A Brazilian Population of the Asexual Fungus-Growing Ant Mycocepurus smithii (Formicidae, Myrmicinae, Attini) Cultivates Fungal Symbionts with Gongylidia-Like Structures

Masiulionis, Virginia E.; Rabeling, Christian; de Fine Licht, Henrik Hjarvard; Schultz, Ted R.; Bacci Jr., Mauricio; Santos Bezerra, Cintia M.; Pagnocca, Fernando C.

Published in: PLOS ONE

DOI: 10.1371/journal.pone.0103800

Publication date: 2014

Document version Publisher's PDF, also known as Version of record

Citation for published version (APA): Masiulionis, V. E., Rabeling, C., de Fine Licht, H. H., Schultz, T. R., Bacci Jr., M., Santos Bezerra, C. M., & Pagnocca, F. C. (2014). A Brazilian Population of the Asexual Fungus-Growing Ant Mycocepurus smithii (Formicidae, Myrmicinae, Attini) Cultivates Fungal Symbionts with Gongylidia-Like Structures. PLOS ONE, 9(8), 1. [e103800]. https://doi.org/10.1371/journal.pone.0103800

Download date: 21. feb., 2022
A Brazilian Population of the Asexual Fungus-Growing Ant *Mycocepurus smithii* (Formicidae, Myrmicinae, Attini) Cultivates Fungal Symbionts with Gongylidia-Like Structures

Virginia E. Masiulionis¹*, Christian Rabeling²,³*, Henrik H. De Fine Licht⁴, Ted Schultz³, Mauricio Bacci Jr.¹, Cintia M. Santos Bezerra¹, Fernando C. Pagnocca¹

1 Instituto de Biociências, São Paulo State University, Rio Claro, SP, Brazil, 2 Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts, United States of America, 3 Department of Entomology, National Museum of Natural History, Smithsonian Institution, Washington, D.C., United States of America, 4 Section for Organismal Biology, Department of Plant and Environmental Sciences, University of Copenhagen, Copenhagen, Denmark

**Abstract**

Attine ants cultivate fungi as their most important food source and in turn the fungus is nourished, protected against harmful microorganisms, and dispersed by the ants. This symbiosis evolved approximately 50–60 million years ago in the late Paleocene or early Eocene, and since its origin attine ants have acquired a variety of fungal mutualists in the Leucocoprinaceae and the distantly related Pterulaceae. The most specialized symbiotic interaction is referred to as “higher agriculture” and includes leafcutter ant agriculture in which the ants cultivate the single species *Leucoagaricus gongylodorus*. Higher agriculture fungal cultivars are characterized by specialized hyphal tip swellings, so-called gongylidia, which are considered a unique, derived morphological adaptation of higher attine fungi thought to be absent in lower attine fungi. Rare reports of gongylidia-like structures in fungus gardens of lower attines exist, but it was never tested whether these represent rare switches of lower attines to *L. gongylodorus* cultivars or whether lower attine cultivars occasionally produce gongylidia. Here we describe the occurrence of gongylidia-like structures in fungus gardens of the asexual lower attine ant *Mycocepurus smithii*. To test whether *M. smithii* cultivates leafcutter ant fungi or whether lower attine cultivars produce gongylidia, we identified the *M. smithii* fungus utilizing molecular and morphological methods. Results show that the gongylidia-like structures of *M. smithii* gardens are morphologically similar to gongylidia of higher attine fungus gardens and can only be distinguished by their slightly smaller size. A molecular phylogenetic analysis of the fungal ITS sequence indicates that the gongylidia-bearing *M. smithii* cultivar belongs to the so-called “Clade 1” of lower Attini cultivars. Given that *M. smithii* is capable of cultivating a morphologically and genetically diverse array of fungal symbionts, we discuss whether asexuality of the ant host maybe correlated with low partner fidelity and active symbiont choice between fungus and ant mutualists.

