MARCELA CRISTINA SILVA CAIXETA

Escovopsis (ASCOMYCOTA: HYPOCREALES), A PARASITE OF ATTINI ANT FUNGUS GARDENS: NEW SPECIES, VIRULENCE AND A CRITICAL REVIEW

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

Orientador: Simon Luke Elliot

Coorientador: André Rodrigues

VIÇOSA – MINAS GERAIS
2020

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
C138e
2020

Caixeta, Marcela Cristina Silva, 1992-
Escovopsis (Ascomycota: Hypocreales), a parasite of Attini ant fungus garden : new species, virulence and a critical review / Marcela Cristina Silva Caixeta. – Viçosa, MG, 2020.
143 f. : il. (algumas color.) ; 29 cm.

Orientador: Simon Luke Elliot.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. *Escovopsis*. 2. *Escovopsioides*. 3. Micoparasitismo. 4. Formicidae. 5. Simbiose. 6. Virulência. 7. Relação hospedeiro-parasito. I. Universidade Federal de Viçosa. Departamento de Entomologia. Programa de Pós-Graduação em Entomologia. II. Título.

CDD 22. ed. 579.5677

MARCELA CRISTINA SILVA CAIXETA

Escovopsis (ASCOMYCOTA: HYPOCREALES), A PARASITE OF ATTINI ANT FUNGUS GARDENS: NEW SPECIES, VIRULENCE AND A CRITICAL REVIEW

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

APROVADA: 30 de outubro de 2020.

Assentimento:

Marcela Cristina Silva Caixeta
Autora

Simon Luke Elliot
Orientador

AGRADECIMENTOS

Em primeiro lugar, agradeço a Deus por me dar forças nos momentos de dificuldades e por sempre guiar e iluminar os meus caminhos, dando-me sabedoria e serenidade quando necessário.

Agradeço aos meus pais, Marcelo e Cristina, por abrirem mão de qualquer coisa em prol da minha felicidade e realizações. Da mesma forma, agradeço aos meus irmãos, Fernanda e Mateus, pelo carinho e momentos de alegria em casa.

Aos meus avós, tios e primos pela torcida e confiança de sempre.

À Universidade Federal de Viçosa pela oportunidade de conhecimento e amadurecimento.

Ao Departamento de Entomologia, em especial à Eliane, que sempre foi amiga em vários momentos de desespero!

À equipe do Laboratório de Interações Inseto-Microrganismo, pelo apoio e contribuição ao meu crescimento profissional. É muito bom fazer parte de um grupo em que todos se ajudam de alguma forma. Em especial, agradeço ao meu orientador Simon Luke Elliot pelos anos de parceria e confiança e à Verônica por toda sua ajuda como técnica do laboratório. Obrigada a todos os demais colegas que passaram pelo laboratório nesses 9 anos com os quais pude aprender tanto sobre ciência e principalmente por me melhorarem como pessoa, aprendendo a conviver e respeitar as diferenças.

Aos amigos Marcos, Eduardo, Keminy e Elenir pelos vários momentos de alegrias e leveza que vivenciamos juntos. À Aline, Silma, Mayara, Débora e Fernanda, às quais não tenho mais o prazer da convivência diária, mas que estão sempre dispostas a me ajudar de onde quer que seja!

Ao sr. Manoel pela disponibilidade e animação para ir ao campo tantas vezes à espera da revoada.

Ao meu coorientador, professor André Rodrigues, pela receptividade, disponibilidade, auxílio e valiosas discussões, assim como os seus orientados que sempre se mostraram muito receptivos.

Ao meu amigo e parceiro Quimi Montoya por tantos ensinamentos e diversas correções. Nossas discussões foram essenciais para que eu chegasse até aqui. Nunca conseguirei agradecer o suficiente!

Ao Fábio Custódio, integrante do Laboratório de micologia e etiologia de doenças fúngicas de plantas, coordenado pelo professor dr. Olinto Liparini, pela assistência com as imagens de microscopia.

Ao pesquisador Harry Evans, que é fonte de inspiração, pelo incentivo, entusiasmo, conhecimento e valiosas sugestões para a realização desse trabalho.

Aos drs. André Rodrigues, Camila Moreira, Maria Augusta Siqueira e Vinícius Barros por terem aceito o convite de participar da banca dessa tese e por toda contribuição. O meu muito obrigada também às dras. Madelaine Venzon e Verônica Saraiva por terem aceito fazer parte dos membros suplentes.

Aos drs. André Rodrigues, José Henrique Schoereder, Lucas Magalhães Abreu e Vinícius Barros por terem aceito o convite de participar da banca de qualificação e por todas as sugestões.

À minha grande amiga e professora Fernanda Sickert pelos momentos de distração, por todo ensinamento e compreensão de sempre. Obrigada por me deixar fazer parte da sua linda família. E não se esqueça nunca que você é uma grande inspiração para mim! Sou muito grata por ter encontrado vocês!

Ao Cristian por todo amor, cuidado, carinho, paciência e atenção!

Muito obrigada a todos que de alguma forma contribuíram para a realização desse trabalho!!

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

Agradecemos também pelo apoio financeiro recebido através do programa Professor Visitante no Exterior (PVE-CsF-CAPES) e à “Fundação de Amparo à Pesquisa do Estado de São Paulo” (FAPESP 2019/03746-0).

RESUMO

CAIXETA, Marcela Cristina Silva, D.Sc., Universidade Federal de Viçosa, outubro de 2020. ***Escovopsis* (Ascomycota: Hypocreales), parasita do jardim de fungos das formigas Attini: novas espécies, virulência e uma revisão crítica.** Orientador: Simon Luke Elliot. Coorientador: André Rodrigues.

Escovopsis (Ascomycota: Hypocreales) é um fungo encontrado em associação com colônias de formigas que cultivam fungo (Hymenoptera: Formicidae: Tribo Attini, "atines"). Diversos outros micro-organismos são encontrados nas colônias de formigas atines, entre eles, outros fungos filamentosos, bactérias e leveduras. Alguns deles tem papéis ecológicos bem-definidos dentro da interação, mas é provável que a maioria dessas relações ainda seja pouco conhecidas. Esse é o caso de *Escovopsioides* (Ascomycota: Hypocreales). Sabe-se que esse fungo é filogeneticamente próximo a *Escovopsis* e que tem a capacidade de inibir o crescimento do fungo mutualista das formigas, mas de forma menos intensa que *Escovopsis*. Além disso, a partir da recente padronização dos métodos para descrição de novas espécies, duas espécies anteriormente consideradas do gênero *Escovopsis* estão sendo realocadas em dois novos gêneros: *Luteomyces* e *Sympodiorosea*. Esses achados ressaltam a importância de se investigar a diversidade de *Escovopsis* e dos outros fungos associados a ele. Estudos taxonômicos e filogenéticos contribuem para que as investigações ecológicas dentro do complexo sistema que as colônias de formigas atines representam sejam mais precisas. *Escovopsis* tem sido considerado uma ameaça para essas formigas principalmente por sua capacidade de causar danos no fungo consumido pelas formigas, sendo classificado, como micoparasita (fungo que parasita outro fungo). No entanto, esse tipo de relação é descrita em experimentos utilizando subcolônias (geralmente pequenas colônias sem rainha), fragmentos de jardins de fungos sem a presença da rainha e operárias ou ainda em experimentos de co-cultivo, onde os fungos são testados de forma isolada dos outros organismos do sistema. Diante dessas considerações, os objetivos gerais desta tese foram: (i) contribuir para o conhecimento da diversidade de formigas cortadeiras (um clado derivado dentro da tribo Attini); (ii) testar a virulência de *Escovopsis* e *Escovopsioides* em colônias e (iii) oferecer uma crítica revisão das considerações citadas acima sobre *Escovopsis* e os gênero relacionados a ele. No primeiro capítulo, nós realizamos amostragens dos jardins de fungos de duas espécies de formigas cortadeiras *Acromyrmex subterraneus subterraneus* e *Acromyrmex balzani*.

A partir de analises morfológicas e filogenéticas, nós propomos três novas espécies de *Escovopsis*: *Escovopsis* R6, *Escovopsis* R12 (associados a *A. subterraneus subterraneus*) e *Escovopsis* IT4-1 (associado a *A. balzani*). Esses resultados demonstram quão complexa pode ser a rede simbiótica encontrada em formigas atines, com a presença de diferentes espécies de fungos, além disso eles contribuem para nosso entendimento sobre a diversidade e história evolutiva de *Escovopsis*. No capítulo 2, nós testamos a hipótese que *Escovopsis* e *Escovopsioides* apresentam baixa virulência. Para isso, nós dividimos colônias de *A. subterraneus subterraneus* em três condições diferentes: (i) colônias completas – com rainha, operárias e jardim de fungo; (ii) subcolônias – colônias sem rainha e (iii) somente jardim de fungo, sem a presença da rainha e de nenhuma operária. Nossos resultados mostraram que subcolônias e jardins de fungos estão condenados à morte independente da exposição ou não aos fungos. *Escovopsis* e *Escovopsioides* não foram capazes de causar morte de colônias completas, embora *Escovopsis* apresente alguns efeitos prejudiciais nas colônias, principalmente nos primeiros dias após a inoculação. Finalmente, no terceiro capítulo nós revisamos as informações contidas na literatura sobre o micoparasita do jardim de fungos das formigas atines e apresentamos nossas ideias baseadas em recentes resultados. Ao contrário de estudos anteriores, nós propomos que *Escovopsis* possui baixa virulência em colônias saudáveis de formigas atines, exigindo uma mudança fundamental na visão atual deste fungo e seus parentes.

Palavras-chave*: Escovopsis*. *Escovopsioides.* Micoparasitismo. Formigas atines. Simbiose. Virulência. Interações parasita-hospedeiro.

ABSTRACT

CAIXETA, Marcela Cristina Silva, D.Sc., Universidade Federal de Viçosa, October, 2020. ***Escovopsis* (Ascomycota: Hypocreales), a parasite of Attini ant fungus gardens: new species, virulence and a critical review.** Adviser: Simon Luke Elliot. Co-adviser: André Rodrigues

Escovopsis (Ascomycota: Hypocreales) is a fungus found in association with fungus-growing ants (Hymenoptera: Formicidae: Tribe Attini, "attines"). Several other microorganisms are found in the colonies of attine ants, among them, other filamentous fungi, bacteria and yeasts. Some of these have well-defined ecological roles within the interaction, but it is likely that most of these relationships are still poorly understood. This is the case of *Escovopsioides* (Ascomycota: Hypocreales). It is known that this fungus is phylogenetically close to *Escovopsis* and that it has the ability to inhibit the growth of the ants' mutualistic fungus, but less intensely than *Escovopsis*. In addition, from the recent standardization of methods for describing new species, two species previously considered within *Escovopsis* are being relocated into two new genera: *Luteomyces* and *Sympodiorosea*. These findings highlight the importance of investigating the diversity of *Escovopsis* and the other fungi associated with it. Taxonomic and phylogenetic studies contribute accuracy and help define ecological investigations of interactions within the complex systems that attine ant colonies represent. *Escovopsis* has long been considered a threat to these ants mainly because of its ability to damage the fungus consumed by ants, being classified as a mycoparasite (a fungus that parasitizes another fungus). However, this type of relationship has been described in experiments using sub-colonies (usually small colonies without a queen), fungus gardens fragments without the presence of the queen and workers, or even in co-cultivation experiments, where fungi are tested in isolation from other organisms that comprise the system. In the light of these considerations, the general objectives of this thesis were: (i) to contribute to knowledge of the diversity of *Escovopsis* found in association with leaf-cutting ants (a clade derived from the Attini tribe); (ii) to test the virulence of *Escovopsis* and *Escovopsioides* in colonies and (iii) to offer a critical review of the above considerations of *Escovopsis* and its relatives. In the first chapter, we sampled the fungus gardens of two species of leaf-cutting ants *Acromyrmex subterraneus subterraneus* and *Acromyrmex balzani*. From morphological and phylogenetic analyses, we propose three new species: *Escovopsis* R6, *Escovopsis* R12

(associated with *A. subterraneus subterraneus*) and *Escovopsis* IT4-1 (associated with *A. balzani*). These results demonstrate how complex the network symbiotic found in attine ants can be, with the presence of different fungi species; in addition, they contribute to our understanding of the diversity and evolutionary history of *Escovopsis*. In chapter 2, we tested the hypothesis that *Escovopsis* and *Escovopsioides* have low virulence. For this, we divided colonies of *A. subterraneus subterraneus* in three different conditions: (i) queenright colonies – with queen, workers and fungus garden; (ii) queenless colonies – colonies without a queen and (iii) only fungus garden, without the queen and any workers. Our results showed that queenless colonies and fungus garden are fated to death regardless of exposure to fungi. *Escovopsis* and *Escovopsioides* were not able to cause the death of queenright colonies although *Escovopsis* shows some harmful effects on the colonies mainly in the first days after inoculation. Finally, in the third chapter we review the information contained in the literature on the mycoparasite of the attines' fungus garden and present our ideas based on recent results. Contrary to previous studies, we propose that *Escovopsis* has low virulence in healthy colonies of attine ants, requiring a fundamental change in the current view of this fungus and its relatives.

Keywords: *Escovopsis. Escovopsioides.* Mycoparasitism. Attine ants. Symbiosis. Virulence. Host-parasite interactions.

SUMMARY

General Introduction

Colonies of Attine ants (Hymenoptera: Formicidae) are known to maintain an obligatory mutualism with basidiomycete fungi that they cultivate as their main source of food (Weber 1972). Meanwhile, the fungi depend on the ants to ensure their dispersion, nutrition and protection against other microorganisms. However, the symbiotic relationships in this system goes beyond these two organisms. It is known that there are many other filamentous fungi, yeasts and bacteria that share this habitat, maintaining a complex association of mutualists and parasites (Currie et al. 1999a; 1999b; Rodrigues et al. 2005; 2008; Little and Currie 2007). Among these microorganisms, the fungus *Escovopsis* (Ascomycota: Hypocreales), described in 1990 by Muchovej and Della Lucia, is considered a specialized mycoparasite of fungus cultivated by ants (Currie et al. 1999a). *Escovopsioides,* a genus phylogenetically associated to *Escovopsis*, was found in Attini ant colonies and seems to have an antagonistic relationship with attines (Augustin et al. 2003; Varanda-Haifig et al. 2017; Osti and Rodrigues 2018).

Escovopsis is found in healthy colonies that are actively foraging (Rodrigues et al., 2008; Augustin et al. 2013) yet it has been considered a highly virulent parasite (Currie et al. 1999a; Currie 2001). Studies of *Escovopsis* have intensified in the last 20 years, but they have not shown clearly how it is transmitted or how it infects new colonies. To investigate the possibility of vertical transmission, studies have sampled fragments of the fungus garden transported in the infrabuccal cavity of the queens before the nuptial flight (Currie et al. 1999a; Pagnocca et al. 2008; Moreira et al. 2015). *Escovopsis* has never been isolated using that method. Usually, some of these studies are performed by the collection of queens immediately after the nuptial flight. About 24 hours later, the females regurgitate the fungal pellet contained in the infrabuccal cavity, which is then collected and placed in culture medium. We carried out a similar study, however, in an attempt to

stimulate the growth of *Escovopsis*, we added the symbiont fungus of the leafcutter ants on the same plate containing the pellet carried by the ants. As in previous studies, we did not observe *Escovopsis* growth in any sample (unpublished data). However, it may be that culture-dependent methodologies are inadequate to detect the presence of *Escovopsis* (if it is indeed present in the pellet). It is conceivable that vertical transmission is being discarded due to a possible mechanism of dormancy used by the mycoparasite at the beginning of the foundation of the new colony. It could spend time "unnoticed" by the ants causing no apparent damage in the colony.

Recently, some evidence has highlighted the possibility of horizontal transmission in *Acromyrmex* colonies. Experiments carried out in the field and in the laboratory have shown that conidia can fix to workers' legs and thus potentially reach the colony phoretically and establish new infections (Augustin et al. 2017). In addition, in this same study, it was demonstrated that conidia may become dormant and germinate in the presence of the host fungus. From this, conidia that are outside the colony (in the midden piles, for example) can be carried by workers or other arthropods, contaminating other colonies, even of other species. Another suggested form of transmission would be from inquiline arthropods associated with attine ant colonies that can migrate between colonies (Currie 2001; Augustin et al. 2017).

All these transmission possibilities and virulence level may be related to the peculiar morphological characteristics of each species or groups of species and are probably not unique to the genus nor mutually exclusive. Considering that in fact there are variations in morphology and virulence between strains or species (Silva et al. 2006; Wallace et al. 2014; Marfetán et al. 2015), where each group can have its own infection strategy, it becomes essential to describe the taxonomy of this genus as well as establishing its phylogenetic relationships where possible. However, the description of

new species has been performed under different conditions, which has hindered the delimitation of the genus. A recent study has shown that two species previously considered within of genus *Escovopsis* actually belong to two new genera (Montoya et al. 2020 – submitted).

The general objectives of this research were to assess *Escovopsis* diversity, to test its virulence and finally to present a review to discuss and present our considerations and ideas about the possible role of *Escovopsis* in the complex system of the attine ants.

Outline of the Thesis

In chapter 1, we present three new species of *Escovopsis* found from collections in three regions of fragments of Atlantic Forest in the state of Minas Gerais, Brazil. The new species were found in colonies of two species of leafcutter ants: *Acromyrmex subterraneus subterraneus* and *Acromymex balzani*. Although they are of the same genus, these ants cut fresh leaves of dicotyledons and monocotyledons, respectively, a difference that may be important for the parasites´ infection strategies.

In chapter 2, we tested the hypothesis that *Escovopsis* presents low virulence to healthy colonies, in contrast to the widespread to present the supposedly high virulence of *Escovopsis* as a fact. We considered that the methodology used in most studies may not reflect what actually occurs in nature, leading to erroneous conclusions. To see if the virulence of *Escovopsis* virulence can vary with different conditions, we utilized three complexity levels: (i) queenright colonies (including queen, workers and fungus garden), (ii) queenless colonies (workers and fungus garden, without queen) and (iii) only fungus garden (without any ants). From this experiment we confirmed the importance of maintaining all the components involved in the leafcutter ants-fungus symbiosis and showed that *Escovopsis* is not a virulent parasite. (This chapter was also part of the

master's thesis of Débora Mello Furtado de Mendonça titled as "Virulence of the fungi *Escovopsis* and *Escovopsioides* to the leafcutter ant-fungus symbiosis".)

To conclude this thesis, in chapter 3 we review historical, taxonomic, phylogenetic and ecological aspects of *Escovopsis*. Besides this, we describe the steps that led *Escovopsis* fungus to be considered a specialized mycoparasite that is highly virulent to the fungus garden of the attine ants and showed critically why we cannot agree with that conclusion. In this review, we suggest a new view of this topic with broad and critical discussions considering the recent findings carefully. We propose that *Escovopsis* has low virulence in healthy colonies and this strategy can change when the colony is collapsing for other reasons.

References

Augustin, J. O., Groenewald, J. Z., Nascimento, R. J., Mizubuti, E. S. G., Barreto, R.W., Elliot, S. L., & Evans, H. C. (2013). Yet more "weeds" in the garden: fungal novelties from nests of leaf-cutting ants. *PLoS ONE*, 8, e82265. https://doi.org/10.1371/journal.pone.0082265

Augustin, J. O., Simões, T. G., Dijksterhuis, J., Elliot, S. L., & Evans, H. C. (2017). Putting the waste out: a proposed mechanism for transmission of the mycoparasite *Escovopsis* between leafcutter ant colonies. *Royal Society Open Science*, 4, 161013. http://dx.doi.org/10.1098/rsos.161013

Currie, C. R., Mueller, U. G., & Malloch, D. (1999a). The agricultural pathology of ant fungus gardens. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 7998–8002. https://doi.org/10.1073/pnas.96.14.7998

Currie, C. R., Scott, J. A., Summerbell, R. C., & Malloch, D. (1999b). Fungus-growing ants use antibiotic producing bacteria to control garden parasites. *Nature*, 398, 701–704. https://doi.org/10.1038/nature01563

Currie, C. R. (2001). Prevalence and impact of a virulent parasite on a tripartite mutualism. *Oecologia*, 128, 99–106. https://doi.org/10.1007/s004420100630

Silva, A., Rodrigues, A., Bacci Jr., M., Pagnocca, F. C., & Bueno, O. C. (2006). Susceptibility of the ant-cultivated fungus *Leucoagaricus gongylophorus* (Agaricales: Basidiomycota) towards microfungi. *Mycopathologia*, 162, 115–119. http://dx.doi.org/10.1007/s11046-006-0037-6

Little, A. E. F. and Currie, C. R. (2007) Symbiotic complexity: discovery of a fifth symbiont in the attine ant – microbe symbiosis. Biology letters, 3, 501-504. doi: 10.1098/rsbl.2007.0253

Marfetán, J. A., Romero, A. I., & Folgarait, P. J. (2015). Pathogenic interaction between *Escovopsis weberi* and *Leucoagaricus* sp.: mechanisms involved and virulence levels. *Fungal Ecology*, 17, 52–61. http://dx.doi.org/10.1016/j.funeco.2015.04.002

Moreira, S. M., Rodrigues, A., Forti, L. C., & Nagamoto, N. S. (2015). Absence of the parasite *Escovopsis* in fungus garden pellets carried by gynes of *Atta sexdens*. *Sociobiology*, 62, 34–38. http://dx.doi.org/10.13102/sociobiology.v62i1.34-38

Muchovej, J.J., Della Lucia, T. M. C. *Escovopsis*, a new genus from leaf cutting ant nests to replace *Phialocladus* nomen invalidum. *Mycotaxon*, v. 37, p. 191–195.

Pagnocca, F. C; Rodrigues, A. Nagamoto, N. S. Bacci Jr. Yeasts and filamentous fungi carried by the gynes of leaf-cutting ants. *Antonie Van Leeuwenhoek***,** v. 94, n. 4, p. 517-26, 2008.

Rodrigues, A.; Pagnocca, F. C., Bacci Jr., M., Hebling, M. J. A., Bueno, O. C., Pfenning, L. H. (2005a). Variability of non-mutualistic filamentous fungi associated with *Atta sexdens rubropilosa* nests. *Folia Microbiologica*, v. 50, p. 421425,

Rodrigues, A., Pagnocca, F. C., Bueno, O. C., Pfenning, L. H., & Bacci Jr., M. (2005).

Assessment of microfungi in fungus gardens free of the leaf-cutting ant *Atta sexdens rubropilosa* (Hymenoptera: Formicidae). *Sociobiology*, 46, 329–334.

Rodrigues, A., Bacci Jr., M. , Mueller, U. G., Ortiz, A., & Pagnocca, F. C. (2008). Microfungal "weeds" in the leafcutter ant symbiosis. *Microbial Ecology*, 56, 604–614. http://dx.doi.org/10.1007/s00248-008-9380-0

Wallace, D. E. E., Asensio, J. G. V., & Tomás, A. A. P. (2014). Correlation between virulence and genetic structure of *Escovopsis* strains from leaf-cutting ant colonies in Costa Rica. *Microbiology*, 160, 1727–1736. https://doi.org/10.1099/mic.0.073593-0

Weber, N. A. (1972). Gardening Ants, the Attines. *Science***,** v. 153, p. 587-604, 1966.

Chapter 1: Three new species of the hypocrealean fungus *Escovopsis* that confirm the great diversity of the genus

Marcela Cristina Silva Caixeta[1], Daniele de Fátima Alves Venâncio[1], Débora Mendonça Melo Furtado[1], Camila Moreira[1], Quimi Vidaurre Montoya[2], André Rodrigues[2], Simon Luke Elliot[1]

1 Department of Entomology, Federal University of Viçosa, UFV, Viçosa, MG, Brazil

[2] Department of General and Applied Biology, São Paulo State University, UNESP, Rio Claro, SP, Brazil

*Corresponding author: Simon Luke Elliot (selliot@ufv.br)

Universidade Federal de Viçosa – Departamento de Entomologia

Campus Universitário, Viçosa (MG), Brazil, 36570-900

Phone: +55 31 3612-3025

Abstract

Attini ants are known to grow a symbiotic basidiomycete fungus as their main source of nutrients, a mutualistic association that originated about 50 million years ago. Depending on the condition of the ant colonies, this association can be threatened by the fungus *Escovopsis*, considered a specialized mycoparasite of the mutualistic fungus of the ants. While the impact of *Escovopsis* on this mutualism, and the mutualists' defences, have been the object of study, the taxonomy and phylogenetic relationships of *Escovopsis* remain uncertain. In this study, our proposal was to explore *Escovopsis* diversity through sampling of the fungus garden of two species of leafcutter ants. Based on morphological and phylogenetic analysis, we propose three new species: *Escovopsis* R6 and *Escovopsis* R12, isolated from the fungus garden of *Acromyrmex subterraneus subterraneus* and *Escovopsis* IT4-1 found on *Acromyrmex balzani*. *Escovopsis* R6 presented different vesicles shapes and it grouped close to *E. aspergilloides* and *E. lentecrescens* which have predominantly globose vesicles. The closest known clade to *Escovopsis* R12 is *E. moelleri*, yet *E. moelleri* is characterized by the presence of relatively large conidia with a distinct apical cap-like structure, not observed in the new species. *Escovopsis* IT4-1 is phylogenetically related to the *Escovopsis* group characterized predominantly by cylindrical vesicles and considered the most derived clade within the genus. Until 2019, there were only seven species within the genus. Recently, however, seven more species have been described in other studies. The knowledge generated from these studies, as well from this present study, may help in a more detailed understanding of the symbiotic relationships between these fungi besides highlighting the great diversity of this group.

Key-words: Attini, Formicidae, leaf-cutting ants, Hypocreaceae, host-parasite interactions, social insects

Introduction

Complex symbiotic networks found in fungus-growing ants (Formicidae: Myrmicinae: Attini) have been of great interest due to the variety of interactions between completely different organisms (Chapela et al. 1994; Hinkle et al. 1994; North et al. 1997; Mueller et al. 1998). These ants cultivate basidiomycete fungal symbionts as their main source of food and, in turn, the ants promote the growth and dispersion of these fungi, that can be maintained as a mycelium or as masses of yeast (the latter observed in the ant genus *Cyphomyrmex*) (Weber 1972).

This ant-fungus mutualistic coevolution has been exploited by parasitic fungi within the genus *Escovopsis* (Ascomycota: Hypocreales: Hypocreaceae) (Currie et al. 1999b; Currie 2001). Although many studies have investigated the role of this parasite the symbiosis to elucidate the relationship between Attini ants and *Escovopsis*, little is known about its taxonomy and phylogeny. Until 2019, only seven species were formally described, although there is a great diversity of isolates unknown (Gerardo et al. 2006; Taerum et al. 2010; Meirelles et al. 2015).

Considering that isolates of *Esvovopsis* may vary in their morphology, virulence (Silva et al. 2006; Wallace et al. 2014; Marfetán et al. 2015; Meirelles et al. 2015; Montoya et al. 2019) and their mechanisms of infection (as suggested by Augustin et al. 2017), it becomes essential to describe the taxonomy of this genus. Currie et al. (2003a) reported that specific clades of *Escovopsis* are associated with specific clades in the basal attines. However, this association is not widely observed among leafcutter ants (Taerum et al. 2007; 2010; Meirelles et al. 2015b). In 2013, for example, three new species of *Escovopsis* were described associated only with the leafcutter *Acromyrmex subterraneus* (Augustin et al. 2013). Meirelles et al. (2015b) confirmed, in a study of *Escovopsis* isolates from several places in the Americas that some of these fungi that infect higher

attines are not monophyletic. It is important to emphasize that from this study, as well as previous ones (Currie et al. 2003a; Gerardo et al. 2006; Meirelles et al. 2015b), there seems to be no exchange of parasitic fungi between higher and lower attines.

In this study, our proposal was to collect the fungus garden of five species of leafcutter ants to examine the diversity of *Escovopsis*. The possibility of finding new species can contribute to understand and explain the biodiversity of fungi associated with these ants, including morphological diversity, phylogenetic patterns and ecological processes that determine this symbiotic relationship.

Material and methods

Fungus collection

Samples of *Escovopsis* were isolated from 66 colonies of leafcutter ants in three regions of the southeastern state of Minas Gerais, Brazil, during the years 2016 and 2017 (Table S1). Ten fragments of fungus garden of each colony (five per plate) were taken with the aid of a sterile forceps and inoculated on plates containing malt extract and agar (MA) supplemented with 150 μg ml^{-1} of chloramphenicol (Sigma-Aldrich) and incubated at 25ºC. All plates were then observed daily under a stereoscope in order to observe the presence of *Escovopsis*. When *Escovopsis* was detected – through morphological characteristics – hyphal fragments and conidia were transferred to new plates of MA. To obtain pure culture, hyphal tips were removed immediately after the fungus begin its growth (1-3 days after inoculation) and re-isolated. Isolates from pure culture were preserved in 10% glycerol at 3 ℃ at UFV – Laboratory of Insect-Microorganism Interactions (LIIM), Viçosa, Brazil.

Extraction, PCR and sequencing

Fungi were grown for 10 days in plates containing 100 ml of liquid medium (10 g of sucrose, 2 g L-asparagine, 2 g yeast extract, 1 g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$, 0.44 mg $ZnSO_4.7H_2O$, 0.48 mg $FeCl_3.6H_2O$ and 0.36 mg $MnCl_2.H_2O$). DNA of all isolates was extracted using the Wizard Genomic purification kit protocol, following the manufacturer's instructions. Three genomic regions were amplified by PCR: ITS rDNA (Internal Transcribed Spacer) (White et al. 1990, Schoch et al. 2012), LSU rDNA (Large Subunit) (White et al. 1990, Haugland and Heckman 1998, Currie et al. 2003) and *tef*1 (Translation Elongation Factor 1-alpha) (Taerum et al. 2007). Primer pairs and the PCR conditions were set as shown in Table S2. Reaction results of the ITS region were

sequenced in ABI3500 (Life Technologies) and LSU and *tef*1 regions were sent to Macrogen Inc (dna.macrogen.com/eng/, Seoul, S. Korea) for purification and sequencing. Sequences were attached in contigs with BioEdit v. 7.1.3 (Hall 1999).

Morphological characters

The isolates were grown in three culture media at four different temperatures (according to the method established by QVM and AR, *unpublished data*). Culture media proposed for a more effective comparison after evaluation of all type strains are: (i) malt extract agar (MEA): 30 g malt extract, 20 g dextrose, 5 g peptone, 18 g agar, 1 liter sterile water; (ii) corn meal dextrose (CMD): 50 g corn meal infusion, 20 g dextrose, 15 g agar, 1 liter sterile water and (iii) potato, dextrose and agar (PDA, KASVI®). Temperatures are 10°, 20°, 25° and 30°C.

All isolates were previously grown in water agar for seven days, from a spore suspension spread on 9 cm Petri dishes and incubated at 25 °C. Subsequently, plugs of 5 mm diameter were made in the plates of all isolates and each plug containing one of the isolates was placed at all temperatures and culture media proposed. Four replicates of each combination (isolate × culture medium × temperature) were made. Plates were inspected daily and growth analyses were performed on the fourth day.

For the microscopic characters, we also used the method of slide culture adapted by QVM (*unpublished data*). Slides were prepared with the cultures growing in PDA medium for 2-3 days, incubated at 25 °C. The structures were visualized and photographed in LAS EZ v.4.0 (Leica Application Suite) and then measured in ImageJ software. We perform 30 replicates of each structure of interest.

Phylogenetic analysis

A total of 66 sequences were used in the phylogenetic analysis. Of this total, 35 *Escovopsis* sequences were obtained from previous studies (Seifert et al. 1995; Augustin et al. 2013; Meirelles et al. 2015a, b; Masiulionis et al. 2015; Marfetán et al. 2018; Montoya et al. 2019). In addition, this dataset includes 7 *Escovopsis* sequences resultant from this present study, 16 sequences of *Escovopsioides* strains and 8 fungal species that were used as outgroups for all analyses (Chaverri et al. 2003; Põldmaa 2011) (Table S3).

All sequences were aligned using MAFFT v. 7 (Katoh and Standle 2013). Phylogenetic analyses were first carried out individually for each genomic region and then all the data were concatenated in a single file using Winclada v.1.00.08 (Nixon 2002). A nucleotide substitution model was based on the Akaike Information Criterion (AIC) previously calculated in jModelTest (Darriba et al. 2012) 2.3 using a 95% confidence interval. Phylogenetic reconstructions were performed using Bayesian Inference (BI) in MrBayes v.3.2.2 (Ronquist et al. 2012) in which we generated two million trees which were sampled every one hundred generations and 25% of the trees were discarded as a burning fraq. Additionally, Maximum Likelihood (ML) analyses were performed in RAxML v.8 (Stamatakis 2014) with 1,000 independent trees and 1,000 bootstrap replicates (MLB). The GTR + G + I model was used for the all regions using BI and the GTR + G model for ML analyses. Final editions in the trees were done using FigTree v.1.4 and Adobe Photoshop v. 10.0.

Results

Morphological analyses

New isolates were compared on three culture media and at four different temperatures. All species analysed exhibit the characteristic that gives name to the genus: brush-like conidiophores with well-defined phialides on vesicles (Muchovej and Della Lucia et al. 1990), each, however, with specificities that separate them into new morphological species. In general, they present floccose-like mycelium found in other species of the genus (Montoya et al. 2019), but with some peculiarities in relation to the temperatures and culture media. We did not observe floccose-mycelium in the species described here that grew at any temperature on CMD medium, for example. In this culture medium, the mycelium is very shallow, does not cover the entire plate and the fungus rapidly begins its sporulation. Regarding sporulation, in all species, the conidia present are hyaline at first, then becoming different shades of brown.

Escovopsis sp. R6 grew only at 20 and 25 °C and exhibited short and shallow development on CMD. Among the fungi described here, it is the most susceptible to contamination. *Escovopsis* R6 presents variable types of vesicle and conidophores shapes. Phylogenetically, R6 is close to *E. aspergilloides* and *E. lentecrescens*, both characterized by predominance of globose vesicles (Seifert et al. 1995; Augustin et al. 2013), which are not present in new species described here. Augustin (2013) used two different culture media from those used here – oatmeal agar (OA) and potato, carrot and agar (PCA); however, similar to *E. lentecrescens*, *Escovopsis* R6 also grows slowly in relation to most *Escovopsis* strains.

Escovopsis R24 grew at 20 and 25 °C in all the culture media, although in CMD its growth was minimal. Conidia are powdery, an uncommon characteristic for the genus, where conidia tend to stick together (see Augustin et al. 2017), and light brown coloration

when mature. By the seventh day, conidia are still white, but in PDA and at 25 °C they have already grown over all the plate. At 20 °C we could observe that *Escovopsis* R24 sporulation started later than at 25 °C, besides presenting smaller mycelial growth. The closest known group phylogenetically is *E. moelleri*, characterized by large conidia (7–10 × 3.0–3.5 μm) and a distinct cap-like structure apically (Augustin et al. 2013). We could not observe these characters here. Unfortunately, we could not determine in which culture medium these structures were produced by *E. moelleri*.

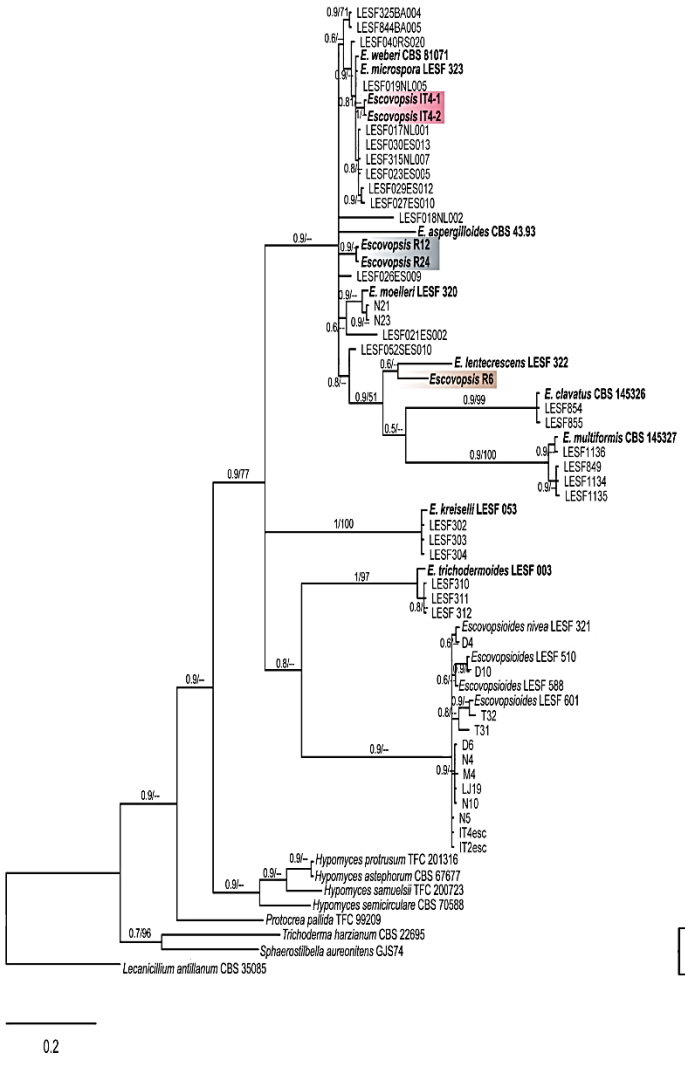
Escovopsis IT4-2 grew in all the temperatures and culture media. Generally, this fungus demonstrated fast growth, dense aerial mycelium reaching the plate lid and dark brown exudate droplets (on PDA and MEA at 25 and 30 °C). On PDA culture medium, colonies vary in coloration from white to light or dark brown (at 25 °C and 30°C, respectively). Colonies on CMD presented a more yellowish brown as the spores mature and a reddish-brown on MEA.

