

Digging into the evolutionary history of the fungus-growing-ant symbiont, *Escovopsis* (*Hypocreaceae*)

Corresponding Author: Professor Andre Rodrigues

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This is a well written manuscript. Unfortunately, as it stands now, it is suitable for journals specializing in phylogenetic studies and/or fungal biology.

Reviewer #2

(Remarks to the Author)

The manuscript by Rodrigues et al. "Digging into the evolutionary history of fungus-growing-ant symbionts in the genus *Escovopsis*" deals with the evolution of the microfungus *Escovopsis* living in fungus gardens of attine ants. Some species of *Escovopsis* are specialized parasites of the fungal cultivars the ants rely on for food and can cause severe damage to the fungus gardens and in consequence to the ant colony.

Rodrigues et al. investigate the evolutionary history of this group of fungi based on a broad sampling indifferent sites in the Americas. They used four loci for their phylogenetic analysis and divergence time analysis. An interesting part is the investigation of the morphological traits of the vesicles since the shape of the vesicles are not only a diagnostic feature in the genus but seems also to be ecologically important. Even more interesting is the analysis of growth rate, conidia production and the viability of conidia. This shows a tremendous increase in *Escovopsis* species associated with Higher Attini and are probably important evolutionary changes towards a more efficient parasitism.

The results of the manuscript are very important to better understand how detrimental symbiosis may evolve. Understanding the evolution of multispecies symbioses in depth is extremely difficult and the authors have added an important piece of the puzzle. I therefore strongly support this study being published.

The methods are adequate and sound, the results section short and concise. The Introduction and discussion are very well written and easy to follow. I would have liked a bit more background information for non-*Escovopsis* specialists: are there any free-living strains known? Fungus growing ants grow different cultivars, are some more at risk to be parasitized by *Escovopsis* than others? As far as I know *Apterostoma* for example does not cultivate *Leucocoprinus*, is it possible that the virulence has evolved especially towards fungal cultivars which may have lost their inventory to produce secondary metabolites while becoming a mutualist? How can ants suppress the detrimental behaviour, is it only by grooming or also by spraying secretions which do not harm the fungal cultivar but the parasite? It's indeed a thrilling topic!

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Have you checked whether the contact with ants is inhibiting germination of conidia in certain *Escovopsis* species? I know

that looking for free-living close relatives is looking for the needle in the haystack but you may find it on leaf surfaces. And once you have been lucky and found it the next question then is: what turns the saprophyte into a parasite?
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Fig. 3 the same applies here as in Fig 2: a symbol to assign it to the respective ant genus would give more information

Reviewer #3

(Remarks to the Author)

In this manuscript, the authors integrate molecular, morphological, and physiological analyses to explore the diversity, host associations, and evolutionary history of the genus *Escovopsis*. The molecular analysis includes 309 fungal isolates from over 400 attine ant colonies collected across six countries (primarily Brazil and Panama). *Escovopsis* was isolated from fungus gardens using established protocols, and strains were preserved under various conditions. DNA was extracted following a modified CTAB method, and five molecular markers (ITS, LSU, *tef1*, *rpb1*, *rpb2*) were amplified for phylogenetic analysis. A Bayesian approach was applied to infer evolutionary relationships and estimate divergence times, incorporating calibrated data from previous studies. For the morphological analysis, the researchers examined 14 representative strains from different clades within the genus, measuring vesicle and conidiophore characteristics under a microscope. The physiological assessment included evaluating fungal growth rate, conidia production, and conidia viability through controlled laboratory experiments. Data were statistically analyzed using ANOVA to identify significant differences among strains. The methodology follows standard protocols in the field, and the authors themselves are recognized experts in these analyses. Therefore, I find no issues or suggestions regarding the methodological approach.