**Citation:** Masiulionis VE, Rabeling C, De Fine Licht HH, Schultz T, Bacci M Jr, et al. (2014) A Brazilian Population of the Asexual Fungus-Growing Ant *Mycocepurus smithii* (Formicidae, Myrmicinae, Attini) Cultivates Fungal Symbionts with Gongylidia-Like Structures. PLoS ONE 9(8): e103800. doi:10.1371/journal.pone.0103800

**Editor:** Nicole M. Gerardo, Emory University, United States of America

**Received** October 17, 2013; **Accepted** July 7, 2014; **Published** August 7, 2014

**Copyright:** © 2014 Masiulionis et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** FCP and MB are grateful to CNPq and Fapesp for their financial support. VEM is a recipient of a CAPES/PEC-PG scholarship. CR was financially supported by a Junior Fellowship from the Harvard Society of Fellows and the HMS Milton Fund. TS was supported by the U.S. National Science Foundation grant DEB 0949689, the Smithsonian Institution Scholarly Studies Program, and the Smithsonian NMNH Small Grants Program. HHDFL was supported by grants from the Danish Research Council and the Carlsberg Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* Current address: Biology Department, University of Rochester, Rochester, New York, United States of America

**Introduction**

Mutualisms, symbiotic interactions between organisms in which each partner benefits, are widespread across the tree of life [1]. Many eukaryotes evolved obligate relationships with symbiotic organelles, such as mitochondria and chloroplasts, and provide stunning examples of ancient, evolutionarily stable mutualisms [2–4]. Co-evolutionary processes, reciprocal genetic changes in one species in response to changes in the partner species, shape these tight relationships, selecting for ecological specialization and resulting in co-diversification and eventually co-speciation [3–10]. Evolutionary patterns of co-speciation can be inferred secondarily from congruent phylogenetic histories (i.e., co-cladogenesis). Unfortunately, however, it is inherently difficult to study currently co-evolving organisms in order to understand the selective processes and proximate mechanisms underlying obligate interdependencies because currently observed patterns may not necessarily reflect the evolutionary interactions that shaped the symbiosis when it originated.

The complex symbiosis of fungus-growing ants with leucocephaline fungi and other associated microorganisms provides a system that is well suited for studying the evolution of mutualistic interactions and the origins of fungiculture in insects [11–17]. The fungus-gardening ants of the tribe Attini comprise a monophyletic
group of more than 250 described species [15,18,19] that are distributed throughout the New World from Argentina in the south to the United States in the north [20–23]. All fungus-growing ant species rely obligately on basidiomycete fungi that they cultivate for food [21,24–26]. To enable the growth of the fungal symbionts, the ants provide nutrition to the fungus garden and prevent the growth of alien macroorganisms [21,27–31].

Originating around 30–60 million years ago, the ancestral attine agricultural system, i.e., “lower agriculture,” is practiced today by the majority of attine ant genera and species, which cultivate a closely related but poly- and paraphyletic group of leucocorpi-neaceous fungi [13,32]. Around 20–30 million years ago, a particular lower-attine ant-fungus association gave rise to “higher agriculture,” which includes the well-known leafcutter ants [15]. The clade of fungi associated with higher attine ants is descended from a lower-attine fungal ancestor, and unlike the lower attine fungi, higher attine fungi are never found free-living apart from their ant hosts, suggesting strong co-evolutionary dynamics between higher attine ants and their cultivars [12,27,33–37]. The most significant morphological adaptations of higher attine fungi are the nutrient-rich hyphal tip swellings, the so-called gongylidia, which serve as the main food source for the ants and their brood [24,26,30–44].

In 1893, the pioneering mycologist Alfred Möller first described these hyphal tip swellings in the fungus gardens of Acromyrmex coronatus, which he called “Kohlrabikopf,” due to their morphological similarity to cabbage turnips (Möller [24], pp. 26). Later Wheeler [25] suggested the Hellenistic version of Möller’s term, “gongyladium, -a” (Greek = gongilos = turnip), for the same structure. In fungus gardens of higher attine ants, gongylidia occur in clusters, which Möller [24] termed “Kohlrabhäufchen,” and Weber [30] later called “staphyla, -ae” (Greek = cluster of grapes). Chemical analyses showed that gongylidia contain glucose, mannitol, trehalose, glycan, arabinose, and glycogen, in addition to lipids, and ergosterol [26,40,45,46], as well as free amino acids [34,45]. In contrast, the filamentous hyphae contain high protein concentrations but only low concentrations of lipids and carbohydrates [26,45]. In addition, a recent study demonstrated how the co-evolutionary adaptations of specific laccase enzymes, which are highly expressed in the gongylidia, participate in the detoxification of secondary plant compounds present in the leaf material [47].