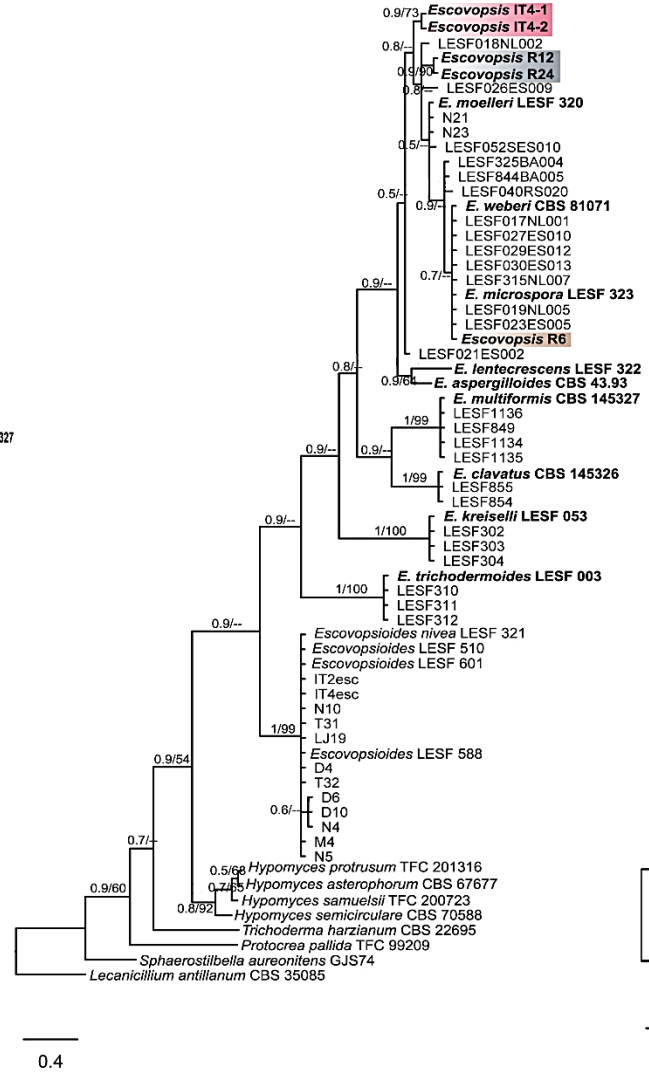
Phylogenetic analyses

We analyzed three partial sequences in the genomes of *Escovopsis* strains: LSU and ITS (non-protein coding genes of the nuclear ribosomal RNA gene operon) plus *tef*1 (part of the protein coding gene). Phylogenetic analyses were realized apart for each region (Fig. 7) and concatenated (Fig. 8), from Bayesian Inference (BI) and Maximum Likelihood (ML) methods. Generally, both phylogenetic construction methods showed similar topologies. Although phylogenetic positions of the three new species proposed here have presented conflicts between the three molecular markers, only *Escovopsis* R6 was not concordant as a new phylogenetic species in the tree inferred from the LSU marker.

The concatenated analysis showed *Escovopsis* R6 (PP=0.9), *Escovopsis* R12 (PP=1; MLB=100%) and *Escovopsis* IT4-1 (PP=1; MLB=100%) as well-supported new

phylogenetic clades (Fig. 8). Both phylogenetic analyses showed that *Escovopsis* R6 shared the same ancestor with *E. aspergilloides* and *E. lentecrescens*; however, the BI and ML analysis placed *E. aspergilloides* and *E. lentecrescens* as its closest relatives, respectively. On the other hand, *Escovopsis* R12 grouped into a monophyletic clade with the strain LESF021. Both strains shared the same ancestor with the clade to which *E. moelleri* belongs. *Escovopsis* IT4-2 grouped close to *E. microspora* and *E. weberi*. Although the new species is actually very morphologically similar to the two species mentioned above, phylogenetically, they have a well-supported clade in both analyses (ML and Bayesian, below). Besides, *Escovopsis* IT4-2 formed a monophyletic clade with the strain LESF 026 in both BI and ML analyses.


LESF325BA004
LESF844BA005
LESF040RS020
E. weberi CBS 81071
E. microspora LESF 323
LESF019NL005
Escovopsis IT4-1
Escovopsis IT4-2
LESF017NL001
LESF030ES013
LESF315NL007
LESF023ES005
LESF029ES012
LESF027ES010
LESF018NL002
E. aspergilloides CBS 43.93
Escovopsis R12
Escovopsis R24
LESF026ES009
E. moelleri LESF 320
N21
N23
LESF021ES002
LESF052SES010
E. lentecrescens LESF 322
Escovopsis R6
E. clavatus CBS 145326
LESF854
LESF855
E. multiformis CBS 145327
LESF1136
LESF849
LESF1134
LESF1135
E. kreiselli LESF 053
LESF302
LESF303
LESF304
E. trichodermoides LESF 003
LESF310
LESF311
LESF 312
Escovopsioides nivea LESF 321
D4
Escovopsioides LESF 510
D10
Escovopsioides LESF 588
Escovopsioides LESF 601
T32
T31
D6
N4
M4
LJ19
N10
N5
IT4esc
IT2esc
Hypomyces protrusum TFC 201316
Hypomyces asteophorum CBS 67677
Hypomyces samuelsii TFC 200723
Hypomyces semicirculare CBS 70588
Protocrea pallida TFC 99209
Trichoderma harzianum CBS 22695
Sphaerostilbella aureonitens GJS74
Lecanicillium antillanum CBS 35085
0.2


Escovopsis IT4-1
Escovopsis IT4-2
LESF018NL002
Escovopsis R12
Escovopsis R24
LESF026ES009
E. moelleri LESF 320
N21
N23
LESF052SES010
LESF325BA004
LESF844BA005
LESF040RS020
E. weberi CBS 81071
LESF017NL001
LESF027ES010
LESF029ES012
LESF030ES013
LESF315NL007
E. microspora LESF 323
LESF019NL005
LESF023ES005
Escovopsis R6
LESF021ES002
E. lentecrescens LESF 322
E. aspergilloides CBS 43.93
E. multiformis CBS 145327
LESF1136
LESF849
LESF1134
LESF1135
E. clavatus CBS 145326
LESF855
LESF854
E. kreiselli LESF 053
LESF302
LESF303
LESF304
E. trichodermoides LESF 003
LESF310
LESF311
LESF312
Escovopsioides nivea LESF 321
Escovopsioides LESF 510
Escovopsioides LESF 601
IT2esc
IT4esc
N10
T31
LJ19
Escovopsioides LESF 588
D4
T32
D6
D10
N4
M4
N5
Hypomyces protrusum TFC 201316
Hypomyces asteophorum CBS 67677
Hypomyces samuelsii TFC 200723
Hypomyces semicirculare CBS 70588
Trichoderma harzianum CBS 22695
Protocrea pallida TFC 99209
Sphaerostilbella aureonitens GJS74
Lecanicillium antillanum CBS 35085
0.4

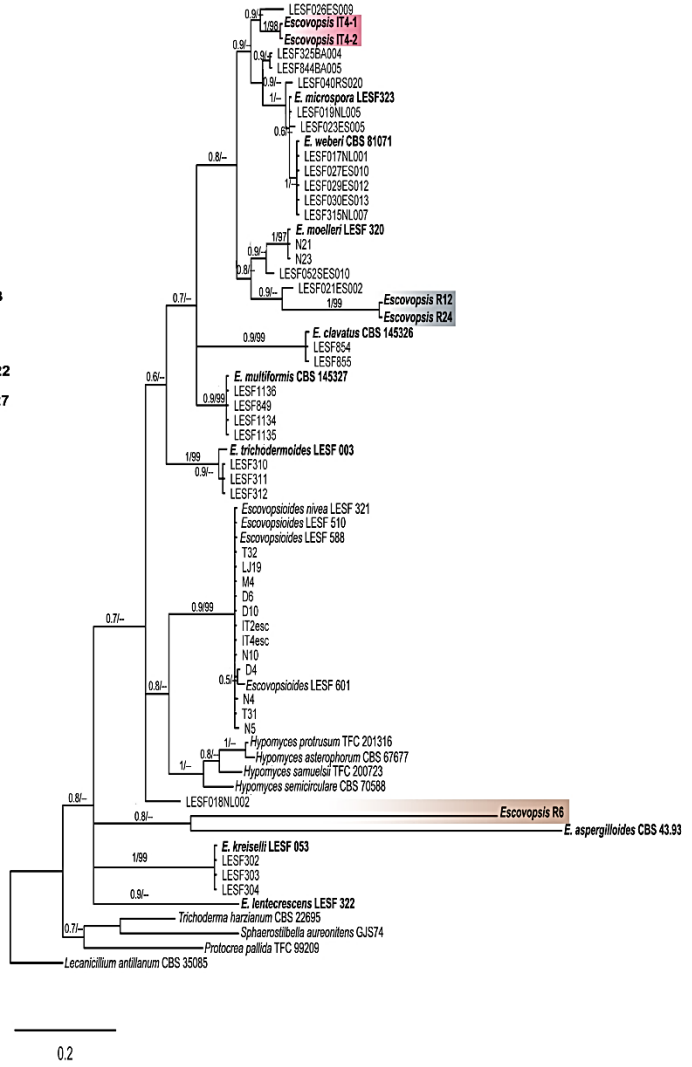

LESF026ES009
Escovopsis IT4-1
Escovopsis IT4-2
LESF325BA004
LESF844BA005
LESF040RS020
E. microspora LESF323
LESF019NL005
LESF023ES005
E. weberi CBS 81071
LESF017NL001
LESF027ES010
LESF029ES012
LESF030ES013
LESF315NL007
E. moelleri LESF 320
N21
N23
LESF052SES010
LESF021ES002
Escovopsis R12
Escovopsis R24
E. clavatus CBS 145326
LESF854
LESF855
E. multiformis CBS 145327
LESF1136
LESF849
LESF1134
LESF1135
E. trichodermoides LESF 003
LESF310
LESF311
LESF312
Escovopsioides nivea LESF 321
Escovopsioides LESF 510
Escovopsioides LESF 588
T32
LJ19
M4
D6
D10
IT2esc
IT4esc
N10
D4
Escovopsioides LESF 601
N4
T31
N5
Hypomyces protrusum TFC 201316
Hypomyces asteophorum CBS 67677
Hypomyces samuelsii TFC 200723
Hypomyces semicirculare CBS 70588
LESF018NL002
Escovopsis R6
E. aspergilloides CBS 43.93
E. kreiselli LESF 053
LESF302
LESF303
LESF304
E. lentecrescens LESF 322
Trichoderma harzianum CBS 22695
Sphaerostilbella aureonitens GJS74
Protocrea pallida TFC 99209
Lecanicillium antillanum CBS 35085
0.2

Figure 7. Phylogenetic analyses considering each molecular marker separately (ITS, LSU and *tef*1) demonstrating position of *Escovopsis* R6, *Escovopsis* R12 and *Escovopsis* IT4-2 (these new species are highlighted in brown, grey and pink, respectively). The trees were reconstructed under Bayesian and Maximum Likelihood inferences and included a total of 68 sequences of each marker (ITS – 652 bp, LSU – 594 bp and *tef*1 – 812 bp). *Escovopsioides*, *Protocrea*, *Trichoderma*, *Sphaerostilbella* and *Hypomyces* were included as the closest phylogenetic relatives of *Escovopsis* and *Lecanicillium antillanum* CBS 350.85 was used as the outgroup. The numbers on branches indicate the posterior probabilities and the bootstrap support values, respectively. The names in bold correspond to described species in other studies. The scale bar shows substitutions per nucleotide position.

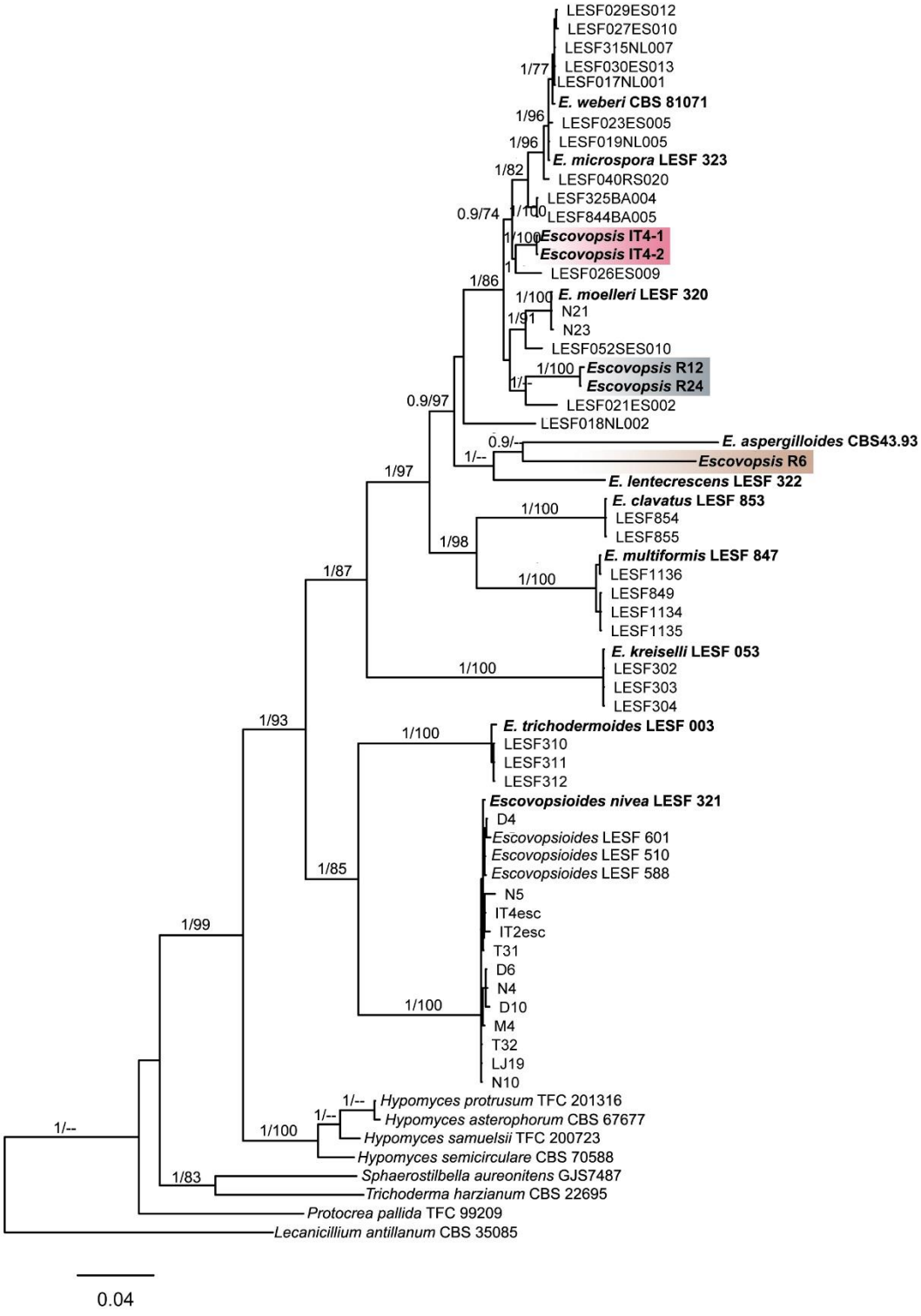


Figure 8. Concatenated phylogenetic analysis of ITS, LSU and *tef*-1 regions showing the position of *Escovopsis* R6, *Escovopsis* R12 e *Escovopsis* IT4-2. The tree was reconstructed under Bayesian and Maximum Likelihood inferences and included 68 sequences (ITS – 652 bp, LSU – 594 bp and *tef*1 – 812 bp). The numbers on branches indicates the posterior probabilities and the bootstrap support values, respectively.

Escovopsioides, *Protocrea*, *Trichoderma*, *Sphaerostilbella* and *Hypomyces* were included as the closest phylogenetic relatives of *Escovopsis* and *Lecanicillium antillanum* CBS 350.85 was used as the outgroup. The names in bold correspond to described species in other studies and the new species are highlight in brown (*Escovopsis* R6), grey (*Escovopsis* R12) and pink (*Escovopsis* IT4-1). The scale bar shows substitutions per nucleotide position.

Taxonomy

***Escovopsis* R6 M.C. S. Caixeta, Q.V. Montoya, A. Rodrigues & S.L. Elliot, sp. nov.**

Figs: 1–2

Typification. Brazil. Minas Gerais, Viçosa (20°45'26"S 42°51'45"W), fungus garden, 06, 2015. D. Venâncio. Ex type strain VIMI-15.0330

Description. Colonies did not grow at 10 and 30° C (Fig. 1). Growth began on the second day at 25 °C on MEA. All growth measurements were taken on the fourth day. The best growth temperature was 25°C. At this temperature, the fungus grew similarly on PDA (0.55–1.0 cm) and MEA (0.7–0.75 cm), with the presence of aerial mycelium; on the other hand, growth was slower on CMD (0.15–0.25 cm), which was restricted only to the agar-water disk used as the initial inoculum. At 20 °C, mycelium development was slower. As at the temperature 25 °C, growth was similar on PDA and MEA (0.4–0.6 cm and 0.4–0.7 cm, respectively) at 20 °C; on CMD it reached 0.15–0.25 cm. This specimen did not reach the edge of the Petri dish during the evaluation period (14 days). It is worth mentioning that although the fungus grew at 20 °C, its morphological characteristics were very different when grown at 25 °C: the isolate did not present aerial mycelium and growth was basically limited to initial inoculum. No soluble pigmentation was observed in any of the conditions evaluated.

Conidiophores monocephalous (mono-vesiculated) and poly-vesiculated (only one level of branching) (Fig. 2). The branches can be opposite or alternating and form a

right angle with the main axis (75–296 µm). Only one or two branches leave the same point. *Vesicles* present different shapes: globose (1.5%), obclavate (1.5%), ovoid (1.5%), broadly-ellipsoidal (4.4%), subglobose (7.3%), clavate (8.8%), ellipsoidal (25%) and cylindrical (50%) predominantly; reaching 18–54 µm × 10–25 µm wide. *Phialides* lageniform formed on vesicles (Fig. 2), with short and narrow base (0.5–1.5 × 1.–2.5 µm), followed by a swollen section (3.5–7.5 × 2–4 µm) and a thin long neck (3.5–8 × 0.5–1.5 µm). *Conidia* are small (1.5–3 µm long × 1.5–2.5 µm wide), smooth and in chains; predominantly globose (36.6%), subglobose (33.3%), broadly-ellipsoidal (16.6%) and ellipsoidal (13.3%). Apparently, they do not have specific ornamentation in light microscopy and present brown coloration when mature.

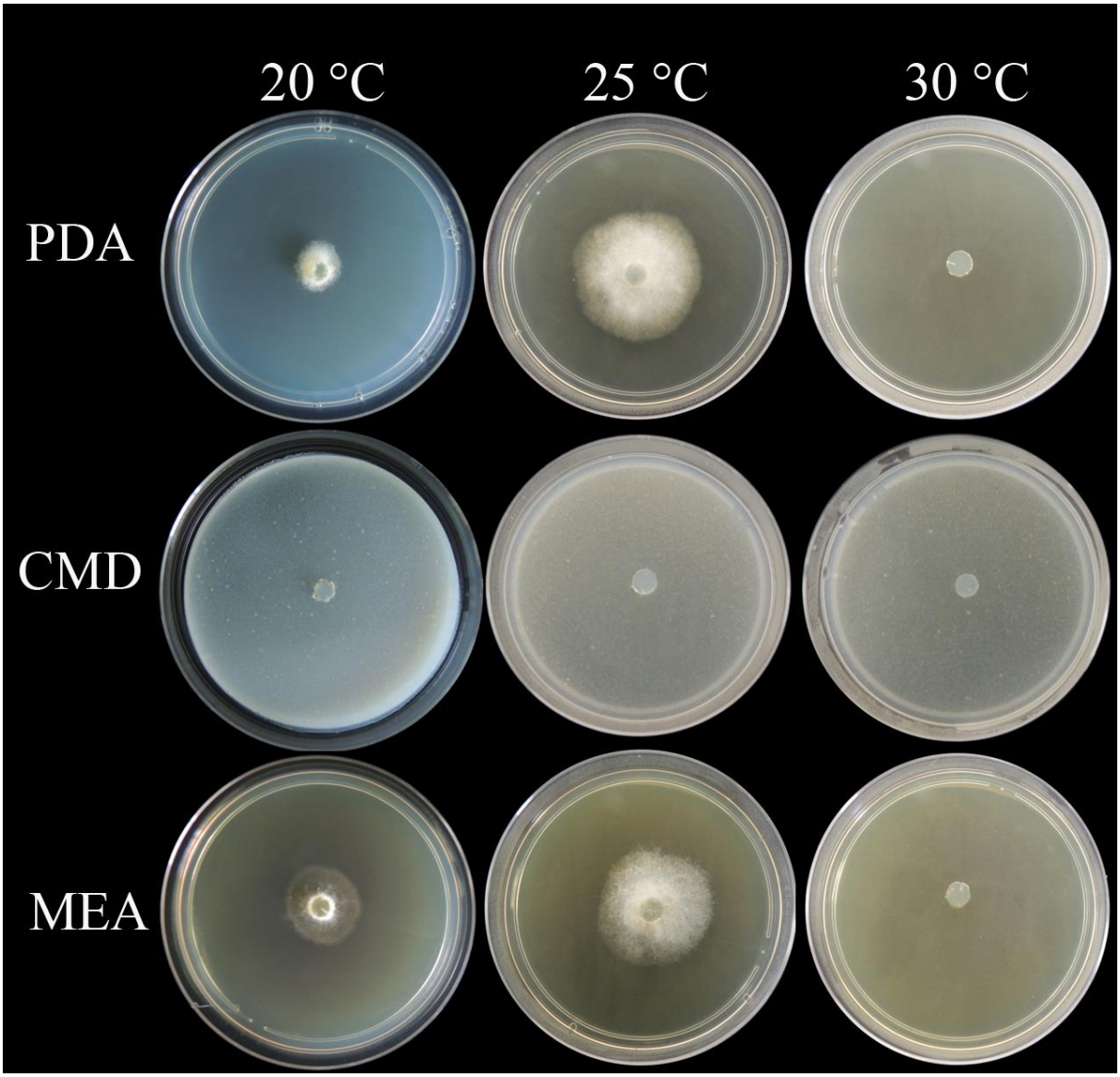


Figure 1. Colony growth and macroscopic characters of *Escovopsis* R6 on PDA, CMD and MEA culture media at 20, 25 and 30 °C after 7 days of inoculation. Fungus was inoculated from the disk of water-agar (5 mm) on 9 cm Petri dishes containing conidia suspension cultivated previously for 7 days at 25°C.

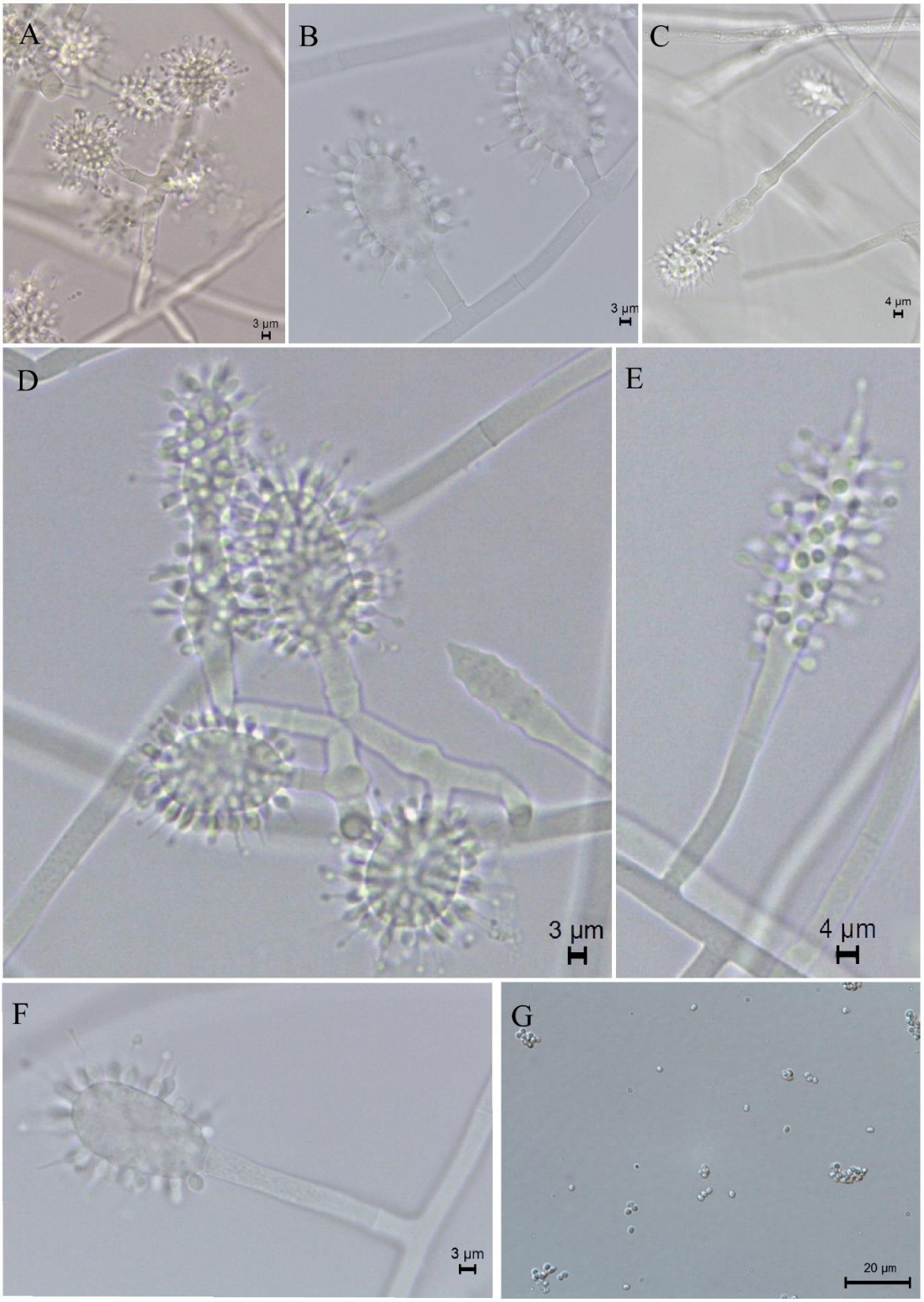


Figure 2. Microscopic characters of *Escovopsis* R6. (A) Conidiophores poly-vesiculated (only one level of branching); (B and C) conidiophores monocephalous (mono-

vesiculated); (D) vesicles in various shapes with lageniform phialides; (E-F) details of vesicles and phialides formation; (G) mature conidia.

***Escovopsis* R24 M.C.S. Caixeta, Q.V. Montoya, A. Rodrigues & S.L. Elliot, sp. nov.**

Figs: 3–4

Typification. Brazil. Minas Gerais, Viçosa (20°45'26"S 42°51'45"W), fungus garden, 06, 2015. D. Venâncio. Ex type strain VIMI-15.0336

Description. Colonies did not grow at 10 and 30 °C (Fig.3). Growth began on the second day after inoculation at both temperatures on PDA and MEA. The best growth temperature was 25 °C. At this temperature, fungus reached 2.5–3 cm on PDA; 0.05–0.2 cm on CMD, where it only sporulated around the agar water disk containing the inoculum, and 0.8–1.3 cm on MEA. On the seventh day, fungus grew to the edge of the plates and started sporulation on PDA. At 20°C, growth was slower reaching 0.7–1.4 cm on PDA, 0.1–0.3 cm on CMD and 0.1–0.2 cm on MEA. On the seventh day, at 20 °C, it was possible to observe mature conidia (characterized by light brown coloration), although radial mycelial growth was small, principally on CMD.

Conidiophores not very long, main axis ranging from 19.5–86.5 µm; they can be monocephalous or exhibit orthogonal branching, two to four arising from the same point (Fig. 4). *Vesicles* principally cylindrical (94%), but it was possible to observe some ellipsoidal (6%); they are produced laterally and apically on the branches, reaching 20.5–40 × 5.5–18.5 µm. *Phialides* lageniform formed from the vesicles following the genus pattern with short base (0.5–2 × 1–2 µm) followed by a swollen section (3–3.5 × 2–3 µm) and a long narrow neck (2.5–5 × 0.5–1 µm). *Conidia* produced in basipetal chains; brown, with smooth walls when mature; dimensions can reach 2–4.5 µm long × 1.5–3 µm wide

in various shapes: broadly ellipsoidal (57.5%), ellipsoidal (24.2%), subglobose (9.1%) e globose (9.1%).

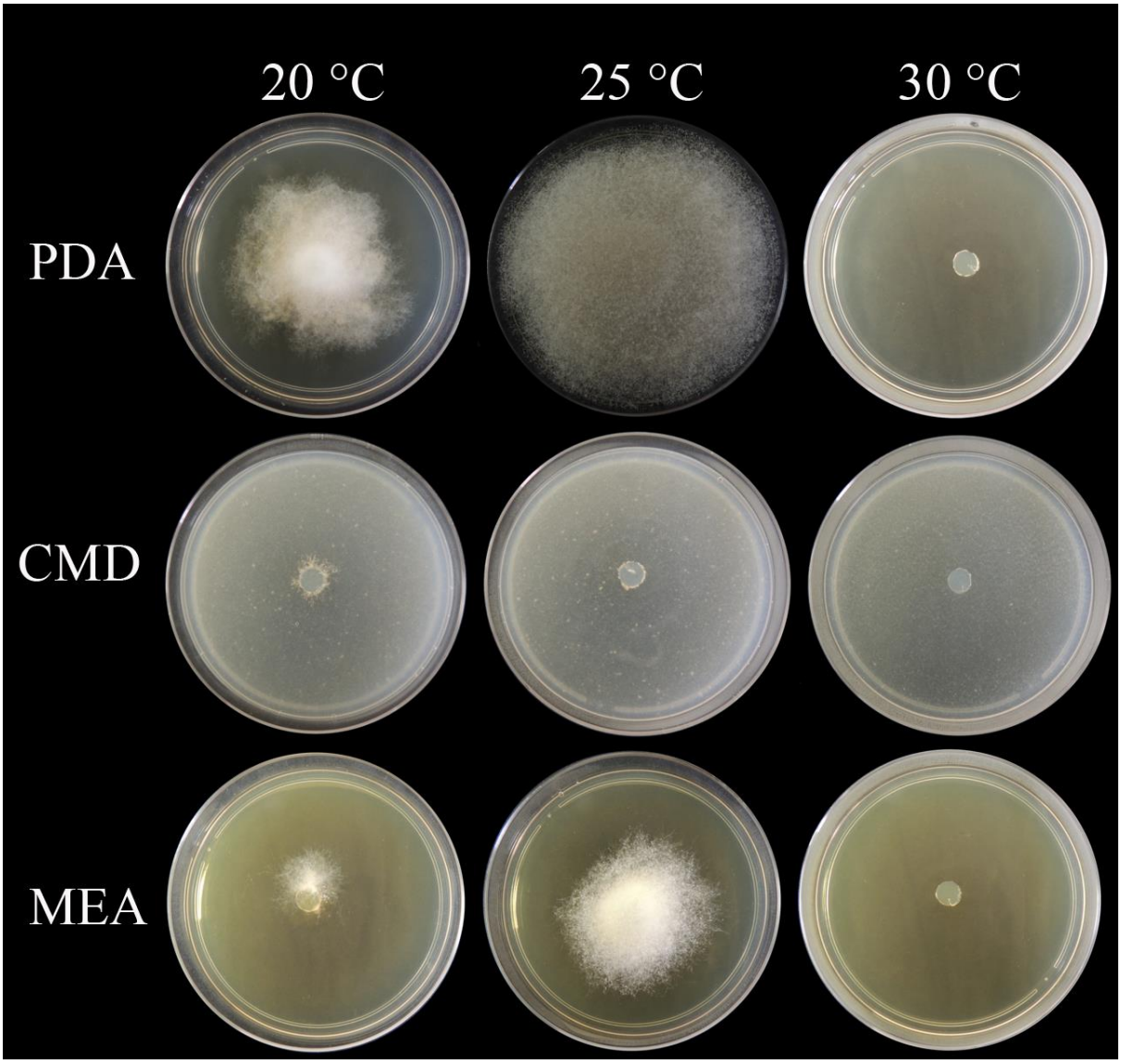


Figure 3. Colony growth and macroscopic characters of *Escovopsis* R24 on PDA, CMD and MEA culture media at 20, 25 and 30 °C after 7 days of inoculation. Fungus was inoculated from the disk of water-agar (5 mm) on 9 cm Petri dishes containing conidia suspension cultivated previously for 7 days at 25°C.

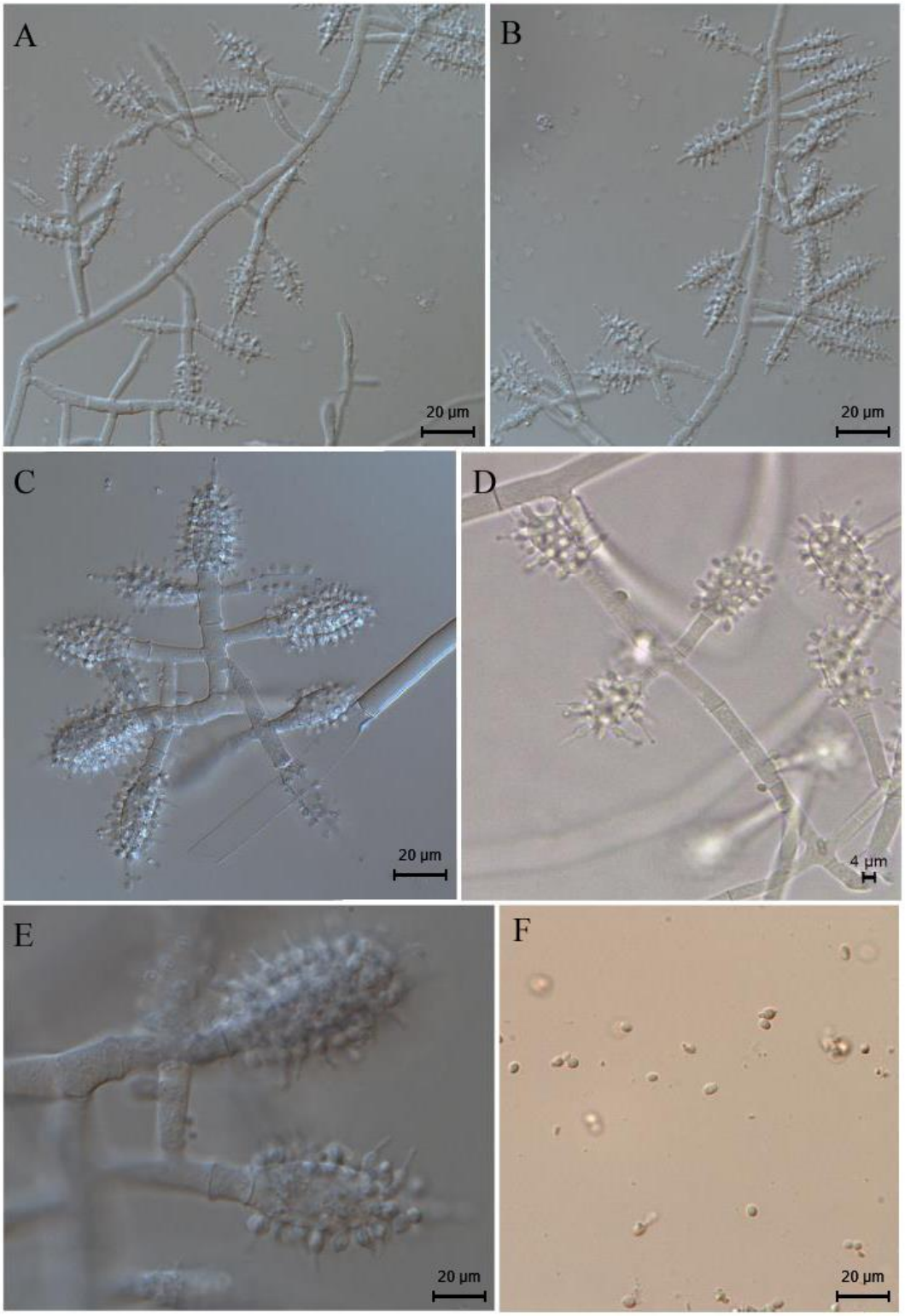


Figure 4. Microscopic characters of *Escovopsis* R12. (A-D) Conidiophores polycephalous predominantly, exhibiting alternated or opposite branches; (E) details of vesicles and phialides formation; (F) mature conidia.

***Escovopsis* IT4-2 M.C.S. Caixeta, Q.V. Montoya, A. Rodrigues & S.L. Elliot, sp. nov.**

Figs: 5 – 6

Typification. Brazil. Minas Gerais, Ouro Preto (20°26'49.5"S 43°27'19.0"W), fungus garden, 05, 2016. D. Venâncio.

Description. Colonies grew in all media tested and at all temperatures, except 10 °C (Fig. 5). The best growth temperatures were 25 and 30 °C, growth initiating in 24 hours on PDA. On the fourth day after inoculation, the isolate placed on the PDA and MEA media had already grown throughout the plate at temperatures of 25 °C and 30 °C (4 cm in all replicates), with small differences in media. On CMD, the growth of the fungus was slower than the other two media (0.7 cm at 25 ° C and 0.4–0.7 cm at 30 °C). At 20 °C, fungus reached 1.8–3.6 cm on PDA and 0.7–2 cm on MEA. In addition, on PDA the development of the aerial mycelium is denser and the coloration of the mature conidia is different, being more pink on MEA than on PDA, where they have brown color, possible to be observed on the seventh day. On CMD, although the growth of the fungus was slower than the other two media at 20 °C (0.1–0.5 cm), no differences were observed either between temperatures for this culture medium.