The study's major findings include that *Escovopsis* is specifically associated with certain fungus-growing ant genera, particularly the "higher" attines (*Atta*, *Acromyrmex*, *Mycetomoellerius*, *Paratrachymyrmex*, *Sericomyrmex*) and the "lower" attine *Apterostigma*. In contrast, it is absent from other fungus-growing ant genera, which are instead associated with sister clades such as *Luteomyces* and *Sympodiorosea*. While *Escovopsis* is linked to specific ant genera, its evolutionary history does not strictly parallel that of its host ants. However, the most derived *Escovopsis* clades tend to be associated with the most derived attine ants. The study estimates that *Escovopsis* originated approximately 56.9 million years ago, shortly after the emergence of the attine ant-fungus mutualism (~66 Mya), aligning with previous studies (De Man et al 2016 & Gotting et al 2022). The authors propose a sequential association of *Escovopsis* with different attine genera, beginning with *Apterostigma* and later expanding to other groups. Regarding its geographic distribution, the results indicate that *Escovopsis* is widespread across Central and South America. However, its phylogeographic patterns remain incomplete, likely due to uneven sampling efforts. Finally, the authors found evidence of a shift in vesicle shape from globose (ancestral state) to cylindrical (derived state). More derived *Escovopsis* species exhibit increased growth rates, conidia production, and viability, suggesting adaptive advantages over time.

These findings improve our understanding of *Escovopsis* evolution and its association with attine ants, expanding on previous work but without radically altering the known framework. The evolutionary sequence proposed (first *Apterostigma*, then *Mycetomoellerius* / *Paratrachymyrmex* / *Sericomyrmex*, finally *Atta* and *Acromyrmex*) is a significant contribution and will likely be of interest to evolutionary biologists studying host-parasite interactions. The morphological and physiological evolution of *Escovopsis* is a compelling aspect that connects fungal adaptation to ecological pressures, which is relevant beyond myrmecology. While the broad claim that *Escovopsis* is primarily associated with certain attine ants is not new (e.g., Currie et al., 2003; Gerardo et al., 2006; Taerum et al., 2007), the detailed evolutionary trajectory and morphological shifts provide novel insights.

The study is largely convincing, although one weak point is the uneven sampling, which weakens its broad biogeographic conclusions. The authors acknowledge this limitation and discuss it within the text. While the paper is unlikely to dramatically shift the field, it refines and reinforces our understanding of *Escovopsis* evolution and its role in ant-fungus symbioses. In addition, insights into morphological and physiological changes in *Escovopsis* could inspire further research on fungal adaptation and evolution in symbiotic systems. If further studies support the proposed sequence of *Escovopsis* host shifts, it could reshape discussions on how specialized parasites diversify alongside their hosts. The manuscript is well-written, engaging, and scientifically sound. I find it highly interesting and support its publication without further changes.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I am satisfied with the revision of this manuscript and recommend its acceptance.

Reviewer #2

(Remarks to the Author)

The authors took all of my comments and questions into account when revising the manuscript. Their thorough revision of the manuscript has made it much more substantial. In my opinion, it is a valuable contribution to the study of the Escovopsis-leafcutter ant interaction and is well-suited for Communications Biology.

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Note: All figures were provided as jpg (9600 x 5742) as the submission system generates a hard-to-ready pdf when providing large and high-resolution images. In the event this manuscript is accepted, we will provide all figures in high-resolution.

Authors' reply to reviewer #1's comments:

Reviewer: This manuscript by Montoya et al tries to identify the evolutionary history of the fungus *Escovopsis*, the well-known garden parasite of fungus-growing ants. They have assembled an impressive array of samples across the Americas, have sequenced 5 different loci, used the calibration estimates of Sung et al (2008) to date the radiation of this group in BEAST. The manuscript is clearly written with state-of-the art methods. I was expecting a little more information on the biology of fungus-growing ants, vis-à-vis, the radiation of this parasite, as one would expect from a Comms. Bio. manuscript. But beside the obvious, (like the headings in the result section: 'Escovopsis is associated with only some fungus-growing ant genera', 'Escovopsis is a late-arriving symbiont in the evolution of higher fungus-growing ants' and 'Escovopsis is distributed across Central and South America'), I could not find any. As it stands now, this manuscript is suitable for journals specializing in phylogenetics, biogeography and/or fungal biology.