The presence of gongylidia in the fungus gardens of higher attine ants is considered an exclusively co-evolved, mutualistic association and gongylidia are not known to convey any fitness benefits to the fungus outside the ant symbiosis [13,15,21,35,37,48]. Here we report the occurrence of gongylidia in fungus gardens of the asexual lower attine ant Mycocepurus smithii from Brazil. M. smithii is distributed across tropical and subtropical habitats in Central and South America [49,50] and consists of a mosaic of sexually and asexually reproducing populations [50]. Populations from southeast Brazil were found to reproduce strictly asexually via thelytokous parthenogenesis [50,51]. Interestingly, M. smithii was previously reported to be one of the very few attine ant species to cultivate a genetically diverse array of fungi [13,32,35,52]. In contrast, some other lower attine populations in Latin America [51,54,55] are known to be faithful to a single cultivar lineage [17,53].

In this study, we test whether M. smithii cultivates leafcutter ant cultivars or, alternatively, whether lower attine cultivars are capable of producing gongylidia. In addition, we explore the hypothesis of whether asexual reproduction in M. smithii may have enabled and/or promoted the cultivation of morphologically and genetically diverse cultivars.

Materials and Methods

Study site and field observations

During a field class taught at São Paolo State University in Rio Claro, Brazil [22.3955’S, 047.5424’W; elevation 608 m], we excavated nests of multiple fungus-growing ant species to illustrate their natural histories and the intricate symbiosis between ants, fungi, and other associated microorganisms. Nest excavations followed the methodology described earlier [51,54]. We excavated a total of three M. smithii fungus chambers, which received the following collection codes: CR110715-01, CR110715-02, and CR110718-01. Fungus-chamber CR110715-01 was found at 25 cm depth. It was 2.5 cm wide and 2 cm high and contained a pendant fungus garden hanging from the chamber ceiling (Fig. 1A), multiple workers, no brood, and no queen. The second chamber, CR110715-02, was located directly underneath the first chamber at 53 cm depth, was slightly larger with a diameter of 3 cm and contained a pendant fungus garden, a single queen, multiple workers, and no brood. We assume that these two chambers belonged to the same nest. The third M. smithii fungus chamber, CR110718-01, was excavated approximately 50 m distant from the first nest. Only a single chamber was encountered at 50 cm depth. It was 5 cm wide and 4 cm high, contained a pendant fungus garden, eleven workers, and neither a queen nor brood. The sizes of the fungus chambers and their locations in the soil are consistent with results reported from other M. smithii populations in Latin America [51,54,55]. The live ant colonies and their fungus gardens were collected with a surface-sterilized spoon and placed into a laboratory nest for further observation. The lab nest consisted of a plastic container with a plaster of Paris bottom; see reference [56] for lab nest setup. Ants were identified using a Leica MS5 stereomicroscope and voucher specimens were deposited in Maurício Bacci’s Molecular Evolution Laboratory at São Paulo State University in Rio Claro. Collections were conducted under collecting permit number 14789-3 issued by the Ministério do Meio Ambiente – MMA and the “Instituto Chico Mendes de Conservação da Biodiversidade” (ICMBio).

Macro and microscopic observations

The presence of staphylae in M. smithii gardens was first noted during observations of the live colonies with a Leica MZ16 stereomicroscope and confirmed under higher magnification with a Leica DM750 bright-field microscope. The fungus garden of colony CR110715-02 was maintained in a dark room at 25°C for two days and measurements of gongylidia, which were grouped into staphylae, were taken with the Leica Application Suite V3 software package under a Leica DM750 bright-field microscope. For microscopic studies, we removed the staphylae from the fungus garden with a pair of acupuncture needles, placed them on a microscope slide, and submerged them in a drop of 15% glycerin solution. Staphylae were collected and 40 gongylidia were measured from a fungus garden of each of the following: lab colonies of Atta sexdens, A. laevigata, and Acromyrmex desiguer, and a field-collected colony of Trachymyrmex fascus. The colonies were collected on the campus of São Paulo State University in Rio Claro between October 2009 and September 2011.