Conidiophores long, from which originate on alternate or opposite branches, with one or more levels of branches, main axis measuring 110–373 µm (Fig. 6). *Vesicles* predominantly cylindrical (27–101.5 µm × 5–10.5 µm) and they can be septate. *Phialides* lageniform originated from the vesicles, with short and narrow base (0.5–1.5 × 1–2 µm), followed by swollen section (2.5–3 µm) and a long narrow neck (2.5–6 × 0.5–1 µm). *Conidia* are produced in chains at the end of phialides, dark brown when mature, with slightly thickened and smooth walls and exhibit different formats: globose (23.4%), subglobose (16.6%), broadly-ellipsoidal (43.4%) and ellipsoidal (16.6%); 2–4 µm long × 1.5–3.5 µm wide.

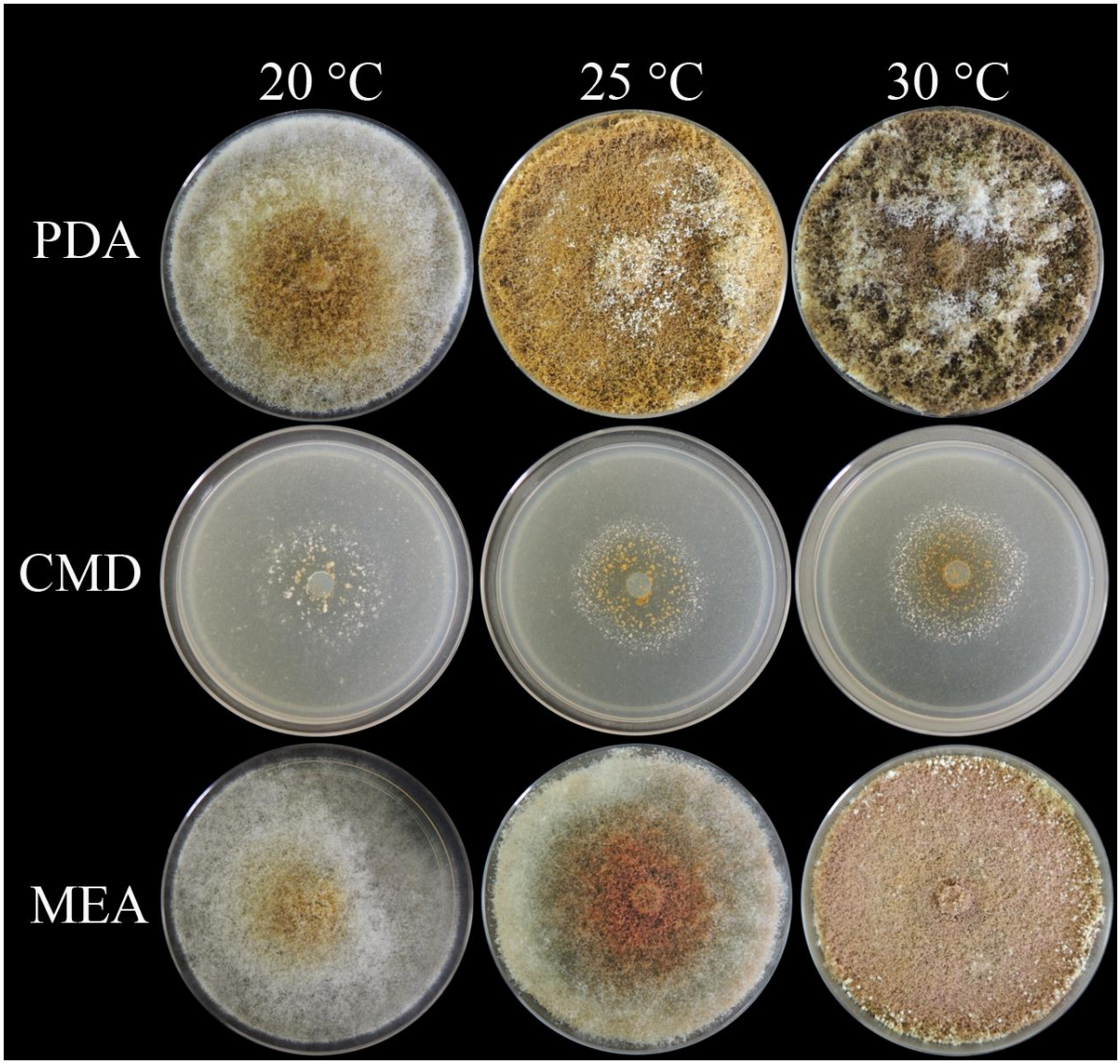


Figure 5. Colony growth and macroscopic characters of *Escovopsis* R6 on PDA, CMD and MEA culture media at 20, 25 and 30 °C after 7 days of inoculation. Fungus was inoculated from the disk of water-agar (5 mm) on 9 cm Petri dishes containing conidia suspension cultivated previously for 7 days at 25°C.

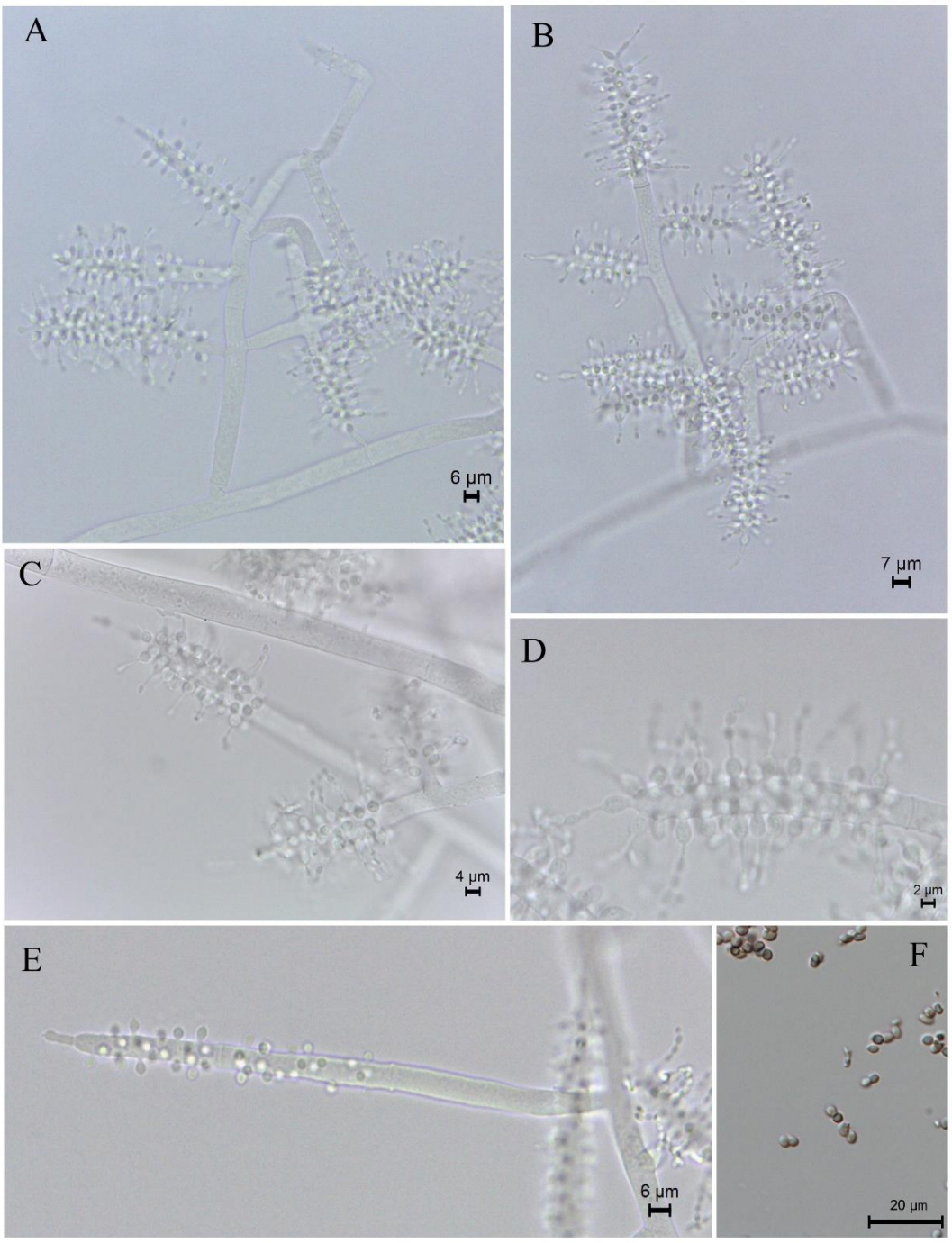


Figure 6. Microscopic characters of *Escovopsis* IT4-2. (A-C) Conidiophores polycephalous predominantly, exhibiting alternated or opposite branches, with two levels of branching; (D-E) details of vesicles, phialides and conidia formation in chains; (F) mature conidia.

Discussion

Despite all the specializations used by leaf-cutting ants to keep their fungus garden healthy and homeostatic, in some specific situations they may be threatened by the mycoparasitic fungus *Escovopsis*. Since the publication of Currie et al. (2001) about the high level of virulence of *Escovopsis*, some studies have suggested its use as a biological control of leaf cutting ants (Reynolds and Currie, 2004; Folgarait et al., 2011a, b; Wallace et al., 2014). However, taxonomic and phylogenetic considerations have been left to the side for some time although it should go without saying that characterizing and studying the relationships between the species of this genus is essential for future studies or practical uses. Here we have described three new species of *Escovopsis*, two of which are found in *Acromyrmex subterraneus subterraneus* (*Escovopsis* R6 and *Escovopsis* R24) in Viçosa, MG, Brazil and the third found in *Acromyrmex balzani* (*Escovopsis* IT4-1), in Ouro Preto, MG, Brazil.

Our results showed that *Escovopsis* R6 did not cluster with any other *Escovopsis* isolate, forming a clade with a single individual. The clade with only one individual makes the morphological characterization of the new species difficult, since it was not possible to compare it to an additional specimen of the same clade. Nonetheless, we can confirm the new species phylogenetically, since this taxon separated from all others in both analyses (ML and Bayesian). *Escovopsis* R6 grouped close to the species *E. lentecrescens* and *E. aspergilloides*, both characterized by the presence of globose vesicles. However, it was possible to observe a variety of vesicle formats, which may suggest that this new taxon can be a transition between the fungi that present predominance of globular vesicles and fungi with predominance of cylindrical vesicles, i.e. "*weberi* group" (see Meirelles et al. 2015b).

Escovopsis IT4-2 was isolated from the fungus garden of *Acromyrmex balzani*, a leaf-cutter ant specialized on grasses. This new species grouped close to *E. microspora* and *E. weberi*, that had been isolated from *A. subterraneus molestans* and *Atta cephalotes*, repectively, corroborating the lack of specificity between *Escovopsis* clades in leafcutter ants (Taerum et al. 2007; Meirelles et al. 2015b). It has been suggested that grass and dicotyledonous-specialized leafcutter species may share fungal cultivars (Mueller et al. 2017) – therefore, it does not seem to depend on the type of substrate used, so it is possible that this *Escovopsis* clade is associated with this cultivar clade and not the ant species, as is already known. Similar to *E. weberi* and *E. microspora*, *Escovopsis* IT4-2 showed a predominance of cylindrical vesicles. It has been suggested that globose vesicles are the ancestral state to cylindrical vesicles (Meirelles et al. 2015b) and here, it is important to observe that IT4-2 seems to produce phialides directly from the hyphae, without the presence of vesicles (see fig. 6). This feature deserves further investigation since the presence of vesicles is one of the original characteristics of the genus. *E. microspora* and *E. weberi* were at no point separated as species in our analyses (except in *tef*1), indicating that new markers are needed to distinguish them or that they may not be two distinct species, since they are morphologically very similar (Augustin et al. 2013). Although vesicle shape is an important character for the morphological identification of the genus (Muchovej and Della Lucia 1990; Seifert et al. 1995; Augustin et al. 2013; Marfetán et al. 2018; Montoya et al. 2019), the characterization is difficult within this group that apparently has a large number of isolates and are very similar morphogically. Therefore, we emphasize the importance of the phylogenetic study for this genus. A more careful analysis of the surface of conidia and their respective ornamentation could also be used to distinguish species morphologically, as Augustin et al. (2017) suggested.

Seven new species of *Escovopsis* have recently been described by Marfetán et al. (2019), and Montoya et al. (2019). Although descriptive studies are extremely important to unravel the genetic and morphological diversity of fungi, contributing to future research, we could unfortunately not include the data of Marfetán et al. (2019) here. The five species introduced by Marfetán et al. (2019) were analysed using different morphological conditions which makes the comparison of the morphological features of the new species impossible. Further, the *tef* sequences deposited by the authors do not align with the sequences deposited by other researchers. So, future studies must re-analyse the morphological features, and re-sequence the *tef* gene to understand the position of these species in relation to the other species described in the phylogenetic tree. The description of *Escovopsis* R6, *Escovopsis* R12, and *Escovopsis* IT4-2 increases our knowledge of *Escovopsis* systematics. As these new species are clustered close to other different undescribed phylogenetic clades it shows us that the diversity of the genus is yet to be fully explored.

References

Augustin, J. O., Groenewald, J. Z., Nascimento, R. J., Mizubuti, E. S. G., Barreto, R.W., Elliot, S. L., & Evans, H. C. (2013). Yet more "weeds" in the garden: fungal novelties from nests of leaf-cutting ants. *PLoS ONE*, 8, e82265. https://doi.org/10.1371/journal.pone.0082265

Augustin, J. O., Simões, T. G., Dijksterhuis, J., Elliot, S. L., & Evans, H. C. (2017). Putting the waste out: a proposed mechanism for transmission of the mycoparasite *Escovopsis* between leafcutter ant colonies. *Royal Society Open Science*, 4, 161013. http://dx.doi.org/10.1098/rsos.161013

Bot, A. N. M., Ortius-Lechner, D., Finster, K., Maile R., & Boomsma, J. J. (2002). Variable sensitivity of fungi and bacteria to compounds produced by the metapleural glands of leaf-cutting ants. *Insectes Sociaux*, 49, 363–370. https://doi.org/10.1007/PL00012660

Chapela, I. H., Rehner, S. A., Schultz, T. R., Mueller, U. G. (1994). Evolutionary history of the symbiosis between fungus-growing ants and their fungi. *Science*, 266, 1691-1694. DOI: 10.1126/science.266.5191.1691

Chaverri, P., Castlebury, L. A., Samuels, G. J., Geiser, D. M. (2003). Multilocus phylogenetic structure within the *Trichoderma harzianum*/*Hypocrea lixiicomplex*. *Molecular Phylogenetics and Evolution*, 27(2): 302–313. https://doi.org/10.1016/S1055-7903(02)00400-1

Currie, C. R., Mueller, U. G., & Malloch, D. (1999a). The agricultural pathology of ant fungus gardens. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 7998–8002. https://doi.org/10.1073/pnas.96.14.7998

Currie, C. R., Scott, J. A., Summerbell, R. C., & Malloch, D. (1999b). Fungus growing ants use antibiotic producing bacteria to control garden parasites. *Nature*, 398, 701–704. https://doi.org/10.1038/nature01563

Currie, C. R. (2001). Prevalence and impact of a virulent parasite on a tripartite mutualism. *Oecologia*, 128, 99–106. https://doi.org/10.1007/s004420100630

Currie, C. R., & Stuart, A. E. (2001). Weeding and grooming of pathogens in agriculture by ants. *Proceedings of the Royal Society B: Biological Sciences*, 268, 1033–1039. https://doi.org/10.1098/rspb.2001.1605

Currie, C. R., Wong, B., Stuart, A. E., Schultz, T. R., Rehner, S. A., Mueller, U.G., Sung, G. H., Spatafora, J.W., & Straus, N. A. (2003a). Ancient tripartite coevolution in the attine ant-microbe symbiosis. *Science*, 299, 386–388. https://doi.org/10.1126/science.1078155

Currie, C. R., Bot, A. N. M., & Boomsma, J. J. (2003b). Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. *Oikos* 101, 91-102. https://doi.org/10.1034/j.1600-0706.2003.12036.x

Darriba, D., Taboada, G. L., Doallo, R., Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, 9: 772. https://doi.org/10.1038/nmeth.2109

Farji-Brener, A. G., Elizalde, L., Fernández-Marín, H., & Amador-Vargas, S. (2016). Social life and sanitary risks: evolutionary and current ecological conditions determine waste management in leaf-cutting ants. *Proceedings of the Royal Society B: Biological Sciences*, 283, 20160625. https://doi.org/10.1098/rspb.2016.0625

Folgarait, P., Gorosito, N., Thomas-Poulsen, M., & Currie, C. R. (2011a). Preliminary *in vitro* insights into the use of natural fungal pathogens of leafcutting ants as biocontrol agents. *Current Microbiology*, 63, 250–258. http://dx.doi.org/10.1007/s00284-011-9944-y

Folgarait, P. J., Marfetán, J. A., & Cafaro, M. J. (2011b). Growth and conidiation response of *Escovopsis weberi* (Ascomycota: Hypocreales) against the fungal cultivar of *Acromyrmex lundii* (Hymenoptera: Formicidae). *Environmental Entomology*, 4040, 342-349. http://dx.doi.org/10.1603/EN10111

Gerardo, N. M., Mueller, U. G. & Currie, C. R. (2006). Complex host-pathogen coevolution in the *Apterostigma* fungus-growing ant-microbe symbiosis. *BMC Evolutionary Biology*, 6, 1-9. https://doi.org/10.1186/1471-2148-6-88

Haugland, R. L., Heckman, J. L. (1998). Identification of putative sequence specific PCR primers for detection of the toxigenic fungal species *Stachybotrys chartarum*. *Molecular and Cellular Probes*, 12, 387–396. https://doi.org/10.1006/mcpr.1998.0197
Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95-98

Hinkle, G., Wetterer, J. K., Schultz, T. R., Sogin, M. L. (1994). Phylogeny of the Attine ant fungi based on analysis of small suunit ribossomal RNA gene sequences. *Science*, 266, 1695-1697. https://doi.org/10.1126/science.7992052

Katoh, K., Standley, D. M. (2013). MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Molecular Biology and Evolution*, 30, 772–780. https://doi.org/10.1093/molbev/mst010

Marfetán, J. A., Romero, A. I., & Folgarait, P. J. (2015). Pathogenic interaction between *Escovopsis weberi* and *Leucoagaricus* sp.: mechanisms involved and virulence levels. *Fungal Ecology*, 17, 52–61. http://dx.doi.org/10.1016/j.funeco.2015.04.002

Marfetán, J. A., Romero, A. I., Cafaro, M. J., Folgarait, P. J. (2018). Five new *Escovopsis* species from Argentina. *Mycotaxon*, 133, 569-589

Masiulionis, V. E., Cabello, M. N., Seifert, K. A., Rodrigues, A., Pagnocca, F. C. (2015). *Escovopsis trichodermoides* sp. nov., isolated from a nest of the lower attine ant *Mycocepurus goeldii*. *Antonie van Leeuwenhoek*, 107, 731–40. https://doi.org/10.1007/sl10482-014-0367-1

Meirelles, L. A., Montoya, Q. V., Solomon, S. E., Rodrigues, A. (2015a). New light on the systematics of fungi associated with attine ant gardens and the description of

Escovopsis kreiselii sp. nov. *PLoS One* 10: e0112067. https://doi.org/10.1371/journal.pone.0112067

Meirelles, L. A., Solomon, S. E., Bacci Jr., M., Wright, A. M., Mueller, U. G., Rodrigues, A. (2015b). Shared *Escovopsis* parasites between leaf-cutting and non-leaf-cutting ants in the higher attine fungus-growing ant symbiosis. *Royal Society Open Science*, 2, 150257. https://doi.org/10.1098/rsos.150257

Montoya, Q. V., Martiarena, M. J. S., Polezel, D. A., Kakazu, S., Rodrigues, A. (2019). More pieces to a huge puzzle: Two new *Escovopsis* species from fungus gardens of attine ants. *MycoKeys*, 46, 97–118. https://doi.org/10.3897/mycokeys.46.30951

Muchovej, J. J., Della Lucia, T. C. (1990). *Escovopsis*, a new genus from leaf-cutting ant nests to replace *Phialocladus* nomen invalidum. *Mycotaxon*, 37, 191–195

Mueller, U. G., Rehner, S. A., Schultz, T. R. (1998). The evolution of agriculture in ants. *Science*, 281, 2034-2038. https://doi.org/10.1126/science.281.5385.2034

Nixon, K. C. (2002). WinClada ver. 1.0000, Ithaca. [Published by the author]

North, R. D., Jackson, C. W., Howse, P. E. (1997) Evolutionary aspects of ant-fungus interactions in leaf-cutting ants. *Trends Ecology Evolution* 12, 386-389. https://doi.org/10.1016/S0169-5347(97)87381-87388

Põldmaa, K. (2011). Tropical species of *Cladobotryum* and *Hypomyces* producing red pigments. *Studies in Mycology*, 68, 1–34. https://doi.org/10.3114/sim.2011.68.01

Poulsen, M., Bot, A. N., Nielsen, M. G., & Boomsma, J. J. (2002). Experimental evidence for the costs and hygienic significance of the antibiotic metapleural gland secretion in leaf-cutting ants. *Behavioral Ecology and Sociobiology*, 52, 151–157. https://doi.org/10.1007/s00265-002-0489-8

Reynolds, H. T., Currie, C. R. (2004). Pathogenicity of *Escovopsis weberi*: The parasite of the attine ant-microbe symbiosis directly consumes the ant-cultivated fungus. *Mycologia*, 96, 955-959

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A., Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. *Systematic Biology*, 61, 539–542. https://doi.org/10.1093/sysbio/sys029

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W. Fungal Barcoding Consortium. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy Sciences of the United States of America*, 109, 6241–6246. https://doi.org/10.1073/pnas.1117018109

Seifert, K. A., Samson, R. A. & Chapela, I. H. (1995). *Escovopsis aspergilloides*, a rediscovered hyphomycete from leaf-cutting ant nests. *Mycologia*, 87, 407–413. https://doi.org/10.2307/3760838

Silva, A., Rodrigues, A., Bacci Jr., M., Pagnocca, F. C., & Bueno, O. C. (2006). Susceptibility of the ant-cultivated fungus *Leucoagaricus gongylophorus* (Agaricales: Basidiomycota) towards microfungi. *Mycopathologia*, 162, 115–119. http://dx.doi.org/10.1007/s11046-006-0037-6

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, 30, 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Taerum, S. J.; Cafaro, M. J.; Little, A. E.; Shultz, T. R. & Currie, C. R. (2007). Low host-pathogen specificity in the leaf-cutting ant-microbe symbiosis. *Proceedings of the Royal Society B*, 274, 1971-1978. https://doi.org/10.1098/rspb.2007.0431

Taerum, S. J., Cafaro, M. J. & Currie, C. R. (2010). Presence of multiparasite infections within individual colonies of leaf-cutter ants. *Environmental Entomology*, 39, 105–113. https://doi.org/10.1603/EN09137

Varanda-Haifig, S. S., Albarici, T. R., Nunes, P. H., Haifig, I., Vieira, P. C., & Rodrigues, A. (2017). Nature of the interactions between hypocrealean fungi and the mutualistic fungus of leaf-cutter ants. *Antonie van Leeuwenhoek*, 110, 593-605. https://doi.org/10.1007/s10482-016-0826-y

White, T.J., Bruns, T., Lee, S. H., Taylor, J. W. (1990). PCR Protocols: A Guide to Methods and Application. *Academic Press*, 315−322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Wallace, D. E. E., Asensio, J. G. V., & Tomás, A. A. P. (2014). Correlation between virulence and genetic structure of *Escovopsis* strains from leaf-cutting ant colonies in Costa Rica. *Microbiology*, 160, 1727–1736. https://doi.org/10.1099/mic.0.073593-0

Weber, N. A. (1972). Gardening Ants, the Attines. *Science*, 153, 587-604

Supplementary Material

Table S1. General information of *Escovopsis* strains isolated. Collections were realized in three cities of state Minas Gerais, Brazil in the years 2015, 2016 and 2017. All the isolates were preserved 10% glycerol at 3 °C at UFV – Laboratory of Interactions Insect Microrganisms (LIIM), Viçosa, Brazil

Isolate code	Ant species	Site	Collection date
R6	*Ac. subterraneus subterraneus*	Recanto das Cigarras, Viçosa – MG	Junho/2015
R12	*Ac. subterraneus subterraneus*	Recanto das Cigarras, Viçosa – MG	Junho/2015
R24	*Ac. subterraneus subterraneus*	Recanto das Cigarras, Viçosa – MG	Junho/2015
M04	*Ac. subterraneus subterraneus*	Mata do Paraíso, Viçosa – MG	Julho/2015
D4	*Ac. subterraneus subterraneus*	Dendrologia, Viçosa – MG	Agosto/2015
D6	*Ac. subterraneus subterraneus*	Dendrologia, Viçosa – MG	Agosto/2015
D10	*Ac. subterraneus subterraneus*	Dendrologia, Viçosa – MG	Agosto/2015
T31	*Ac. subterraneus subterraneus*	Horto, Viçosa – MG	Agosto/2015
T32	*Ac. subterraneus subterraneus*	Horto, Viçosa – MG	Agosto/2015
IT2-esc	*Acromyrmex balzani*	Itacolomi, Ouro Preto – MG	Maio/2016
IT4-1	*Acromyrmex balzani*	Itacolomi, Ouro Preto – MG	Maio/2016
IT4-2	*Acromyrmex balzani*	Itacolomi, Ouro Preto – MG	Maio/2016
IT4-esc	*Acromyrmex balzani*	Itacolomi, Ouro Preto – MG	Maio/2016
LJ19	*Ac. subterraneus subterraneus*	Parque da Lajinha, Juiz de Fora - MG	Maio/2016
N4	*Ac. subterraneus subterraneus*	Dendrologia, Viçosa – MG	Agosto/2016
N5	*Ac. subterraneus subterraneus*	Dendrologia, Viçosa – MG	Agosto/2016
N10	*Ac. subterraneus subterraneus*	Dendrologia, Viçosa – MG	Agosto/2016
N21	*Ac. subterraneus subterraneus*	Recanto das Cigarras, Viçosa – MG	Dezembro/2016
N23	*Ac. subterraneus subterraneus*	Recanto das Cigarras, Viçosa – MG	Dezembro/2016

Table S2. Genomic regions, sequence primers and PCR conditions used for the identification molecular and phylogenetic analysis

Genomic regions	Primers		References	Conditions
ITS		5'CTTGGTCATTTAGAGGAAGTAA3'	Gardes and Bruns, 1993	5 minutes denaturation at 95°C, 30 cycles consisting of 30 seconds at 95°C, 30 seconds at 56,5°C, 90 seconds at 72°C and final extension step at 72°C for 10 min
	ITS4-R	5'TCCTCCGCTTATTGATATGC3'	White et al., 1990	
***tef*1**	EF1-983F	5'GCYCCYGGHCAYCGTGAYTTYA 3'	Currie et al., 2003	2 min at 95°C, 40 cycles of 30s at 95°C, 60s at 63°C, 90s at 72° and 5 minutes of extension at 72°C
	EF1-2218R	5' GACTTGACTTCRGTVGTGAC 3'	Currie et al., 2003	
LSU	CLA-F	5' GCATATCAATAAGCGGAGGA 3'	Currie et al., 2003	2 minutes at 95°C, 40 cycles of 30 seconds at 95°C, 60 seconds at 62°C, 90 seconds at 72°C and 5 minutes of extension at 72°C
	CLA-R	5' GACTCCTTGGTCCGTGTTTCA 3'	Currie et al., 2003	

Table S3. *Escovopsis* sequences and acession number obtained in Genbank used in the phylogenetic analyses. Outgroup sequences of related fungi to *Escovopsis* were acquired as well

Fungal species	GenBank acession number			Strain ID	Reference
	LSU	***tef*1**	**ITS**		
Escovopsis weberi	KF293281	KF293275	KF293285	ATCC64542	Augustin et al., 2013
Escovopsis aspergilloides	KF293283	AY172632	NR_137160	CBS 423.93	Augustin et al. (2013); Currie et al. (2003)
Escovopsis lentecrescens	JQ855717	JQ855714	JQ815079	CBS135750	Augustin et al., 2013
Escovopsis microspora	KF293284	KJ935030	JQ815076	CBS135751	Augustin et al. (2013); Meirelles et al. (2015a)
Escovopsis moelleri	JQ855715	JQ855712	JQ815077	CBS135748	Augustin et al., 2013

Escovopsis kreiselli	KJ808765	KJ808766	KJ808767	CBS139320	Meirelles et al., 2015a
Escovopsis kreiselii	MH715099	MH724259	MH715085	LESF 302	Montoya et al., 2019
Escovopsis kreiselii	MH715100	MH724260	MH715086	LESF 303	Montoya et al., 2019
Escovopsis kreiselii	MH715101	MH724261	MH715087	LESF 304	Montoya et al., 2019
Escovopsis trichodermoides	MF116052	KF033128	KJ485699	CBS137343	Masiulionis et al., 2015
Escovopsis trichodermoides	MH715102	MH724262	MH715088	LESF 310	Montoya et al., 2019
Escovopsis trichodermoides	MH715103	MH724263	MH715089	LESF 311	Montoya et al., 2019
Escovopsis trichodermoides	MH715104	MH724264	MH715090	LESF 312	Montoya et al., 2019
Escovopsis multiformis	MH715105	MH724265	MH715091	CBS145327	Montoya et al., 2019
Escovopsis multiformis	MH715106	MH724266	MH715092	LESF1136	Montoya et al., 2019
Escovopsis clavatus	MH715110	MH724270	MH715096	CBS 145326	Montoya et al., 2019
Escovopsis clavatus	MH715111	MH724271	MH715097	LESF 854	Montoya et al., 2019
Escovopsis clavatus	MH715112	MH724272	MH715098	LESF 855	Montoya et al., 2019
Escovopsis primorosea	_____	KU298278	_____	BAFC-H 52761	Marfetán et al., 2018
Escovopsis longivesica	_____	KU298275	_____	BAFC-H 52762	Marfetán et al., 2018
Escovopsis pseudoweberi	_____	KU298283	_____	BAFC-H 52763	Marfetán et al., 2018
Escovopsis atlas	_____	KU298281	_____	BAFC-H 52764	Marfetán et al., 2018
Escovopsis catenulata	_____	_____	_____	BAFC-H 52765	Marfetán et al., 2018
Escovopsioides nivea	JQ855716	JQ855713	JQ815078	CBS135749	Augustin et al., 2013
Escovopsis sp.	MH715113	KM817142	KM817072	LESF 017	Meirelles et al., 2015b
Escovopsis sp.	MH715114	KM817143	KM817073	LESF 018	Meirelles et al., 2015b
Escovopsis sp.	MH715115	KM817144	KM817074	LESF 019	Meirelles et al., 2015b
Escovopsis sp.	MH715116	KM817123	KM817053	LESF 021	Meirelles et al., 2015b

Escovopsis sp.	MH715117	KM817126	KM817056	LESF 023	Meirelles et al., 2015b
Escovopsis sp.	MH715118	KM817130	KM817060	LESF 026	Meirelles et al., 2015b
Escovopsis sp.	MH715119	KM817131	KM817061	LESF 027	Meirelles et al., 2015b
Escovopsis sp.	MH715120	KM817133	KM817063	LESF 029	Meirelles et al., 2015b
Escovopsis sp.	MH715121	KM817134	KM817064	LESF 030	Meirelles et al., 2015b
Escovopsis sp.	MH715122	EU082803	KM817077	LESF 040	Meirelles et al., 2015b
Escovopsis sp.	MH715123	KM817153	KM817092	LESF 051	Meirelles et al., 2015b
Escovopsis sp.	MH715124	KM817154	KM817093	LESF 052	Meirelles et al., 2015b
Escovopsis sp.	MH715125	KF240730	KM817075	LESF 315	Meirelles et al., 2015b
Escovopsis sp.	MH715126	KM817139	KM817069	LESF 318	Meirelles et al., 2015b
Escovopsis sp.	MH715127	KM817119	KM817049	LESF 325	Meirelles et al., 2015b
Escovopsis sp.	MH715128	KM817120	KM817050	LESF 844	Meirelles et al., 2015b
Hypomyces samuelsii	FN859451	FN868769	FN859451	TFC 2007-23	Põldmaa, K., 2011
Hypomyces samuelsii	FN859445	FN868764	FN859445	C.L.L. 7259	Põldmaa, K., 2011
Hypomyces protrusum	FN859414	FN868732	FN859414	TFC 201316	Põldmaa, K., 2011
Hypomyces semicirculare	FN859417	FN868735	NR_121425	CBS 705. 88	Põldmaa, K., 2011
Hypomyces asterophorum	AJ583469	FN868712	FN859395	CBS 676.77	Põldmaa, K., 2011
Trichoderma harzianum	HM466680	AF534621	AY605713	CBS 226.95	Chaverri et al., 2003
Protocrea pallida	EU710769	EU703903	NR_111329	TFC 99-209	Põldmaa, K., 2011
Sphaerostilbella aureonitens	HM466683	FJ467644	FJ442633	GJS 74-87	Põldmaa, K., 2011
Lecanicillium antillanum	AF339536	DQ522350	NR_111097	CBS 350.85	Põldmaa, K., 2011

Chapter 2: Low virulence of the fungi *Escovopsis* and *Escovopsioides* to a leafcutter ant-fungus symbiosis

Part of this chapter was presented as a masters thesis by Débora Mello Furtado de Mendonça entitled: Virulence of the fungi Escovopsis *and* Escovopsioides *to the leafcutter ant-fungus symbiosis*

Débora Mello Furtado de Mendonça[1], Marcela Cristina Silva Caixeta[1], Gabriel Leite[1], Camila Costa Moreira[1], Tiago Gechel Kloss[2], Simon Luke Elliot[1*]

[1]*Department of Entomology, Universidade Federal de Viçosa, 36570-900, Viçosa, Brazil*

[2]*Department of Biological Sciences, Universidade do Estado de Minas Gerais, 36500-000, Ubá, Brazil*

*Correspondence: Simon L. Elliot, tel: +55 (31) 3612-3025, e-mail: selliot@ufv.br

Type of Paper: Primary Research Article

Running Head: Low virulence of *Escovopsis* and *Escovopsioides*

Abstract

Eusocial insects interact with a diversity of parasites that can threaten their survival and reproduction. The amount of harm these parasites cause to their hosts (i.e. their virulence) can be influenced by numerous factors, such as the ecological context in which the parasite and its host are inserted. Leafcutter ants (genera *Atta* and *Acromyrmex*, Attini: Formicidae) are an example of a eusocial insect whose colonies are constantly threatened by parasites. The fungi *Escovopsis* and *Escovopsioides* (Ascomycota: Hypocreales) are considered a highly virulent parasite and an antagonist, respectively, to the leafcutter ants' fungal cultivar, *Leucoagaricus gongylophorus* (Basidiomycota: Agaricales). Since *Escovopsis* and *Escovopsioides* are common inhabitants of healthy colonies that can live for years, we expect them to have low levels of virulence. However, this virulence could vary depending on the ecological context that the colonies are inserted. We therefore tested two hypotheses: (i) *Escovopsis* and *Escovopsioides* are of low virulence to colonies; (ii) virulence increases as colony complexity decreases. For this, we used three levels of complexity: queenright colonies (fungus garden with queen and workers), queenless colonies (fungus garden and workers, without queen) and fungus gardens (without any ants). Each was inoculated with extremely high concentrations of conidia of *Escovopsis moelleri*, *Escovopsioides nivea*, the mycoparasitic fungus *Trichoderma longibrachiatum* or to a blank control. We found that these fungi were of low virulence to queenright colonies, while the queenless colonies and fungus gardens were suppressed. Moreover, *E. nivea* and *T. longibrachiatum* seemed to be less aggressive than *E. moelleri*, observed both *in vivo* and *in vitro*. The results highlight the importance of each element (queen, workers and fungus garden) in the leafcutter ants-fungus symbiosis. Most importantly, we showed that *Escovopsis* may not be virulent to healthy colonies, despite

commonly being described as such, with the reported virulence of *Escovopsis* being due to poor colony conditions in the field or in laboratory experiments.

Keywords: host-parasite interactions**,** parasitism, *Atta*, *Acromyrmex*, symbiosis

Introduction

Parasites can play an important role in many aspects of their hosts' life, threatening their survival and reproduction. The harm that parasites cause to their hosts, referred to as virulence (Frank 1996), can be influenced by numerous factors, involving traits related to the parasite and to its host, in addition to the environmental conditions in which both are to be found.

Theory about virulence evolution predicts that there is a relationship between parasites' virulence and their mode of transmission to new hosts (Ewald 1987; Alizon et al. 2009). It has been suggested that vertically transmitted parasites tend to be less virulent in relation to horizontally transmitted parasites, because their fitness depends on their host´s reproductive success (Clayton & Tompkins 1994). This has been demonstrated in some empirical studies (Bull et al. 1991; Clayton &Tompkins 1994; Tompkins et al. 1996; Agnew & Koella 1997; Stewart et al. 2005; Pagán et al. 2014) although it cannot be taken as a general rule, especially as many parasites can present both modes of transmission (Ebert 2013; Cressler et al. 2015). Host lifespan can be an important factor in understanding the evolution of a parasite also (Watson 2013). It is predicted that hosts with a shorter lifespan reduce the future opportunities of parasite transmission, thus parasites should grow faster within the host and be transmitted earlier, which is reflected in a higher virulence (Nidelet et al. 2009). In addition, some parasites may present a virulence dependent on the context in which they are inserted, for example increasing their virulence when their hosts are under stressful conditions (Brown et al. 2000; Brown et al. 2003; Jokela et al. 2005; Manley et al. 2017).