R. We appreciate your kind words regarding the sampling effort assembled in this manuscript. Regarding increasing information about the biology of fungus-growing ants, we had already included a brief information on the biology of these insects (L 43-46). Per your request, we expanded this information to make the text more suitable for the journal (L 43-50). We hope that satisfies. On the other hand, since 1999 several hypotheses on the taxonomy, ecology and evolution of the genus *Escovopsis* were put forward with few supported evidence. Thus, in many studies it was simply assumed that the genus: i) was highly diverse (with few phylogenetic evidence and no taxonomic evidence), ii) was the only highly virulent specialized parasite of the mutualistic fungus of the ants (based on the analysis of few strains of only one species – see Elliot et al. 2025 for a detailed background of the genus), and iii) that the genus had coevolved in a close relationship with both ants and their mutualistic fungus. While some of these assumptions, such as the diversity of the genus, were subsequently corroborated with taxonomic studies (Montoya et al. 2021, 2023), more recent studies revealed some aspects that are not aligned with what was originally hypothesized. For instance: i) not all fungi that were thought to be *Escovopsis* belong to the same genus (Montoya et al. 2019, 2021); ii) not the entire genus, but rather a small number of species, exhibit a mycoparasitic lifestyle (Jiménez-Gómez et al. 2021, Furtado de Mendoca et al, 2021, Elliot et al. 2025); iii) not all attines, but only the most derived clades and one basal genus (*Apterostigma*) are evolutionarily associated with *Escovopsis* (this study); iv) *Escovopsis* did not evolve with all attines since the origin of the attine ant-fungus mutualism, but it is a late-evolving symbiont in the evolution of these ants (this study); and v) in addition to genomic traits (de Man et al. 2016, Gotting et al. 2022, Berasategui et al, 2024), *Escovopsis* has morphological and physiological characters that have changed throughout its evolutionary history (this study). Based on these facts, we are extremely surprised that the reviewer categorizes our results as obvious and fails to see the advance that our study is contributing to the evolutionary history of this group of fungi (which is the main conclusion also highlighted by the other two reviewers).

Regarding the geographic distribution of the genus and its co-cladogenetic relationship with the ants and their mutualists, we know that there are some hypotheses in the literature about this topic. Notwithstanding, no study has demonstrated with factual evidence, the distribution of this group of fungi, much less concluded that they have a similar distribution as their attine symbionts. Although we amassed a large sampling size in the present study, we were quite conservative with our conclusions on this topic, because we know that even with 309 samples from five different countries, our data set does not cover all the regions where the ants are distributed (explained in L 156-167 and positively highlighted by the other reviewers). To fill this knowledge gap and draw more robust conclusions, we need more field work, as well as many more trained researchers to study these fungi.

Reviewer: I was also confused with the ‘Trait evolution analysis’ with vesicle shape. It is considered to be a diagnostic character for fungal taxonomy. But such diagnostic characters are not necessarily the most important, evolutionarily speaking, character for a taxon which shows such specialized parasitic lifestyle. The authors consider this to be an important feature for the parasitic adaptation of *Escovopsis* (lines 216-240) and state that ‘*Escovopsis* is evolving in such a way as to increase asexual reproductive efficiency’. Also, the authors conclude that these fungi have evolved to grow quickly which they demonstrate by growing them on a rich media of potato dextrose agar. A previous study by Mueller et al (2011) showed that the cultivar fungi seem to have adapted to withstand cold conditions in the relatively Northern climes of their range. Could this also explain the differential growths of the co-evolved parasitic fungi when grown at a constant 25° C? Needless to say, that I found both of these conjectures to be very speculative.

R. In our manuscript we have not highlighted the vesicles or any other feature as the **most important** evolutionary trait of this genus, neither we considered them to be important features for the parasitic adaptation of *Escovopsis* (as you mention in your comment). In the text it is literally written: ‘Therefore, we hypothesize that these traits may have evolved specifically to facilitate persistence in attine gardens, and particularly in the presence of the higher attine ants, which employ suppression strategies such as mechanically weeding out *Escovopsis* in laboratory infections and employing antibiotic-producing bacteria that can suppress *Escovopsis* growth’ (L 272-276). We agree that taxonomic diagnostic characters sometimes might not be the most important for the evolution of a fungus. However, over several years, we observed that these structures change in shape, size, and abundance throughout the phylogeny of the genus. Biologically speaking, vesicles are directly related to the production of the asexual structures in this group of fungi [conidia - well explained in the text (L 80-83, 257-267, and in the Figure S1)]. Considering that reproduction is one of the main purposes that preserves the existence of living organisms (East, 1918; García et al. 2024), then, the vesicles appear to be not only important structures for the morphological description of the new species, but also for evolutionary studies of this group of fungi. Based on this idea, we decided to conduct a more in-depth evolutionary analysis in combination with physiological tests of the growth and conidia production of these organisms. Future studies will possibly study other characters (perhaps more important than the vesicles) but these were the ones we chose and the reasons why are clearly explained in the text.