The normality of the morphological measures was tested using Shapiro-Wilk’s test and the homogeneity of variance was assessed with Levene’s test of homoscedasticity. To test for variance of size distribution in gongylidia of different fungi, we conducted an analysis of variance (one-way ANOVA and Tukey test) with a significance level of less than 1% (p<0.01) using the software package BioEstat 5.0 [57].
Genotyping and molecular phylogenetic analyses

To genotype the gongylidia-bearing *M. smithii* cultivar CRI10715-02, we extracted genomic DNA from tissue samples of the staphylae following the methodology described in Martins Jr. et al. [58]. The ITS region was amplified according to Manter and Vivanco [59] using the forward primer ITS 5 (5′-GGAGGTAAGACGATTAGAAGG-3′) and reverse primer ITS 4 (5′-TCTTCCGCTTATTGATATGCG-3′) [60]. The PCR reaction consisted of an initial 2-min incubation at 96°C, followed by 28 cycles of 46 s at 96°C, 30 s at 50°C and 4-min at 60°C. The PCR product was gel-purified using an Illustra™ GEX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, UK). The same primer pair was utilized for amplification and sequencing. The sequencing reaction was prepared with 100 ng of PCR template, 6 pmols primers, 2.0 μl BigDye Terminator (Applied Biosystems), 1.0 μl buffer (20 μM Tris.HCl, 5 mM MgCl₂) and ddH₂O. Sequencing products were purified and then analyzed on an automated sequencer ABI3500 (Applied Biosystems). The consensus sequence was edited with the program BIOEDIT 7.0.5 [61] and aligned using CLUSTALW [62]. The obtained sequence was deposited at NCBI’s GenBank (www.ncbi.nih.gov) as accession number JX027477 and was compared to other ITS sequences deposited in GenBank. The resulting ITS DNA sequence consisted of 606 base pairs and nucleotides at positions 76 and 514 bp were unknown. The ITS DNA sequence of *M. smithii* cultivar CRI10715-02 is identical to ITS sequences of *M. smithii* cultivars from Panama, Costa Rica, and Trinidad; *M. tedus* from Panama; *Myrmicocrypta ednaella* and a new *Mycocepurus* species from Guyana (erroneously identified as *M. cf. goeldii*), all of which were previously published in Mueller et al. [13] and Kellner et al. [63].

To determine the molecular phylogenetic placement of the gongylidia-bearing *M. smithii* fungus, we added this sample to a data matrix of attine and free-living leucocoprineous fungal ITS DNA sequences consisting of 305 fungal taxa and 1042 nucleotide sites (including indels), which was previously published by Mehdiabadi et al. [17]. Sequences were aligned in MAFFT v7.017 [64] using the E-INS-I algorithm, a 200PAM/k = 2 scoring matrix, a gap opening penalty of 1.53, and an offset value of 0.

Maximum likelihood analyses. Initially, a best-fit model of sequence evolution was selected for the entire unpartitioned fungal ITS alignment under the Akaike information criterion as calculated in jModel Test 2.1.1 [65]. Using the resulting model, GTR+H+G (with 6 rate categories), an initial ML “best tree” analysis was conducted in Garli 2.0.1019 [66] consisting of 100 replicates using parallel processing and default parameter values. The results of this initial analysis were used to divide the sequence data into faster- and slower-evolving character sets. This was done by evaluating character evolution on the ML tree in MacClade 4.08 [67] under the parsimony criterion and assigning characters requiring 3 or fewer steps to a “slow” partition (596 characters) and characters requiring 4 or more steps to a “fast” partition (446 characters). Each of these two partitions was fit, again using the AIC in jModel Test, to an evolutionary model. Based on the results, a second “best tree” search was conducted with two partitions, a “slow” partition under the JC model and a “fast” partition under the HKY+G model, consisting of 100 searches and deviating from the default settings as follows: topopweight = 0.01; brlenweight = 0.002. ML bootstrap analyses in Garli, also employing the same two partitions and models, consisted of 1000 pseudoreplicates, deviating from default settings as follows: genthreshold = 5000; scorethreshold = 0.10; startoptprec = 0.5; miinoptprec = 0.01; numberofprecomputations = 1; treerejectionthreshold = 20.0; topopweight = 0.01; brlenweight = 0.002.

