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SYSTEMATICS AND EVOLUTION OF *ESCOVOPSIS* AND HYPOCREALEAN RELATIVES ASSOCIATED WITH ATTINE ANT COLONIES

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SYSTEMATICS AND EVOLUTION OF *ESCOVOPSIS* AND HYPOCREALEAN RELATIVES ASSOCIATED WITH ATTINE ANT COLONIES

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To my mother…

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Science is born from ideas, crawls and pronounces its first words on the arms of hypothesis, feeds and nourishes itself on discussion, and makes its way guided by the light of the evidence.

ABSTRACT

Escovopsis is a group of fungi that inhabit the colonies of fungus-growing ants. The study of this fungal genus has been based on several assumptions that became dogmas. For instance, i) many fungi associated with attines were treated as *Escovopsis*, without taxonomic support, ii) the genus was considered a specialized mycoparasite of the attines' mutualistic fungi, and iii) it was thought that the genus coevolved with attines based on the assumption of its parasitic lifestyle. However, for many years the *Escovopsis* taxonomy, its relationship with the ants and its phylogeographic distribution were almost an empty space for the scientific community. In addition, the origin of the genus was never addressed. Therefore, the main objective of this study was to build the systematics of the *Escovopsis* and shed light on its origin, evolution, diversification, and phylogeographic distribution. Three manuscripts are the result of this study and are presented here as chapters. The first manuscript presents the description of two new *Escovopsis* species (published in MycoKeys). The second manuscript brings the reassessment of the *Escovopsis* taxonomy, provides a suitable taxonomic and phylogenetic framework for the systematics of the genus, and describes two new Hypocreaceae genera (submitted to IMA Fungus). The third manuscript shows the origin, evolution, phylogeographic distribution and the trait adaptations experienced by *Escovopsis* since its entry in fungus-growing ant colonies. This study fills an important gap in the taxonomy, systematics, and evolution of *Escovopsis* and related genera which certainly will help researchers to better understand the evolution of the attines' system.

Key-words: Hypocreales, Taxonomy, Phylogeny, Symbiosis, Evolution

RESUMO

Escovopsis é um interessante grupo de fungos que habita as colônias das formigas atíneas. O estudo desse fungo se baseou em diversos pressupostos que, por muito tempo, se tornaram dogmas. Por exemplo, i) vários fungos associados à essas formigas foram tratados como *Escovopsis*, porém sem nenhum suporte taxonômico, ii) o gênero foi considerado um micoparasita especializado da associação formiga - fungo cultivado, e iii) acreditou-se que o gênero co-evoluiu com as atíneas, pois parasita o parceiro fúngico dessas formigas desde a origem da associação. No entanto, a taxonomia de *Escovopsis*, bem como sua relação com as formigas e sua distribuição filogeográfica foram quase um espaço vazio para a comunidade científica. Além disso, a origem do gênero nunca foi abordada. Nesse contexto, o objetivo deste estudo foi construir a sistemática do gênero *Escovopsis* e angariar evidências sobre sua origem, evolução, diversificação e distribuição filogeográfica. Três manuscritos são o resultado deste estudo e são apresentados como capítulos. O primeiro manuscrito traz a descrição de duas novas espécies de *Escovopsis* (publicado na MycoKeys). O segundo manuscrito apresenta a reavaliação da taxonomia de *Escovopsis*, fornece um marco taxonômico e filogenético robusto para a sistemática do gênero e descreve dois novos gêneros dentro da família Hypocreaceae (submetido na IMA Fungus). O terceiro manuscrito mostra a origem, evolução, distribuição filogeográfica e as adaptações experimentadas pelo gênero desde seu ingresso nas colônias das atíneas. Este estudo preenche uma lacuna importante na taxonomia, sistemática e evolução de *Escovopsis* e gêneros próximos e, certamente, ajudará os pesquisadores a compreender melhor a evolução do sistema das formigas atíneas.