On another topic, the ‘specialized parasitic lifestyle concept’ of *Escovopsis* has become controversial, as it seems that not all species in genus exhibit a mycoparasitic lifestyle (Jiménez-Gómez et al. 2021, de Mendonça et al. 2021, Simon et al. 2025). Based on this literature, we can conclude that some species of the genus can exhibit a mycoparasitic lifestyle but others may act as opportunists with low virulence for the ant colony, and that there are other species whose lifestyles are completely unknown. We have carefully reviewed this point throughout the manuscript, and we confirmed that our results and discussion do not take sides on whether this group of fungi is a parasite or not, and this is just to avoid speculations. Finally, regarding the growth test performed on PDA, we used this culture medium because it is a standard medium for the growth of species in this genus (Montoya et al. 2019, 2021, 2023). However, we also performed the assays on SNA and CMD (which are media used to describe new species in the genus), and the conclusion was the same. So, we included these results in figure 4. Finally, as you just mentioned in your comment above, speculating about whether these tests respond to Mueller et al. (2011) findings, or whether they explain the differential growth of co-evolved parasitic fungi at 25 °C, would be entirely subjective. That's why our manuscript neither presents nor suggests such conjectures.

This is a well written manuscript. Unfortunately, as it stands now, it is suitable for journals specializing in phylogenetic studies and/or fungal biology.

R. We thank you for your comments on the manuscript. However, we regret that you did not find any novelty on our study. As we argued above, our study brings several well-supported novelties that will help future research outlining and understanding the evolutionary history of the genus *Escovopsis* (as acknowledged by the other reviewers).

Authors' reply to reviewer #2's comments:

Reviewer: The manuscript by Rodriguez et al. “Digging into the evolutionary history of fungus-growing-ant symbionts in the genus *Escovopsis*” deals with the evolution of the microfungus *Escovopsis* living in fungus gardens of attine ants. Some species of *Escovopsis* are specialized parasites of the fungal cultivars the ants rely on for food and can cause severe damage to the fungus gardens and in consequence to the ant colony. Rodriguez et al. investigate the evolutionary history of this group of fungi based on a broad sampling indifferent sites in the Americas. They used four loci for their phylogenetic analysis and divergence time analysis. An interesting part is the investigation of the morphological traits of the vesicles since the shape of the vesicles are not only a diagnostic feature in the genus but seems also to be ecologically important. Even more interesting is the analysis of growth rate, conidia production and the viability of conidia. This shows a tremendous increase in *Escovopsis* species associated with Higher Attini and are probably important evolutionary changes towards a more efficient parasitism. The results of the manuscript are very important to better understand how detrimental symbiosis may evolve. Understanding the evolution of multispecies symbioses in depth is extremely difficult and the authors have added an important piece of the puzzle. I therefore strongly support this study being published.

R. We appreciate your comments about this manuscript and your conclusions regarding the future contributions that it will make to our understanding of the evolution and ecological role of this group of fungi. We understand that the *Escovopsis*-mutualistic fungus

interaction is of particular interest to many researchers studying the fungus-growing ant system. However, as you have pointed out, we also believe our results are just the tip of the iceberg regarding the evolutionary history of Escovopsis and we are convinced that there is still much to learn about their ecology and taxonomy.

Reviewer: The methods are adequate and sound, the results section short and concise. The Introduction and discussion are very well written and easy to follow. I would have liked a bit more background information for non-Escovopsis specialists: are there any free-living strains known? Fungus growing ants grow different cultivars, are some more at risk to be parasitized by Escovopsis than others? As far as I know *Apterostigma* for example does not cultivate *Leucocoprinus*, is it possible that the virulence has evolved especially towards fungal cultivars which may have lost their inventory to produce secondary metabolites while becoming a mutualist? How can ants suppress the detrimental behaviour, is it only by grooming or also by spraying secretions which do not harm the fungal cultivar but the parasite? It's indeed a thrilling topic!