Bayesian analyses. The fungal alignment was analyzed under Bayesian criteria as implemented in MrBayes v3.2.1 [68] with the two partitions and models described above and with 10 million generations and samplefreq = 1000. All parameters were unlinked across partitions except for branch-lengths and topology. All analyses were carried out using parallel processing (one chain per CPU) with mncumodel = 4by4, nnruns = 2, nchains = 8, and samplefreq = 1000. To address known problems with branch-length estimation in MrBayes, we reduced the branch-length priors using brlnsampler = unconstrained:Exp(100) based on the procedure suggested in Brown et al. [69] and as applied in Ward et al. [70] and Rabeling et al. [50]. Burn-in and convergence were assessed using Tracer v1.5 [71], by examining potential scale reduction factor (PSRF) values in MrBayes.stat output files, and by using Bayes factor comparisons of marginal likelihoods of pairs of runs in Tracer, which employs the weighted likelihood bootstrap estimator of Newton and Raftery [72] as modified by Suchard et al. [73], with standard error estimated using 1,000 bootstrap pseudoreplicates.

**Results**

The fungal cultivars collected from three fungus chambers belonging to at least two *M. smithii* colonies contained gongylidia-like structures that were organized in staphylae (Figs. 1B–D). Our microscopic examination at 400x magnification showed that the gongylidia of this *M. smithii* fungal cultivar were morphologically very similar to the gongylidia found in fungal cultivars of *T. fuscus*, *Ac. disciger*, *A. laevigata*, and *A. sexdens* (Fig. 1 E,F,G,H), differing only in their smaller size (Fig. 2).

The diameter of gongylidia found in the *M. smithii* garden varied between 16.3 μm and 25.41 μm, which was significantly smaller than the gongylidia diameters in the studied *Trachymyrmex*, *Acromyrmex*, and *Atta* species (one-way ANOVA and Tukey test, p<0.01; Fig. 2). Gongylidia diameter in the higher attine cultivars was distributed along a continuum, on which *A. sexdens* gongylidia were the smallest, ranging from 21.04 μm to 39.99 μm in diameter, and on which *T. fuscus* gongylidia were the largest, ranging between 42.01 μm and 68.26 μm in diameter. The cultivars of all five examined ant species had gongylidia diameters significantly different from one another, except for *Ac. disciger* and *A. laevigata* (Fig. 2).

Gongylidia are thought to be highly specialized morphological adaptations of higher attine cultivars. The detection of gongylidia in the fungus garden of one of the most “primitive” attine lineages therefore suggests at least three alternative explanations: (i) *M. smithii* is capable of growing leafcutter ant cultivars; (ii) gongylidia production arose independently in the cultivars of lower and higher attine ants; or (iii) gongylidia are ancestrally present in at least some lower attine fungi, including presumably, the lineage that gave rise to higher attine fungi. To distinguish between these evolutionary scenarios, we conducted molecular phylogenetic
analyses of the ITS region of attine cultivars to determine the phylogenetic position of the gongylidia-producing *M. smithii* fungus.

Both Bayesian and maximum likelihood phylogenetic analyses agree that the gongylidia-bearing *M. smithii* fungal cultivar of the Rio Claro population is embedded in the so-called “Clade 1” of the fungus tribe Leucocoprineae (Fig. 3) and is sequence identical with fungi cultivated by *M. smithii* (from Trinidad, Panama, Costa Rica), *M. tardus* (Panama), an undescribed species of *Mycocepurus* from Guyana, and *Myrmicocrypta edncaa* (Panama) (MLBP = 95, BPP = 100). Interestingly, a clade only a couple of nodes removed from the gongylidia-producing *M. smithii* cultivar contains a fungus cultivated by *T. papulatus* (Fig. 3), which is a member of the higher Attini. The highly derived higher attine fungal clade, which includes the cultivars of the leafcutter ants, is shown in our analysis to have arisen from a lower-attine fungal ancestor in Clade 1, which is consistent with earlier results [12,16].

**Discussion**

Although somewhat smaller in diameter, the gongylidia found in the fungus gardens of the asexual fungus-growing ant *M. smithii* occurring in Rio Claro are similar in shape to gongylidia found in the cultivars of higher attine species. This is surprising because gongylidia are thought to be a derived morphological specialization in higher attine fungi that originated as a result of the ant-fungus co-evolution that produced the higher attine ants. **Figure 2. Comparison of gongylidia diameter from fungi cultivated by Mycocepurus smithii and four species of higher Attini.** The diameter of each gongylidum (n = 40 gongylidia per colony per ant species) was measured at its widest point. Mean values that are annotated with different letters are significantly different from each other (one-way ANOVA and Tukey test, p<0.01). doi:10.1371/journal.pone.0103800.g002