As with all living organisms, eusocial insects such as ants, termites and some bees and wasps may be exposed to a diversity of parasites. These parasites include viruses, bacteria and fungi, among others (Schmid-Hempel 1998) and the manner in which

sociality could affect their evolution has been discussed in the literature. Some authors argue that long-lived colonies of insect societies represent a buffered and homeostatic environment, which tends to lead to a reduction in parasite's virulence (e.g. Hughes et al. 2008). Additionally, highly genetically diverse colonies of some eusocial insects, that have *polyandrous* queens (i. e multiple mating queen with several males), have been associated with a lower incidence of disease and better resistance to parasite infection (Hughes & Boomsma 2004; Tarpy & Seeley 2006; Seeley & Tarpy 2007). Furthermore, the level of a parasite´s virulence can increase in some stressful stages of a eusocial insects' life history, such as the colony-founding period (Brown et al. 2003).

Among eusocial insects, the leafcutter ants (genera *Atta* and *Acromyrmex*, Attini: Formicidae) are well-known to interact with a diversity of microorganisms that are present in their colonies, including parasites and mutualists (Pagnocca et al. 2012). These ants cultivate the fungus *Leucoagaricus gongylophorus* (Basidiomycota: Leucocoprini, Agaricales), using it as food, while providing nutrients, protection and dispersion in return. The fungal cultivar of leafcutter ants is farmed in subterraneous chambers that shelter the "fungus garden". This structure is composed of the fungal cultivar mycelium and fragments of leaves and flowers that serve as a substrate to the fungus' growth. It is important to note that fungus gardens can also harbor a range of bacteria, yeasts and filamentous fungi (Carreiro et al. 1997; Currie et al. 1999a; Rodrigues et al. 2005; Rodrigues et al. 2008; Suen et al. 2010). Fungi of the genera *Escovopsioides* and *Escovopsis* (Ascomycota: Hypocreales) are examples of microorganisms that are commonly found in the fungus gardens (Augustin et al. 2013; Reis et al. 2015).

The genus *Escovopsioides* includes only one species described to date, *Escovopsioides nivea* (Augustin et al. 2013) and its role in colonies of leafcutter ants is as yet poorly understood. A study performed by Varanda-Haifig et al. (2017)

demonstrated that this fungus is an antagonist of *L. gongylophorus* capable of inhibiting the growth of the fungal cultivar in culture medium. Negative effects of *E. nivea* were also demonstrated in fungus garden fragments (Osti and Rodrigues 2018). The genus *Escovopsis* is phylogenetically related to *Escovopsioides* (Augustin et al. 2013) and is considered a specialized parasite of the fungal cultivar of leafcutter ants (Currie et al. 1999a). Some early studies suggested that this parasite is highly virulent to its host, capable of causing the death of infected colonies and reducing the fungus garden biomass as well as the production of new ant individuals (Currie et al. 1999a; Currie 2001). Based on the results of these studies, it has become disseminated in the literature that this fungus actually represents a highly virulent parasite (Currie et al. 1999a; Currie 2001; Currie & Stuart 2001; Currie et al. 2003a, b; Stearns & Hoekstra 2005; Hölldobler & Wilson 2009; Farji-Brener et al. 2016; Verza et al. 2017). However, it is important to consider some points related to this. Firstly, *Escovopsis* is often found in the fungus garden of healthy colonies that remain foraging and growing (Currie et al. 1999a; Gerardo et al. 2004; Rodrigues et al. 2005a; Augustin 2011). Secondly, one of the pioneering studies that investigated the impact of *Escovopsis* on the ant-fungus symbiosis was conducted using newly-founded colonies of 10-12 weeks (Currie 2001). Thus, it is possible these colonies were still fragile, and in this condition, could suffer a heightened negative impact because of this parasite. Thirdly, some studies investigated the mode of *Escovopsis* transmission between colonies of leafcutter ants and suggested that the parasite seems to be horizontally transmitted (Currie et al. 1999a; Moreira et al. 2015; Augustin et al. 2017) which has been taken spuriously as evidence of high virulence (as vertical transmission selects for lower virulence – see above) (Currie et al. 1999a). Nevertheless, the results that indicated horizontal transmission of *Escovopsis* do not exclude the possibility of vertical transmission, which could support a low virulence consistent with theory of

virulence evolution. Fourth, horizontal transmission does not automatically indicate high virulence as in the case of the common cold of humans for example. Finally, another important issue is that, like other parasites, the level of virulence presented by *Escovopsis* may also vary depending on the ecological context in which it is inserted.

It is also important to consider that colonies of leafcutter ants function as superorganisms. Besides the fungus gardens, the reproductive and non-reproductive castes are involved in different functions, such as reproduction, defence and foraging. These individuals act similarly to the germ and somatic cells in the body of multicellular organisms (Cremer & Sixt 2009). Thus, all elements that comprise the colonies act as a cooperative unit (Detrain & Deneubourg 2006) and its components cannot survive and reproduce without one another (Cremer et al. 2017). In this sense, studies that investigate the impact of *Escovopsis* and *Escovopsioides* to the fungal cultivar of leafcutter ants must be conducted considering the role of the ants in the symbiosis as well as the other elements that comprise the colonies. Despite the importance of this factor, some studies that evaluated the impact of *Escovopsis* have been performed using *in vitro* assays (Silva et al. 2016; Folgarait et al. 2011a; b; Marfetán et al. 2015; Varanda-Haifig et al. 2017), colonies without queens (referred to as queenless colonies) or the fungus gardens without ants (Wallace et al. 2014). These studies are very important to understand the antagonism of *Escovopsis* and *Escovopsioides* with *L. gongylophorus* and how this may affect leafcutter ant colonies. However, since the virulence of parasites can be influenced by several factors, it may be that the use of only parts of the superorganism to conduct experiments also influences the level of virulence presented by these antagonistic fungi.

In this context, our objective was to test two hypotheses: first, *Escovopsis* and *Escovopsioides* are of low virulence to healthy colonies of leafcutter ants; second, the virulence of these fungi varies according to the level of complexity that a colony presents.

Here, we considered that a parasite with low virulence can cause some negative impact to its host, yet not enough to compromise its survival. On the other hand, a highly virulent parasite is able to cause its host´s death. In addition, we also considered that a higher level of colony complexity involves the interaction between the queen and workers as well as the fungus gardens (which include the fungal cultivar and the other microorganisms that are also present). When we disregard one or more components of this symbiosis, the level of complexity of this interaction decreases. The fungal cultivar *L. gongylophorus* cultivated in culture medium (*in vitro*) was considered the simplest level of the interaction since it excludes all the other elements that compromise a colony. Therefore, we considered those colonies, queenless colonies, fungus gardens and only *L. gongylophorus in vitro* represent decreasing levels of complexity. Our expectation was that *Escovopsis* and *Escovopsioides* would not represent a threat to a higher level of complexity, however, the virulence of these fungi would increase as the complexity levels of the colonies decrease.

Material and methods

Experimental approach

We conducted two experiments to investigate the impact of *Escovopsis* and *Escovopsioides* on the leafcutter ant-fungus symbiosis. In the first experiment (Experiment I), we evaluated the impact of these fungi, *in vivo*, on leafcutter ant colonies and in three different levels of complexity: (i) queenright colonies (fungus garden + queen + workers; Fig.1A), (ii) queenless colonies (fungus garden + workers, without queen; Fig.1B) and (iii) fungus gardens (fungus gardens without any ants; Fig.1C). For this, we exposed the different levels of colonies to the following treatments: (a) conidial suspensions of *Escovopsis moelleri*, (b) *Escovopsioides nivea*, (c) *Trichoderma longibrachiatum* and (d) blank control (0.01% Tween 80® + NaCl 0.85% solution). In the second experiment (Experiment II), we evaluated the interaction of *E moelleri*, *E. nivea* and *T. longibrachiatum* with the fungal cultivar, *in vitro*, through a paired culture bioassay (Fig.1D). The purpose of this experiment was to exclude most of the elements that comprise a colony, resulting in the simplest level of complexity, composed of only the fungal cultivar, *L. gongylophorus*. Thus, from these two experiments, we tested the first hypothesis, that *Escovopsis* and *Escovopsioides* are of low virulence for colonies and the second, that this virulence increases as colony complexity decreases.

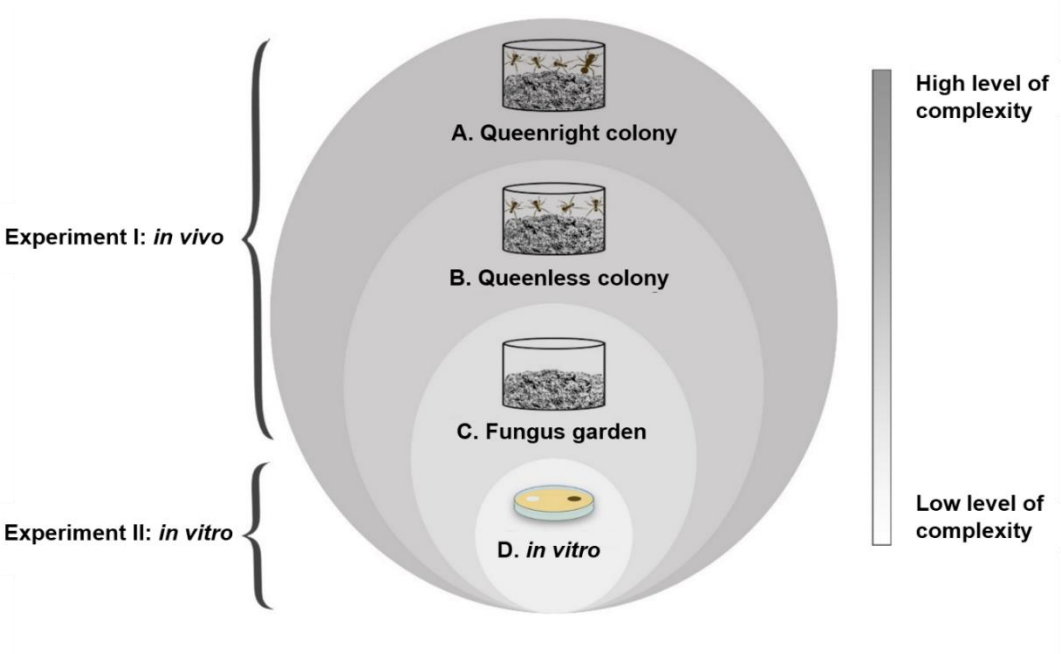


Figure 1. Schematic representation of experiments I and II showing the complexity levels of leafcutter ant-fungus symbiosis: **(A)** queenright colony; **(B)** queenless colony; **(C)** fungus garden; **(D)** fungal cultivar (*in vitro*). Note that a queenright colony is composed of the fungus garden, the queen and specialized workers with different functions. Therefore, we considered in our study that a queenright colony encompasses all these elements. On the other hand, a queenless colony do not contain an essential component of the queenright colony, the queen. As we disregard one or more organisms involved in the association between leafcutter ants and its fungal cultivar, the complexity levels of this interaction decrease. This can also be observed in fungus gardens without ants as well in the cultivation of the fungal cultivar *in vitro*, which we considered the simplest level of this interaction. From these considerations, we evaluated the impact of the fungi *Escovopsis* and *Escovopsioides* on *Acromyrmex subterraneus subterraneus* colonies and their fungal cultivar in different complexity levels. This schematic representation is also present in the master's thesis of Débora Mello Furtado de Mendonça.

Organisms

Twelve colonies of *Acromyrmex subterraneus subterraneus* (~1-year-old) were collected in three areas on the Campus of Universidade Federal de Viçosa (UFV), Viçosa, Minas

Gerais, southeastern Brazil: *Dendrologia* (20º 46'21"S 42º52'25"W), *Recanto das Cigarras* (20º 45'26"S 42º51'45"W) and *Horto Botânico* (20º 45'25"S 42º52'23"W). The first two areas are fragments of secondary Atlantic forest while *Horto Botânico* is a living plant collection comprising native and exotic plant specimens. The fungus garden of each colony was transferred to a plastic pot (500 ml) and then placed in a plastic tray (43 × 29 × 7cm). In the base of the pots, we made a 2 cm diameter exit hole to allow the passage of workers for the foraging arena in the tray. The inner sides of each tray were covered with neutral talcum powder (magnesium silicate) to prevent the ants from escaping. We offered fresh leaves of *Acalypha wilkesiana* (Euphorbiaceae) daily as forage for the ants. The colonies were maintained under controlled temperature (25±2°C) and humidity conditions (75±3% RH). In order to check if *Escovopsis* and *Escovopsioides* were naturally present in the collected colonies, we sampled fragments from fungus gardens and plated these on growth media. The presence of these fungi was recorded and is presented in Supplementary Material. *Escovopsis* was found in five of the twelve colonies while *Escovopsioides* was found in just one, and the colonies were considered suitable for conducting the experiment since that they were apparently healthy, for example, foraging normally.

In both experiments, the same fungal isolates were used: *Escovopsis moelleri* (VIMI-10.0001), *Escovopsioides nivea* (VIMI-17.0136) and *Trichoderma longibrachiatum* (VIMI-17.0135). In the paired culture bioassay (*in vitro*) we also used a *Leucoagaricus gongylophorus* isolate (VIMI-17.0137). *Trichoderma longibrachiatum* was used in the assays for comparison with *E. moelleri* and *E. nivea*. This allowed us to evaluate if the possible effects observed in our experiments is due to *E. moelleri* and *E. nivea* or can be caused by the presence of any other fungi. The genus *Trichoderma* constitutes a group close to *Escovopsis* and *Escovopsioides*, belonging also to the family

Hypocreaceae and is found in leafcutter ant colonies (Rocha et al. 2014; Montoya et al. 2016).

Escovopsis moelleri was obtained from the fungus collection of the Laboratory of Insect-Microorganism-Interactions, Universidade Federal de Viçosa (LIIM-UFV). This isolate was collected from the fungus garden of *Acromyrmex subterraneus molestans* (Augustin et al. 2013) and stored on silica gel at 5°C. *Leucoagaricus gongylophorus*, *E. nivea* and *T. longibrachiatum* (all isolated from this study by D.M.F.M.) were collected from *Ac. subterraneus subterraneus* fungus gardens, the last one from the fungus garden of a dead colony kept in the lab.

Leucoagaricus gongylophorus and *E. nivea* were cultivated on MEA (20 g malt extract and 15 g agar l^{-1}), while *T. longibrachiatum* was cultivated on PDA 20% (7.8 g PDA, KASVI® plus 12 g agar l^{-1}). Subsequently, these fungi were incubated at 25°C for seven days. After this period, they were re-isolated (ca. 2 times) until we obtained a pure culture. The isolate of *L. gongylophorus* was identified through its morphological characteristics, such as the production of gongylidia. *Escovopsioides nivea* and *T. longibrachiatum* were identified by their morphological characteristics and at the molecular level. For morphological identifications, we made slides and examined these with a Nikon (Eclipse E200) light microscope. The molecular characterization was conducted by sequencing the genomic region translation elongation factor (*tef*). The obtained sequences were compared with other sequences available at GenBank through Basic Local Alignment Search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The isolates were stored in 10% glycerol at -80°C and included in the LIIM mycological collection.

Experiment I (*in vivo*) – Impact of fungi and at different levels of complexity on colonies of leafcutter ants

The aim of this experiment was to evaluate the impact caused by *Escovopsis* and *Escovopsioides* in colonies of leafcutter ants divided into different levels of colony complexity. For this, we exposed queenright colonies, queenless colonies and fungus gardens to *Escovopsis* and *Escovopsioides,* individually.

Conidial suspensions

To prepare the conidial suspensions, we first grew the isolates *E. moelleri* and *E. nivea* on plates containing MEA, while *T. longibrachiatum* was grown on PDA 20%. These fungi were incubated at 25°C for 15 days. After this period, fragments of fungus were removed from the plates and individually inserted into a Falcon tube containing sterile distilled 0.01% of Tween 80® + NaCl 0.85% solution. The suspensions were stirred for 3 min and then filtered using sterile gauze. This procedure allowed the separation of the conidia from hyphal fragments, resulting in suspensions containing only conidia. We prepared the suspensions according to the maximum conidial concentrations we could obtain for each fungus. The concentrations of the suspensions were determined using a Neubauer chamber. *Escovopsis moelleri* and *T. longibrachiatum* suspensions contained 1 $\times 10^8$ conidia ml^{-1} while *E. nivea* suspension contained 1×10^6 ml^{-1}. These were the greatest concentrations we could obtain for each fungus. All suspensions were kept at 5°C overnight.

Experimental set-up

In order to verify the effect of the three different levels of colony complexity, we performed the following procedure: each initial colony was divided into three fragments

(Fig. 2A), one fragment consisted of a queenright colony and the two remaining fragments consisted of queenless colonies, totalizing 12 queenright colonies and 24 queenless colonies (Fig. 2B). Each fragment was placed in 250 ml plastic pots (Fig. 2B). The fragments were maintained in the separate pots in the same tray for 30 days; by this time the fungus garden fragment reached the top of the 250 ml pot. To obtain the treatment that consisted only of fungus garden, we carefully removed the ants from 12 queenless colonies using forceps one day before the start of the experiment (Fig. 2C). For this, the fungus gardens were fragmented to get access to all their regions so we could remove all the ants. We observed in preliminary tests that fungus gardens decreased in weight and suffered changes in their physical structure when the ants were removed, presumably due to the manipulation during the procedure. In order to apply the same conditions to all fragments, the fungus gardens of the 12 queenright colonies and 12 queenless colonies were fragmented similarly, without damaging the queens or the workers. This step was important also to observe if queenright colonies were indeed queenright and queenless colonies were queenless. We opted not to use insecticides to remove the ants from fungus gardens as some insecticides may have inhibitory effects on fungal growth (Cowley & Lichtenstein 1970; Olmert & Kenneth 1974; Ali et al. 2012), which could interfere in our experiment. Thus, we considered mechanical removal the most adequate procedure. After this period, each pot that contained each complexity level was placed individually in a tray (343 × 200 × 66 mm). The inner sides of the trays were covered with neutral talcum powder to prevent the ants from escaping. Thus, we had in total 12 queenright colonies (fungus garden + queen + workers), 12 queenlees colonies (fungus garden + workers) and 12 pots containing only fungus garden (Fig. 2D). To assemble and conduct the experiment many steps were required and are listed in Table S1 of Supplementary Material.

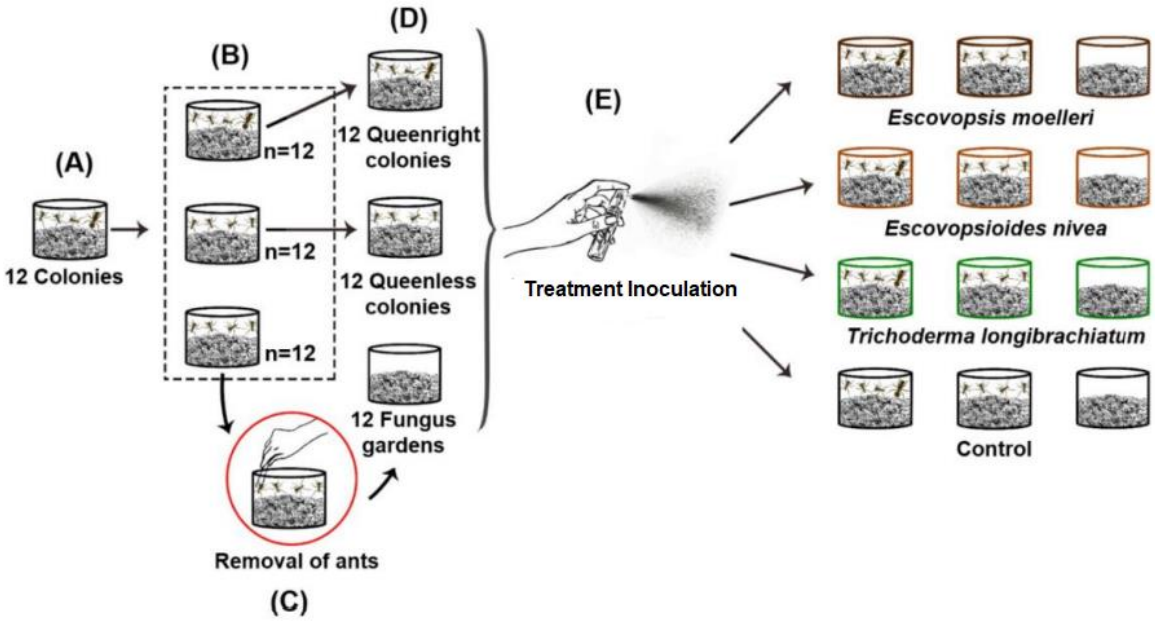


Figure 2. Schematic representation of experimental set-up. Twelve colonies of the leafcutter ant *Acromyrmex subterraneus subterraneus* were necessary to assemble the experiment. **(A)** Each of these 12 initial colonies was separated in three fragments. **(B)** One fragment consisted of a queenright colony (fungus garden + workers + queen), and the two other fragments consisted of a queenless colony (fungus garden + workers, without queen), resulting in the total of 12 queenright colonies and 24 queenless colonies. Each queenright colony with their respective queenless colonies were placed in 250 ml individual pots and maintained together in the same tray for 30 days to allow the growth of the fungus gardens. **(C)** After this period, in order to obtain the fungus gardens without any ants, we removed using tweezers, the workers from 12 queenless colonies. **(D)** Thus, we obtained our three colonies complexity levels: queenright colony (n=12), queenless colony (n=12) and fungus garden (n=12). **(E)** We exposed each of these colonies complexity levels to one of the three fungal treatments or control: conidial suspension of *Escovopsis moelleri* (1×10^8 conidia ml^{-1}), *Escovopsioides nivea* (1×10^6 conidia ml^{-1}), *Trichoderma longibrachiatum* (1×10^8 conidia ml^{-1}) or control (0.01% Tween 80® + NaCl 0.85% solution). The inoculation of suspensions was done through a sterile spray bottle. We sprayed 4 ml of conidial suspensions or the control on the surface of fungus gardens. The queenright colonies, queenless colonies and fungus gardens that originated from the same initial colony were exposed to the same treatment. Each treatment was replicated three times (combination of a fungus treatment + a colony complexity level). This

schematic representation is also present in the master's thesis of Débora Mello Furtado de Mendonça.

Fungal inoculation

We inoculated 4 ml of conidial suspensions of *E. moelleri* (1×10^8 conidia ml^{-1}), *E. nivea* (1×10^6 conidia ml^{-1}) and *T. longibrachiatum* (1×10^8 conidia ml^{-1}) individually, on each pot (Fig. 2E). For the control blank, we inoculated 4 ml of 0.01% Tween 80® + NaCl 0.85% solution (Fig. 2E). The queenright colonies, queenless colonies and fungus gardens that originated from the same initial colony were exposed to the same treatment. Each combination of fungus treatment with a colony complexity level was replicated three times. The inoculation of conidial suspensions was done using a sterile spray bottle (Fig. 2E). We sprayed the suspensions on the surface of fungus gardens until all the 4 ml were dispensed (Fig. 2E). The trays that contained each treatment were placed randomly in three shelves. Each shelf contained one repetition of each treatment. During the experiment, laboratory conditions were 25±2°C, 75±3% RH and 12 hours photoperiod.

Data collection

Survival of colonies divided in complexity levels and exposed to different fungal treatment

The survival of queenright colonies, queenless colonies and fungus gardens was checked every day. These records were made for 118 days after fungal inoculation. We considered fungus gardens without ants to be dead when they were completely covered by other fungi. The queenright colonies and queenless colonies were considered dead when they did not contain any live ants, their fungus gardens presented a dry texture, almost crumbling or if they were overgrown by other fungi. We kept the colonies for seven months after the end of the experiment to see if they remained alive.

Weights of colonies divided in complexity levels and exposed to different fungal treatments To evaluate if there was variation in the weight according to each complexity level or if all of them succumbed to fungi treatment after fungal inoculation, the weights of queenright colonies, queenless colonies and fungus gardens from each treatment were measured. We weighed the queenright colonies and queenless colonies two days before the experiment start while the fungus gardens were weighed one day before. We repeated this procedure 48 hours after inoculations of conidial suspensions and then every 72 hours for 20 days. The initial weight of each queenright colony presented some variation and this was also observed between the queenless colonies and among the fungus gardens. These variations are natural since each colony presents particular characteristics and thus it is difficult to find colonies with equal weights.

Midden weights

Middens from each queenright colony and from queenless colonies were weighed to evaluate if ants produced different quantities of midden depending on complexity level and fungal treatment. We weighed the midden produced 24 hours after fungal inoculation and then every 72 hours for 20 days.

Weights of leaves cut by ants

The weights of leaves cut by workers were determined to evaluate if ants altered the amount of food supplied to the fungal cultivar due to the possible detrimental effects of the fungal treatments and the absence of queen (in the case of queenless colonies). For this purpose, we offered, daily, 3 g of fresh *Acalypha wilkesiana* leaves and after 24 hours, we weighed the leaves that were not cut (leaves remaining in the trays) for 20 days. To calculate the real quantity of leaves that were cut, we considered the percentage of water

loss of these leaves. Thus, we daily maintained on each shelf that had the treatments, one tray containing only fresh *A. wilkesiana* leaves (3 g) that were also weighed in order to evaluate the water loss. Therefore, we calculated the weight of leaves that were cut according to the adapted formula from Antunes & Della Lucia (1999) and Gandra (2014).

Cr= QFi – QFf – (%PA)

Cr= weight of cut leaves

QFi= Quantity of leaves that we offered

QFf= Quantity of leaves that were not cut by ants

%PA= Percentage weight of water loss

Sampling from fungus gardens and middens

We sampled the fungus gardens from each queenright colony, queenless colony and fungus garden without ants to verify the presence of *Escovopsis*, *Escovopsioides* or *Trichoderma*. We also sampled the middens produced by queenright colonies and queenless colonies to observe if the ants were removing these fungi from their fungus gardens (Supplementary Material). This sampling was always carried out after weighing the midden.

Data analysis

We compared colony survivals related to three complexity levels (queenright and queenless colonies and fungus garden) and to fungal treatment (*E. moelleri*, *E. nivea*, *T. longibrachiatum* or blank control), using survival analyses with a Weibull hazard distribution (Crawley, 2013). We adjusted a full model with treatment and complexity levels, and an interaction term between these variables. The full model was simplified by deletion of nonsignificant effects. The colony of origin was included as a frailty factor.

Analysis was performed in R software (R Core Team, 2020) with survival package v. 2.38 (Therneau, 2015).

We adjusted linear mixed models (LMM) with normal distributions and random intercepts to evaluate the significance of two explanatory factors: the colony complexity levels and fungal treatments. The response variable consisted of: (i) weight of queenright colonies, queenless colonies, fungus gardens and weight of all these complexity levels together submitted to fungal treatment, (ii) midden weight produced by queenright colonies and queenless colonies and weight of all of them related to fungal treatment and (iii) weight of leaves cut by ants from queenright colonies and queenless colonies and their weight associated to fungal treatment. Response variables were analysed in separate models. To each response variable we adjusted a full model with treatment and complexity levels, and interactions between treatment and time, as well as complexity levels and time of evaluation. The full model was simplified by deletion of nonsignificant effects. The models were compared using Chi-squared test (χ^2) (P<0.05) and all these analyses were conducted in the R statistical software (R Core Team, 2017).

Experiment II (*in vitro*) – Interaction of fungi with the fungal cultivar of leafcutter ants

The aim of this experiment was to evaluate the interaction between *L. gongylophorus* with *Escovopsis*, *Escovopsioides* and *Trichoderma* with, *in vitro*, that we considered the simplest level of complexity. We evaluated the growth of *L. gongylophorus* through a paired culture assay (co-culture) in Petri dishes with *Escovopsis, Escovopsioides, Trichoderma* and blank control.

Paired culture bioassay

The isolates of *E. moelleri*, *E. nivea* and *T. longibrachiatum* were previously grown in Petri dishes (9 cm in diameter) containing MEA and incubated at 25°C for 10 days. The isolate of *L. gongylophorus* was previously grown on MEA for 15 days, because it has slower growth. After this period, 8 mm diameter disks of *L. gongylophorus* were cut and plated 5 mm from the border of Petri dishes (90 × 15 mm) containing 20 ml of MEA. The plates were incubated at 25°C for 15 days, the period required for the growth of this fungus, as described above. After this, mycelium disks of *E. moelleri*, *E. nivea* and *T. longibrachiatum* were placed individually in plates containing the disk of *L. gongylophorus*. These fungi were inoculated on the opposite side of the fungal cultivar, also at 5 mm from the plate edge. For the control treatment, we used an 8 mm diameter disk of plain MEA instead of the fungal cultivar. Thus, we obtained the four following combinations: (i) *L. gongylophorus* × *E. moelleri* (n=10), (ii) *L. gongylophorus* × *E. nivea* (n=10), (iii) *L. gongylophorus* × *T. longibrachiatum* (n=10), (iv) *L. gongylophorus* × control (n=10). During the experiment, the plates were maintained incubated at 25°C and distributed on five shelves of an incubator. Each shelf contained two blocks with one repetition of each treatment per block. The growth of fungi was evaluated every 12 hours, for 10 days. However, we conducted the analysis using the data of day 5, when the first fungal isolate reached the fungal cultivar on the opposite side of the plate. We photographed and scanned the plates using a digital camera (Nikon D2000) and a multifunction printer (HP LaserJet Pro CM1415fnw). From the images, we measured the growth of fungal cultivar *L. gongylophorus* using ImageJ 1.49v software.

Data analysis

To compare the growth of the *Leucoagaricus gongylophorus* isolate in the presence of *Escovopsis moelleri*, *Escovopsioides nivea*, *Trichoderma longibrachiatum* fungi and control (agar), we adjusted generalized linear mixed models (GLMM) with normal distributions and random intercepts. For this, we considered presence of fungi (*E. moelleri*, *E. nivea* and *T. longibrachiatum* and control) and time as explanatory factors, and colony radius (cm) of *L. gongylophorus* as response factor. The identity of each plate was considered as a random factor in both analyses. We then tested the interaction between the explanatory factors. Also, we compared *L. gongylophorus* growth, performing multiple comparisons with multcomp package v. 1.4-13 (Hothorn *et al.*, 2008), to verify the potential differences of *L. gongylophorus* growth in presence of *E. moelleri*, *E. nivea*, *T. longibrachiatum* and control before inoculation of fungi and control (after 15 days of *L. gongylophorus* inoculation) and at the end of the experiment (after 108 hours). We performed all analyses in R Software (R Core Team, 2020).

Results

Experiment I (*in vivo*) – Impact of fungi on colonies of leafcutter ants at different levels of complexity

Survival of colonies divided in complexity levels and exposed to different fungal treatments

All queenright colonies remained alive during the 118 days evaluated. Seven months after the end of the experiment, the queenright colonies were still alive and at this juncture were discarded. At the other extreme, all fungus gardens (without queen and workers) died 11 days after the inoculation of fungal treatments, including the blank control, independent of the fungus that was inoculated. Therefore, between-treatment variation in survival was only observed in the queenless colonies (Fig. 3A).

The survival times of queenless colonies did not differ between the blank control (115.66 ± 1.85 days; mean ± S.E.), *E. nivea* (86.33 ± 19.5 days; mean ± S.E.) and *T. longibrachiatum* (74.66 ± 19.6 days; mean ± S.E.) ($\chi^2_{[1]} = 1.868$, $P = 0.393$; Fig. 3B). However, queenless colonies submitted to *E. moelleri* died more quickly (36 ± 8.3 days; mean ± S.E.) than control, *E. nivea* and *T. longibrachiatum* (queenless) colonies ($\chi^2_{[1]} = 9.582$, $P = 0.002$; Fig. 3B).

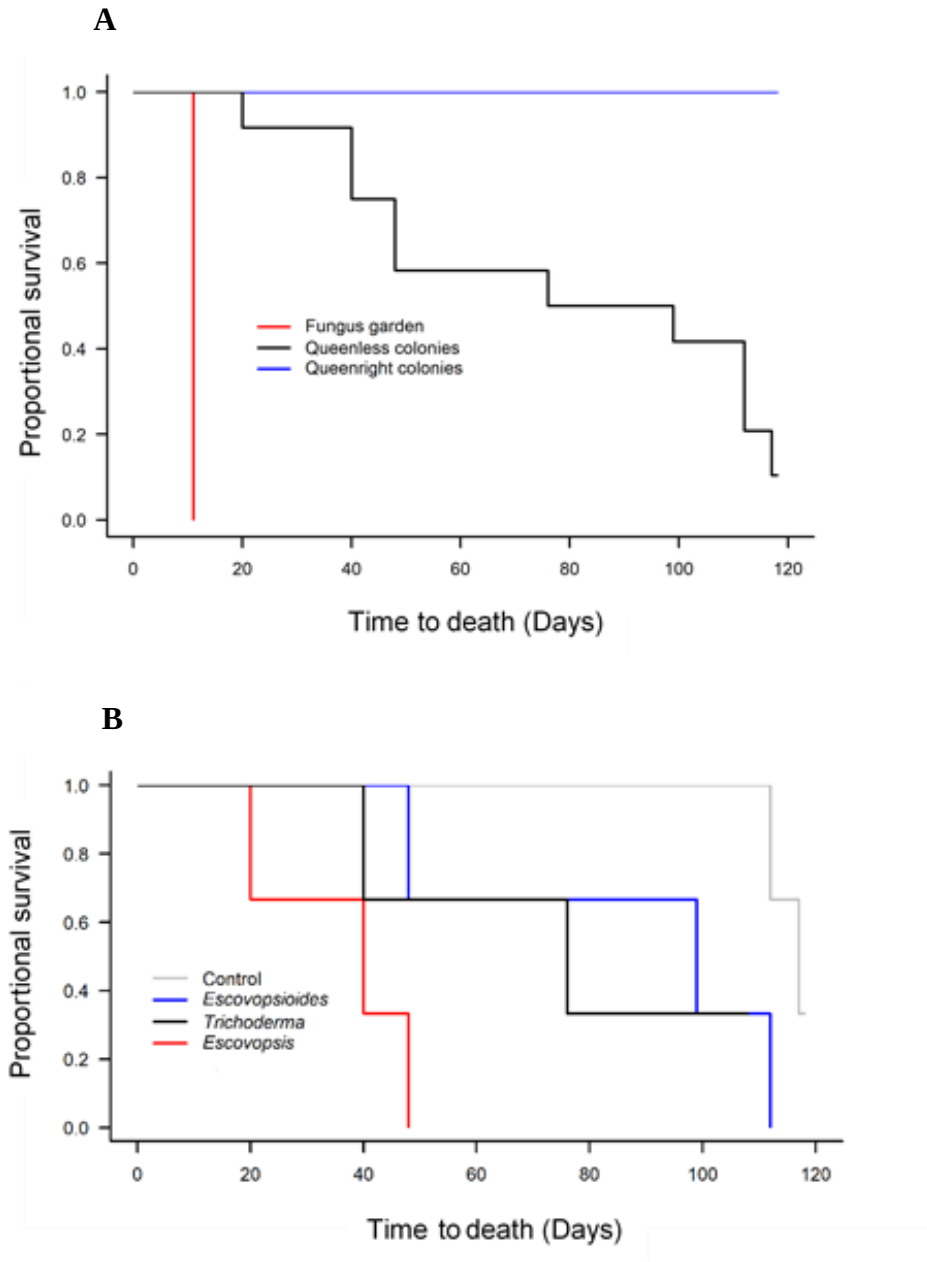


Figure 3. (A) Survival of *Acromyrmex subterraneus subterraneus* leafcutter ant queenright colonies (black line), queenless colonies (gray line) and fungus gardens (red line) exposed to fungal treatments: conidial suspension of the fungi *Escovopsis moelleri*; *Escovopsioides nivea; Trichoderma longibrachiatum*; blank control (0.01% Tween 80® solution + saline solution - NaCl 0.85%). Survival was checked every day from the day of inoculation with fungal treatments (Day 0) until 118 days post-inoculation. The fungus gardens were considered dead when they were completely covered by other fungi and presented a dry texture. We considered the queenright colonies and queenless colonies dead when they did not contain any live ants. **(B)** Survival of *Acromyrmex subterraneus*

subterraneus leafcutter queenless colonies exposed to *Escovopsis moelleri*; *Escovopsioides nivea; Trichoderma longibrachiatum* or blank control (0.01% Tween 80® solution + saline solution – NaCl 0.85%). From the analyses comparing survival between the three complexity levels, we observed that only queenless colonies suffered variation in mortality over time (Fig. 3A). For this reason, we compared within this level the effect of each fungal treatment individually (Fig. 3B). There was no difference between queenless colonies inoculated with *E. nivea*, *T. longibrachiatum* and blank control ($\chi^2_{[1]} = 1.868$, $P = 0.393$). However, queenless colonies inoculated with *E. moelleri* treatment died faster ($\chi^2_{[1]} = 9.582$, $P = 0.002$). A survival analysis with a Weibull distribution was conducted and the models compared using χ^2 test ($P < 0.05$).

Weights of colonies divided in complexity levels and exposed to different fungal treatment

There was an interaction between complexity level and time ($\chi2_{[11]} = 290.8$; $P < 0.001$) as well as between fungal treatment and time ($\chi2_{[21]} = 164.5$; $P < 0.001$). Both complexity level and timewere influenced and modified over time according to interaction analyses. These results probably are due to the first 11 days of evaluation in which different treatments had similar values (mainly in relation to fungal treatments), with greater divergence subsequently occurring between treatments.

We observed that all queenright colonies lost weight the day after the inoculation of conidial suspensions, independent of the fungal treatment to which they were exposed and then recovered weight over subsequent days (Fig. 4A). Queenless colonies showed a similar behaviour after fungal inoculation, but the weight continued to drop over time until the end of the experiment (Fig. 4A). In contrast, we observed on the first evaluation day that fungus gardens suffered an increase in their weight. This was probably because the fungus garden without any workers and queen is quickly colonized by other fungi, increasing its biomass. Over time, the entire fungus garden is consumed and the weight starts to decrease. All fungus gardens died in the day 11 of the experiment (Fig. 4A).