R. Thank you for this comment and questions. We have included now a paragraph for the 'non-Escovopsis specialists' (L 235-252). Briefly, there are still few information about the biology, diversity, and ecology of the sister clades of Escovopsis that inhabit the colonies of the early derived attines. So far, there are no evidence about free-living strains of these genera and whether they are associated to a specific cultivar or cultivars are still poorly addressed. Regarding their 'parasitic lifestyle', or the possible virulence evolution towards fungal cultivars' we have no information about that topic. There are some experiments on the literature that show certain degree of attraction to the ant cultivars in *Sympodiorosea* strains, no attraction in *Luteomyces*, a certain degree of antagonism in *Escovopsioides*, and we have no information, so far, about the other clades (this information is now added in the new paragraph- L 235-252). As you mentioned, we also believe that this is indeed a thrilling topic, thus we: i) added the information on these genera just as a reminder of the lack of information about them, and ii) highlighted how important it would be to develop new studies about these fungi to the evolutionary story of the *Hypocreaceae* genera that inhabit the attine colonies. Finally, we preferred not raising hypotheses or conclusions about the ecology or lifestyles of these *Hypocreaceae* fungi, as we could create more speculations or confuse the readers (who in some cases still consider all these genera as species within Escovopsis).

More specific remarks:

Line 30: I guess there is "ago" missing.

R. Thanks for noticing that. We amended this now (L 30)

Line 55: altered and redirected the research in which direction? Towards microbial one?

R. Thank you for this question. It redirected our understanding in the way that many researchers drew their conclusions based in the hypothesis that the ant system was influenced by a single, highly specialized parasite with the potential to devastate the mutualistic fungi of the fungus-growing ants. We added this information in the text (L 60 - 61)

Line 71: what exactly do you mean by “that may shape interactions within the system”?

R. Thank you for noticing this mistake. What we meant was: 'Escovopsis also differs from its sister clades in terms of gene content, which may be the result of the genus's interactions with other organisms within the system'. We amended this sentence in the new version (L 83 - 86)

Line 76: does this means that there is no indication of host specificity neither ants or fungi?

R. Thank you for raising this concern. We do not deny this possibility; however, we highlight the fact that there is no robust evidence to support co-cladogenesis between the three individuals of the proposed tripartite symbiosis. This is because: 1) Not all species of the genus *Escovopsis* are mycoparasites (Jiménez-Gómez et al. 2021, de Mendoca, et al. 2021, Elliot et al. 2025). This means that even if the hypothetical picture of a strict co-cladogenesis between the three individuals (ants + mutualistic fungus + *Escovopsis*) was true, it would not necessarily be linked to a parasite-host coevolution model; 2) Not all species of the *Attines* coevolved with the genus *Escovopsis* (results of this study), which means that there are ant colonies in which the tripartite coevolution model with *Escovopsis* cannot be fulfilled; and 3) To date, there are very few cases in which it has been possible to isolate the three individuals (ants + mutualistic fungus + *Escovopsis*) from the same ant colony. Commonly, when samples are available from the ants, we have no samples from the mutualist neither *Escovopsis*, or if there are samples from the ants and the mutualist, we have no *Escovopsis* samples, or there are many *Escovopsis* strains without the respective ant or the mutualist samples. This implies that any conclusion about co-cladogenesis within the tripartite model should be inconclusive due to a lack of support (we added this information in the introduction section to make it clearer and more understandable for readers L 68-77). We are currently joining forces with several laboratories to fill this gap.

Line 85: you mention in line 48 that many strains/species live without any detrimental impact in the nests. Is it because they are not able to be detrimental or because the colony is vital enough to control it?

R. Good point. We believe either way. However, what we do have factual evidence about is that the system (where ants are the main protagonists in the care of the system) manages to maintain the balance even with *Escovopsis* and other fungi living in the same fungal garden. Whether they are active or in a dormant stage is poorly known and it is still matter of discussion. On the other hand, as we discuss in this manuscript, the literature shows that *Escovopsis* appear to have distinct lifestyles compared to their siblings in the same family (L 235-252). Furthermore, evidence also supports the possibility that these fungi may be living within the garden as opportunists. Therefore, the *Escovopsis* strains that are present within the colonies (without causing apparent damage) could simply be sitting and waiting for some type of dysbiosis in the system to emerge.