dol:10.1371/journal.pone.0103800.g002

It remains uncertain whether the tubular swellings in *Apterostigma* and *Cyphomyrmex* gardens observed by Møller, the gongylidia of higher attine cultivars, and the gongylidia of the *M. smithii* cultivar reported here are developmentally homologous. Interestingly, the developmental origins of gongylidia remain entirely uninvestigated. One hypothesis suggests that gongylidia are modified cystidia, the hyphal swellings found in the hymenium of many free-living basidiocarps (J.A. Scott pers. obs., cited in [39]) and a hypothesis of the origin of ant fungivory suggests that “proto-gongylidia” may have served as the fungal analogue of ant-attractant elaiosomes of plant seeds, which provide a food reward in return for ant dispersal [14,24].

The mean diameter of the gongylidia in the *M. smithii* cultivar was smaller than the mean diameter of gongylidia of higher attine species collected in Rio Claro. Earlier mycological studies by Møller [24] on the fungus gardens of *Acromyrmex coronatus* and *Ac. disciger* reported relatively small gongylidia sizes (10–24 μm), which are comparable in size to those found in *M. smithii* gardens. *Ac. disciger* was also studied by us and the fungi from the Rio Claro population had significantly larger gongylidia than those from Blumenau reported by Møller [24]. Gongylidia size could differ significantly between colonies of the same ant species
because the same species may utilize different cultivar species or strains in different nests and in different geographic locations. Gongylidia size could also differ in the same cultivar strain depending on the developmental status of the ant colony and the amount of nutrition provided by the ants.

The mechanisms underlying the production of gongylidia and the factors determining their size remain poorly understood. Previous studies of staphylae in higher attine nests suggested that the ants’ constant pruning stimulated the formation of gongylidia [74,75]. In addition, Powell and Stradling [76] showed that the quality of the substrate, the pH, and the temperature affect the growth and size of gongylidia in cultivars of three higher attine species (A. cephalotes, Ac. octospinosus, and T. urichi from Trinidad) when cultivated in-vitro on agar plates. A recent genetic expression study added a functional dimension to our understanding of gongylidia by showing that one laccase enzyme, which is highly expressed in the gongylidia of leafcutter ants, digests plant defensive phenolic compounds [46]. The ability to up-regulate laccase expression in the presence of fresh leaf material is thought to be a pre-adaptation of the fungus that evolved prior to the symbiosis with attine ants [46].

The phylogenetic position of the gongylidia-producing *M. smithii* cultivar in Clade 1 of the fungal phylogeny, the uncertainty surrounding the phylogenetic position of the higher attine fungi within Clade 1, and the lack of information regarding the distribution of gongylidia in lower attine fungi leave open the question of whether gongylidia evolved repeatedly in leucocoprineaceous fungi or whether they had a single origin. The fungal phylogeny further shows that *M. smithii* additionally cultivates fungi in Clade 2 (not shown here; see Mehdiabadi et al. [17]). The phylogram results from maximum-likelihood analyses of ITS sequence data of 305 fungal taxa. In the inset, numbers above branches are Bayesian posterior probabilities; numbers below branches are maximum-likelihood bootstrap proportions.