In relation to fungal treatments, all the complexity levels submitted to *Escovopsis* suffered a weight reduction over time (Fig. 4B). This result was not observed in any other treatment. Queenright colonies, queenless colonies and fungus gardens inoculated with the blank control, *E. nivea* e *T. longibrachiatum* suffered an initial reduction in weight as well *Escovopsis*, but by day 11 after fungal inoculation, they began to recover.

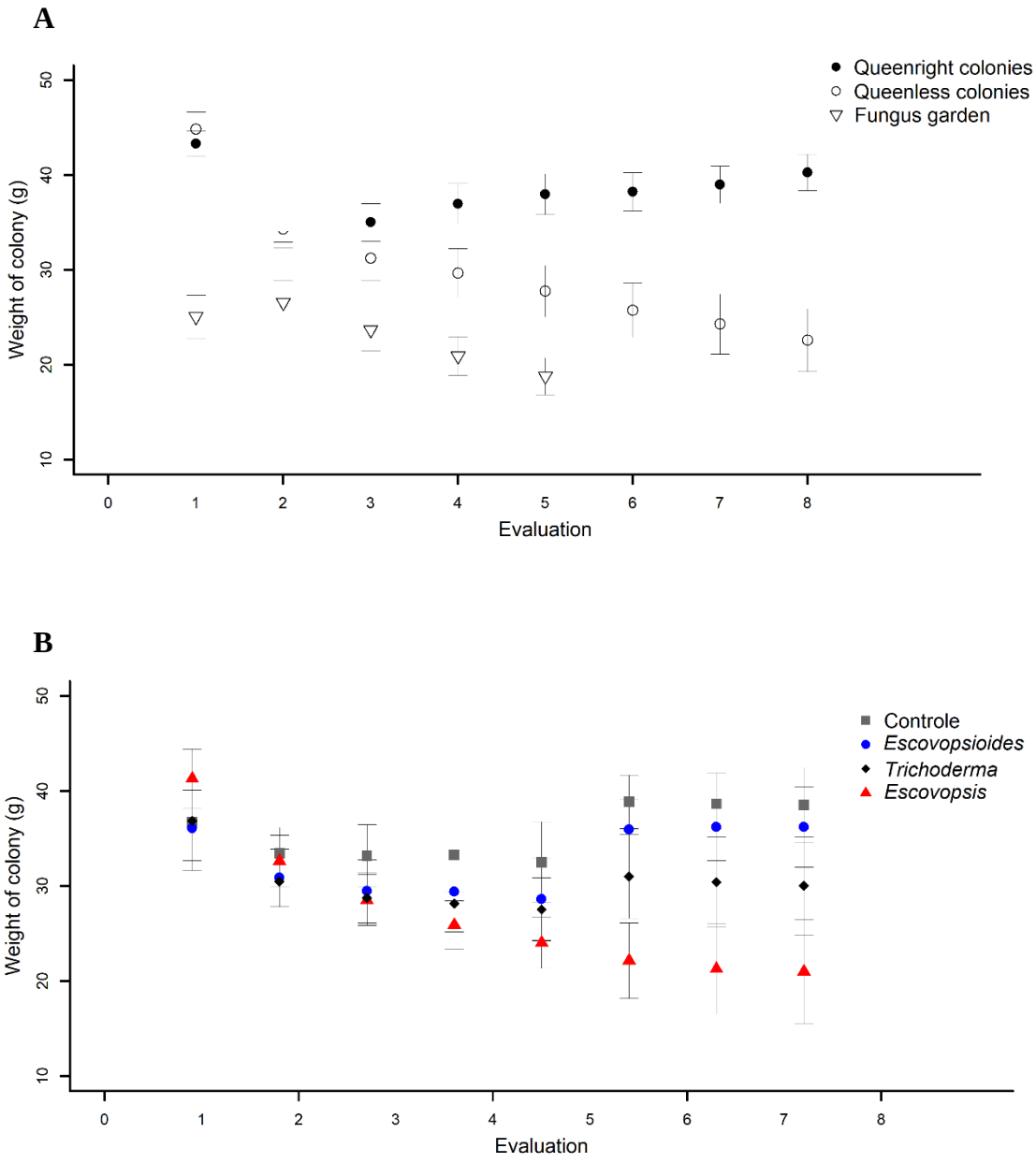


Figure 4. (A) Weights of *Acromyrmex subterraneus subterraneus* leafcutter ant colonies in the three complexity levels: queenright colonies (closed circle), queenless colonies and (open circle) and fungus gardens (open triangle) exposed to fungal treatments *Escovopsis*

moelleri; *Escovopsioides nivea; Trichoderma longibrachiatum*) and to blank control (0.01% Tween 80® solution + saline solution - NaCl 0.85%). The weight of queenright colonies and queenless colonies was measured two days before inoculation (day -2) while fungus gardens were weighed one day before inoculation (day -1). Weighing was repeated 48 hours after inoculation of conidial suspensions for all treatments (corresponding to second evaluation in the figure), and then every 72 hours until day 20 (evaluations 3, 4, 5, 6, 7 and 8). The fungus gardens were evaluated until day 11 (fourth evaluation), when all parcels were considered dead. There was an interaction between the complexity level of colonies and the days ($\chi^2_{[11]} = 290.8$*;* $P < 0.001$), indicating that the difference observed between different complexity levels changes over time. **(B)** Weights of *Acromyrmex subterraneus subterraneus* leafcutter ant colonies of three complexity levels exposed to *Escovopsis moelleri* (red triangle); *Escovopsioides nivea* (blue circle)*; Trichoderma longibrachiatum* (black diamond) or blank control (0.01% Tween 80® solution + saline solution - NaCl 0.85%) (grey square). Conidial suspensions and blank control were inoculated on day 0 and the queenright colonies and queenless colonies were evaluated 7 times until day 20 (seventh evaluation) after inoculation. Weighing was repeated 48 hours after inoculation of conidial suspensions for all treatments, and then every 72 hours. There was an interaction between the complexity level of colonies and time ($\chi^2_{[21]} = 164.5$; $P < 0.001$), indicating that the difference observed between fungal treatments undergoes changes over time. Significance was evaluated using χ^2 test ($P < 0.05$).

Midden weights

There was no interaction between complexity level and time ($\chi^2_{[6]} = 5.251$; $P = 0.512$; Fig. 5A). However, there was an interaction between fungal treatment and time ($\chi^2_{[18]} = 102.84$; $P < 0.001$; Fig. 5B).

The weights of middens produced by ants in queenright and queenless colonies inoculated with fungal treatments and blank control did not differ from each other over time ($\chi^2_{[1]} = 0.773$, $P = 0.379$; Fig. 5A). On the first day of evaluation (24 hours after

inoculation), both produced a large amount of midden which decreased and remained stable until the end of the experiment.

Midden production was high on the first day after inoculation of *E. moelleri* and this treatment was larger than blank control, *E. nivea* and *T. longibrachiatum* (Fig. 5B). Over time, midden production was similar, independent of the fungus applied, except on the second day of evaluation, on which queenright and queenless colonies of the control group presented midden production smaller than other fungal treatments (Fig. 5B).

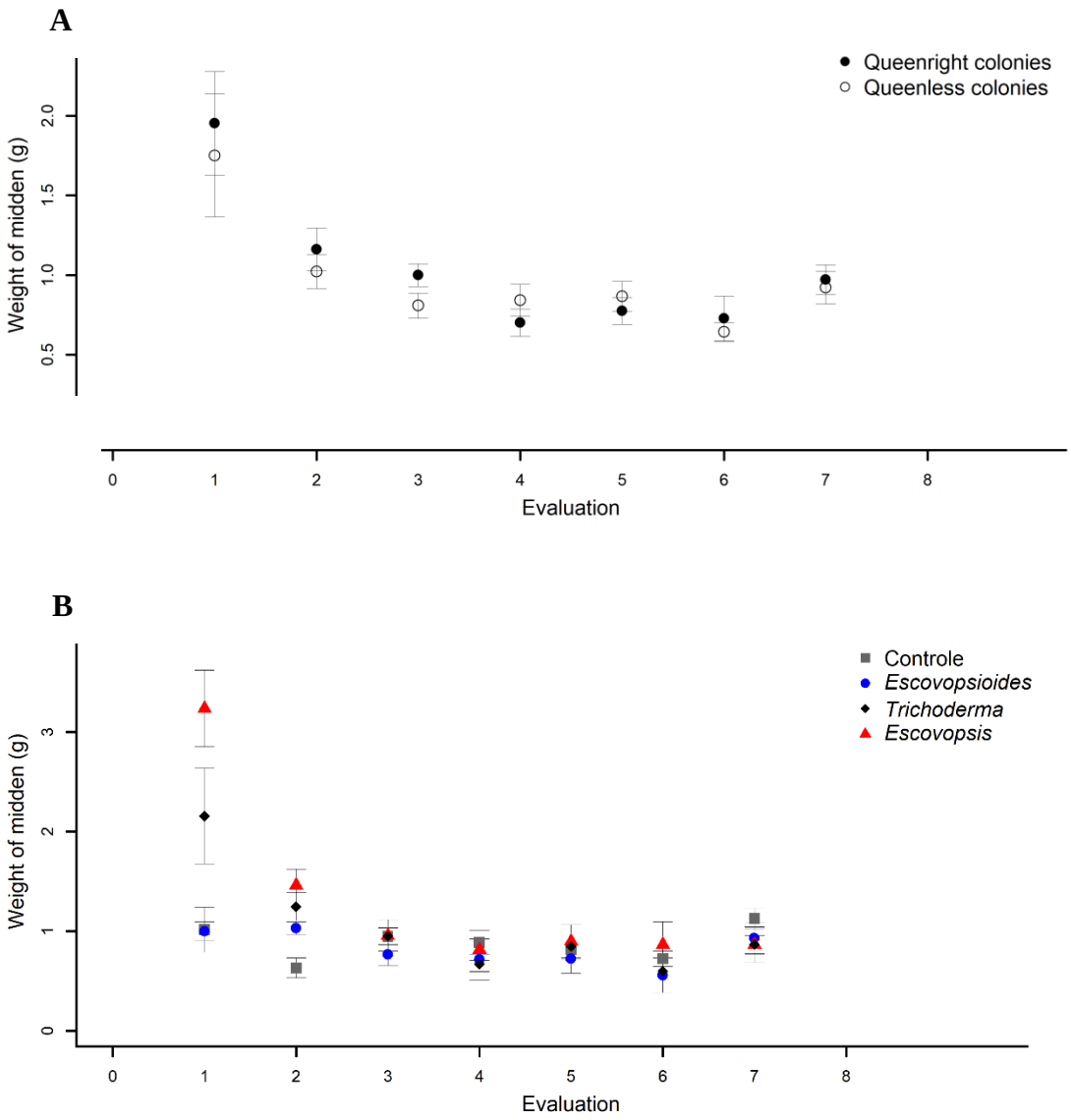


Figure 5. (A) Weights of midden produced by *Acromyrmex subterraneus subterraneus* leafcutter ant queenright colonies (closed circle) and queenless colonies (open circle)

exposed to three fungal treatments and to blank control. To conduct the analysis, we adjusted linear mixed models (LMM) considering each sample as a repeated measure. The weight of midden was measured 24 hours after inoculation of conidial suspensions for all treatments (evaluation 1) and then, this procedure was repeated every 72 hours. The differences between treatments were evaluated by comparing the complete and simplified models. There was no difference in midden production between queenright colonies and queenless colonies ($\chi^2_{[1]} = 0.773$, $P = 0.379$). **(B)** Weights of midden produced by *Acromyrmex subterraneus subterraneus* leafcutter exposed to one of three treatments or to blank control: conidial suspension of the fungi *Escovopsis moelleri* (red triangle); *Escovopsioides nivea* (blue circle)*; Trichoderma longibrachiatum* (black diamond); blank control (0.01% Tween 80® solution + saline solution - NaCl 0.85%) (grey square). Conidial suspensions and blank control were inoculated on day 0 and the weight of midden produced by the queenright colonies and queenless colonies was evaluated until day 20 after inoculation. The weight of midden was measured 24 hours after inoculation of conidial suspensions for all treatments (day 1) and then, this procedure was repeated every 72 hours. To conduct the analysis, we adjusted linear mixed models (LMM) considering each sample as a repeated measure. There was an interaction between fungal treatments and days ($\chi^2_{[18]} = 102.84$; $P < 0.001$)., indicating that the fungal treatments affected midden production in different manners over time. Significance was evaluated using χ^2 tests ($P < 0.05$).

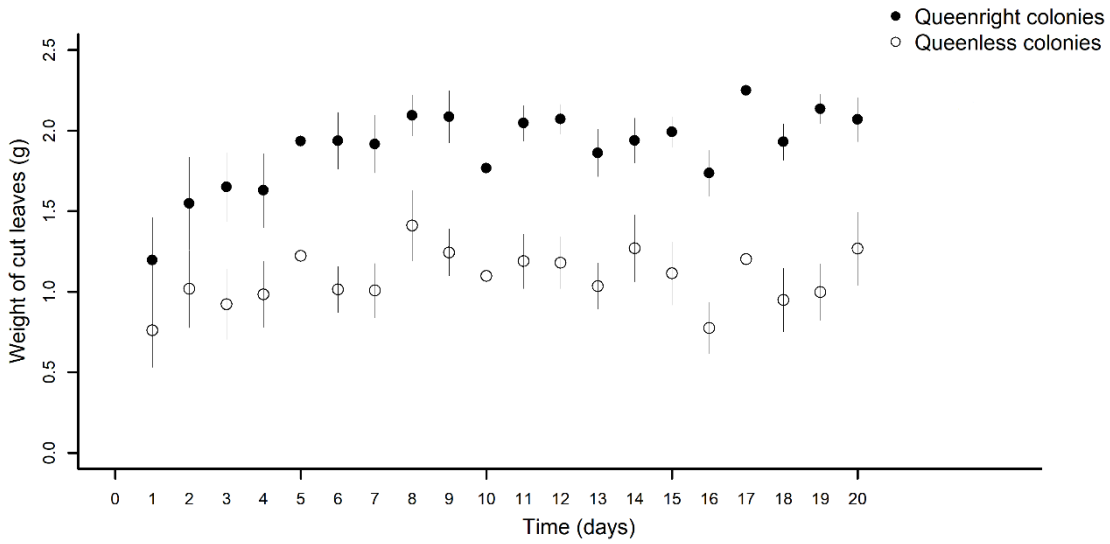
Weights of leaves cut by ants

There was an interaction between the two complexity levels (queenright and queenless) and the days of evaluation ($\chi^2_{[19]} = 30.161$; $P = 0.049$). We also found an interaction between fungal treatment and time ($\chi^2_{[57]} = 77.863$; $P = 0.034$).

In the first two days after exposure to fungal treatments, there was a similarity in the weights of leaves cut by queenright and queenless colonies. From the third day, queenright colonies started to cut a larger amount of leaves than queenless colonies (Fig.6A). Probably because of this variation over time, we observed an interaction between the complexity levels and time.

In relation to fungal treatments, in general, all the complexity levels inoculated with *Escovopsis* cut less leaves than those inoculated with other fungi or blank control over the 20 days (Fig. 6B). We can observe a fluctuation within each treatment, but without changing the main pattern.

A



B

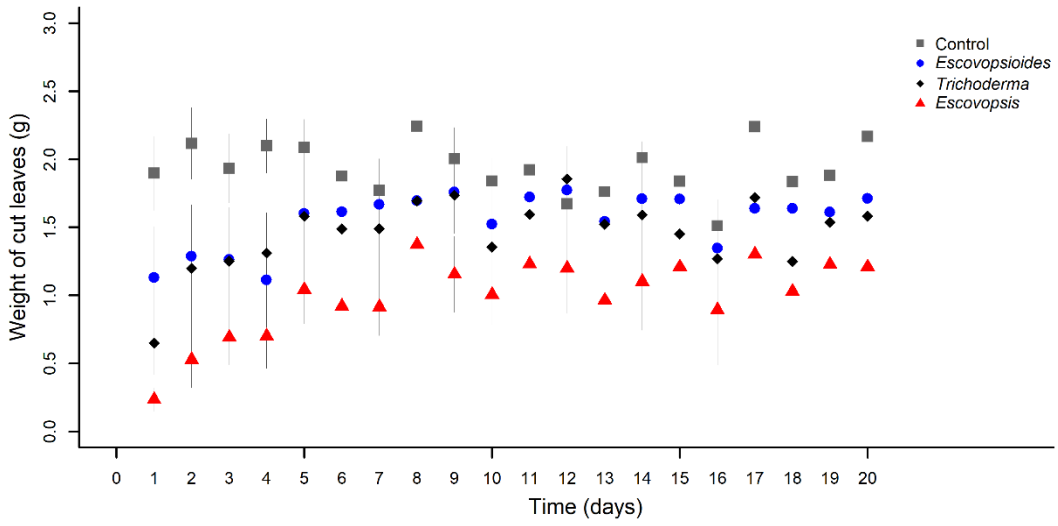


Figure 6. (A) Weights of leaves cut by *Acromyrmex subterraneus subterraneus* leafcutter ant queenright colonies (closed circle) and queenless colonies (open circle) exposed to three fungal treatments and blank control. The weight of cut leaves was measured every day after inoculation of conidial suspensions for all treatments (day 1) until day 20 after

inoculation. To conduct the analysis, we adjusted linear mixed models (LMM) considering each sample as a repeated measure. There was interaction between complexity levels and days ($\chi^2_{[19]}$ = 30.161; P = 0.049), indicating that the different complexity levels affect the cut of leaves of distinct way over time. **(B)** Weights of leaves cut by *Acromyrmex subterraneus subterraneus* leafcutter ant exposed to one of three treatments or to blank control: conidial suspension of the fungi *Escovopsis moelleri* (red triangle); *Escovopsioides nivea* (blue circle)*; Trichoderma longibrachiatum* (black square); blank control (0.01% Tween 80® solution + saline solution - NaCl 0.85%) (grey square). Conidial suspensions and blank control were inoculated on day 0 and the weight of cut leaves by ants of the queenright colonies and queenless colonies was evaluated until day 20 after inoculation. The weight of cut leaves was measured daily after inoculation of conidial suspensions for all treatments. To conduct the analysis, we adjusted linear mixed models (LMM) considering each sample as a repeated measure. There was interaction between complexity levels and time ($\chi^2_{[57]}$ = 77.863; P = 0.034), indicating that the fungal treatments cause different effects in the cut of leaves over time. The significance was evaluated using χ^2 test ($P < 0.05$).

Experiment II (*In vitro*) – Interaction of fungi with the fungal cultivar of leafcutter ants *Paired culture bioassay*

The growth of *L. gongylophorus in vitro* varied with the presence of *E. moelleri*, *E. nivea*, *T. longibrachiatum* and control over time ($\chi^2_{[3]}$ = 13.81, P = 0.003, Fig. 7). Before the inoculation of other fungi on the plates (time 0), there was no difference in growth of *L. gongylophorus* among plates of control and *E. moelleri* (z = -0.43, P = 0.66), *E. nivea* (z = 1.53, P = 0.12) and *T. longibrachiatum* (z = -0.006, P = 0.99; Fig. 7). After 108 hours, the colony radius of *L. gongylophorus* in the presence of *T. longibrachiatum* (0.86 ± 0.01cm; mean ± S.E.) and *E. moelleri* (0.94 ± 0.03cm) was smaller than the colony radius observed in control (1.01 ± 0.03cm) (*T. longibrachiatum*: z = 4.43, $P < 0.001$; *E. moelleri*:

z = 2.03, *P* = 0.04). However, there was no difference between the growth of *L. gongylophorus* in the presence of control and *E. nivea* (0.98 ± 0.02cm) (z = 1.0, *P* = 0.31).

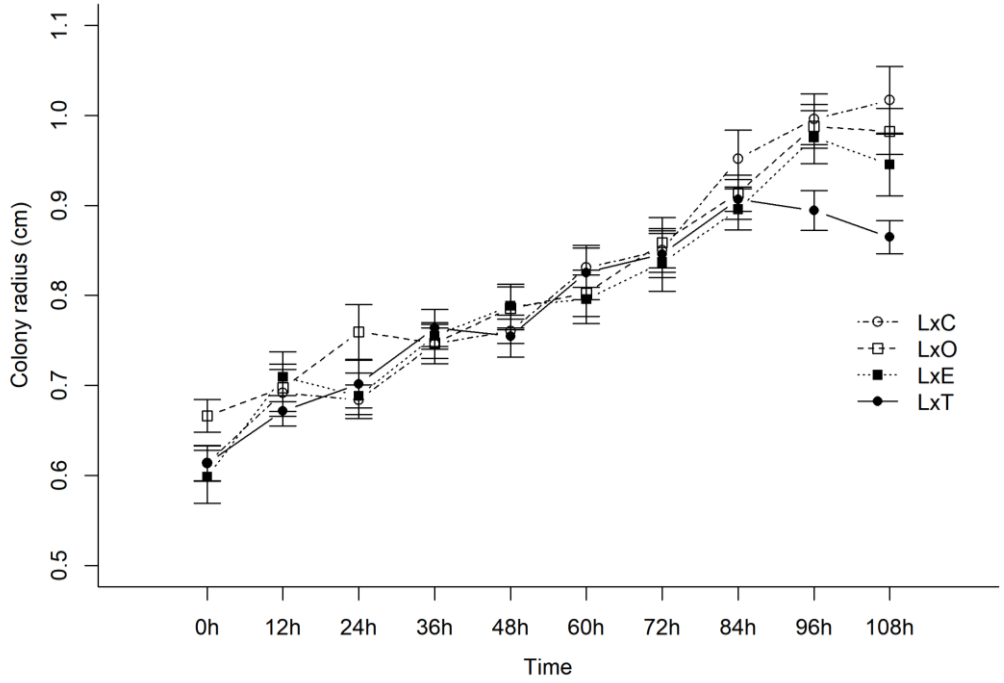


Figure 7. Growth of *Leucoagaricus gongylophorus* (L) in paired culture bioassays with *Escovopsis moelleri* (E), *Escovopsioides nivea* (O), *Trichoderma longibrachiatum* (T) fungi or control (C). We adjusted generalized linear mixed models (GLMM) with normal distributions and random intercepts. The interaction between the factors presence of fungi and time was also tested. The *in vitro* growth of *L. gongylophorus* was different in the presence of *E. moelleri*, *E. nivea*, *T. longibrachiatum* and control over time ($\chi^2_{[3]}$ = 13.81, P = 0.003). There was no difference between the growth of *L. gongylophorus* in the presence of control and *E. nivea* (0.98±0.02cm) (z = 1.0, P = 0.31) after 108 hours. Nevertheless, in the presence of *T. longibrachiatum* (0.86± 0.01cm) (mean±S.E.) and *E. moelleri* (0.94±0.03cm), colony radius of *L. gongylophorus* was smaller than colony radius observed in control (1.01±0.03cm) (*T. longibrachiatum*: z = 4.43, P < 0.001; *E. moelleri*: z = 2.03, P = 0.04).

Discussion

Our objective was to investigate whether the parasitic fungus *Escovopsis moelleri* and the antagonist *Escovopsioides nivea* represent threats to the health of leafcutter ants' colonies. We also tested whether the virulence level of these fungi varied according to levels of colony complexity. The *in vivo* experiment showed that although the colonies exposed to *E. moelleri* suffered a reduction in their weight, this was not enough to compromise their survival. This indicates that *Escovopsis* may not always present a high virulence to colonies as has been previously suggested (Currie et al. 1999a; Currie 2001). We hypothesized that workers controlled the growth of this fungus inside the colonies through their defence mechanisms. Some of these mechanisms are already well-known, such as the production of antimicrobial compounds by the metapleural glands (Poulsen et al. 2002; Bot et al. 2002; Fernández-Marín et al. 2006; Fernández-Marín et al. 2009; Yek et al. 2012), hygienic behaviours such as grooming and weeding (Currie & Stuart,2001) and a symbiotic association with a filamentous bacteria (actinobacteria) that secretes antimicrobial substances (Currie et al. 1999b). This control of alien fungal growth by the workers may also have occurred in relation to *E. nivea* and *T. longibrachiatum*, since neither fungus affected negatively the weight of the colonies nor were they lethal to them. Moreover, according to Frank (1996) it is predicted that some parasites can obtain nutritional resources from their hosts in a sustainable manner, not causing great harm to them, i.e. with low virulence. We propose that this is the case for both *E. moelleri* and *E. nivea*.

In relation to the queenless colonies, most suffered a decrease in their weight leading to their subsequent death, independent of the fungal treatment or control. Some studies have shown that the queen is not only important for reproduction but can also influence colony cohesion (Vienne et al. 1998; Della Lucia et al. 2003). According to

Sousa-Souto & Souza (2006), the absence of the queen in a colony of leafcutter ants *Atta sexdens rubropilosa* increased the workers' mortality and decreased the refuse disposal that could indicate a disruption in colony´s internal tasks. Considering this and based on our results, it seems that the absence of the queen naturally causes a negative impact on queenless colonies. As demonstrated here, *E. moelleri* promoted a great decrease in the weight of colonies. In this manner, since the queenless colonies seem to be already debilitated, probably rendering them limited resources, it is possible that a better strategy of *E. moelleri* would be increase the exploitation of its host resource, reflected in an increase in virulence. This strategy may allow an early parasite reproduction and transmission to new hosts in a natural setting.

Similar to queenless colonies, the fungus gardens without ants also reduced their weight. However, they died more rapidly, with only eleven days after the inoculation of fungi treatments. It is important to note that we did not observe any fungal sporulation in queenright colonies and queenless colonies. In contrast, *Escovopsis*, *Escovopsioides*, *Trichoderma* and other fungi were observed growing in fungus gardens without ants. These results indicate that the ants are very important to the protection of a colony against parasites as the fungal cultivar seems not to defend itself alone. Thus, the absence of ants probably rendered the fungus gardens more susceptible to infection. Our results clearly demonstrated that ant-free fungus gardens represent a completely unreal condition. In addition, these results are in line with other studies that showed the growth of alien fungi and signals of infection in fungus gardens not tended by workers (Currie et al 1999a; Augustin 2011; Wallace et al. 2014; Kellner et al. 2017).

The protection of the fungal cultivar by leafcutter ants can be performed through two main hygienic behaviours, fungus grooming and weeding (Currie & Stuart 2001). Fungus grooming is characterized by the removal of parasite spores from the fungus

gardens while weeding is the removal of infected garden pieces or of vegetal material contaminated (Currie & Stuart 2001). The infected material is discarded in the midden, located in specific underground chambers or outside the colony (Hölldobler & Wilson 2009; Lacerda et al. 2011). The removal of contaminants from the fungus gardens is very important to control infections and consequently the maintenance of colony health. In our study, we found that queenright colonies and queenless colonies responded similarly to the presence of alien fungi on their fungus gardens in terms of midden production. In both of these colonies' complexity levels, the amount of midden produced by workers was high in the day post-inoculation of *E. moelleri* and *T. longibrachiatum*. We hypothesized that workers tried to remove both fungi from their fungus gardens to control its growth inside of their fungus gardens. On the other hand, both queenright colonies and queenless colonies exposed to control and *E. nivea* maintained the production of midden similar throughout the evaluation of the experiment. This may indicate that workers do not put much effort into removing *E. nivea* from their fungus gardens, probably because this fungus does not pose a great threat to them.

On the day after the inoculation of fungal treatments in the queenright colonies, workers cut smaller amounts of leaves than in the following days. We suspect that the workers initially invested time and effort removing the parasites from their queenright colonies rather than cutting leaves to be incorporated into the fungus gardens. This result seems to be supported by the results of midden production, because on this same day the amount of midden produced by colonies (mainly those inoculated with *E. moelleri* and *T. longibrachiatum*) was high, as discussed above. After this period, the queenright colonies exposed to fungi increased the amount of cut leaves. It is possible that workers increase the cutting of vegetal material to incorporate them in the fungus gardens, promoting the fungal cultivar grow. Similar results were found by Augustin (2011) when colonies of

Atta sexdens rubropilosa were exposed to *Escovopsis microspora* and it was observed that workers increased the incorporation of leaf fragments in the fungus gardens 50 hours post fungal inoculation. According to this author, this could act as a mechanism that circumvents the possible negative effects caused by this fungus on the colonies and we believe this same hypothesis can be applied to our results. In contrast, the amount of cut leaves, in queenright and queenless colonies treated with *E. nivea*, were similar to *T. longibrachiatum* and control with some variation over time. This can indicate once again that fungus does not pose a risk to the health of colonies and the ants do not alter the foraging activity to overcome its presence in their fungus gardens.

The results of the *in vitro* bioassay have shown that growth of *L. gongylophorus* is slowed in the presence of *E. moelleri* and *T. longibrachiatum* in paired cultures. We observed that *E. moelleri* may easily overgrow the fungal cultivar, probably because there are no workers to control it in the culture medium. Furthermore, the absence of other microorganisms in this interaction, which could compete for the same nutritional resources, may have facilitated its growth as well. In fact, this was observed in our assay after approximately seven days post-inoculation of *E. moelleri* in the culture medium with the fungal cultivar, in contrast to what we observed in queenright colonies. The overgrowth on the fungal cultivar by *Escovopsis in vitro* has also been shown in previous studies (Folgarait et al. 2011a; Varanda-Haifig et al. 2017).

The antagonist fungus *E. nivea* did not have any effect on the *L. gongylophorus*. Also, we observed that *E. nivea* only overgrew the fungal cultivar after approximately 10 days post-inoculation in the culture medium with *L. gongylophorus*. This result, associated with the data obtained from the *in vivo* experiment, may suggest that this fungus has a low virulence strategy toward the fungal cultivar of leafcutter ants. According to Varanda-Haifig et al. (2017) and Osti and Rodrigues (2018), *E. nivea*

isolates were capable of inhibiting the growth of *L. gongylophorus* in the culture medium, however, their isolates seem to be less aggressive compared to *Escovopsis* sp. (unidentified isolates) similar to what we observed here.

In general, *Trichoderma* caused a decrease in the growth of *L. gongylophorus* without overgrowing the mutualistic fungus of the attines. This is a common effect observed by *Trichoderma* against plant-pathogenic fungi and that is exploited for biological control of plant diseases. This fungus can compete for nutrients in the environment, release substances that decrease growth or cause the death of the other fungus (antibiosis) or it can directly parasitize the other fungus, obtaining resources from the plant-pathogenic fungus (parasitism) (reviewed by Gajera et al. 2013). In this case, we do not know what led to decrease of *L. gongylophorus* growth specifically.

Our study aimed to investigate the virulence of fungi commonly found in colonies of leafcutter ants. From this study, we showed how the virulence of these microorganisms can be influenced by the complexity of interactions that composed a colony. Our results showed that, in general, the fungi *E. moelleri*, *E. nivea* and *T. longibrachiatum* were not capable of causing the death of queenright colonies of leafcutter ants. However, most of the queenless colonies and fungus gardens died, which suggests that the queen and workers are very important to the maintenance of colony health and stability. These results highlight the importance of considering the whole superorganism in studies that investigate the virulence of parasites in colonies of eusocial insects. It is important to point out that *Escovopsis* has been suggested as a potential biological control agent of leafcutter ants (Folgarait et al. 2011a, b; Wallace et al. 2014). In this manner, we emphasize the importance of conducting experiments to test this possibility using queenright colonies, since *in vitro* experiments may not represent a realistic approach. In fact, a colony of leafcutter ants is not only composed of the fungal cultivar or the queen

and workers but is a complex of interactions involving all these organisms and this must be considered.

In addition, we observed that *E. nivea* and *T. longibrachiatum* seems to be less aggressive than *E. moelleri*, and this was observed both *in vivo* and *in vitro*. Our study is one of the first to investigate the impact of *Escovopsioides* on colonies of leafcutter ants. This may open new avenues for future research that seek to understand the role of this fungus in the leafcutter ants-fungus symbiosis. It would be interesting to develop new studies that investigate other factors related to the virulence of each these fungi species. This could help us to understand the diversity of strategies and virulence these fungi can present, especially because some studies have shown that different strains can vary in their virulence level (Silva et al. 2006; Wallace et al. 2014; Marfetán et al. 2015).

To conclude, we propose that *Escovopsis* is far from being the highly virulent parasite it has been cast as in the literature and that this is also true of its sister genus *Escovopsioides*. This would make evolutionary sense if one considers the comparatively long-lived nature of its (superorganism) hosts. Where *Escovopsis* may be virulent, we propose that this is a change in strategy when its host is weakened, which would seem not be true for *Escovopsioides*.

References

Agnew, P., & Koella, J. C. (1997). Virulence, parasite mode of transmission, and host fluctuating asymmetry. *Proceedings of the Royal Society B*, 264, 9–15. https://doi.org/10.1098/rspb.1997.0002

Ali, S., Huang, Z., Zou, S., Bashir, M. H., Wang, Z., & Ren, S. (2012). The effect of insecticides on growth, germination and cuticle-degrading enzyme production by *Isaria fumosorosea*. *Biocontrol Science and Technology*, 22, 1047–1058. https://doi.org/10.1080/09583157.2012.708394

Alizon, S., Hurford, A., Mideo, N., & Van Baalen, M. (2009). Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. *Journal of Evolutionary Biology*, 22, 245–259. https://doi.org/10.1111/j.1420-9101.2008.01658

Antunes, E. C., & Della Lucia, T. M. C. (1999). Consumo foliar em *Eucalyptus urophylla* por *Acromyrmex laticeps nigrosetosus* Forel (Hymenoptera-Formicidae). *Ciência e Agrotecnologia*, 23, 208-211.

Augustin, J. O. (2011). Novas espécies de *Escovopsis*, sua dispersão e virulência na simbiose formigas Attini - *Leucoagaricus gongylophorus*. D.Sc. Thesis. Viçosa, MG, Brazil: Universidade Federal de Viçosa, p.131. http://locus.ufv.br/handle/123456789/897

Augustin, J. O., Groenewald, J. Z., Nascimento, R. J., Mizubuti, E. S. G., Barreto, R.W., Elliot, S. L., & Evans, H. C. (2013). Yet more "weeds" in the garden: fungal novelties from nests of leaf-cutting ants. *PLoS ONE*, 8, e82265. https://doi.org/10.1371/journal.pone.0082265

Augustin, J. O., Simões, T. G., Dijksterhuis, J., Elliot, S. L., & Evans, H. C. (2017). Putting the waste out: a proposed mechanism for transmission of the mycoparasite *Escovopsis* between leafcutter ant colonies. *Royal Society Open Science*, 4, 161013. http://dx.doi.org/10.1098/rsos.161013

Bot, A. N. M., Ortius-Lechner, D., Finster, K., Maile R., & Boomsma, J. J. (2002). Variable sensitivity of fungi and bacteria to compounds produced by the metapleural glands of leaf-cutting ants. *Insectes Sociaux*, 49, 363–370. https://doi.org/10.1007/PL00012660

Brown, M. J. F., Loosli, R., & Schmid-Hempel, P. (2000). Condition-dependent expression of virulence in a trypanosome infecting bumblebees. *Oikos*, 91, 421–427. https://doi.org/10.1034/j.1600-0706.2000.910302.x

Brown, M. J. F., Schmid-Hempel, R., & Schmid-Hempel, P. (2003). Strong context-dependent virulence in a host – parasite system: reconciling genetic evidence with theory. *Journal of Animal Ecology*, 72, 994–1002. https://doi.org/10.1046/j.1365-2656.2003.00770.x

Bull, J. J., Molineux, I. J., & Rice, W. R. (1991). Selection of benevolence in a host-parasite system. *Evolution*, 45, 875-882. https://doi.org/10.2307/2409695

Carreiro, S. C., Pagnocca, F. C., Bueno, O. C., Bacci Jr., M., Hebling, M. J. A., & Silva, O. A. (1997). Yeasts associated with nests of the leaf-cutting ant *Atta sexdens rubropilosa*. *Antonie van Leeuwenhoek*, 71, 243–248. https://doi.org/10.1023/A:1000182108648

Clayton, D. H., & Tompkins, D. M. (1994). Ectoparasite virulence is linked to mode of transmission. *Proceedings of the Royal Society B: Biological Sciences,* 256, 211–217. https://doi.org/10.1098/rspb.1994.0072

Cowley, B. G. T., & Lichtenstein, E. P. (1970). Growth inhibition of soil fungi by insecticides and annulment of inhibition by yeast extract or nitrogenous nutrients. *Journal of General Microbiology*, 62, 27-34. https://doi.org/10.1099/00221287-62-1-27

Cremer, S., Pull, C. D., & Fürst, M. A. (2017). Social immunity: emergence and evolution of colony-level disease protection. *Annual Review of Entomology*, 63, 105–23. https://doi.org/10.1146/annurev-ento-020117-043110

Cremer, S., & Sixt, M. (2009). Analogies in the evolution of individual and social immunity. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364, 129–142. https://doi.org/10.1098/rstb.2008.0166

Cressler, C. E., Mcleod, D.V., Rozins, R., Hoogen, J. V. D., & Day, T. (2015). The adaptive evolution of virulence: a review of theoretical predictions and empirical tests. *Parasitology*, 143, 915–930. https://doi.org/10.1017/S003118201500092X

Currie, C. R., Mueller, U. G., & Malloch, D. (1999a). The agricultural pathology of ant fungus gardens. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 7998–8002. https://doi.org/10.1073/pnas.96.14.7998

Currie, C. R., Scott, J. A., Summerbell, R. C., & Malloch, D. (1999b). Fungus-growing ants use antibiotic producing bacteria to control garden parasites. *Nature*, 398, 701–704. https://doi.org/10.1038/nature01563

Currie, C. R., & Stuart, A. E. (2001). Weeding and grooming of pathogens in agriculture by ants. *Proceedings of the Royal Society B: Biological Sciences,* 268, 1033–1039. https://doi.org/10.1098/rspb.2001.1605

Currie, C. R. (2001). Prevalence and impact of a virulent parasite on a tripartite mutualism. *Oecologia*, 128, 99–106. https://doi.org/10.1007/s004420100630

Currie, C. R., Wong, B., Stuart, A. E., Schultz, T. R., Rehner, S. A., Mueller, U.G., Sung, G. H., Spatafora, J.W., & Straus, N. A. (2003a). Ancient tripartite coevolution in the attine ant-microbe symbiosis. *Science*, 299, 386–388. https://doi.org/10.1126/science.1078155

Currie, C. R., Bot, A. N. M., & Boomsma, J. J. (2003b). Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. *Oikos* 101, 91-102. https://doi.org/10.1034/j.1600-0706.2003.12036.x

Della Lucia, T. M. C., Peternelli, E. F. O., Lacerda, F. G., & Peternelli, L. A. (2003). Colony behavior of *Atta sexdens rubropilosa* (Hymenoptera: Formicidae) in the absence of the queen under laboratory conditions. *Behavioural Processes*, 64, 49–55.

https://doi.org/10.1016/S0376-6357(03)00078-0

Detrain, C., & Deneubourg, J. L. (2006). Self-organized structures in a superorganism: do ants "behave" like molecules? *Physics of Life Reviews*, 3, 162–187. https://doi.org/10.1016/j.plrev.2006.07.001

Ebert, D. (2013). The epidemiology and evolution of symbionts with mixed-mode transmission. *Annual Review of Ecology, Evolution, and Systematics*, 44, 623–643. https://doi.org/10.1146/annurev-ecolsys-032513-100555

Ewald, P. W. (1987). Transmission modes and evolution of the parasitism-mutualism continuum. *Annals of the New York Academy of Science*, 503, 295-306. https://doi.org/10.1111/j.1749-6632.1987.tb40616.x

Farji-brener, A. G., Elizalde, L., Fernández-Marín, H., & Amador-Vargas, S. (2016). Social life and sanitary risks: evolutionary and current ecological conditions determine waste management in leaf-cutting ants. *Proceedings of the Royal Society B: Biological Sciences*, 283, 20160625. http://dx.doi.org/10.1098/rspb.2016.0625

Fernández-Marín, H., Zimmerman, J. K., Rehner, S. A., & Wcislo, W. T. (2006). Active use of the metapleural glands by ants in controlling fungal infection. *Proceedings of the Royal Society B: Biological Sciences*, 273, 1689–1695. http://dx.doi.org/10.1098/rspb.2006.3492

Fernández-Marín, H., Zimmerman, J. K., Nash, D. R., Boomsma, J. J., & Wcislo, W. T. (2009). Reduced biological control and enhanced chemical pest management in the evolution of fungus farming in ants. *Proceedings of the Royal Society B: Biological Sciences*, rspb-2009. http://dx.doi.org/10.1098/rspb.2009.0184

Folgarait, P., Gorosito, N., Thomas-Poulsen, M., & Currie, C.R. (2011a). Preliminary *in vitro* insights into the use of natural fungal pathogens of leaf-cutting ants as biocontrol agents. *Current Microbiology*, 63, 250–258. http://dx.doi.org/10.1007/s00284-011-9944-y

Folgarait, P. J., Marfetán, J. A., & Cafaro, M. J. (2011b). Growth and conidiation response of *Escovopsis weberi* (Ascomycota: Hypocreales) against the fungal cultivar of *Acromyrmex lundii* (Hymenoptera: Formicidae). *Environmental Entomology*, 4040, 342-349. http://dx.doi.org/10.1603/EN10111

Frank, S. A. (1996). Models of parasite virulence. *The Quarterly Review of Biology*, 71, 37-78. https://doi.org/10.1086/419267

Gajera, H., Domadiya, R., Patel, S., Kapopara, M., Golakiya (2013). Molecular mechanism of Trichoderma as bio-control agents against phytopathogen system – a review. *Current Research in Microbiology and Biotechnology*, 4, 133-142. http://crmb.aizeonpublishers.net/content/2013/4/crmb133-142.pdf

Gandra, L. C. (2014). Mecanismo de supressão de colônias da formiga cortadeira *Acromyrmex subterraneus subterraneus* por fipronil. M.Sc. Thesis. Viçosa, MG, Brazil: Universidade Federal de Viçosa, p.37. http://locus.ufv.br/handle/123456789/3988

Gerardo, N. M., Mueller, U. G., Price, S. L., Currie, C. R (2004). Exploiting a mutualism: parasite specialization on cultivars within the fungus-growing ant symbiosis, *Proceedings of the Royal Society B: Biological Sciences*, v. 271, n. 1550, p. 1791–1798.

