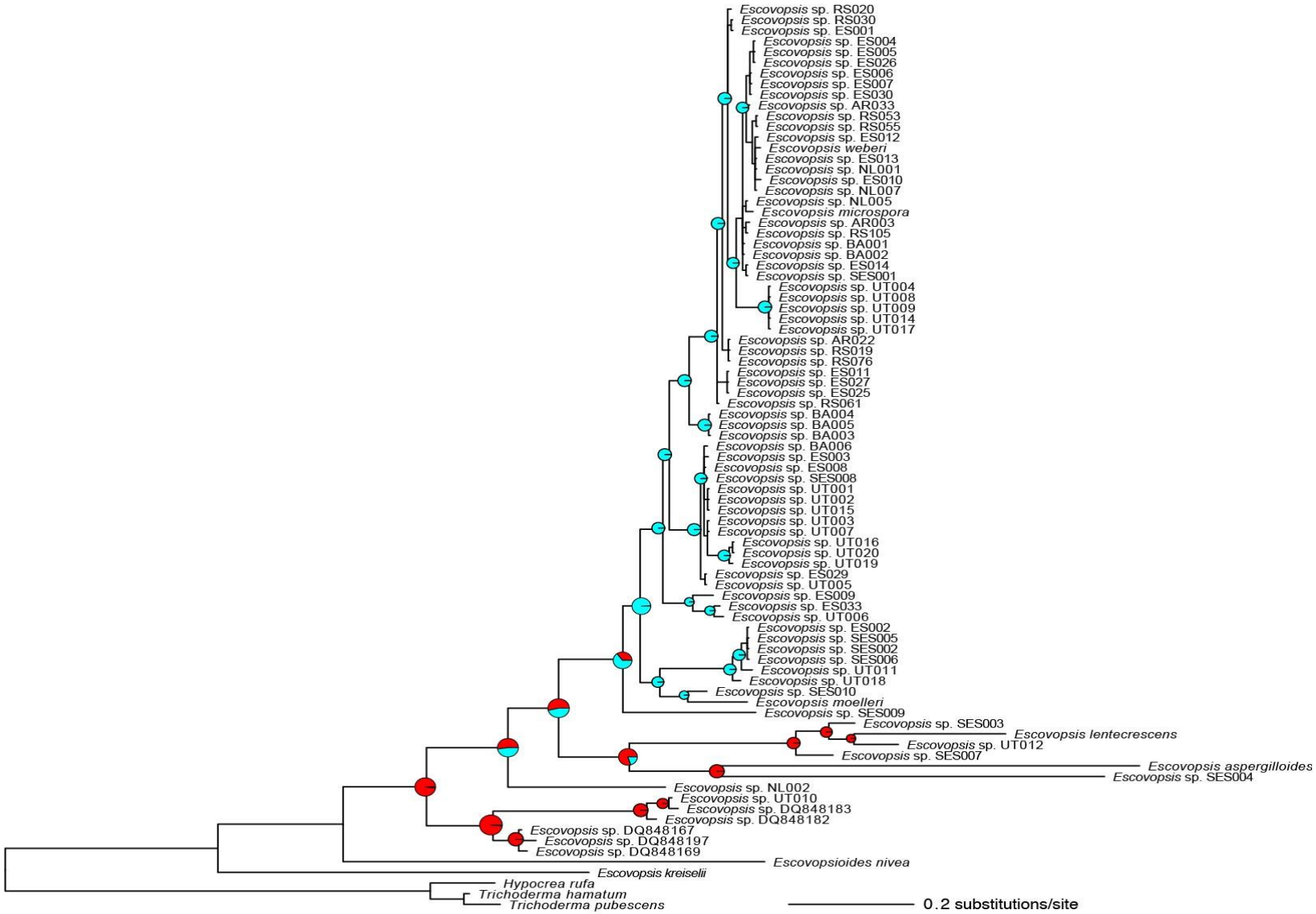


Table S1. *Escovopsis* strains analyzed in this study. Data from described species of brown-spored *Escovopsis* were derived from previous studies [16, 20]. It is also informed molecular data for *Escovopsioides nivea* and *Escovopsis kreiselii* [20, 21] as well as other brown-spored *Escovopsis* strains derived from Gerardo et al. [19]. Data from species of Hypocreaceae used as outgroup are also shown.