Palavras-chave: Hipocreales, Taxonomia, Filogenia, Simbiose, Evolução

SUMMARY

Introduction

Sixty-five million years ago, after the massive extinction of the dinosaurs, fungus-growing ants (subfamily Myrmicinae, Attini tribe, Attina subtribe, "the attines") started producing their own food. Finding food is a constant and demanding struggle to organisms, and sometimes it leads them to certain death. Thus, the emergence of fungus-growing behaviour was not only a safer way to deal with lack of food, but directly influenced the evolutionary success of the attines and established the beginning of one of the most interesting and complex insect-fungus associations we know on earth.

Fungiculture performed by attine ants can be divided into five types: i) the fungiculture practiced by the genera in the basal attines, *Cyphomyrmex*, *Mycetophylax*, *Mycocepurus*, *Apterostigma auriculatum* group that grow fungi from the tribe Leucocoprinae (Agaricaceae); ii) the fungiculture practiced by species of the *Apterostigma pilosum* group that cultivate fungi of the Pterulaceae family, named coral fungi; iii) the fungiculture practiced by ant species of the *Cyphomyrmex rimosus* group, which cultivate yeasts (in Leucocoprinae); iv) the fungiculture practiced by the genera *Trachymyrmex* and *Sericomyrmex* that cultivate other fungi in Leucocoprinae, and v) the fungiculture of leaf-cutting ants, which cultivate *Leucoagaricus gongylophorus*.

Attine ants are restricted to the American continent and comprise 17 genera with more than 250 species phylogenetically divided into Neoattini and Paleoattini. The genera *Atta* and *Acromyrmex*, in the Neoattini group (more derived clades) are known as leaf-cutting ants, and they are important crop pests, because the large amounts of fresh plant material they collect to feed their fungal partner. In return, the fungal cultivar *L. gongylophorus* produces vesicles called gongylidia which are used by the ants as food source. On the other hand, the

less derived attini genera, known as non-leaf-cutting ants (comprising ant genera in both Neoattini and Paleoattini) use other substrates like seeds, insect carcasses, and dry plant material to nourish their fungal partners. In addition, for millions of years attines have been introducing into their colonies a great diversity of microorganisms along with the substrates they collect and also microorganisms attached to their bodies. Consequently, complex symbiotic networks made their way slowly in attine colonies.

Though several symbiotic relationships have been described between the microorganisms living in the attine ant fungus garden, the mutualism between attines and the fungi they cultivate is the backbone of this ecosystem. With the constant entrance of microorganisms into the colonies, attine's environment is prone to parasites that are able to take advantage of the system. Indeed, ecological factors suggest that several parasites could have arrived at the fungus-farming ant colonies. However, the genus *Escovopsis* (Ascomycota: Hypocreales, Hypocreaceae) was proposed as the only and specialized parasite in this system.

Due to the ecological importance that a parasite represents within the attine system, many studies turned their efforts to the study of the *Escovopsis*-mutualistic fungus interaction. However, the study of this diverse and interesting group of fungi has been based on several assumptions that became dogmas, since its discovery. For instance, it was proposed that: i) all fungi producing brown conidia found in attine colonies belong to *Escovopsis*. However, the *Escovopsis* taxonomy was almost an empty space for the scientific community and most that we know about its taxonomy was raised by non-taxonomic studies; ii) the genus is a specific and specialized mycoparasite. Nonetheless, our knowledge on the parasitic mechanisms used by *Escovopsis* to overcome the fungal cultivar defences and the

ant colonies barriers is still limited. The conclusion that *Escovopsis* is a parasite was based on the evaluation of few strains from one of the 26 clades of the genus; and iii) *Escovopsis* had coevolved with the attines and their mutualistic fungi. However, the origin of *Escovopsis* is a complete mystery, as it is its phylogenetic relationship with the attine and the fungal cultivars.

Under this scenario, this study aimed to build the systematics of the *Escovopsis* showing its origin, diversification, and phylogeographic distribution. To reach this goal we used the following approach: i) increase the number of *Escovopsis* strains in our collection, ii) standardize the taxonomy of the genus to access its morphological features, iii) create a phylogenetic framework to perform the *Escovopsis* tree of life, iv) combine the taxonomic and phylogenetic analysis to build the *Escovopsis* systematics, v) estimate the origin of the genus, vi) evaluate its relationship with the ants, and vii) unravel its phylogeographic distribution.