Hölldobler, B., & Wilson, E. O. (2009). *The Superorganism: The beauty, elegance and strangeness of insect societies*, WW Norton & Company.

Hughes, W. O. H., & Boomsma, J. J. (2004). Genetic diversity and disease resistance in leaf-cutting ant societies. *Evolution*, 58, 1251–1260. http://dx.doi.org/10.1554/03-546

Hughes, D. P., Pierce, N. E., & Boomsma, J. J. (2008). Social insect symbionts: evolution in homeostatic fortresses. *Trends in Ecology and Evolution*, 23, 672–677. http://dx.doi.org/10.1016/j.tree.2008.07.011

Jokela, J., Taskinen, J., Mutikainen, P., & Kopp, K. (2005). Virulence of parasites in hosts under environmental stress: experiments with anoxia and starvation. *Oikos*, 108, 156–164. https://doi.org/10.1111/j.0030-1299.2005.13185.x

Kellner, K., Kardish, M. R., Sea, J. N., Linksvayer, T. A., &. Mueller, U. G. (2017). Symbiont-mediated host-parasite dynamics in a fungus-gardening ant. *Microbial Ecology*, 1–14. https://doi.org/10.1007/s00248-017-1124-6

Lacerda, F. G., Della Lucia, T. M. C., & Souza, D. J. (2011). Biologia comportamental das operárias do lixo das colônias de formigas-cortadeiras. In: Della Lucia, T. M. C. (Ed.), *Formigas-cortadeiras: da bioecologia ao manejo*. (pp. 226-235). Editora UFV

Manley, R., Boots, M., & Wilfert, L. (2017). Condition-dependent virulence of slow bee paralysis virus in *Bombus terrestris*: are the impacts of honeybee viruses in wild pollinators underestimated? *Oecologia*, 184, 305–315. https://doi.org/10.1007/s00442-017-3851-2

Marfetán, J. A., Romero, A. I., & Folgarait, P. J. (2015). Pathogenic interaction between *Escovopsis weberi* and *Leucoagaricus* sp.: mechanisms involved and virulence levels. *Fungal Ecology*, 17, 52–61. http://dx.doi.org/10.1016/j.funeco.2015.04.002

Mendonça, D. M. F. (2018). Virulence of the fungi *Escovopsis* and *Escovopsioides* to the leafcutter ant-fungus symbiosis. M.Sc. Thesis. Viçosa, MG, Brazil: Universidade Federal de Viçosa, 48p.

Montoya, Q. V., Meirelles, L. A., Chaverri, P., & Rodrigues, A. (2016). Unraveling *Trichoderma* species in the attine ant environment: description of three new taxa. *Antonie van Leeuwenhoek*, 109, 633–651. http://dx.doi.org/10.1007/s10482-016-0666-9

Moreira, S. M., Rodrigues, A., Forti, L. C., & Nagamoto, N. S. (2015). Absence of the parasite *Escovopsis* in fungus garden pellets carried by gynes of *Atta sexdens*. *Sociobiology*, 62, 34–38. http://dx.doi.org/10.13102/sociobiology.v62i1.34-38

Mueller, U. G., Ishak, H. D., Bruschi, S. M., Smith, C. C., Herman, J. J., Solomon, S. E., Mikheyev, A. S., Rabeling, C., Scott, J. J., Cooper, M., Rodrigues, A., Ortiz, A., Brandão, C. R. F., Lattke, J. E., Pagnocca, F. C., Rehner, S. A., Schultz, T. R., Vasconcelos, H. L, Adams, R. M. M., Bollazzi, M., Clark, R. M., Himler, A. G., LaPolla, J. S., Leal, I. R.,

Johnson, R. A., Roces, F., Sosa-Calvo, J., Wirth, R., & Bacci Jr, M. (2017). Biogeography of mutualistic fungi cultivated by leafcutter ants. *Molecular Ecology*, 26, 6921-6937. http://dx.doi.org/10.1111/mec.14431

Nidelet, T., Koella, J. C., & Kaltz, O. (2009). Effects of shortened host life span on the evolution of parasite life history and virulence in a microbial host-parasite system. *BMC Evolutionary Biology*, 9, 1–10. http://dx.doi.org/10.1186/1471-2148-9-65

Olmert, I., & Kenneth, R. G. (1972). Sensitivity of the entomopathogenic fungi, *Beauveria bassiana*, *Verticillium lecanii*, and *Verticillium* sp. to fungicides and insecticides. *Environmental Entomology*, 3, 33–38. https://doi.org/10.1093/ee/3.1.33

Pagán, I., Montes, N., Milgroom, M. G., & García-Arenal, F. (2014). Vertical transmission selects for reduced virulence in a plant virus and for increased resistance in the host. *PLoS Pathogens*, 10, 23–25. https://doi.org/10.1371/journal.ppat.1004293

Pagnocca, F. C., Masiulionis, V. E., & Rodrigues, A. (2012). Specialized fungal parasites and opportunistic fungi in gardens of attine ants. *Psyche*, 2012. https://doi.org/10.1155/2012/905109

Poulsen, M., Bot, A. N., Nielsen, M. G., & Boomsma, J. J. (2002). Experimental evidence for the costs and hygienic significance of the antibiotic metapleural gland secretion in leaf-cutting ants. *Behavioral Ecology and Sociobiology*, 52, 151–157. https://doi.org/10.1007/s00265-002-0489-8

R Core Team (2017). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Reis, B. M. S., Silva, A., Alvarez, M. R., Oliveira, T. B., & Rodrigues, A. (2015). Fungal communities in gardens of the leafcutter ant *Atta cephalotes* in forest and cabruca agrosystems of southern Bahia State (Brazil). *Fungal Biology*, 119, 1170–1178. http://dx.doi.org/10.1016/j.funbio.2015.09.001

Rocha, S. L., Jorge, V. L., Della Lucia, T. M. C., Barreto, R.W., Evans, H. C., Elliot, S. L. (2014). Quality control by leaf-cutting ants: evidence from communities of endophytic fungi in foraged and rejected vegetation. *Arthropod-Plant Interactions*, 8, 485–493. http://dx.doi.org/10.1007/s11829-014-9329-9

Rocha, S. L., Evans, H. C., Jorge, V. L., Cardoso, L. A. O., Pereira, F. S. T., Rocha, F. B., Barreto, R. W., Hart, A. G., & Elliot, S. L. (2017). Recognition of endophytic *Trichoderma* species by leaf-cutting ants and their potential in a Trojan-horse management strategy. *Royal Society Open Science*, 4, 160628. http://dx.doi.org/10.1098/rsos.160628

Rodrigues, A.; Pagnocca, F. C., Bacci Jr., M., Hebling, M. J. A., Bueno, O. C., Pfenning, L. H. (2005a). Variability of non-mutualistic filamentous fungi associated with *Atta sexdens rubropilosa* nests. *Folia Microbiologica*, v. 50, p. 421425,

Rodrigues, A., Pagnocca, F. C., Bueno, O. C., Pfenning, L. H., & Bacci Jr., M. (2005). Assessment of microfungi in fungus gardens free of the leaf-cutting ant *Atta sexdens rubropilosa* (Hymenoptera: Formicidae). *Sociobiology*, 46, 329–334.

Rodrigues, A., Bacci Jr., M, Mueller, U. G., Ortiz, A., & Pagnocca, F. C. (2008). Microfungal "weeds" in the leafcutter ant symbiosis. *Microbial Ecology*, 56, 604–614. http://dx.doi.org/10.1007/s00248-008-9380-0

Schmid-Hempel, P. (1998). *Parasites in social insects*. Princeton University Press.

Seeley, T. D., & Tarpy, D. R. (2007). Queen promiscuity lowers disease within honeybee colonies. *Proceedings of the Royal Society B: Biological Sciences*, 274, 67–72. http://dx.doi.org/10.1098/rspb.2006.3702

Silva, A., Rodrigues, A., Bacci Jr., M., Pagnocca, F. C., & Bueno, O. C. (2006). Susceptibility of the ant-cultivated fungus *Leucoagaricus gongylophorus* (Agaricales: Basidiomycota) towards microfungi. *Mycopathologia*, 162, 115–119. http://dx.doi.org/10.1007/s11046-006-0037-6

Sousa-Souto, L., & Souza, D. J. (2006). Queen influence on workers behavior of the leaf-cutting ant *Atta sexdens rubropilosa* Forel, 1908. *Brazilian Journal of Biology*, 66, 503–508. http://dx.doi.org/10.1590/S1519-69842006000300016

Stearns, S. C., & Hoekstra, R. F. (2005). *Evolution: an introduction*, 2nd ed. Oxford University Press.

Stewart, A. D., Logsdon Jr., J. M., & Kelley, S. E. (2005). An empirical study of the evolution of virulence under both horizontal and vertical transmission. *Evolution*, 59, 730–739. https://doi.org/10.1554/03-330

Suen, G., Scott, J. J., Aylward F. O., Adams, S. M., Tringe, S. G., Pinto-Tomás, A. A., Foster, C. E., Pauly, M., Weimer, P. J., Barry, K. W., Goodwin, L. A., Bouffard, P., Lewyn, L., Osterberger, J., Harkins, T. T., Slater, S. C., Donohue, T. J., & Currie, C. R. (2010). An insect herbivore microbiome with high plant biomass-degrading capacity. *PLoS Genetics*, 6, e1001129. https://doi:10.1371/journal.pgen.1001129

Tarpy, D. R., & Seeley, T. D. (2006). Lower disease infections in honeybee (*Apis mellifera*) colonies headed by polyandrous vs monandrous queens. *Naturwissenschaften*, 93, 195–199. https://doi.org/10.1007/s00114-006-0091-4

Tompkins, D. M., Jones, T., & Clayton, D. H. (1996). Effect of vertically transmitted ectoparasites on the reproductive success of Swifts (*Apus apus*). *Functional Ecology*, 10, 733–740. https://doi.org/10.2307/2390508.

Varanda-Haifig, S. S., Albarici, T. R., Nunes, P. H., Haifig, I., Vieira, P. C., & Rodrigues, A. (2017). Nature of the interactions between hypocrealean fungi and the mutualistic fungus of leaf-cutter ants. *Antonie van Leeuwenhoek*. 110, 593-605. https://doi.org/10.1007/s10482-016-0826-y

Verza, S. S., Diniz, E. A., Chiarelli, M. F., Mussury, R. M., & Bueno, O. C. (2017). Waste of leaf-cutting ants: disposal, nest structure, and abiotic soil factors around internal waste chambers. *acta ethologica*, 20, 119-126. https://doi.org/10.1007/s10211-017-0255-6

Vienne, C., Errard, C., & Lenoir, A. (1998). Influence of the queen on worker behaviour and queen recognition behaviour in ants. *Ethology*, 104, 431–446. https://doi.org/10.1111/j.1439-0310.1998.tb00081.x

Wallace, D. E. E., Asensio, J. G. V., & Tomás, A. A. P. (2014). Correlation between virulence and genetic structure of *Escovopsis* strains from leaf-cutting ant colonies in Costa Rica. *Microbiology*, 160, 1727–1736. https://doi.org/10.1099/mic.0.073593-0

Watson, M. J. (2013). What drives population-level effects of parasites? Meta-analysis meets life-history. *International Journal for Parasitology: Parasites and Wildlife*, 2, 190–196. http://dx.doi.org/10.1016/j.ijppaw.2013.05.001

Yek, S. H., Nash, D. R., Jensen, A. B., & Boomsma, J. J. (2012). Regulation and specificity of antifungal metapleural gland secretion in leaf-cutting ants. *Proceedings of the Royal Society B: Biological Sciences*, 279, 4215–4222. http:// doi:10.1098/rspb.2012.1458

Supplementary Material

Sampling of fungus gardens before the experiments

We conducted a sampling of the fungus gardens from the 12 colonies collected to check whether they naturally presented *Escovopsis* and *Escovopsioides*. For each colony, we collected ten fragments of fungus garden from three regions: top, middle and base, totalling 30 fragments. For this, we first sampled the top and the base region of the fungus gardens. Subsequently, each fungus garden was carefully split in half with the aid of a spatula to allow access to the middle region and then we conducted our sampling of this region. The fragments of each region were plated onto Petri dishes containing PDA 20% following incubation at 25ºC for 15 days. The plates were checked daily to verify the presence of *Escovopsis* and *Escovopsioides* on fragments.

None of the colonies presented *Escovopsis* and *Escovopsioides* growing on the top region of their fungus gardens. We found *Escovopsis* growing in the middle region from only one colony, while *Escovopsioides* was not observed in this region in any of colonies. The presence of *Escovopsis* and *Escovopsioides* in the base region was detected in four

colonies and one colony, respectively. In total, *Escovopsis* was found in five of the 12 colonies and *Escovopsioides* was found in one.

Sampling from middens

We also sampled the middens produced by queenright colonies and queenless colonies to observe if the ants were removing these fungi from their fungus gardens.

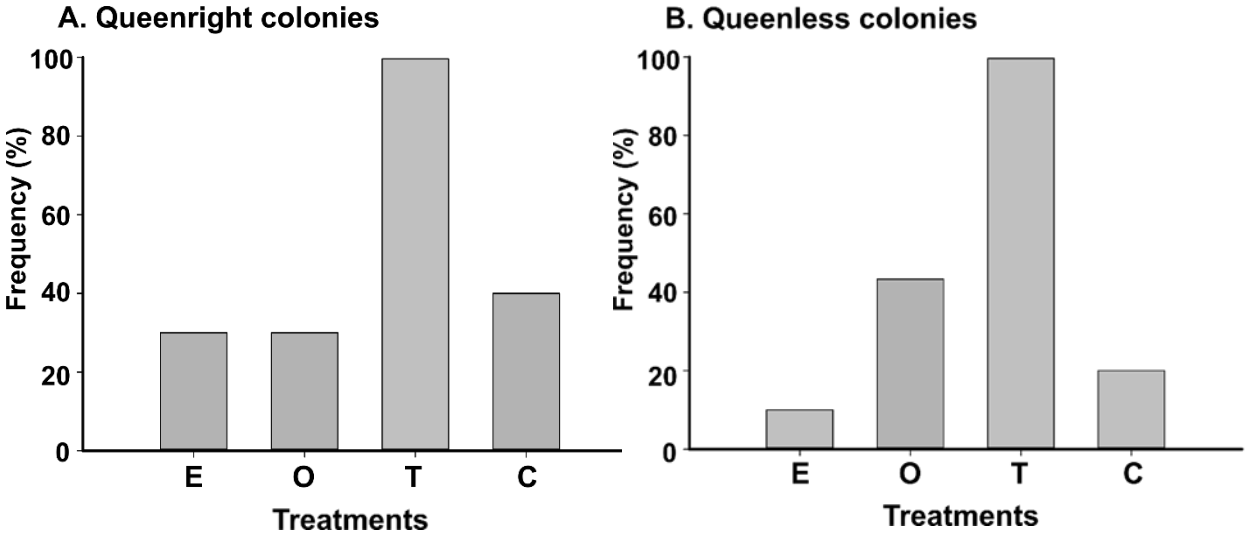


Figure S1. Frequency of the fungi genera *Escovopsis*, *Escovopsioides* and *Trichoderma* found in the midden of **(A)** queenright colonies and **(B)** queenless colonies of *Acromyrmex subterraneus subterraneus* leafcutter ants exposed to one of three treatments or to control: conidial suspension of the fungi *Escovopsis moelleri* (E); *Escovopsioides nivea* (O); *Trichoderma longibrachiatum* (T); blank control (0.01% Tween 80® solution + saline solution - NaCl 0.85%; C). We plated five fragments of midden from each queenright colony and queenless colony on Petri dishes containing PDA 20% and then incubated at 25°C for 15 days. This procedure was conducted 24 hours after inoculation of conidial suspensions for all treatments (Day 1) and then every 72 hours, totalling 10 days of sampling. We checked the plates daily to verify the occurrence of *Escovopsis, Escovopsioides* and *Trichoderma*. The bars represent the medium frequency of these fungi presence in the 10 days of sampling. In the same manner as the previous parameter (sampling of fungus gardens), we recognized that the ideal would identify these fungi genera at a species level, however, this was not possible due to practical reasons. No

growth of *Escovopsis* and *Escovopsioides* was observed in the midden of queenright colonies and queenless colonies. We suspect that midden could present these fungi, nevertheless, other microorganisms that are also present may have inhibited their growth in the culture medium. On the other hand, *Trichoderma* was found growing the midden from the queenright colonies and queenless colonies, independent of the treatment they were exposed.

Table S1. Summary of the days and the respective activities conducted for the assembly and evaluation of the experiment

Summary of experiment set up days	
Day -28	Sampling of the colonies' fungus gardens; Division of each initial colony into three fragments (totalling 12 queenright colonies and 24 queenless colonies);
Day -2	Weighing of the 12 queenright colonies and 12 queenless colonies;
Day -1	Removal of ants from the other 12 queenless colonies; Weighing fungus gardens; Preparation of fungal suspensions;
Day 0	Inoculation of fungal treatments; Survival evaluation;
Day 1	Weighing and sampling of midden; Weighing of the leaves cut by ants from queenright colonies and queenless colonies;
Day 2	Sampling of queenright colonies, queenless colonies and fungus gardens;
Day 11	Last day of weighing fungus gardens;
Day 28	Last day of weighing and sampling of midden;
Day 29	Last day of weighing and sampling of queenright colonies and queenless colonies; Last day of weighing the leaves cut by the ants from queenright colonies and queenless colonies;
Day 118	End of the experiment: last day of survival evaluation.

Chapter 3: The fungus *Escovopsis* (Ascomycota: Hypocreales): A critical review of its biology, life style and interactions with other organisms in attine ant colonies

Marcela Cristina Silva Caixeta[1*], Quimi Vidaurre Montoya[2*], Nicole Marie Gerardo[3], André Rodrigues[2], Simon Luke Elliot[1]

[1] Department of Entomology, UFV – University of Viçosa, Viçosa, MG, Brazil.

[2] Department of General and Applied Biology, UNESP – Sao Paulo State University, Rio Claro, SP, Brazil.

[3] Department of Biology, Emory University, O. Wayne Rollins Research Center, Atlanta, USA

* Corresponding authors:

Marcela Cristina Silva Caixeta[a], Quimi Vidaurre Montoya[b] (marcela.cristina.bio@gmail.com, quimivimo@gmail.com)

[a] Department of Entomology, Universidade Federal deViçosa, Viçosa, MG, CEP 36.570-900, Brazil

Phone #: 55 31 3612-3026

[b] Department of Biochemistry and Microbiology, UNESP – Sao Paulo State University, Rio Claro, SP, CEP 13.506-900, Brazil

Phone #: 55 19 3526-4364

Abstract

Fungus-growing insects such as ants, beetles, termites and bees are part of an interesting group that have symbiotic relationships with several other organisms in addition to mutualistic fungi, that they use mainly as a nutritional source. For example, the mutualism fungus – Attine ants (Hymenoptera: Formicidae) can often be threatened by other organisms such as *Escovopsis* (Hypocreales: Hypocreaceae), a specialized mycoparasite of this system. There are many other organisms involved in this system such as bacteria, yeasts and filamentous fungi that establish different types of interactions with eachother, but here we focus mainly on *Escovopsis*. As a common inhabitant of attine nests and, although it has been considered a mycoparasite with high virulence, the ants continue to perform their main functions in its presence. The damage that *Escovopsis* causes in the colonies is often investigated in highly simplified experiments, for example without the presence of the queen. While simpler experiments are the basis for more elaborate studies, since *Escovopsis* is part of the system of a social organism, the conclusions can be uncertain. In recent years, studies have focused more on ecological aspects of *Escovopsis* than on diversity. Moreover, it is not known for certain the delimitation of the genus due to divergences in methods used on the description of new species. The taxonomic uncertainty has impacted mainly on studies that evaluate the effect of *Escovopsis* on colonies, because it is not really known if findings are applicable to the entire genus. Besides that, taxonomic and phylogenetics surveys can be useful to give clues as to how *Escovopsis* reaches new colonies, a crucial topic to understanding the (still far from clear) biological cycle of this fungus. We also compared the known characteristics of *Escovopsis* species related to mycoparasitic lifestyle with other well-studied mycoparasites, such as some species of *Trichoderma*. Considering the conclusions of previous studies and analysing recent results, we present a new view of the strategy used

by *Escovopsis* to infect colonies of attine ants. We propose that it remains at a low virulence level in the nests and takes advantage of adverse conditions in the colonies such as the queen's death due to other factors to overgrow the fungus garden. In addition, we consider other genera of filamentous fungi phylogenetically related to *Escovopsis* that are being described, as *Escovopsioides* and two new genera separated recently of *Escovopsis* clade (*Luteomyces* and *Sympodiorosea*). It is unclear yet what type of interaction they establish with attines ants or what their distribution is among these ants.

Key-words: Attine ants, fungus-growing insects, *Escovopsis*, *Escovopsioides*, mycoparasitism, virulence

Introduction

Symbiotic relationships are of great importance as they guide the entire evolutionary history of the organisms that are involved in them. While symbioses may be parasitic, almost all organisms on Earth rely on mutualistic symbioses, often to the point that endosymbionts are incorporated as organelles (mitochondria and chloroplasts being the obvious examples). In considering insect societies, symbiotic mutualists may be considered as being parallel to obligate symbionts such as mitochondria – a classic example of this is fungus-growing ants (subfamily Myrmicinae, Attini tribe, "attine") that cultivate a Basidiomycete fungus in a nutritional mutualism (Weber 1972; De Fine Licht et al. 2013). As with any organism that relies on mutualistic symbionts, colonies of fungus-growing ants and their fungal partners can be used as a resource by other organisms, potentially in a parasitic manner. Here, we consider fungi that are generally considered to be parasitic on the fungi that are cultivated by the ants (i.e. mycoparasites; Table 1). We focus on *Escovopsis* (Ascomycota: Hypocreales) as it is the best known and most studied of these, but consider its close relatives such as *Escovopsioides* and also other fungi that may be exploiting this resource-rich niche in a similar fashion (notably *Syncephalastrum*; Mucoromycota: Mucorales).

Between the years 1999 and 2004, a flurry of studies highlighted *Escovopsis* as a component of attine systems. Studies conducted principally with leafcutter ants (a more derived group within the attine ants), indicated it to be a parasite of the fungus gardens cultivated by these insects (Currie et al. 1999a; Currie 2001; Currie and Stuart 2001; Currie et al. 2003a; Gerardo et al. 2004; Reynolds and Currie 2004). Over the following years, studies continued on ecological and evolutionary aspects of *Escovopsis*, some of them suggesting a potential applied role of this fungus in the biological control of leafcutter ants (Folgarait et al. 2011a; b; Wallace et al. 2014).

Although these publications have contributed to a deeper understanding of this system and some of its interactions, these findings may be specific to certain species or combinations of species and may not be representative of the whole genus. This may be because taxonomic and phylogenetic studies of *Escovopsis* have not kept pace with other studies, so it has been considered almost as a single entity. This problem has not been improved by the fact we are only now approaching a taxonomic view of this genus and its close relatives (Montoya et al. 2020, submitted). This effort began in 2013 and it was clear from then that different species of *Escovopisis* have diverse patterns of growth and morphological structures that seem to indicate different strategies to respond to and exploit their environments. We wish to discuss here some methodological criteria used in previous study that could have been conducted under a broader view of the system (considering the eusocial organism in a more conspicuous way).

This review starts with an outline of fungiculture practiced by insects to provide some context. We then move specifically to attine fungiculture, presenting a historical view of how our understanding of these systems has developed, including other organisms that form part of these systems. We highlight the importance of remembering that there may be countless relationships that are still unknown and will eventually modify our current thinking. We then consider specifically the genus *Escovopsis*, detailing what we know of its history – early descriptions of the genus, when and how it was associated with attines and how its parasitic nature was revealed; diversity – how many species are currently known; taxonomy – what are the characteristics that delimit the genus and what are the methodological conditions in which studies are being carried out to describe new species; geographical distribution – what is the range of *Escovopsis*; biological cycle – what is accepted about the life cycle of *Escovopsis* species, what is

known of transmission to new colonies; and its relationship with the fungi cultivated by attine ants.

While presenting and discussing previous studies, we offer critical appraisals of some areas of study, in particular some methodological questions that we feel may have led to some confusing interpretations of the true role of *Escovopsis* in attine-ants' colonies. Here we consider *Escovopsis* as a diverse group classified currently in *Escovopsis,* but are aware that some species are being placed in other genera (see the Table 1 for this and other fundamental definitions). We compare what we know about its possible mechanisms of infection of colonies to other well-recognized mycoparasites, paying attention to genetic, physiological and chemical aspects. We go into depth in discussing the evidence, considerations and ideas, raised in previous studies, that have led *Escovopsis* to be considered a highly virulent parasite of the attine's mutualistic fungus. We raise some new hypotheses and suggest approaches that could help future researchers to decipher this fascinating system and also consider the future of studies of other insect fungicultural systems.

Table 1. Definitions

Symbiosis. An interspecific interaction in which two organisms live together (de Bary 1879) for a considerable part of the lifespan* of at least one of the organisms, irrespective of effects of this association on either organism´s fitness. In this interaction, the natural lifespan of the **host** is usually longer than that of its **symbiont** (excluding resting phases) while the host is also usually the larger of the two. * "A considerable part of the lifespan" is deliberately left loose in this definition.
Parasite. A symbiont that on balance reduces its hosts fitness. This reduction in host fitness can be termed **virulence**.
Mutualism. An interspecific interaction (symbiotic or not) in which the fitness benefits outweigh the fitness costs for both organisms. This association can be symbiotic or not and can be facultative or obligatory.
Mycoparasitism. An interaction which a fungus (the mycoparasite) parasitizes another fungus (host or mycohost) (Barnet 1963). The latter has its fitness decreased on balance.
Endophytism. A relationship established by any microorganism that lives in plant tissues (De Bary 1866). Usually, these microorganisms do not cause damage to their hosts, distinguishing them from plant pathogens.
Virulence. The harm that parasites cause to their hosts (Frank 1996). A strict definition would rely upon a reduction in host fitness due to the association, but this is often not measured.
Escovopsis. Here, we use *Escovopsis* to refer to a diverse group of parasites that are currently mostly classified in *Escovopsis*, though some have now been placed in *Escovopsiodes*.
***Escovopsis* (*stricto sensu*).** *Escovopsis* species have the brush-like anamorph with phialides (vase-shaped cells from which conidia arise) on well-defined vesicles (Muchovej and Della Lucia 1990).
Eusocial organisms. "True" **s**ocial organisms according to the prefix 'eu-'. This is the highest degree of social organization and must have three characteristics: (i) adults separated in reproductive casts, in which the workers are partially or totally nonreproductive; (ii) overlapping generations in the colony; (iii) parental care of young individuals by nonreproductive or less reproductive workers (Wilson 1971).
Semisocial organisms. These differ from eusocial organisms in the absence of overlapping generations (Wilson 1971).
Vertical transmission. Transfer of symbionts from parents to offspring (Ewald 1994). In the case of social insects, this can be applied to the transmission of symbionts to new colonies.
Horizontal transmission. Transmission of symbionts among individuals of the same generation (Ewald 1994). In the case of social insects, this can be applied to the transmission of symbionts between colonies

An overview of fungiculture in insects

Many arthropods eat fungi ("mycophagy"). Examples exist among mites, springtails, beetles, flies, moths, termites, wood wasps, bees and ants (Wheeler and Blackwell 1984; Martin 1987; Hammond and Lawrence, 1989; Smrž and Čatská 2010; Menezes et al. 2015). Some of these mycophagous arthropods may actually cultivate the fungi on which they feed (fungiculture). This may lead them to have a close relationship with this microorganism that can form the basis for a symbiotic mutualism. In such instances, the basis of the mutualism is nutritional, but the fungal partners may gain benefits such as dispersion and protection (Weber 1972; Norris 1979; Leuthold et al. 1989).

Fungiculture has been developed mainly by insects are either semisocial or eusocial (Table 1). Some groups of beetles (Coleoptera: Curculionidae: subfamilies Scolytinae and Platypodinae; Kok et al. 1970; Woody 1982; Beaver 1989; Farrell et al. 2001; Harrington et al. 2005; Hulcr and Stelinski 2017), termites (Isoptera: Termitidae: Macrotermitinae; Johnson et al. 1981; Bignell and Eggleton 2000; Aanen et al. 2002), ants (Hymenoptera: Formicidae: Myrmicinae: Attini tribe; Weber 1972a; Mueller et al. 2001) and, more recently discovered, social bees (Hymenoptera: Apidae: Trigonini tribe; Menezes et al. 2015) are examples of insects that maintain a close relation with fungi. In the case of beetles, interestingly, there is a non-social species (Coleoptera: Erotylidae) that also relies on mutualism with a fungus for growth and development of the larvae (Toki et al. 2012).

These fungicultural systems share many features. One is the nutrition provided by the fungus to the insect. Another is the insect providing the substrate upon which the fungus grows and develops; thus, this nutritional mutualism can be described as an 'external gut' in which the insect can digest material such as polysaccharides, such as starch or cellulose, from which it may otherwise be unable to obtain nutrients (Siqueira

et al. 1998; Silva et al. 2006). Besides this, the fungal partners in these systems can contribute to detoxification and degradation of allelochemicals present in the plant tissue (Zhao et al. 2017; Davis et al. 2018; De Fine Licht et al. 2013). In general, the microbiota associated with fungus-growing insects seem to be adjusted to fungiculture with similar taxonomic and functional composition among the system of fungus-growing insects and different from those found in other hosts (Barcoto et al. 2020). These findings suggest that these bacteria play important roles in this micro-habitat, including metabolism of simple sugars, lysis of the fungal cell wall and biosynthesis of antimicrobial compounds among other functions that contribute to the ecological success of fungiculture (Barcoto et al. 2020). Another feature is that the establishment of new colonies or breeding sites by the insect may be accompanied by the dispersal and vertical transmission of the fungus by the insects (here, vertical transmission is at the level of the group or colony of insect) (Batra 1963; Weber 1966; Johnson 1981). The insect is generally considered to offer a suitable location for the fungus to grow, through excavating chambers underground or galleries in trees and providing a homeostatic and protective environment for the fungus (Odling-Smee et al. 2003). All these topics have been reviewed in detail recently by Biedermann and Vega (2019).

A further feature common to these systems is the presence of other organisms related to these associations. This is perhaps best known from studies of Attini but investigations are accelerating with coleopteran systems and more recently termites. Work on bee fungiculture is very recent but it will be interesting also to see what other organisms coexist in these systems and their importance. In the case of Attini, bacteria, yeasts, other fungi and arthropods (especially mites and other ants) have been recorded from fungus gardens. The filamentous bacterium *Pseudonocardia* (Actinobacteria) has become a classic instance of this; in this case, it is involved in a tripartite mutualism with

some attine ants, especially leafcutters of the genus *Acromyrmex* (Hymenoptera: Formicinae: Attini), providing fungicidal or fungistatic substances that the ants use to protect their fungus gardens and themselves (Currie et al. 1999b). This mutualism appears to be obligatory for *Pseudonocardia*, but facultative interactions have been shown with other bacteria such as *Burkholderia* (Betaproteobacteria) (Santos et al. 2004) and *Streptomyces* (Kost et al. 2007). Meanwhile, other members of the community present in fungus gardens may have what seems to be a more negative interaction with the ant-fungus mutualism, as is the case with *Escovopsis* or other fungi such as *Trichoderma* (Ascomycota: Hypocreales) and *Syncephalastrum* (Reynolds and Currie 2004; Rocha et al. 2014; Barcoto et al. 2017). What emerges from these studies is the understanding that there may be many organisms acting in many different ways within an attine fungus garden. Examples are also seen in the termites, such as *Pseudoxylaria* (Ascomycota: Xylariales) (Visser et al. 2011). This fungus appears to have an antagonist relationship with the termite-fungus mutualism (at the colony level), since it can compete for resources and damage the mutualistic-termite fungus, depending on colony condition. It is worth noting that the fungus *Xylaria* (Ascomycota: Xylariales) has also been found associated with leafcutters and deteriorating nest termites (Thomas 1987; Rocha et al. 2014). Southern pine beetle fungiculture is also threatened by an additional symbiont of the system. The fungus *Ophiostoma minus* (Ascomycota: Ophiostomatales) is carried by a phoretic mite (*Tarsonemus* sp.; Trombidiformes: Tarsonemidae) in the galleries built by *Dendroctonus frontalis* (Coleoptera: Curculionidae) (Bridges and Moser 1983). This pathogenic fungus can surpass the mutualistic fungus' growth, damaging the development of beetle larvae (Klepzig and Wilkens 1997; Klepzig et al. 2004). The association of these different organisms in the examples above demonstrates how complex the symbiotic network of fungus-growing insects can be. Each of these harbours

a particular ecosystem responsible for the ecological and evolutionary success of the involved species.

Fungus-farming in ants

Attines have been growing fungus for millions of years, but one of the first reports that demonstrated this behaviour was in 1874. During a field expedition to Central America, the English naturalist Thomas Belt was impressed by the amount of leaves cut and carried by the ants and wondered why he never found a corresponding amount of vegetative material inside the nests. After more careful investigation, he observed that the chambers inside the colonies were filled "with a speckled brown, flocculent, spongy-looking mass of a light and loosely connected substance" (Belt 1874). Belt also observed that among the spongy material, which he identified as a fungus, were the pupae, larvae and countless pieces of very small leaves incorporated into the spongy mass. He showed for the first time that leafcutter ants are, in fact, mycophagous and therefore they use the leaves as fertilizer for the subterranean fungus garden and not for direct consumption.