Line 105: you list the ants but not the fungi they may cultivate. Do all higher *Attini* cultivate *Leucocoprinus* or *Leucoagaricus*? At least the representative of the lower *Attini* does not.

R. Thank you for bringing this concern to our attention. It is an interesting idea; however, we fear that it can cause misinterpretations of our study. Our study was not focused on co-cladogenesis between fungal cultivars and *Escovopsis*. We understand that for questions of host-parasites co-cladogenesis, information on the fungiculture of each group of fungi would be quite appropriate (this, of course, assuming that we consider the genus *Escovopsis* as a species-specific parasite of the mutualistic fungus). However, as we mentioned previously, we would like to avoid any controversy or misunderstanding about this topic and by listing the different fungicultures associated with each group of ants would only cause confusion among readers. mainly among those who have a reluctant stance on the lifestyle and taxonomy of this group of fungi and those who are just beginning to understand how the system works.

Line 188: another scenario could be that a free-living ancestor found a suitable niche in *Attine* nests because of the ability to degrade ant debris, insect carcasses and plant material and to deal with the antifungal substances produced by the ants. Have you checked whether the contact with ants is inhibiting germination of conidia in certain *Escovopsis* species? I know that looking for free-living close relatives is looking for the needle in the haystack but you may find it on leaf surfaces. And once you have been lucky and found it the next question then is: what turns the saprophyte into a parasite?

R. We are happy that you pointed this out. We had the same idea and hypothesis and we wrote about it in a previous version of the manuscript. Yet, because we have no factual evidence to support it, previous reviewers asked to remove it, and we agreed with them. Regarding the germination of *Escovopsis* conidia in the presence of the ants, we never try it. But we do have information when *Escovopsis* is inoculated in ant fungus garden and when it is confronted with the mutualist. In the first scenario, the ants can remove the conidia and the colonies remain functional as if nothing had happened. The number of colonies experiencing dysbiosis due to the presence of experimentally introduced *Escovopsis* is statistically not significant (Jiménez-Gómez et al. 2021). In the second scenario, some *Escovopsis* are positively influenced for the mutualist and others can inhibit it. Finally, where is *Escovopsis* when it is not inside of the ant colonies? This is a question that is poorly addressed overall. We can only speculate that *Escovopsis* is entering to the ant colonies together with the material used to feed the mutualistic fungus, but this remains to be verified.

Lines 545, 570 and 600: the journal is missing in these citations, please check all of them carefully whether they are complete. Also, the way of citing is not consistent regarding capital letters (sometimes yes sometimes no) and abbreviations of the journal (sometimes abbreviated, sometimes not)

R. Thanks for noticing that. We amended all the citations now.

Fig 1: perhaps you could add the sample size per collection site, this would help to see at once why there are many connecting lines in some sampling sites but in others not

R. Thank you for this suggestion. We added the percentages to each collection point on the figure. In addition, we increased the size of some names, organized the names of the geographical points by country names and regions, and amended some information that was

mistakenly placed. This information was also added to the figure legend. Besides, we realized that the taxonomic names of some ants were mistakenly written (*Paratrachymyrmex zeteki* is in fact *Mycetomoellerius zeteki*). This does not change our conclusions but makes the manuscript more accurate. So, we take this opportunity to fix these mistakes in the figure and in the table of the supplementary material (highlighted in grey).

Fig. 2 it would help the ant people if you could add the respective ant genus next to the *Escovopsis* sample in the tree, a symbol for example

R. Thank you for this suggestion. We added to the figure the phylogenetic tree of the ants and we connected, with dotted lines, the species of *Escovopsis* with the ant genera to which each fungal species is associated. This information was also added to the figure legend.

Fig. 3 the same applies here as in Fig 2: a symbol to assign it to the respective ant genus would give more information

R. Thank you for this suggestion. In this case, it was not possible to add the ant phylogeny due to didactic purposes. Instead, we added some colored circles representing the ant genera. Each color represents a genus of ants, and it is detailed in the figure legend.