STRAIN ID	ANT	NEST ID	SOURCE	LOCAL	LOCAL ID	*tef1*	ITS
NL001[1]	*Atta capiguara*	N66	Midden	Botucatu - SP, Brazil	1	KM817142	KM817072
NL002[1]	*Atta capiguara*	N66	Fungus garden	Botucatu - SP, Brazil	1	KM817143	KM817073
NL005[2]	*Atta sexdens*	N68	Fungus garden	Botucatu - SP, Brazil	1	KM817144	KM817074
NL007[2]	*Atta sexdens*	N68	Fungus garden	Botucatu - SP, Brazil	1	KF240730	KM817075
ES002	*Atta sexdens*	N-Ale	Fungus garden	Rio Claro - SP, Brazil	2	KM817123	KM817053
ES011	*Atta sexdens*	6B	Fungus garden	Corumbataí - SP, Brazil	3	KM817132	KM817062
ES012	*Atta sexdens*	13B	Fungus garden	Corumbataí - SP, Brazil	3	KM817133	KM817063
ES013	*Atta sexdens*	1	Fungus garden	Corumbataí - SP, Brazil	3	KM817134	KM817064
ES014	*Atta sexdens*	4	Fungus garden	Corumbataí - SP, Brazil	3	KM817135	KM817065
ES003	*Atta cephalotes*	SES040121-01	Fungus garden	Frei Caneca - PE, Brazil	4	KM817124	KM817054
ES005	*Atta cephalotes*	SES040129-06	Fungus garden	Alta Floresta - MT, Brazil	5	KM817126	KM817056
ES009	*Atta cephalotes*	SES040220-01	Fungus garden	Carreiro da Varzea - AM, Brazil	6	KM817130	KM817060
ES033	*Atta cephalotes*	CTL110912-05	Fungus garden	Parauapebas - PA, Brazil	7	KM817141	KM817071
RS105	*Atta laevigata*	AOMB170904-02	Fungus garden	T. de Santa Bárbara - SP, Brazil	8	KM817145	KM817083
BA001	*Atta cephalotes*	BMSR120702-01(FL1)	Fungus garden	Camacan - BA, Brazil	9	KM817116	KM817046
BA002	*Atta cephalotes*	BMSR120803-02(CA7)	Fungus garden	Camacan - BA, Brazil	9	KM817117	KM817047
BA003[3]	*Atta cephalotes*	BMSR120803-01(CA10)	Fungus garden	Camacan - BA, Brazil	9	KM817118	KM817048
BA004[4]	*Atta cephalotes*	BMSR120703-01(FL5)	Fungus garden	Camacan - BA, Brazil	9	KM817119	KM817049
BA005[4]	*Atta cephalotes*	BMSR120703-01(FL5)	Fungus garden	Camacan - BA, Brazil	9	KM817120	KM817050
BA006[3]	*Atta cephalotes*	BMSR120803-01(CA10)	Fungus garden	Camacan - BA, Brazil	9	KM817121	KM817051
ES004	*Acromyrmex* sp.	SES040125-08	Fungus garden	Camacan - BA, Brazil	9	KM817125	KM817055
ES006	*Ac. coronatus*	SES040130-11	Fungus garden	Alta Floresta - MT, Brazil	6	KM817127	KM817057
ES007	*Ac. coronatus*	SES040131-07	Fungus garden	Alta Floresta - MT, Brazil	6	KM817128	KM817058
ES008	*Acromyrmex* sp.	SES040216-02	Fungus garden	Santarem - PA, Brazil	10	KM817129	KM817059
ES010	*Ac. landolti*	-	Fungus garden	Rio Claro - SP, Brazil	2	KM817131	KM817061
ES025	*Ac. balzanii*	AR05	Fungus garden	Botucatu - SP, Brazil	1	KM817136	KM817066
ES027	*Ac. rug. rugosus*	-	Midden	Rio Claro - SP, Brazil	2	KM817138	KM817068
RS019	*Ac. ambiguus*	AOMB060904-04	Fungus garden	Nova Petropolis - RS, Brazil	11	EU082802	KM817076
RS020	*Ac. laticeps*	AOMB060904-05	Fungus garden	Nova Petropolis -RS, Brazil	11	EU082803	KM817077