Fungus-farming ants constitute a diversified group and have created a complex symbiotic system. It is not known how attine ants began farming their own food; however, it is clear that this event has contributed to the group´s ecological and evolutionary success. There are two theories to explain the origin of fungiculture in the tribe Attini proposed by Weber (1958; 1972a) and termed by Mueller et al. (2001): (i) the most accepted hypothesis is that from accidental growth of an unspecialized fungus in ant colonies which later became part of the diet – "Consumption first" model and (ii) specialized fungi were dispersed by ants that subsequently domesticated the fungi – "Transmission first" model. The attine ants maintain the association with the mutualistic fungus through different substrates. Most species of attines practice 'lower agriculture', in which a domesticated fungus (Basidiomycota: Agaricales: Leucocoprineae tribe) is

cultivated by incorporating dead parts of plants, invertebrate carcasses and / or insect faeces (Mueller et al. 2001; Shultz and Brady 2008, Mueller et al. 2017; 2018). In this type of association, the fungus can be free-living (Mueller et al. 1998). On the other hand, in 'higher agriculture', the mutualistic fungus – ant association is more specialized (Chapela et al. 1994; Shultz and Brady 2008). The fungus produces modified hyphae, called gongylidia, which are consumed by ants (Quinlan and Cherret 1979; Chapela et al. 1994). Members of this group use fresh plant parts as a substrate and in addition, the fungus is not found outside the attine nests. However, broader samplings of the fungus garden of attine ants have shown that higher-attine mutualisms are less specialized that previously thought (Mueller et al. 2018). There is an exchange of mutualistic fungus between lower and higher attines (apparently less frequent than in lower attines) (Mueller et al. 1998; Green et al. 2002; Mueller et al. 2017; 2018). In a few cases reported in the literature, colonies of the higher attines can cultivate a lower-attine fungus while a species of the lower-attine ant was found growing a type of fungus previously known only from leafcutter ants (i.e. higher attines) (Shultz et al. 2015; Mueller et al. 2018). Interestingly, in this latter case, the lower-attine ant grows their mutualistic fungus using arthropod frass instead of fresh leaves like leafcutter ants.

Initially, molecular-dating analyses indicated that fungiculture in the Attini had a single origin on the South American continent (Shultz and Brady 2008). Recently, however, it has been suggested that the domestication of free-living fungal populations occurred in dry habitats (Branstetter et al. 2017). There are some inconsistencies about which ant group is closest to the Attini, and also which was the first attine agricultural system. Some studies indicate that fungiculture in ants started from farming yeast (Wilson 1971; Hölldobler and Wilson 1990), others from coral fungi (Agaricales: Pterulaceae) (Sánchez Peña 2005) or even from lower agriculture (Mueller et al. 2001; Shultz and

Brady 2008). Yeast cultivars (Basidiomycota: Agaricales: Leucocoprineae tribe) and fungi associated with lower attines can be free-living, not associated with ants (Mueller et al. 1998). Interestingly, yeast-phase growth is an exception in Agaricales and is only observed when the fungus is associated with attines. Coral fungi are cultivated by a specific clade of *Apterostigma* (Hymenoptera: Formicinae: Attini) and the most recent analyses shows that the acquisition of coral fungi was more recent in the evolution of this attine group (Shultz and Brady 2008).

For a long time, it was assumed that the attine fungus garden was a pure culture, with only the mutualistic fungus present. However, laboratory colonies often exhibited overgrowth of alien microorganisms (Möller 1893; Stahel and Geijskes 1941; Weber 1966; 1979). Most of these organisms were considered air contaminants or were associated with plant fragments used as a substrate for the growth of the mutualistic fungus. By now, we know that, by becoming fungus farmers, the attines initiated a complex symbiotic network of microorganisms within their colonies. These relationships began thanks to the wide variety of food provided to their symbionts, over millions of years. It is known, indeed, that leafcutters take a wide diversity of endophytic fungi (Table 1) into their nests within cut leaf fragments (Rocha et al. 2014). Although some symbiotic relationships have already been studied (attine – mutualistic fungus – filamentous bacteria (actinobacteria) – black yeast *Phialophora* (Ascomycota:

Chaetothyriales) – *Escovopsis* (Ascomycota: Hypocreales), the vast majority of the relationships that make up the attine's fungus gardens are still unclear.

Considering that the attine – mutualistic fungus relationship is the main part of the attine’s colonies, the knowledge of a parasite´s able to affect this relationship is indispensable to understand the evolution and ecological success of this insects. That is why the mutualistic fungus – *Escovopsis* symbiosis is the most investigated relationships

found in the attines' fungus garden. *Escovopsis* was considered as a parasite highly virulent of the mutualistic fungus for many years, this notion even finding its way into undergraduate textbooks (Stearns & Hoekstra 2005) and popular science texts (Holldobler and Wilson 2009). Nowadays, however, this seems to have been an oversimplification of the interaction and this hypothesis of high virulence merits re-examination. This question forms the basis for much of the following sections of this review.

The genus *Escovopsis*

The history of *Escovopsis* (although it was yet to be named such) began with Möller (1893), who mistakenly considered it an anamorph (i.e. asexual phase) of the mutualistic fungus of the attines. Subsequently, other scientists produced illustrations of this mysterious fungus. Stahel and Geijskes (1941) and Weber (1966) observed it growing in nests of fungus-growing ants, the latter correlating its presence with "abnormal circumstances" in a colony of *Trachymyrmex septentrionalis* (Hymenoptera: Formicinae: Attini). Afterwards, this same fungus was identified and described by Kreisel (1972) as *Phialocladus zsoltii*, using an isolate associated with an *Atta insularis* (Hymenoptera: Formicinae: Attini) colony in Cuba. About a century after its discovery, the genus *Escovopsis* was formally described by Muchovej and Della Lucia (1990). Since Kreisel had not determined a holotype at the time, Muchovej and Della Lucia renamed it as *Escovopsis* referring to the brush-like anamorph with phialides on well-defined vesicles (Table 1).

Escovopsis has probably been involved in the Attini – mutualistic fungus interaction since the beginning of the fungus' domestication by ants (Currie et al. 2003; Mehdiabadi and Shultz 2010). The scenario accepted so far is that this genus was probably

a parasite of free-living leucocoprineous fungi and has followed the evolution of fungiculture practiced by the attines since then (Currie et al. 2003). This hypothesis is mainly based on the fact that *Escovopsis* belongs to the family Hypocreaceae, which contains others mycoparasitic fungi such as *Hypomyces* and *Trichoderma*. However, Hypocreaceae also contains parasites of insects and parasites and endophytes of plants, with evidence for host-switching through evolutionary history (Spatafora et al. 2007). Although *Escovopsis* has only been found inside colonies of fungus farming-ants (or discarded with waste from within them) and therefore its distribution depends on attine ants, it is important that future surveys consider other hypotheses to elucidate the beginning of this symbiosis. It could be interesting to investigate whether some *Escovopsis* species retain the ability to associate with plants, for example.

The genus *Escovopsis* – Geographical distribution

The geographical distribution of *Escovopsis* has been little explored. *Escovopsis* has never been found unless associated with the fungus garden of Attine ants and is present in colonies of basal and higher attines. Thus, it is expected that *Escovopsis* species are limited to the distribution of fungus-growing ants – exclusively New World and mainly Neotropical, since it is a specialized parasite of attines (Schultz and Meier 1995; Mayhé-Nunes and Jaffé 1998).

Meirelles et al. (2015b) investigated the phylogenetic specificity of the association between *Escovopsis* clades and higher attines from broad sampling on the American continent. Although the focus of that study was not geographical distribution, it is the only study to date to show in any detail the occurrence of *Escovopsis* among different species of attine ants (see figure 2 in Meirelles et al. 2015b). *Escovopsis* has been found from all regions of Brazil, part of Central America, Mexico and the Caribbean island of

Guadeloupe. This study separated *Escovopsis* into 9 different clades, including some strains from the basal attine *Apterostigma* sp. isolated in a previous study (Gerardo et al. 2006b).

The data of *Escovopsis* diversity in higher attines gives a possible insight into the pattern of geographical distribution (Meirelles et al. 2015b). These authors suggested that *Escovopsis* species could present a latitudinal diversity gradient, in which there is a reduction of diversity at higher latitudes. However, a greater sampling effort is needed to test this possibility, in addition to including larger samples from basal attines species.

The genus *Escovopsis* – Taxonomy and systematics

Between 1990 and 2013 only two species of *Escovopsis* had been described (Muchovej and Della Lucia 1990; Seifert et al. 1995) and it was common for different morphotypes to be described by their colouration (e.g. Gerardo et al. 2006a). This changed from 2013 with the descriptions of five new species and the genus *Escovopsioides* which is phylogenetically close to *Escovopsis* (Augustin et al. 2013; Masiulionis et al. 2015; Meirelles et al. 2015a). During this nearly three-decade interval to 2013, studies with *Escovopsis* had been focused on their possible parasitic nature, addressing more ecological aspects rather than taxonomy and diversity. In late 2018, a group of researchers described five more species in Argentina (Marfetán et al. 2018) and in 2019 two other species were described from Brazil (Montoya et al. 2019).

Although we do not have many species described, species descriptions have not followed a standard of morphological evaluation or molecular analyses. Therefore, after standardization to identify and describe new species of *Escovopsis*, two species described earlier (*E. kreiselli* and *E. trichodermoides*) were renamed and placed in new genera (*Sympodiorosea* and *Luteomyces*, respectively) (Montoya et al. *submitted*). The addition

of two new molecular markers and the most detailed macro- and microscopic morphological evaluation indicated that the two species cited above did not belong to *Escovopsis* species. Following the recommendations to identify new species, three new species are being described (Caixeta et al., Chapter 1).

Escovopsioides Evans and Augustin (Ascomycota: Hypocreales), as well as *Sympodiorosea* and *Luteomyces*, already have been identified as sister genera related to *Escovopsis* (Augustin et al. 2013; Montoya et al. 2020 – submitted). These four genera belong to the family Hypocreaceae, but they form separated monophyletic clades, *Luteomyces* being the group closest to *Escovopsis*, *Sympodiorosea* closest to *Escovopsioides* (Montoya et al. *submitted*). In addition to phylogenetic division, these species also have morphological peculiarities that place them in distinct genera. Although *Escovopsioides* produces phialides on vesicles like *Escovopsis,* it differentiates itself by presenting lageniform (flask-shaped) phialides arranged in terminal and intercalary vesicles, in addition to differences in the form of conidia. *Sympodiorosea* has sympodial (side-branching) conidiogenous cells as the main characteristic of the genus and *Luteomyces* presents conidiophores with synchronous conidiogenous cells and yellow coloured colonies. (Note that some studies to date have described *Escovopsis* isolates as "brown", "pink" or yellow", so some may actually belong to these new genera – Gerardo et al. 2006a; 2006b). Intriguingly, these morphological characteristics are not observed in any other genus of the Hypocreaceae, so it may be that they have arisen as a result of selection to the particular life styles of these fungi in association with the ants.

These recent findings open interesting possibilities for the study of this system. The formal description of new genera expands the known diversity of fungi that associate with the attine system and can exploit it. Future studies may reveal if these fungi have a specific relationship with attine ant nests and if they affect the mutualism. It would also

be interesting to investigate the relationships of these genera to each other to see if they can co-occur in the same colony or if they have any inhibition mechanisms in relation to each other.

The genus *Escovopsis* – Diversity

As highlighted by Montoya et al. (2019), the lack of standardization for the description of new species within the genus has contributed to the scarcity of taxonomic information over the past few years. For this reason, many studies may have generalized their results without considering the peculiarities of species or groups of species that are closely related phylogenetically. Our concern is that this fact may have caused a cascade of misinterpretations that are passed on as facts (consequently including ecological aspects).

Morphological diversity is also being revealed through the description of new species. *Escovopsis* (*senso stricto* in this case, as specified in Table 1) was determined to have well-defined vesicles and this morphological character allowed the differentiation of some clades and the determination of the phylogenetic position of the groups (Della Lucia and Muchovej 1990; Montoya et al., *submitted*). Initially, there were clades with basically two types of vesicles: globose and cylindrical, the first ones were considered the ancestral state in relation to the latter (Meirelles et al. 2015b). However, we already know of species that possess both types of vesicles concurrently (*E. multiformis*) and still other formats that had until then not been observed (Montoya et al. *submitted*, Caixeta et al., Chapter 1).

Morphological characterization of species is indispensable to their classification, but it can likewise be fundamental to give indications about their relationship with the host fungus and strategy for exploitation of available resources. *Escovopsis moelleri* conidia, for example, are larger (approx. 10 µm in length) than those observed in other

species and present a distinct apical cap-like structure (Augustin et al. 2013). After description of this species, another study raised the possibility that these characteristics confer a phoretic ability on arthropods in *E. moelleri*, that might suggest a means of horizontal transmission between colonies (Augustin et al. 2017). Likewise as particularity in conidia morphology, others aspect such as dormancy, production of soluble and volatile compounds and growth rate can be differences that give us clues about the strategy used by this fungus in the association with attine colonies and in their relationship with each other (see below).

The genus *Escovopsis* – Biological Cycle

The life cycle of *Escovopsis* species is far from understood, let alone those of its sister genera. Considering that *Escovopsis* has never been isolated from newly founded colonies, horizontal transmission has always been considered to be the main and most likely way in which the fungus can reach new colonies and complete its life cycle (Currie 1999a). It is important to note, however, that horizontal transmission has never actually been demonstrated, even in the laboratory. Only one study has addressed this, showing perhaps the first part of the story with waste being discarded from the colony containing *Escovopsis*, some of it sporulating, and the potential for phoresy in the species *E. moelleri* (Augustin et al. 2017). This area requires much more study, especially if we take into account that there are few species of higher atines that have waste external to the colony.

Escovopsis is known from nests of *Atta*, *Acromyrmex*, *Trachymyrmex*, *Sericomyrmex*, *Mycetophylax* and *Apterostigma*. Examination of infrabuccal pellets, however, has only been done with *Atta* species. The nuptial flight in *Atta* sp. is a phenomenon that can be observed with the naked eye and, therefore, it is relatively easy to conduct this type of experiment, which probably explains this fact. Perhaps vertical

transmission occurs in other attine species that have yet to be examined. This seems to be an area of great importance for future study so as to draw any sort of general conclusion regarding the life cycle of this group of fungi. This is even more so in generating hypotheses about how transmission may affect selection on virulence.

Before nuptial flight, alate female reproductives remove fragments of the garden fungus of their natal nest and store these as pellets in an infrabuccal cavity. After they have mated, fallen to the ground and dug their own nest, the pellet is regurgitated and cultivated using faecal material provided by the reproductive female, now a foundress queen, until the first workers can emerge and start foraging. Driven mainly by a desire to know which fungi are carried by foundress queens before the nuptial flight, these pellets have been sampled from *Atta* spp., in addition to gardens of incipient laboratory colonies and cuticle of foundress *Atta* queens (Currie et al. 1999a; Pagnocca et al. 2008; Moreira et al. 2015). From cultivation-dependent methods, these results have never detected *Escovopsis s.l.* in the pellets. Meanwhile, in a field study of *Atta sexdens*, the first detection of its presence in the colony coincided with the moment when the first workers started foraging, suggesting that infection arose from an external source (Moreira et al. 2015). Despite this weight of evidence, it is still possible that at least some species of *Escovopsis s.l.* do effect vertical transmission. If dormant spores are taken into the infrabuccal pellet, they would not be detected by cultivation methods and could have a fixed dormancy period or else respond to a stimulus associated with the opening of the colony for dormancy to break.

The apparent absence of vertical transmission in *Escovopsis s.l.* has been interpreted in the light of theory on the evolution of virulence, to explain the apparently high virulence of this fungus (Currie 1999a). In a general and simplified way, horizontally transmitted parasites tend to be more virulent than vertically transmitted parasites as the

latter rely on their hosts for transmission (Ewald 1994). This in no way means, however, that horizontally transmitted parasites need be highly virulent, as was implied in that initial application of the theory. The common cold of humans, exclusively transmitted horizontally, is proof of this. One factor, in fact, that should select for low virulence, is long-lived hosts (Watson 2013) and ant colonies could easily be described as such, especially when compared to individual insects. A simple examination of extant theory, therefore, suggests that *Escovopsis* s.l. should have low virulence, especially if it is able to effect constant transmission through the host colony´s life (see Augustin et al. 2017).

We already know that at least *E. moelleri* has a dormancy mechanism (Augustin et al. 2017), a fact that would support the idea that it can be transmitted vertically. *Escovopsis* could strategically remain dormant at the beginning of the development of the colonies until it finds suitable conditions to grow. Likewise, that same fact could invalidate the vertical transmission proposal. If dormancy is broken by the presence of the host, why was it never detected from the moment that fungus garden begins to grow by the queen's exclusive cultivation? We suggest several possibilities: the first is that the queen releases substances that can inhibit the growth of *Escovopsis.* It is known that during the foundation of the nest in *Acromyrmex octospinosus*, a leafcutter ant species, foundresses have behaviours such as autogrooming and addition of faecal liquids when incorporating plant substrate into the fungus garden, thereby preventing the growth of pathogens (Fernández-Márin et al. 2003). Furthermore, secretions from the metapleural gland are also used by the queens as a prophylactic measure against the invasion of pathogenic bacteria and fungi (Hölldobler and Wilson 1990). Therefore, it is possible that these measures might prevent this initial *Escovopsis* growth. Second, all attempts to identify possible vertical transmission of this fungus are culture-dependent methods. In other words, it is likely that the other fungi present in the pellet, which are saprotrophic

fungi, air contaminants, soil-born fungi, endophytes or other mycoparasites (Rodrigues et al. 2005; 2008), can prevent *Escovopsis* growth on culture medium. Third, breaking of dormancy could be an endogenous process that depends on the passage of time. Finally, we cannot discard the possibility that the dormancy-breaking mechanism involves processes and conditions that are much more complex and specific than just the presence of the mutualistic fungus of the attine ants. To investigate this, we would initially need to expose incipient colonies in different to different conditions of light, temperature and humidity for example.

Escovopsis as a mycoparasite

Fungicolous (or mycophyllic) fungi maintain some type of relationship (symbiotic, mycoparasitic, saprotrophic) with another fungus (Barnett 1963; Rudakov 1978; Jeffries 1995). Most of the representatives of these fungi are mycoparasites or hyperparasites. Mycoparasitic fungi are found in diverse phyla such as Ascomycota, Basidiomycota, Blastocladiomycota, Chytridiomycota, Entomophthoromycota (the latter mostly known as animal parasites, but two species have been reported as mycoparasites), Kickxellomycota, Mucoromycota and Rozellomycota (recently reviewed by Sun et al., 2019).

According to the mode of action, mycoparasites can be divided into biotrophs and necrotrophs (Jeffries 1995). Biotrophic mycoparasites usually have slow growth and are less competitive than necrotrophs (Deacon and Berry 1992). They have an obligatory relationship with their hosts and usually a narrow host range, using living cytoplasm as their source of nutrition without causing much damage (Barnett and Binder 1963). They penetrate the tissue of their hosts through specialized hyphae, then obtaining nutrients released by the host (Deacon and Berry 1992; Jeffries and Young 1994). On the other

hand, necrotrophic fungi kill their host (at least locally, considering that fungi are modular organisms), by the action of enzymatic and anti-fungal chemicals, and subsequently use the dead biomass as their source of nutrients (Deacon and Berry 1992, Krauss et al. 2013; Borges et al. 2015; Karlsson et al. 2015). These fungi generally have a comparatively broad host range (Jeffries 1994; Viterbo and Horwitz. 2010).

Mycoparasitic ascomycetes are generally necrotrophic, prominent examples being *Trichoderma* (Hypocreales: Hypocreaceae) and *Clonostachys* (Hypocreales: Bionectriaceae) (see table 2). These genera are recognized for their usefulness as biocontrol agents because of their mycoparasitic nature. *Escovopsis weberi* was initially described as a necrotrophic mycoparasite as well (Currie and Reynolds, 2004). However, *E. weberi* develops structures such as appressorium-like bodies and hyphal loops during its interaction with the host, characteristics that have led it to be described as a biotrophic mycoparasite (Marfetán et al. 2015). The difference in classification is probably due to the fact that each study followed different references to classify the mode of action of *E. weberi* and investigated distinct isolates.

Here, we wish to highlight the mechanism itself and the probability that it depends on the isolate or species of *Escovopsis* studied as is the case with *Trichoderma* (Sivan & Chet 1986; Atanasova et al. 2013). The parasitic mechanisms of *Escovopsis* were tested once again recently and some strains (unidentified species) isolated from *Atta* and *Acromyrmex* leafcutter ant species showed that all isolates tested use chemical compounds and physical mechanisms to parasitize their host, without specialized structures (Varanda-Haifig et al. 2017).

Chemical interactions of *Escovopsis* have been better studied recently. Like other mycoparasites, *Escovopsis* species produce chemical compounds known to inhibit the growth of the fungus grown by ants. In addition, these compounds can inhibit mutualistic

bacteria and some that cause damage even to workers (Boya et al. 2017; Varanda-Haifig, 2017; Dhodary et al. 2018; Heine et al. 2018). However, there are no reports so far that *Escovopsis* influences the growth of other fungi beyond the ants' mutualistic fungi. This specialization is reflected in the small genome of *E. weberi*, which is among the smallest within the Pezizomycotina (approximately 29 Mb). Genome sequencing showed that *E. weberi* lost genes related to carbohydrate active enzymes (de Man et al. 2016). On the other hand, similarly to *Trichoderma reesei*, *E. weberi* has chitinases, responsible for degradation of host cell walls (de Man et al. 2016).

Volatile compounds are also to be found in *Escovopsis* – *Leucoagaricus gongylophorus* interaction. Some chemical signals released by fungus grown by ants can accelerate the growth of *Escovopsis* growth whereas the volatile compounds produced by *Escovopsis* can damage both *L. gongylophorus* and ants (Masiulionis and Pagnocca 2020). An interesting observation was made by Masiulionis and Pagnocca (2020), indicating that volatile compounds produced by *L. gongylophorus* could nourish *Escovopsis*, which would explain its rapid growth in the presence of its host. This hypothesis was based on the parallel with volatile vitamins produced by soil micro-organisms that can be used by other micro-organisms as a nutritional source (Stotzky and Schenck 1976). We stress that this would be extremely important especially because, as far as we know, there are no studies proving the nutrient transfer from host fungus. Besides that, it would be interesting to test if volatiles produced by *L. gongylophorus* (mainly when it is dying in the nest or dead in the midden piles) can break dormancy of *Escovopsis*. It is important to emphasize that dormancy has been observed only in *E. moelleri*, but it cannot be disregarded as a possibility in other species. Although the effects of the interaction of volatile compounds have been previously noted (Reynolds and Currie 2004; Gerardo et al. 2006; Custodio and Rodrigues 2018), the identification of

compounds involved in the interaction is very recent (Masiulionis and Pagnocca 2020). The volatile profile of *L. gongylophorus* and *Escovopsis* can be useful for many future surveys involving specificity in the relationship between these two fungi from different species of attines or even as additional tools for taxonomic and phylogenetic studies (see Croxato et al. 2012).

Table 2. Characteristics of other mycoparasites compared to *Escovopsis* species

Characteristics	Examples	Has this been observed in any *Escovopsis* species?
Biotrophic mycoparasites. Parasitic fungi that obtain nutrients from live mycelium of the host (Barnett, 1964)	*Ampelomyces quisqualis* (Kiss et al. 2004)	Yes – shown in *E. weberi* (Marfetán et al. 2015). However, this species was considered a biotrophic mycoparasite because of the penetration of the host hyphae from the presence of structures such as hooks (Boosalis, 1964)
Necrotrophic mycoparasites. Destructive parasitic fungi that kill their host to obtain nutrients (Barnet, 1964)	*Clonostachys* sp., *Trichoderma* sp.	Yes – shown in *E. weberi* (Reynolds and Currie, 2004)
Wide range of hosts.	*Arthrobotrys oligospora*, *Clonostachys rosea*, *Trichoderma viride* (Krauss et al. 2013; Mukherjee et al. 2013; Borges et al. 2015; Karlsson et al. 2015)	No
Host-specificity in genus or family level	*Hypomyces* that parasitize agarics (Rogerson and Samuels 1989; Tamm and Põldmaa 2013)	Yes – (Currie et al. 2003)
Formation of appressoria-like infection structures or hyphal swellings at the points of interaction with host	*Trichoderma* sp. (Chet et al. 1981; Lu et al. 2004)	No
Specialized structures to penetrate the host. Typical of invasive necrotrophic fungi (Jeffries, 1995)	*Trichoderma* sp. (Chet et al. 1981)	Yes – shown in *E. weberi* (Marfetán et al. 2015)
Coiling of parasite hyphae on host hyphae. Typical of contact necrotrophic fungi (Jeffries, 1995)	*Arthrobotrys oligospora* (Olsson and Persson 1994; Singh et al. 2012), *Clonostachys rosea* (Li et al. 2002), *Trichoderma* sp. (Lu et al. 2004)	Yes* (Varanda-Haifig et al. 2016) and *E. kreiselli*** (Custodio and Rodrigues 2018)
Production of anti-fungal chemicals during parasitism. Typical of non-contact necrotrophic fungi (Jeffries, 1995)	*Clonostachys* sp. (Karlsson et al. 2015)	Yes – shown in *E. weberi* (Reynolds and Currie 2004; Varanda-Haifig et al. 2016) and *Escovopsioides nivea* (Varanda-Haifig et al. 2016)

Chitinases present. Enzymes important for degradation of cell wall of host fungus during mycoparasitism.	*Trichoderma reesei* (Kubicek et al. 2011); *T. harzianum* (Zeilinger et al. 1999); *T. atroviride* (Reithner et al. 2005)	Yes – shown in *E. weberi* (de Man et al. 2016)
Volatile compounds.	*Trichoderma atroviride* (Stoppacher et al. 2010)	Yes* (Masiulionis and Pagnocca, 2020)
Nutrient transfer from host fungus.	*Arthrobotrys oligospora* (Olsson and Persson 1994)	No

* The *Escovopsis* isolates used in these studies were not identified.
** *Escovopsis kreiselli* is being renamed and placed in the genus *Sympodiorosea*.

Escovopsis ant-cultivar relationships

Escovopsis is a common inhabitant of attine ant gardens. Several studies have shown its prevalence that vary depending on the ant species and location (18 to 75%) (Currie 2001; Gerardo et al. 2004; Rodrigues et al. 2005; Rodrigues et al. 2008; Augustin et al. 2013). *Escovopsis* is presumed to have coevolved with the attines and their symbiotic fungus (Currie et al. 2003) and the first studies of interactions between it and the mutualistic fungus assumed a tight association between the groups: *Escovopsis* clades were specifically associated with certain ant clades. However, it was known that different higher attines can share the same *Escovopsis* (Taerum et al. 2007; Meirelles et al. 2015b). Meanwhile a single fungus garden may have multiple *Escovopsis* strains (Taerum et al. 2010; Augustin et al. 2013).

Escovopsis began to become of particular interest after publication of the study by Currie et al. (1999) and that study warrants particular attention. In it, the frequent isolation of *Escovopsis s.l.* from attine ant colonies (26% of all contaminants found in more than 2,400 garden pieces) and the verification of Koch's postulates, led *Escovopsis* to be considered a specialized pathogen. Although Koch´s postulates were used, it is questionable whether the step of proving that a disease has been caused was really satisfied. Meanwhile, some specific methodological aspects may have influenced the

conclusions of this work, such as the age and size of the colonies that were used. Koch's postulates are important steps to indicate the causal agent of a disease, but Robert Koch himself recognized the limitation of his method, especially for cases of non-cultivable pathogens (which is not the case here). These barriers were not discussed or questioned in the original text that suggested the pathogenicity of *Escovopsis* through the postulates (Currie et al. 1999). Perhaps the most problematic issue is the fact that we are not dealing with an individual, but rather a eusocial organism and its symbiont. Although *Escovopsis* is considered a specialized mycoparasite of the mutualistic fungus of the attine ants, the effect caused by it in certain situations (especially in those where the colony is already suffering a disorder – Mendonça et al.; Chapter 2), affects the entire system. Besides, it is very common to isolate *Escovopsis* from healthy colonies that are normally foraging, both in the field and in the laboratory (Currie et al. 1999a; Gerardo et al. 2004; Rodrigues et al. 2005a; Augustin 2011). Consequently, it is rarely possible to identify if a nest is infected by *Escovopsis s.l.* – this can only be determined when it is being overgrown or by isolating the fungus – there are no 'symptoms' beyond the presence of the fungus that can be attributed to a 'disease' caused by the fungus. Meanwhile, it is impossible to verify whether a colony is free of *Escovopsis* by culture-dependent methods as total sampling of a fungus garden would require its destruction. Perhaps in the future a sampling plan of fungus gardens could be devised based on extensive sampling of gardens with different rates of infection, that might allow one to determine the probable infection status of a fungus garden or colony. This would be a major effort but would be of tremendous use for guiding future studies.

During this same experiment (Currie et al. 1999), young colonies of *Atta colombica*, between 6 and 8 weeks old, containing garden volumes of 60 to 75 ml were used. Such incipient colonies are fragile and do not have the same defence capability as

mature colonies. The impact of *Escovopsis* infection in this study could well be ascribed to this fact. Also, *Trichoderma*, a well-known necrotrophic mycoparasite fungus, was used as a positive control for high inoculation of a proven aggressive fungus. However, the authors reported that they were unable to recover either it or *Escovopsis* at the end of the experiment. Two further issues require addressing: firstly, the authors did not mention whether they tested the viability of the conidia of both fungi. This test is common and essential in infection experiments to confirm if the conidia are capable of infecting the host. Therefore, it is likely that *Trichoderma* conidia were not able to infect colonies. Secondly, it is not possible to know whether the *Escovopsis* recovered after spread is the same as that which was inoculated in the nests. Even though the colonies have been labeled as *Escovopsis*-free, as explained above, it is not possible to state this by the methods used. In the face of everything that we have discussed here, we consider that evaluating the virulence of a probable parasite under these conditions is not the most appropriate method, especially because it is a complex system that involves different symbiotic associations. In addition, the results of that single study had great reach and served as the basis for several others, without concern for some details that may be crucial for understanding this parasite-host relationship.

Because we can find different strains of *Escovopsis* growing on the same ant species and even sharing the same colony (Taerum et al. 2010; Augustin et al. 2013), different strategies (e.g. infection, transmission or virulence) are important for the survival and persistence of each species. Therefore, it is expected that not all species or isolates present the same virulence level or aggressivity. The generalization that is made for the whole genus, assuming it as a virulent parasite and disregarding factors as colony condition, as is often observed, is also questionable.

For *Escovopsioides* and the two newly constructed genera, *Luteomyces* and *Sympodiorosea*, we are almost entirely ignorant as to their roles in the symbioses of the Attini. Preliminary studies have shown that *Escovopsioides* is an antagonist of the basidiomycete mutualist, but it appears to be less virulent than *Escovopsis*, causing no remarkable negative effect on colonies (Varanda-Haifig et al. 2017; Mendonça et al.; Chapter 2). The other two genera are very recent and there is only one study that evaluated the interaction between *E. kreiselli* (now *Sympodiorosea* genus) and the fungus garden of a lower attine, *Mycetophylax morschii*, its native host (Custodio and Rodrigues 2018). The results showed that *E. kreiselli* was able to inhibit the mutualistic fungus in dual culture assays.

Phylogenetics analyses indicate that *Escovopsis* co-evolved with fungus growing ants (Currie et al. 2003), so it is expected that defensive strategies of attine ants and their mutualistic fungus against this parasite have been shaped by evolution. The social organization of the ants, hygienic behaviour and association with the actinobacterium *Pseudonocardia* are strategies that contribute to *Escovopsis* control within nest. In addition, *in vitro* results have shown that the fungus cultivated by ants can itself inhibit the growth of pathogens (Gerardo et al. 2006a; Van Bael et al. 2009). These features can contribute to the reduction of parasite's virulence in social insects, in general, as discussed by Hughes et al. (2008). Perhaps the evolutionary pressures have been shaping *Escovopsis* for a strategy in which it remains in the colonies causing minimal damage and waiting for the most propitious moment (e.g. queen's death for any other reason) to actually overgrow inside nest in an aggressive way.

Task divisions within the colonies of ants and other social insects can be separated by individuals with different morphologies (polyphenism or polymorphism) and ages (age polyethism) (Wilson 1980; Hinze & Leuthold 1999). Schmid-Hempel (1998)

compared the separation of tasks by age to a conveyor belt model, where young workers are responsible for safer duties inside colonies, and as these workers get older, they start to perform tasks outside the nest that have higher risks. If they do not return to the centre of the nest they are less likely to bring pathogens in with them. This time schedule is very well studied in bees and it is known that it can be accelerated if the colony is under stress (Natsopoulou et al. 2016). In other words, some workers may have a reduced life expectancy and, therefore, begin to perform more risky tasks, depending on the stress factors that the colony is suffering from. Furthermore, in bees it appears that less virulent parasites influence host behaviour less, in terms of accelerating the change in the performance of nest activities, than more virulent pathogens (Natsopoulou et al. 2016). It would be interesting to investigate whether this occurs with colonies experimentally infected with *Escovopsis* and other fungi found in ant colonies of the Attini tribe. This response can give us evidence of the host-pathogen fidelity, pathogen's virulence and the stability of the interaction.

Considering that we now know that what we thought was one genus, with one described species (*Escovopsis weberi*) is now actually four genera with 14 species described to date from just one of these, and a range of morphologies and growth patterns, it seems that we have more lacunae regarding the interactions of this group of fungi with the ant-fungus mutualism than actual knowledge. Additionally, there is considerable diversity within the attine ants and the basidiomycetes involved, the substrate brought into the nests, the sizes of these colonies and their ecological contexts. We suggest therefore that the virulence of *Escovopsis sensu latu* towards its host is far more complex than a simple description as highly virulent for the all genera, especially context-dependency. While simplifying this system facilitates research and makes it possible to

carry out numerous studies, it can be a long way from reality. We need to consider at least the main known interactions present in this symbiosis to obtain more realistic results.

Conclusion and perspectives

The tribe Attini, ants that grow fungus as a nutritional source, are inserted in an environment full of symbiotic interactions, as observed in other fungus-growing insects. Some of these relationships are well-studied, but there are probably countless other relationships of which we are not even yet aware, which may even influence the interactions already established.

Between the late '90s and early 2000's, the fungus of the genus *Escovopsis* emerged as an important mycoparasite of the fungus garden of this complex system of the attine ants. In fact, most species within Hypocreales contain fungi that are consistently associated with other fungi (Sun et al. 2019), such as mycoparasites, saprotrophic or maintaining neutral relationships, referred to as fungicolous fungi (Jeffries 1995). Perhaps, neutral relationships are transient organisms and, therefore, do not play a prominent role within the interaction. There is no consensus regarding the origin of the association of Hypocreales fungi with other fungi, but it is believed to have a multi-ancestor origin: from plant pathogens (Nectriaceae) to insect pathogens (Cordycipitaceae and Ophiocordycipitaceae). Studies that investigate the way in which *Escovopsis* is transmitted can give us clues about the ancestry of this fungus as well as research on molecular evolution.

Although there are divergences, *Escovopsis* has characteristics also observed in other mycoparasitic fungi, such as its closest relatives. These characteristics have basically been studied in two different species so far (*E. weberi* – the vast majority of studies and *E. moelleri* – Mendonça et al., Chapter 2), beyond other isolates not formally

described. However, the strategy used by a given *Escovopsis* species, as well as its virulence, is dependent on the species or isolate. Therefore, we emphasize the importance of taxonomic and phylogenetic studies so that the genus is delimited and inferences about the ecological role of *Escovopsis* are more assertive. Future research can evaluate the parasitic nature of new species, comparing the strategies of the morphologically different isolates. Likewise, it is possible to compare isolates that are more phylogenetically related to those that are more distant. In addition, the species' morphology can be compared with the way in which they reach new colonies: are there any that can be associated with plants carried by ants? Which of them are possibly transmitted by small arthropods and other insects phoretically? There are many questions that still need further investigation.

The *Escovopsis* – mutualistic-fungus – ant interaction occurs only in nests of the Attini tribe and it seems that *Escovopsis* is unable to infect and overtake the entire system under normal conditions. *Pseudoxylaria*, a genus associated with fungus-growing termites, has a similar strategy to what we are proposing for *Escovopsis* (Visser et al. 2011). Even being present in termite nests, *Pseudoxylaria* species are imperceptible until the activity of termites is reduced for some external reason such as death of the queen or presence of entomopathogenic fungi, allowing *Pseudoxylaria* to overgrow fungus cultivated by termites.

Environmental context must also be considered in future studies. What are the circumstances under which *Escovopsis* is being inoculated? It is important that this factor is made clear. We have seen that regardless of fungal inoculation, colonies without a queen or abandoned fungus gardens are fated to die (Mendonça et al.; Chapter 2). The addition of fungi in this type of scenario, be it *Escovopsis* or the other fungi phylogenetically related to it, does not reveal the real effect of a pathogen on ant colonies that grow fungus. Another important point is the unknown relationship between

Escovopsis, *Escovopsioides* and new genera that are being proposed. We know that *Escovopsioides* is an antagonist of fungi cultivated by ants, but is not aggressive when compared to *Escovopsis in vitro* (Varanda-Haifig et al. 2017; Mendonça et al.; Chapter 2). We do not have additional information about *Escovopsioides* nor of the other fungi which are related to *Escovopsis*. Future surveys can reveal the diversity of fungi associated with attine ants and then discover in more details about the transmission, virulence levels, type of interactions established and evolution of these fungi in the attine-ants system.

Considering the entirety of this review, we conclude that *Escovopsis s.l.* is indeed a mycoparasite of *Leucoagaricus gongylophorus*, but at the colony level, it is probably a parasite with a very low virulence and/or an opportunist that is sitting and waiting to overgrow a weakened nest and then effect transmission.