Authors' reply to reviewer #3's comments:

In this manuscript, the authors integrate molecular, morphological, and physiological analyses to explore the diversity, host associations, and evolutionary history of the genus *Escovopsis*. The molecular analysis includes 309 fungal isolates from over 400 attine ant colonies collected across six countries (primarily Brazil and Panama). *Escovopsis* was isolated from fungus gardens using established protocols, and strains were preserved under various conditions. DNA was extracted following a modified CTAB method, and five molecular markers (ITS, LSU, *tef1*, *rpb1*, *rpb2*) were amplified for phylogenetic analysis. A Bayesian approach was applied to infer evolutionary relationships and estimate divergence times, incorporating calibrated data from previous studies. For the morphological analysis, the researchers examined 14 representative strains from different clades within the genus, measuring vesicle and conidiophore characteristics under a microscope. The physiological assessment included evaluating fungal growth rate, conidia production, and conidia viability through controlled laboratory experiments. Data were statistically analyzed using ANOVA to identify significant differences among strains. The methodology follows standard protocols in the field, and the authors themselves are recognized experts in these analyses. Therefore, I find no issues or suggestions regarding the methodological approach.

The study's major findings include that *Escovopsis* is specifically associated with certain fungus-growing ant genera, particularly the "higher" attines (*Atta*, *Acromyrmex*, *Mycetomoellerius*, *Paratrachymyrmex*, *Sericomyrmex*) and the "lower" attine *Apterostigma*. In contrast, it is absent from other fungus-growing ant genera, which are instead associated with sister clades such as *Luteomyces* and *Sympodiorosea*. While *Escovopsis* is linked to specific ant genera, its evolutionary history does not strictly parallel that of its host ants. However, the most derived *Escovopsis* clades tend to be associated with the most derived attine ants. The study estimates that *Escovopsis* originated approximately 56.9 million years

ago, shortly after the emergence of the attine ant-fungus mutualism (~66 Mya), aligning with previous studies (De Man et al 2016 & Gotting et al 2022). The authors propose a sequential association of Escovopsis with different attine genera, beginning with *Apterostigma* and later expanding to other groups. Regarding its geographic distribution, the results indicate that Escovopsis is widespread across Central and South America. However, its phylogeographic patterns remain incomplete, likely due to uneven sampling efforts. Finally, the authors found evidence of a shift in vesicle shape from globose (ancestral state) to cylindrical (derived state). More derived Escovopsis species exhibit increased growth rates, conidia production, and viability, suggesting adaptive advantages over time.

These findings improve our understanding of Escovopsis evolution and its association with attine ants, expanding on previous work but without radically altering the known framework. The evolutionary sequence proposed (first *Apterostigma*, then *Mycetomoellerius* / *Paratrachymyrmex* / *Sericomyrmex*, finally *Atta* and *Acromyrmex*) is a significant contribution and will likely be of interest to evolutionary biologists studying host-parasite interactions. The morphological and physiological evolution of Escovopsis is a compelling aspect that connects fungal adaptation to ecological pressures, which is relevant beyond myrmecology. While the broad claim that Escovopsis is primarily associated with certain attine ants is not new (e.g., Currie et al., 2003; Gerardo et al., 2006; Taerum et al., 2007), the detailed evolutionary trajectory and morphological shifts provide novel insights.

The study is largely convincing, although one weak point is the uneven sampling, which weakens its broad biogeographic conclusions. The authors acknowledge this limitation and discuss it within the text. While the paper is unlikely to dramatically shift the field, it refines and reinforces our understanding of Escovopsis evolution and its role in ant-fungus symbioses. In addition, insights into morphological and physiological changes in Escovopsis could inspire further research on fungal adaptation and evolution in symbiotic systems. If further studies support the proposed sequence of Escovopsis host shifts, it could reshape discussions on how specialized parasites diversify alongside their hosts. The manuscript is well-written, engaging, and scientifically sound. I find it highly interesting and support its publication without further changes.

R. We appreciate your detailed analysis of our manuscript, as well as sharing your point of view about the geographic distribution analysis. Your comments are in line with those of the second reviewer, who pointed out some specific questions that were addressed in this new version of our manuscript. Specifically, we thank the reviewer for pointing out the uneven sampling for the biogeographic part of the study. As the reviewer pointed out we tried to be conservative in our conclusion, as we are aware that are data set does not fully cover the entire range of attine ants. This is acknowledged in L. 156, 167.