Figure 3. ITS phylogeny of Clade 1 of the fungal tribe Leucocoprineae. Clade 1 of the Leucocoprineae includes four primary clades of attine cultivars and closely related free-living fungi, here indicated as subclades A–D (sensu Mehdiabadi et al. [17]). The clade within subclade B that contains the gongylidia-bearing fungal cultivar of *M. smithii* from Rio Claro is indicated by dashed lines and is enlarged in the inset. Within the inset, the name of the gongylidia-bearing cultivar is indicated in red. The names of other cultivars of *M. smithii* in subclades A and B are indicated in red; *M. smithii* additionally cultivates fungi in Clade 2 (not shown here; see Mehdiabadi et al. [17]). The phylogram results from maximum-likelihood analyses of ITS sequence data of 305 fungal taxa. In the inset, numbers above branches are Bayesian posterior probabilities; numbers below branches are maximum-likelihood bootstrap proportions.
have evolved largely via diffuse co-evolution, in which groups of species of ants and fungi interact [16]. However, particular ant and fungal species in the *Cyphomyrmex wheeleri* group were shown to have been exclusively associated over long periods of time, potentially for millions of years [17]. The opposite pattern is observed in *M. smithii*, which is capable of cultivating a high diversity of fungal species in both Clades 1 and 2. It has been hypothesized that this pattern of fungal association indicates that symbiont choice behavior (e.g., choosing cultivars adapted for particular microhabitats) is more important for *M. smithii* colonies than being faithful to a specific cultivar type (i.e., high partner fidelity) [63]. In the absence of a cultivar/habitat correlation, the observation that *M. smithii* cultivates an unprecedented genetic diversity of cultivars is also consistent with weakened symbiont choice. *M. smithii* could only have acquired such a high diversity of cultivars by frequently domesticating free-living cultivars *de novo* and/or by acquiring novel cultivars from sympatric fungus-growing ants. A local study of Panamanian *M. smithii* colonies and their associated fungi hypothesizes that, because ants and fungi reproduce asexually and accumulate deleterious mutations (according to Muller’s ratchet), the ants’ frequent acquisition of novel cultivars functions as a kind of recombination, purging the ants from “deleterious fungi” and vice versa [63,77]. It remains to be demonstrated that the fungi cultivated by *M. smithii* suffer from Muller’s ratchet because the large feral populations from which the cultivars are domesticated experience frequent sexual recombination [13,32].

An alternative null hypothesis is that, with regard to lower attine fungi, patterns of ant-fungus association in *M. smithii* are random. One hypothesis that would predict such randomness is that, due to the lack of genetic recombination, the ants’ genomes accumulate deleterious mutations, some of which affect their olfactory recognition and mate recognition are expected to become obsolete in an asexually reproducing species such as *M. smithii* and, if olfaction genes are not actively maintained by natural selection, these genes will accumulate non-synonymous mutations. Consequently, the ants’ ability to recognize nestmates, or a specific fungal symbiont for that matter, may then deteriorate, and the ants may become unable to distinguish between cultivar types. In contrast, sexually reproducing attine species in the genus *Cyphomyrmex* have been experimentally shown to prefer the fungal cultivars they grew up with [78]. If olfactory abilities deteriorate in asexual fungus growing ants, which still needs to be demonstrated, the predicted outcome would be congruent with the pattern we observe in nature, in which an asexual fungus-growing ant cultivates a wide variety of morphologically and genetically different fungi. Future studies will first test whether the genes involved in olfaction are under relaxed natural selection in asexual populations of *M. smithii* and, second, comprehensively analyze the co-evolutionary interactions between *M. smithii* and its fungal cultivators on a global scale (Rabeling, Bacci, Schultz, unpublished data).

**Acknowledgments**

The authors are grateful to Dr. Silvio J. Govone for statistical advice, to both anonymous reviewers as well as the journal editor Dr. Nicole G. Gerardo for their valuable comments. A research permit to conduct fieldwork in Brazil was issued by the Ministério do MeioAmbiente and the Instituto Chico Mendes de Conservação da Biodiversidade (permit number 14709–3).

**Author Contributions**

Conceived and designed the experiments: VEM CR FCP. Performed the experiments: VEM CMSB. Analyzed the data: VEM CR HHDFL TS MB. Contributed reagents/materials/analysis tools: FCP CR MB. Wrote the paper: VEM FCP CR MB. Contributed reagents/materials/analysis tools: FCP CR MB. Conceived and designed the experiments: VEM CR FCP. Performed the experiments: VEM CMSB. Analyzed the data: VEM CR HHDFL TS MB. Contributed reagents/materials/analysis tools: FCP CR MB. Wrote the paper: VEM FCP CR HHDFL TS MB.