RS030	*Ac. lundi*	AOMB080904-02	Fungus garden	São Marcos - RS, Brazil	12	EU082795	KM817078
RS053	*Ac. lundi*	AOMB110904-02	Fungus garden	Chuvisca - RS, Brazil	13	EU082797	KM817079
RS055	*Ac. heyeri*	AOMB110904-07	Fungus garden	Chuvisca - RS, Brazil	13	EU082796	KM817080

RS061	*Ac. heyeri*	AOMB110904-15	Fungus garden	Pelotas - RS, Brazil	14	EU082799	KM817081
RS076	*Ac. coronatus*	AOMB130904-04	Fungus garden	Vacaria - RS, Brazil	15	EU082801	KM817082
SES008	*Acromyrmex* sp.	SES081007-01	Fungus garden	Faz. São Sebastião - RO, Brazil	16	KM817152	KM817091
AR003	*Ac. balzanii*	AR110511-01	Fungus garden	Ilhéus - BA, Brazil	17	KM817113	KM817043
AR022	*Acromyrmex* sp.	AR110515-01	Fungus garden	Camacan - BA, Brazil	9	KM817114	KM817044
AR033	*Acromyrmex* sp.	ARFV6110517-01	Fungus garden	Camacan - BA, Brazil	9	KM817115	KM817045
ES001	*Trachymyrmex* sp.	TR-117	Fungus garden	Rio Claro - SP, Brazil	2	KM817122	KM817052
ES026	*Trachymyrmex* sp.	ARTD030908-02	Fungus garden	Rio Claro - SP, Brazil	2	KM817137	KM817067
ES029	*Trachymyrmex* sp.	WGPM091021-01	Fungus garden	Palmas - TO, Brazil	18	KM817139	KM817069
ES030	*Trachymyrmex* sp.	AR091020-01	Fungus garden	Palmas - TO, Brazil	18	KM817140	KM817070
SES001	*Trachymyrmex* sp.	SES080402-03	Fungus garden	Rio Claro - SP, Brazil	2	KM817146	KM817084
SES002	*Trachymyrmex* sp.	SES080408-02	Fungus garden	Fazenda Pau Brasil - GO, Brazil	19	KM817147	KM817085
SES003	*Trachymyrmex* sp.	CTL080820-02	Fungus garden	E.E.Panga, Uberlândia - MG, Brazil	20	KM817148	KM817086
SES005	*Trachymyrmex* sp.	SES080922-03	Fungus garden	E.E.Panga, Uberlândia - MG, Brazil	20	KF240731	KM817088
SES006	*T. dichrous*	SES080922-02	Fungus garden	E.E.Panga, Uberlândia - MG, Brazil	20	KM817150	KM817089
SES007	*Trachymyrmex* sp.	SES080921-03	Fungus garden	E.E.Panga, Uberlândia - MG, Brazil	20	KM817151	KM817090
SES009	*Trachymyrmex* sp.	SES081108-04	Fungus garden	Palmeiras - BA, Brazil	21	KM817153	KM817092
SES010	*T. diversus*	SES090109-04	Fungus garden	Camp 41, Manaus - AM, Brazil	22	KM817154	KM817093
SES004	*S. luederwaldti*	CTL080825-02	Fungus garden	E.E.Panga, Uberlândia - MG, Brazil	20	KM817149	KM817087
Escovopsis weberi	Ant nest	-	-	Brazil	-	AY172623	KF293286
Escovopsis microspora	*Ac. sub. molestans.*	-	Fungus garden	Viçosa - MG, Brazil	23	KJ935030	JQ815076
Escovopsis moelleri	*Ac. sub. molestans.*	-	Fungus garden	Viçosa - MG, Brazil	23	JQ855712	JQ815077
Escovopsis lentecrescens	*Ac. sub. subterraneus*	-	Fungus garden	Viçosa - MG, Brazil	23	JQ855714	JQ815079
UT001	*Ac. octospinosus*	ASM031224-02	Fungus garden	Caribbean island of Guadeloupe	24	KM817155	KM817094
UT002	*Acromyrmex* sp.	ASM021224-07	Fungus garden	Caribbean island of Guadeloupe	24	KM817156	KM817095