The first two years, we gathered a collection of 365 strains [153 strains already deposited in the Laboratory of Fungal Ecology and Systematics (LESF - UNESP, Rio Claro, SP) and 212 obtained in this study]. In addition, we carried out an in-depth study of the taxonomy of this genus to know more about its taxonomic and to plan a strategy to standardize it. The main issues of the *Escovopsis* taxonomy were presented in the paper "**More pieces to a huge puzzle: Two new *Escovopsis* species from fungus gardens of attine ants**" which is the first chapter of this thesis. This study introduced *Escovopsis clavata* and *E. multiformis* within a well detailed taxonomic and phylogenetic framework, and emphasized the non-standardized taxonomy and the phylogenetic incongruities of the genus.

Some suggestions to reach a better view of the *Escovopsis* systematics were proposed in this paper.

Moving on to which was suggested in that study, we sequenced five molecular markers (ITS, LSU, *tef1*, *rpb*1, and *rpb*2) to start building the phylogenetic tree of life of *Escovopsis*. Our results suggested that the *Escovopsis* clade is composed by more than one genus. Based on these results we raised the hypothesis that: If more than one genus is living in attines fungus garden, then it would suppose the possibility that more than one fungus would have co-evolved with the attines colonies in a multiparasitism relationship. To answer these questions, we aimed to build the most comprehensive phylogenetic tree of *Escovopsis* and infer the divergence time of the genus to access its evolutionary history and to shed light on our hypothesis. This part of the study was developed at Emory University, Department of Biology, O. Wayne Rollins Research Center, Atlanta, USA.

We assembled samples from LESF and from Dr. Nicole M. Gerardo' Laboratory (Emory University, Atlanta, USA) and carried out the broadest phylogenetic analysis of the genus *Escovopsis*. This analysis comprehended a total of 584 strains from different regions across America (Argentina, Brazil, Ecuador, Panama, and Costa Rica) spanning several biomes. The results supported our previous hypothesis that *Escovopsis* comprehends more than one genus. Then, we reassessed the taxonomy of *Escovopsis* by using standardized parameters including a select and informative set of morphological characters and a comprehensive multilocus phylogeny which is explained in the manuscript **"Fungi of the strong conidial form" in attine ant colonies: taxonomic and phylogenetic reassessment of the genus *Escovopsis***". This manuscript was submitted to IMA Fungus and is presented here as the second chapter.

The reassessment of *Escovopsis* and the comprehensive phylogeny of the genus provided the ground to estimate the origin of the genus as well as its phylogenetic relationship with the attines and its phylogeographic distribution. Our findings support that the origin of *Escovopsis* and the beginning of the attine fungus domestication correspond in time. However, *Escovopsis* appears to have reached the ant colonies of just few attine genera during the last 38 million of years, along with the plant material collected to nourish the fungus gardens. These results are presented in the manuscript "**Digging into the past of a fungus-growing ant guest: origin and evolution of *Escovopsis***", which is presented here as the third chapter. Interesting fungal trait adaptations and hypothesis about the origin and the evolutionary history of the genus are discussed in this manuscript.

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Overall thesis conclusion

Fungiculture by attine ants have fascinated scientists since its discovery about 150 years ago. In addition to the economic importance of some attine species, colonies of these social insects are models to study evolution and symbiosis. Parasites directly influence the evolution of ecosystems, so that the discovery of *Escovopsis* was of great importance to understand the evolution of the fungus-growing ants' environment. The lack of a standardized taxonomy as well as an unresolved phylogeny of *Escovopsis*, studies on its origin, evolution, and ecology (lifestyle) have clouded our understanding of the impact of this fungus in the evolution of fungus-growing ants. By providing grounds for the systematics of *Escovopsis*, inferring its origin and raising hypothesis on its evolution, this study not only provides a stable foundation from which to build future research on the taxonomic diversity, ecology and the evolutionary history of the genus, but raises a different point of view on the evolution of the *Escovopsis*-attine ant symbiosis. We hope to open new windows for discussion to understand the evolution of both *Escovopsis* and the attine ants' ecosystem.