References

Aanen, D. K., Eggleton, P., Rouland-Lefèvre, C., Guldberg-Frøslev, T., Rosendahl, S., and Boomsma, J. J. (2002). The evolution of fungus-growing termites and their mutualistic fungal symbionts. *Proceedings of the National Academy of Sciences*, 99, 14887–14892. https://doi.org/10.1073/pnas.222313099

Atanasova, L., Le Crom, S., Gruber, S., Coulpier, F., SeidlSeiboth, V., Kubicek, C. P., Druzhinina, I. S. (2013). Comparative transcriptomics reveals different strategies of *Trichoderma* mycoparasitism. *BMC Genomics*, 14, 121. https://doi.org/10.1186/1471-2164-14-121

Augustin, J. O. (2011). Novas espécies de *Escovopsis*, sua dispersão e virulência na simbiose formigas Attini - *Leucoagaricus gongylophorus*. D.Sc. Thesis. Viçosa, MG, Brazil: Universidade Federal de Viçosa, p.131. http:// locus.ufv.br/handle/123456789/897

Augustin, J. O., Groenewald, J. Z., Nascimento, R. J., Mizubuti, E. S. G., Barreto, R.W., Elliot, S. L., & Evans, H. C. (2013). Yet more "weeds" in the garden: fungal novelties from nests of leaf-cutting ants. *PLoS ONE*, 8, e82265. https://doi.org/10.1371/journal.pone.0082265

Augustin, J. O., Simões, T. G., Dijksterhuis, J., Elliot, S. L., & Evans, H. C. (2017). Putting the waste out: a proposed mechanism for transmission of the mycoparasite *Escovopsis* between leafcutter ant colonies. *Royal Society Open Science*, 4(5), 161013. https://doi.org/10.1098/rsos.161013

Barcoto, M. O., Pedrosa, F., Bueno, O. C., Rodrigues, A. (2016). Pathogenic nature of *Syncephalastrum* in *Atta sexdens rubropilosa* fungus gardens. *Pest Management Science*, 73, 999–1009

Barcoto, M. O., Carlos-Shanley, C., Fan, H., Ferro, M., Nagamoto, N. S., Bacci Jr., M., Currie, C. R., Rodrigues, A. (2020). Fungus-growing insects host a distinctive microbiota apparently adapted to the fungiculture environment. *Science Reports*, 10**,** 12384. https://doi.org/10.1038/s41598-020-68448-7

Barnett, H. L. (1963). The nature of mycoparasitism by fungi. *Annual Review of Microbiology*, 17, 1–14. https://doi.org/10.1146/annurev.mi.17.100163.000245

Batra, L. R. (1963). Ecology of ambrosia fungi and their dissemination by beetles. *Transactions of the Kansas Academy of Science*, 66, 213–236. https://doi.org/10.2307/3626562

Beaver R. A. (1989). Insect-fungus relationships in the bark and ambrosia beetles. In: *Insect-fungus Interactions*, 121-143, eds. Wilding, N., Collins, N. M., Hammond, P. M., Webber, J. F. NATO ASI Series (Series A: Life Sciences), 230, Springer, Boston, MA. https://doi.org/10.1016/B978-0-12-751800-8.50011-2

Belt, T. (1874). *The naturalist in Nicaragua*. London, John Murray, Albermale Street

Biedermann, P., & Vega, F. E. (2020). Ecology and Evolution of Insect-Fungus Mutualisms. *Annual review of entomology*, *65*, 431–455. https://doi.org/10.1146/annurev-ento-011019-024910

Bignell, D. & Eggleton, P. (2000). Termites in Ecosystems. In *Termites: Evolution, Sociality, Symbioses, Ecology*, 363–388, eds. Abe, T., Bignell, D. E. & Higashi, M. Kluwer Academic, Dordrecht, The Netherlands. https://doi.org/10.1007/978-94-017-3223-9_17

Boosalis, M. G. (1964). Hyperparasitism. *Annual Review of Phytopathology, 2(1), 363–376.* https://doi:10.1146/annurev.py.02.090164.002051

Borges, A. V., Saraiva, R. M., Maffia, L. A. (2015). Biocontrol of gray mold in tomato plants by *Clonostachys rosea*. *Tropical Plant Pathology*, 40, 71–76. https://doi.org/10.1007/s40858-015-0010-3

Boya P, C. A., Fernández-Marín, H., Mejía, L. C., Spadafora, C., Dorrestein, P. C., & Gutiérrez, M. (2017). Imaging mass spectrometry and MS/MS molecular networking reveals chemical interactions among cuticular bacteria and pathogenic fungi associated with fungus-growing ants. *Scientific reports*, *7*(1), 5604. https://doi.org/10.1038/s41598-017-05515-6

Branstetter, M. G., Ješovnik, A., Sosa-Calvo, J., Lloyd, M. W., Faircloth, B. C., Brady, S. G., & Schultz, T. R. (2017). Dry habitats were crucibles of domestication in the evolution of agriculture in ants. *Proceedings of the Royal Society B: Biological Sciences, 284(1852).* https://doi:10.1098/rspb.2017.0095

Bridges, R. (1983). Mycangial fungi of *Dendroctonus frontalis* (Coleoptera: Scolytidae) and their relationship to beetle population trends. *Environmental Entomology*, 12, 858–861. https://doi.org/10.1093/ee/12.3.858

Chapela, I. H., Rehner, S. A., Schultz, T. R., & Mueller, U. G. (1994). Evolutionary history of the symbiosis between fungus-growing ants and their fungi. *Science*, 266(5191), 1691–1694. https://doi.org/10.1126/science.266.5191.1691

Chet, I., Harman, G. E., Baker, R. (1981). *Trichoderma hamatum*: its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. *Microbial Ecology*, 7, 29–38. https://doi.org/10.1007/BF02010476

Croxatto, A, Prod'hom, G., Greub, G. (2012). Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiology Reviews*, 36, 380–407. https://doi.org/10.1111/j.1574-6976.2011.00298.x

Currie, C. R. (2001). Prevalence and impact of a virulent parasite on a tripartite mutualism. *Oecologia*, 128, 99–106. https://doi.org/10.1007/s004420100630

Currie, C. R., Mueller, U. G. & Malloch, D. (1999a). The agricultural pathology of ant fungus gardens. *Proceedings of the National Academy of Sciences.* 96, 7998–8002. https://doi.org/10.1073/pnas.96.14.7998

Currie, C. R., Scott, J. A., Summerbell, R. C. & Malloch, D. (1999b). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* 398, 701–704. https://doi.org/10.1038/19519

Currie, C. R., Stuart, A. E. (2001). Weeding and grooming of pathogens in agriculture by ants. *Proceedings of the Royal Society B: Biological Sciences*, 268, 1033–1039. https://doi.org/10.1098/rspb.2001.1605

Currie, C. R., Wong, B., Stuart, A. E., Schultz, T. R., Rehner, S. A., Mueller, U.G., Sung, G. H., Spatafora, J.W., & Straus, N. A. (2003a). Ancient tripartite coevolution in the attine ant-microbe symbiosis. *Science*, 299, 386–388. https://doi.org/10.1126/science.1078155

Custodio, B. C., Rodrigues, A. (2019). *Escovopsis kreiselii* specialization to its native hosts in the fungiculture of the lower attine ant *Mycetophylax morschi*. *Antonie van Leeuwenhoek*, 112(2), 305–317. https://doi.org/10.1007/s10482-018-1158-x

Davis, T. S., Stewart, J. E., Mann, A., Bradley, C., Hofstetter, R. W. (2018). Evidence for multiple ecological roles of *Leptographium abietinum*, a symbiotic fungus associated with the North American spruce beetle. *Fungal Ecology*, 38, 62–70. https://doi.org/10.1016/j.funeco.2018.04.008

de Bary, A. (1866). Morphologie und Physiologie der Pilze, Flechten und Myxomyceten. In: *Handbuch der Physiologischen Botanik*, 1831–1838, eds: de Bary, A., Irmisch, T., Pringsheim, N., Sachs, J., 2. Bd., 1. Abt. Leipzig. https://doi.org/10.5962/bhl.title.120970

de Bary, A. (1879). Die Symbiose als Mittel der Synthese von Organismen. *Biologisches Centralblatt*, 26, 353–363.

Deacon, J. W., Berry, L. A. (1992). Modes of action of mycoparasites in relation to biocontrol of soilborne plant pathogens. In *Biological control of plant diseases; progress and challenges for the future*, 157–167, eds. Tjamos, E. C., Papavizas, G. C., Cook, R. J., London, Plenum Press. https://doi.org/10.1007/978-1-4757-9468-7_21

de Fine Licht, H. H., Schiøtt, M., Rogowska-Wrzesinska, A., Nygaard, S., Roepstorff, P., Boomsma, J. J. (2013). Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts. *Proceedings of the National Academy of Sciences*, 110 (2), 583–587. doi: 10.1073/pnas.1212709110.

de Man, T. J., Stajich, J. E., Kubicek, C. P., Teiling, C., Chenthamara, K., Atanasova, L., Druzhinina, I. S., Levenkova, N., Birnbaum, S. S., Barribeau, S. M., Bozick, B. A., Suen, G., Currie, C. R., & Gerardo, N. M. (2016). Small genome of the fungus Escovopsis weberi, a specialized disease agent of ant agriculture. *Proceedings of the National Academy of Sciences of the United States of America*, 113(13), 3567–3572. https://doi.org/10.1073/pnas.1518501113

Dhodary, B., Schilg, M., Wirth, R., & Spiteller, D. (2018). Secondary Metabolites from *Escovopsis weberi* and their role in attacking the garden fungus of leaf-cutting Ants. *Chemistry (Weinheim an der Bergstrasse, Germany)*, 24(17), 4445–4452. https://doi.org/10.1002/chem.201706071

Ewald, P. W. (1994). *Evolution of infectious disease*. Oxford University Press, Oxford, 320p.

Farrell, B. D., Sesqueira, A. S., O'Meara, B. C, Normark, B. B, Chung, J. H., Jordal, B. H. (2001). The evolution of agriculture in beetles (Curculionidae: Scolytinae and Platypodinae). *Evolution* 55, 2011–2027. https://doi.org/10.1111/j.0014-3820.2001.tb01318.x

Fernandéz-Marín, H; Zimmerman, J. K.; Wcislo, W. T. (2003). Nest-founding in *Acromyrmex octospinosus* (Hymenoptera, Formicidae, Attini): demography and putative prophylactic behaviors. *Insectes Sociaux*, 50, 304–308. https://doi.org/10.1007/s00040-003-0687-z

Folgarait, P., Gorosito, N., Thomas-Poulsen, M., & Currie, C.R. (2011a). Preliminary *in vitro* insights into the use of natural fungal pathogens of leaf-cutting ants as biocontrol agents. *Current Microbiology*, 63, 250–258. http://dx.doi.org/10.1007/s00284-011-9944-y

Folgarait, P. J., Marfetán, J. A., & Cafaro, M. J. (2011b). Growth and conidiation response of *Escovopsis weberi* (Ascomycota: Hypocreales) against the fungal cultivar of *Acromyrmex lundii* (Hymenoptera: Formicidae). *Environmental Entomology*, 4040, 342–349. http://dx.doi.org/10.1603/EN10111

Frank, S. A. (1996). Models of parasite virulence. *The Quarterly Review of Biology*, 71, 37–78. https://doi.org/10.1086/419267

Gerardo, N. M., Mueller, U. G., Price, S. L., Currie, C. R. (2004). Exploiting a mutualism: parasite specialization on cultivars within the fungus-growing ant symbiosis. *Proceedings of the Royal Society B: Biological Sciences*, v. 271, n. 1550, p. 1791–1798. https://doi.org/10.1098/rspb.2004.2792

Gerardo, N. M., Jacobs, S. R., Currie, C. R., Mueller, U. G. (2006a) Ancient host-pathogen associations maintained by specificity of chemotaxis and antibiosis. *PLoS Biology*, 4(8), e235. https:// doi.org/10.1371/journal.pbio.0040235

Gerardo, N. M., Mueller, U. G. & Currie, C. R. (2006b). Complex host-pathogen coevolution in the *Apterostigma* fungus-growing ant-microbe symbiosis. *BMC Evolotionary Biology*, 6, 88. https://doi.org/10.1186/1471-2148-6-88

Harrington, T. W., Vega, F., & Blackwell, M. (2005). Ecology and evolution of mycophagous bark beetles and their fungal partners.

Heine, D., Holmes, N. A., Worsley, S. F., Santos, A., Innocent, T. M., Scherlach, K., Patrick, E. H., Yu, D. W., Murrell, J. C., Vieria, P. C., Boomsma, J. J., Hertweck, C., Hutchings, M. I., & Wilkinson, B. (2018). Chemical warfare between leafcutter ant symbionts and a co-evolved pathogen. *Nature communications*, *9*(1), 2208. https://doi.org/10.1038/s41467-018-04520-1

Hinze, B. & Leuthold. (1999). Age related polyethism and activity rhythms in the nest of the termite *Macrotermes bellicosus* (Isoptera, Termitidae). *Insectes Sociaux*, 46, 392-397. https://doi.org/10.1007/s000400050162

Hölldobler, B., Wilson, E. O. (1990). *The ants*. Cambridge, Harvard University Press, Cambridge, MA, xiv, 732 p.

Hulcr, J., and Stelinski, L. L. (2017). The ambrosia symbiosis: from evolutionary ecology to practical management. *Annual Review Entomology*, 62, 285–303. doi: https://doi.org/10.1146/annurev-ento-031616-035105

Hughes, D. P., Pierce, N. E., & Boomsma, J. J. (2008). Social insect symbionts: evolution in homeostatic fortresses. *Trends in ecology & evolution*, 23(12), 672–677. https://doi.org/10.1016/j.tree.2008.07.011

Jeffries, P. (1995). Biology and ecology of mycoparasitism. *Canadian Journal of Botany*, 73, S1284–S1290. https://doi.org/10.1139/b95-389

Jeffries, P., Young, T. W. K. (1994). *Interfungal parasitic relationships.* Oxford University Press, Wallingford, Oxon UK: CAB International, 318p.

Johnson, R. A., Thomas, R. J., Wood, T. G. & Swift, M. J. (1981). The inoculation of the fungus comb in newly founded colonies of some species of the Macrotermitinae (Isoptera) from Nigeria. *Journal of Natural History*, 15, 751–756. http://dx.doi.org/10.1080/00222938100770541

Karlsson, M., Durling, M. B., Choi, J., Kosawang, C., Lackner, G., Tzelepis, G. D, Nygren, K., Dubey, M. K., Kamou, N., Levasseur, A. (2015). Insights on the evolution of mycoparasitism from the genome of *Clonostachys rosea*. *Genome Biology and Evolution*, 7, 465–480. https://doi.org/10.1093/gbe/evu292

Kiss, L., Russell, J. C., Szentiványi, O., Xu, X., Jeffries, P. (2004). Biology and biocontrol potential of *Ampelomyces* mycoparasites, natural antagonists of powdery mildew fungi. *Biocontrol Science Technology*, 14(7), 635–651. https://doi.org/10.1080/09583150410001683600

Klepzig, K. D., Flores-Otero, J., Hofstetter, R.W., Aryes, M.P. (2004). Effects of available water on growth and competition of southern pine beetle associated fungi. *Mycological Research*, 108, 183–188. https://doi.org/10.1017/S0953756203009055

Klepzig, K. D & Wilkens, R. T. (1997). Competitive Interactions among Symbiotic Fungi of the Southern Pine Beetle. *Applied and Environmental Microbiology*, 63, 621–627. https://doi.org/10.1128/AEM.63.2.621-627.1997

Kok, L. T., Norris, D. M., Chu, H. M. (1970). Sterol metabolism as a basis for a mutualistic symbiosis. *Nature*, 225, 661–662. https://doi.org/10.1038/225661b0

Korb, J., Aanen. D. (2003). The evolution of uniparental transmission of fungal symbionts in fungus rowing termites (Macrotermitinae). *Behavioral Ecology and Sociobiology*. 53, 65–71. https://doi.org/10.1007/s00265-002-0559-y

Kost, C., Lakatos, T., Böttcher, I., Arendholz, W. R., Redenbach, M., & Wirth, R. (2007). Non-specific association between filamentous bacteria and fungus-growing ants. *Die Naturwissenschaften*, *94*(10), 821–828. https://doi.org/10.1007/s00114-007-0262-y

Krauss, U., Ten, H. M., Rees, R., Stirrup, T., Argyle, T., George, A., Arroyo, C., Corrales, E., Casanoves, F. (2013). Mycoparasitism by *Clonostachys byssicola* and *Clonostachys rosea* on *Trichoderma* spp. from cocoa (*Theobroma cacao*) and implication for the design of mixed biocontrol agents. *Biological Control*, 67, 317–327. https://doi.org/10.1016/j.biocontrol.2013.09.011

Kreisel, H. (1972). Pilze aus Pilzgärten von *Atta insularis* in Kuba [Fungi from fungus gardens of *Atta insularis* in Cuba]. *Zeitschrift fur allgemeine Mikrobiologie*, *12*(8), 643–654. https://doi.org/10.1002/jobm.19720120805

Kubicek, C. P., Herrera-Estrella, A., Seidl-Seiboth, V., Martinez, D. A., Druzhinina, I. S, Thon, M., Zeilinger, S., Casas-Flores, S., Horwitz, B. A., Mukherjee, P. K. (2011) Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. Genome Biology, 12(4), R40. https://doi.org/10. 1186/gb-2011-12-4-r40

Leuthold, R. H., Badertscher, S., Imboden, H. (1989). The inoculation of newly formed fungus comb with *Termitomyces* in Macrotermes colonies (Isoptera, Macrotermitinae). *Insectes Sociaux*, 36, 328–338. https://doi.org/10.1007/BF02224884

Little, A. E., Currie, C. R. (2007). Symbiotic complexity: discovery of a fifth symbiont in the attine ant-microbe symbiosis. *Biollogy Letters*, 3(5), 501–504. https://doi.org/10.1098/rsbl.2007.0253

Lu, Z., Tombolini, R., Woo, S., Zeilinger, S., Lorito, M., Jansson, J. K. (2004). In vivo study of *Trichoderma* pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. *Applied and Environmental Microbiology*, 70, 3073–3081. https://doi.org/10.1128/aem.70.5.3073-3081.2004

Marfetán, J. A., Romero, A. I., Folgarait, P. J. (2015). Pathogenic interaction between *Escovopsis weberi* and *Leucoagaricus* sp.: mechanisms involved and virulence levels. *Fungal Ecology*, 17, 52–61. https://doi.org/10.1016/j.funeco.2015.04.002

Marfetán, J. A., Romero, A. I., Cafaro, M. J., & Folgarait, P. J. (2019). Five new *Escovopsis* species from Argentina. *Mycotaxon*, 133(4), 569–589. https://doi.org/10.5248/133.569

Martin MM. (1979). Biochemical implications of insect mycophagy. *Biological Reviews*, 54,11–21. https://doi.org/10.1111/j.1469-185X.1979.tb00865.x

Masiulionis, V. E., Cabello, M. N., Seifert, K. A., Rodrigues, A., Pagnocca, F. C. (2015) *Escovopsis trichodermoides* sp. nov., isolated from a nest of the lower attine ant *Mycocepurus goeldii*. *Antonie van Leeuwenhoek*, 107(3), 731–40. https://doi.org/10.1007/s10482-014-0367-1

Masiulionis, V. E., & Pagnocca, F. C. (2020). In vitro study of volatile organic compounds produced by the mutualistic fungus of leaf-cutter ants and the antagonist *Escovopsis*. *Fungal Ecology*, 48, 100986. https://doi.org//10.1016/j.funeco.2020.100986

Mayhé-Nunes, A. J., Jafeé, K. (1998). On the biogeography of Attini (Hymenoptera: Formicidae). *Ecotropicos*, 11, 45–54.

Mehdiabadi, N. J., Schultz, T. R. (2010). Natural history and phylogeny of the fungus-farming ants (Hymenoptera: Formicidae: Myrmicinae: Attini). *Myrmecological News*, 13, 37-55

Meirelles, L. A., Montoya, Q. V., Solomon, S. E., Rodrigues, A. (2015a). New light on the systematics of fungi associated with attine ant gardens and the description of *Escovopsis kreiselii* sp. nov. *PLoS One*, 10(1), e0112067. https://doi.org/10.1371/journal.pone.0112067

Meirelles, L. A., Solomon, S. E., Bacci, M., Wright, A. M., Mueller, U. G., Rodrigues, A. (2015b). Shared *Escovopsis* parasites between leaf-cutting and non-leaf-cutting ants in the higher attine fungus-growing ant symbiosis. *Royal Society Open Science*, 2(9), 150257. https://doi. org/10.1098/rsos.150257

Menezes, C., Vollet-Neto, A., Marsaioli, A. J., Zampieri, D., Fontoura, I. C., Luchessi, A. D., & Imperatriz-Fonseca, V. L. (2015). A Brazilian social bee must cultivate fungus to survive. *Current Biology*, *25*(21), 2851–2855. https://doi.org/10.1016/j.cub.2015.09.028

Möller, A. F. W. (1893). *Die Pilzgarten Einiger Sudamerikanischer Ameisen*. Jena, Gustav Fischer, 127p.

Montoya, Q. V., Martiarena, M. J. S., Polezel, D. A., Kakazu, S., Rodrigues, A. (2019). More pieces to a huge puzzle: Two new *Escovopsis* species from fungus gardens of attine ants. *MycoKeys*, 46, 1–22. https://doi.org/10.3897/mycokeys.46.30951

Moreira, S. M., Rodrigues, A., Forti, L. C., Nagamoto, N. S. Absence of the parasite *Escovopsis* in fungus garden pellets carried by gynes of *Atta sexdens*. (2015). *Sociobiology*, 62, 34–38. http://dx.doi.org/10.13102/sociobiology.v62i1.34-38

Muchovej, J. J., Della Lucia, T. M. C. (1990). *Escovopsis*, a new genus from leaf cutting ant nests to replace *Phialocladus* nomen invalidum. *Mycotaxon*, 37, 191–195.

Mueller, U. G., Rehner, S. A., Schultz, T. R. (1998). The evolution of agriculture in ants. *Science*, 281, 2034–2038. https://doi.org/10.1126/science.281.5385.2034

Mueller, U. G., Schultz, T. R., Currie, C. R., Adams, R. M. M., Malloch, D. (2001). The origin of the attine ant-fungus mutualism. *The Quartely review of Biology*, 76,169–197. https://doi.org/10.1086/393867

Mueller, U. G., Kardish, M. R., Ishak, H. D., Wright, A. M., Solomon, S. E., Bruschi, S. M., Carlson, A. L., & Bacci, M., Jr. (2018). Phylogenetic patterns of ant-fungus associations indicate that farming strategies, not only a superior fungal cultivar, explain the ecological success of leafcutter ants. *Molecular Ecology*, *27*(10), 2414–2434. https://doi.org/10.1111/mec.14588

Mukherjee, P. K., Horwitz, B. A., Singh, U. S., Mukherjee, M., Schmoll, M. (2013). *Trichoderma* in agriculture, industry and medicine: an overview. In: *Trichoderma: biology and applications* 1–9, eds. Mukherjee, P. K., Horwitz, B. A., Singh, U. S., Mukherjee, M. Schmoll, M. CABI Publishing, Central Institute for Cotton Research, Shankar, Nagpur 440 010, India.

Natsopoulou, M. E, McMahon, D. P, Paxton, R. J. (2016). Parasites modulate within-colony activity and accelerate the temporal polyethism schedule of a social insect, the honey bee. *Behavioral Ecology and Sociobiology*, 70, 1019–1031. https://doi.org/10.1007/s00265-015-2019-5

Norris, D. M. (1979) The mutualistic fungi of the Xyleborini beetles. In: *Insect-Fungus Symbiosis: Nutrition, Mutualism, and Commensalism*, 53–63, ed. L. R. Batra. John Wiley & Sons, New York.

Ewald, P. W. (1994). *Evolution of infectious disease*. Oxford University Press, Oxford, 320p.

Odling-Smee, F. J., Laland, K. N., Feldman, M. W. (2003). *Niche Construction: The Neglected Process in Evolution*. Princeton University Press, v. 37, 472 p.

Olsson, S., Persson, Y. (1994). Transfer of phosphorus from *Rhizoctonia solani* to the mycoparasite *Arthrobotrys oligospora*. *Mycological Research*, 98, 1065–1068. https://doi.org/10.1016/S0953-7562(09)80434-6

Pagnocca, F. C., Rodrigues, A., Nagamoto, N. S., Bacci Jr., M. (2008) Yeasts and filamentous fungi carried by the gynes of leaf-cutting ants. *Antonie Van Leeuwenhoek*, 94, 517-526. https://doi.org/10.1007/s10482-008-9268-5

Quinlan, R. J., Cherrett, J. M. (1979). The role of the fungus in the diet of the leafcutting ant *Atta cephalotes* (L.). *Ecological Entomology*, 4, 151 – 160. https://doi.org/10.1111/j.1365-2311.1979.tb00570.x

Reithner, B., Brunner, K., Schuhmacher, R., Peissl, I., Seidl, V., Krska, R., Zeilinger, S. (2005). The G protein alpha subunit Tga1 of *Trichoderma atroviride* is involved in chitinase formation and differential production of antifungal metabolites. *Fungal Genetics and Biology*, 42, 749–760. https://doi.org/10.1016/j.fgb.2005.04.009

Reynolds, H. T., & Currie, C. R. (2004). Pathogenicity of *Escovopsis weberi*: The parasite of the attine ant-microbe symbiosis directly consumes the ant-cultivated fungus. *Mycologia*, 96(5), 955–959. https://doi.org/10.2307/3762079

Rocha, S. L., Jorge, V. L., Della Lucia, T. M. C., Barreto, R. W., Evans, H. C., Elliot, S. L. (2014). Quality control by leaf-cutting ants: evidence from communities of endophytic fungi in foraged and rejected vegetation. *Arthropod-Plant Interactions* (Print), 8, 485-493. https://doi.org/10.1007/s11829-014-9329-9

Rocha, S. L., Evans, H. C., Jorge, V. L., Cardoso, L. A. O., Pereira, F. S. T., Rocha, F. B., Barreto, R. W., Hart, A. G., Elliot, S. L. (2017). Recognition of endophytic Trichoderma species by leaf-cutting ants and their potential in a Trojan-horse management strategy. *Royal Society Open Science*, 4, 160628. https://doi.org/10.1098/rsos.160628

Rodrigues, A., Pagnocca, F. C., Bacci, M., Hebling, M. J. A., Bueno, O. C., Pfenning, L. H. (2005). Variability of non-mutualistic filamentous fungi associated with *Atta sexdens rubropilosa* nests. *Folia Microbiologica*, 50, 421(2005). https://doi.org/10.1007/BF02931424

Rodrigues, A., Bacci Jr., M., Mueller, U. G., Ortiz, A., Pagnocca, F. C. (2008). Microfungal "Weeds" in the Leafcutter Ant Symbiosis. *Microbial Ecology*, 56, 604–614. https://doi.org/10.1007/s00248-008-9380-0

Rogerson, C. T., Samuels, G. J. (1989). Boleticolous species of *Hypomyces*. *Mycologia* 81, 413–432. https://doi.org/10.2307/3760079

Rouland-Lefèvre, C. (2000). Symbiosis with fungi. In: *Termites: Evolution, Sociality, Symbioses, Ecology*, 289–306, eds. Abe, T., Bignell, D. E. & Higashi, M. (Kluwer Academic, Dordrecht, The Netherlands). https://doi.org/10.1007/978-94-017-3223-9_14

Rudakov, O. L. (1978). Physiological groups in mycophilic fungi. *Mycologia*, 70, 150–159. https://doi.org/10.2307/3758695

Sánchez-Peña, S. R. (2005). New view on origin of attine ant-fungus mutualism: Exploitation of a prexisting insect–fungus symbiosis (Hymenoptera: Formicidae). *Annals of the Entomological Society of America*, 98, 151–164. https://doi.org/10.1603/0013-8746(2005)098[0151:NVOOOA]2.0.CO;2

Santos, A. V., Dillon, R. J., Dillon, V. M., Reynolds, S. E., & Samuels, R. I. (2004). Ocurrence of the antibiotic producing bacterium *Burkholderia* sp. in colonies of the leaf-cutting ant *Atta sexdens rubropilosa*. *FEMS microbiology letters*, 239(2), 319–323. https://doi.org/10.1016/j.femsle.2004.09.005

Schmid-Hempel, P. (1998). Host-Parasite Dynamics. In *Parasites in Social Insects*, 204-236. Princeton, New Jersey: Princeton University Press.

Schultz TR, Meier R (1995) A phylogenetic analysis of the fungus-growing ants (Hymenoptera: Formicidae: Attini) based on morphological characters of the larvae. *Systematic Entomology*, 20, 337–370. https://doi.org/10.1006/mpev.1997.0466

Schultz, T. R., & Brady, S. G. (2008). Major evolutionary transitions in ant agriculture. *Proceedings of the National Academy of Sciences of the United States of America*, 105(14), 5435–5440. https://doi.org/10.1073/pnas.0711024105

Scott, J. J., Oh, D. C., Yuceer, M. C., Klepzig, K. D, Clardy, J., Currie, C. R. (2008). Bacterial protection of beetle-fungus mutualism. *Science*, 322(5898), 63-63. https://doi.org/10.1126/science.1160423

Silva, A., Bacci, M., Jr., Pagnocca, F. C., Bueno, O. C., & Hebling, M. J. (2006). Production of polysaccharidases in different carbon sources by *Leucoagaricus gongylophorus* Möller (Singer), the symbiotic fungus of the leaf-cutting ant *Atta sexdens* Linnaeus. *Current microbiology*, 53(1), 68–71. https://doi.org/10.1007/s00284-005-0431-1

Siqueira, C. G., Bacci Jr., M., Pagnocca, F. C., et al. (1998). Metabolism of plant polysaccharides by *Leucoagaricus gongylophorus*, the symbiotic fungus of leaf-cutting ant *Atta sexdens* L. *Applied and Environmental Microbiology*, 64, 4820–4822. https://doi.org/10.1128/AEM.64.12.4820-4822.1998

Sivan A, Chet I. (1986). Biological control of *Fusarium* spp. in cotton, wheat and muskmelon by *Trichoderma harzianum*. *Journal of Phytopathology*, 116, 39–47. https://doi.org/10.1111/j.1439-0434.1986.tb00892.x

Smrž J., Čatská V. (2010). Mycophagous mites and their internal associated bacteria cooperate to digest chitin in soil. *Symbiosis*, 52, 33–40. https://doi.org/10.1007/s13199-010-0099-6

Spatafora, J. W., Sung, G. H., Sung, J. M., Hywel-Jones, N. L., & White, J. F., Jr (2007). Phylogenetic evidence for an animal pathogen origin of ergot and the grass endophytes. *Molecular Ecology*, 16(8), 1701–1711. https://doi.org/10.1111/j.1365-294X.2007.03225.x

Stahel, G., Geijskes, D. C. (1941). Weitere Untersuchungen über Nestbau und Gartenpilz von *Atta cephalotes* L. und *Atta sexdens* L. (Hym. Formicidae). *Review Entomology*, 12, 243–268.

Stearns, S. C., Hoekstra, R. F. (2005). *Evolution: an introduction*. OUP Oxford, Second edition, 600p.

Stoppacher, N., Kluger, B., Zeilinger, S., Krska, R., Schuhmacher, R. (2010). Identification and profiling of volatile metabolites of the biocontrol fungus *Trichoderma atroviride* by HS-SPME-GC-MS. *Journal of Microbiological Methods*, 81, 187–193. https://doi.org/10.1016/j.mimet.2010.03.011

Stotzky, G., Schenck, S., & Papavizas, G. C. (1976). Volatile Organic Compounds and Microorganisms. *CRC Critical Reviews in Microbiology*, 4(4), 333–382. https://doi.org/10.3109/10408417609102303

Sun, J., Liu, X., McKenzie, E. H. C., Jeewon, R., Liu, J. K. J., Zhang, X. L., Zhao, Q., Hyde, K. D. (2019) Fungicolous fungi: terminology, diversity, distribution, evolution, and species checklist. *Fungal Diversity*, 95, 337–430. https://doi.org/10.1007/s13225-019-00422-9

Skelton, J., Johnson, A. J., Jusino, M. A., Bateman, C. C, Li, Y., Hulcr, J. (2019). A selective fungal transport organ (mycangium) maintains coarse phylogenetic congruence between fungus-farming ambrosia beetles and their symbionts. *Proceedings of the Royal Society B: Biological Sciences*, 286, 20182127. https://doi.org/10.1098/rspb.2018.2127

Taerum, S. J., Cafaro, M. J., Little, A. E., Schultz, T. R., & Currie, C. R. (2007). Low host-pathogen specificity in the leaf-cutting ant-microbe symbiosis. *Proceedings of the Royal Society B: Biological Sciences*, 274(1621), 1971–1978. https://doi.org/10.1098/rspb.2007.0431

Taerum, S. J., Cafaro, M. J., Currie, C. R. (2010). Presence of multiparasite infections within individual colonies of leaf-cutter ants. *Environmental Entomology*, 39, 105–113. http://dx.doi.org/10.1603/EN09137

Tamm, H., Põldmaa, K. (2013). Diversity, host associations, and phylogeography of temperate aurofusarin-producing *Hypomyces*/*Cladobotryum* including causal agents of cobweb disease of cultivated mushrooms. *Fungal Biology*, 117, 348–367. https://doi.org/10.1016/j.funbio.2013.03.005

Thomas, R. J. (1987). Factors affecting the distribution and activity of fungi in the nests of Macrotermitinae (Isoptera). *Soil Biology and Biochemistry*, 19(3), 343–349. https://doi.org/10.1016/0038-0717(87)90020-4

Toki, W., Tanahashi, M., Togashi, K., Fukatsu, T. (2012). Fungal Farming in a Non-Social Beetle. *PLoS ONE*, 7(7), e41893. htpps://doi.org/10.1371/journal.pone.0041893

Van Bael, S. A., Fernández-Marín, H., Valencia, M. C., Rojas, E. I., Wcislo, W. T., & Herre, E. A. (2009). Two fungal symbioses collide: endophytic fungi are not welcome in leaf-cutting ant gardens. *Proceedings Biological Sciences*, 2419–2426. https://doi.org/10.1098/rspb.2009.0196

Varanda-Haifig, S. S., Albarici, T. R., Nunes, P. H., Haifig, I., Vieira, P. C., & Rodrigues, A. (2017). Nature of the interactions between hypocrealean fungi and the mutualistic fungus of leaf-cutter ants. *Antonie van Leeuwenhoek*, 110(4), 593–605. https://doi.org/10.1007/s10482-016-0826-y

Visser, A.A.; Kooij, P.W.; Debets, A.J.M.; Kuyper, T.W.; Aanen, D. K. (2011). *Pseudoxylaria* as stowaway of the fungus-growing termite nest: Interaction asymmetry between *Pseudoxylaria*, *Termitomyces* and free-living relatives. *Fungal Ecology*. 2011, 4, 322–332. https://doi.org/10.1016/j.funeco.2011.05.003

Visser, A. A., Nobre, T., Currie, C. R., Aanen, D. K., & Poulsen, M. (2012). Exploring the potential for actinobacteria as defensive symbionts in fungus-growing termites. Microbial ecology, 63(4), 975–985. https://doi.org/10.1007/s00248-011-9987-4

Viterbo, A., Horwitz, B. A. (2010). Mycoparasitism. In *Cellular and Molecular Biology of Filamentous Fungi*, 676–693, ed. Borkovich, K. A., Ebbole, D. J. ASM Press, Washington, DC.

Wallace, D. E. E., Asensio, J. G. V., & Tomás, A. A. P. (2014). Correlation between virulence and genetic structure of *Escovopsis* strains from leaf-cutting ant colonies in Costa Rica. *Microbiology*, 160, 1727–1736. https://doi.org/10.1099/mic.0.073593-0

Watson M. J. (2013). What drives population-level effects of parasites? Meta-analysis meets life-history. *International journal for parasitology*. Parasites and wildlife, 2, 190–196. https://doi.org/10.1016/j.ijppaw.2013.05.001

Weber, N. A. (1958). Evolution in fungus-growing ants. *Proceedings of the 10th International Congress of Entomology*, 2, 459–473.

Weber, N. A. (1966). The fungus-growing ants. *Science*, 121, 587–604. http://doi.org/10.1126/science.153.3736.587

Weber, N. A. (1979). Fungus culturing by ants. In: *Insect Fungus Symbiosis, Mutualism and Commensalism*, 77–116, ed. Batra, L. R. New York: Allanheld, Osmun & Co/Halsted Press.

Weber, N. A. (1972a). Gardening Ants, the Attines. *Science*, 153, 587-604. http://doi.org/10.1126/science.178.4063.856

Weber, N. A. (1972b). The fungus-culturing behaviour of ants. *American Zoologist*, 12, 577–587.

Wheeler, Q. D. & Blackwell, M. (eds) (1984). *Fungus – Insect relationships: Perspectives in ecology and evolution.* Columbia University Press, New York. 514p.

Wilson, E. O. (1971). *The insect societies*. Harvard University Press, Cambridge, Massachusetts. 584p.

Wilson, E. O. (1980). Caste and division of labor in leaf-cutter ants (Hymenoptera, Formicidae: *Atta*). I: The overall pattern in *A. sexdens*. *Behavioral Ecology and Sociobiology*, 7, 143-156. https://doi.org/10.1007/BF00299520

Wood, S. L. (1982). *The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph*. Great Basin naturalist memoirs, 6, 1359p.

Zhao T, Kandasamy D, Krokene P, Chen J, Gershenzon J, Hammerbacher A. (2019). Fungal associates of the tree-killing bark beetle, *Ips typographus*, vary in virulence, ability to degrade conifer phenolics and influence bark beetle tunneling behavior. *Fungal Ecology*, 38, 71–79. https://doi.org/10.1016/j.funeco.2018.06.003

Zeilinger, S., Galhaup, C., Payer, K., Woo, S. L., Mach, R. L., Fekete, C., Lorito, M., Kubicek, C. P. (1999). Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. *Fungal Genetics and Biology*, 26, 131–140. https://doi.org/10.1006/fgbi.1998.1111