UT003	*Acromyrmex* sp.	CC020602-06	Fungus garden	Gamboa, Panama	25	KM817157	KM817096
UT004[5]	*Atta colombica*	NMG010319-24	External dump	Gamboa, Panama	25	KM817158	KM817097
UT005	*Acromyrmex* sp.	SP030327-01	Fungus garden	Misiones, Argentina	26	KM817159	KM817098
UT006	*Atta cephalotes*	CC010324-14	Fungus garden	Gamboa, Panama	25	KM817160	KM817099
UT007	*Atta colombica*	NMG010319-25	External dump	Gamboa, Panama	25	KM817161	KM817100
UT008	*Atta colombica*	NMG010319-22	External dump	Gamboa, Panama	25	KM817162	KM817101
UT009[5]	*Atta colombica*	NMG010319-24	External dump	Gamboa, Panama	25	KM817163	KM817102
UT010	*Atta sexdens*	CC020529-01	Fungus garden	Coclecito, Panama	27	KM817164	KM817103
UT011	*Trachymyrmex* sp.	SES020522-02	Fungus garden	Gamboa, Panama	25	KM817165	KM817104
UT012	*Trachymyrmex* sp.	AL030106-09	Fungus garden	"Canal Zone", Panama	28	KM817166	KM817105
UT014	*Atta colombica*	UGM030106-16	Fungus garden	Darien, Panama	29	KM817167	KM817106
UT015	*Atta colombica*	UGM030106-17	Fungus garden	Darien, Panama	29	KM817168	KM817107
UT016	*Trachymyrmex* sp.	JS030114-01	Fungus garden	Palenque, Mexico	30	KM817169	KM817108
UT017	*Atta colombica*	NMG010319-22	External dump	Gamboa, Panama	25	KM817170	KM817109
UT018	*Trachymyrmex* sp.	SP011102-01	Fungus garden	Gamboa, Panama	25	KM817171	KM817110
UT019	*Atta cephalotes*	SES030113-01	Fungus garden	Palenque, Mexico	30	KM817172	KM817111
UT020	*Trachymyrmex* sp.	JS030113-01	Fungus garden	Palenque, Mexico	30	KM817173	KM817112
Escovopsis aspergilloides	*Trachymyrmex ruthae*	-	Fungus garden	Trinidad and Tobago	31	AY172632	KF293287
Escovopsioides nivea	*Ac. sub. subterraneus*	-	Fungus garden	Viçosa - MG, Brasil	23	JQ855713	JQ815078

Escovopsis kreiselii	*Mycetophylax morschi*	AR090306-01	Fungus garden	Florianópolis, SC, Brasil	-	KJ808766	KJ808767
Brown-spored *Escovopsis* derived from Gerardo et al. [19]							
Escovopsis sp.	*Apterostigma dentigerum*	AGH020629-02	Fungus garden	Costa Rica	-	DQ848167	-
Escovopsis sp.	*Apterostigma* sp.	AGH030627-03	Fungus garden	Ecuador	-	DQ848197	-
Escovopsis sp.	*Apterostigma dentigerum*	AGH020709-10	Fungus garden	Costa Rica	-	DQ848169	-
Escovopsis sp.	*Apterostigma dentigerum*	UGM020531-01	Fungus garden	Panama	-	DQ848183	-
Escovopsis sp.	*Apterostigma* sp.	AGH030627-08	Fungus garden	Ecuador	-	DQ848182	-
Other species of Hypocreaceae used as outgroup							
Hypocrea rufa	-	-		-	-	AF534585	X93980
Trichoderma hamaum	-	-		-	-	AF534620	EU280124
Trichoderma pubens	-	-		-	-	AF534624	NR_077179

Abbreviations: *Ac.* = *Acromyrmex*; *rug.* = *rugosus*; *sub.* = *subterraneus*; *S.* = *Sericomyrmex*; *T.* = *Trachymyrmex*.
[1,2,3,4,5] Numbers after the strain ID indicate *Escovopsis* isolated from the same nest.