**References**

1. Sachs JL, Eisenberg CJ, Trucotte M (2011) New paradigms for the evolution of beneficial infections. Trends Ecol Evol 26(4): 202–209.
2. Blackstone NW (1995) A unit-of-evolution perspective on the endosymbiotic theory of the origin of the mitochondrion. Evolution 49: 785–796.
3. Maynard Smith J, Szathmáry E (1998) The major transitions in evolution. Oxford: Oxford University Press. 346 p.
4. Timmis JN, Ayliffe MA, Huang CY, Martin W (2004) Endosymbiotic gene transfer: organelle genomes forge eukaryotic communities. Nature Rev Genet 5: 125–135.
5. Ehrlich PR, Raven PH (1964) Butterflies and plants: a study in coevolution. Evolution 18: 586–608.
6. Benson WW, Brown Jr KS, Gilbert LE (1975) Coevolution of plants and herbivores: passion flower butterflies. Evolution 29: 659–680.
7. Janzen DH (1980) When is it coevolution? Evolution 34: 611–612.
8. Herre EA, Machado CA, Berrinham E, Nason JD, Windsor DM, et al. (1996) Molecular phylogenies of figs and their pollinator wasps. J Biogeography 23: 521–530.
9. Pellmyr O, Thompson JN, Nason JD, Brown Jr KS, Gilbert LE (1996) Evolution of pollination and mutualism in the yucca moth lineage. Ann Nat Hist 23: 827–847.
10. Mueller UG (2012) Symbiotic recruitment versus ant-symbiont co-evolution in the attine ant-microbe symbiosis. Curr Opin Microbiol 15: 1–9.
11. Zhang Z, Hanner R, Hultmark D, Weisser WW (2004) Associations between ants and their fungal symbionts. Ecol Entomol 29: 437–443.
12. Platt JD, Levine DJ (2000) A review of the fungal associations of ants. Ecol Entomol 25: 35–47.
13. Mueller UG, Rhener SA, Schultz TR (2012) Symbiotic fidelity and the origin of species in fungus-growing ants. Nat Commun 3: 840. doi: 10.1038/ncomms1844.
14. Schultz TR, Meier R (1995) A phylogenetic analysis of the fungus-growing ants (Hymenoptera: Formicidae: Attini) based on morphological characters of the larvae. Syst Entomol 20: 337–370.
15. Sous-Calvo J, Schwartz TR, Brandão CRF, Klingenberg C, Ferrari RM, et al. (2013) *Cyatta abscondita*: Taxonomy, evolution, and natural history of a new fungus-farming ant genus from Brazil. PLoS ONE 8(11): e70498.
16. Kempf WW (1972) Catálogo abreviado das formigas da Regiao Neotropical. Studa Ent 15: 1–344.
17. Weber NA (1972) Gardening Ants: The Attines. Philadelphia: American Philosophical Society. 146 p.
18. Brandão CRF (1991) Adendos ao catálogo abreviado das formigas da Região Neotropical (Hymenoptera: Formicidae). Rev Bras Entomol 35: 319–412.
19. Mayhew AJ, Jaffe K (1998) On the biogeography of Attini (Hymenoptera: Formicidae): Ecotropicos 11(1): 45–54.
20. Moller A (1893) Die Pilzgärten einiger Südamerikaner Ameisen. Bet Mitt Trop 6: 1–127.
21. Wheeler WM (1907) The fungus-gardening ants of North America. Bull Amer Mus Nat Hist 23: 689–697.
22. Quinlan RJ, Cherrett JM (1979) The role of fungus in the diet of the leaf-cutter ants *Atta cephalotes*. Ecol Entomol 4: 151–160.
23. Mayatt RF, Cherrett JM (1979) The role of fungus in the diet of the leaf-cutter ants *Atta cephalotes*. Ecol Entomol 4: 151–160.
24. Chen JY, Kuroiwa T (1996) The evolution of the fungus garden of the leafcutter ant *Atta cephalotes*. Science 272: 536–539.
25. Wheeler WM (1907) The fungus-growing ants of North America. Bull Amer Mus Nat Hist 23: 689–697.
26. Quinlan RJ, Cherrett JM (1979) The role of fungus in the diet of the leaf-cutting ants *Atte cephalotus*. Ecol Entomol 4: 151–160.
27. Cherrett JM (1979) The role of fungus in the diet of the leafcutter ants *Atta cephalotes*. Ecol Entomol 4: 151–160.
28. Quinlan RJ, Cherrett JM (1979) The role of substrate preparation in the symbiosis between the leaf-cutting ant *Acromyrmex octospinosus* (Reich) and its food fungus. Ecol Entomol 2: 161–170.
29. Carrie CR, Mueller UG, Malloch D (1999) The agricultural pathology of ant fungus gardens. Proc Natl Acad Sci USA 96: 7998–8002.
30. Carrie CR (2001) Prevalence and impact of a virulent parasite on a tripartite mutualism. Oecologia 126: 99–106.
Gongylidia in Fungus Garden of Mycocepurus smithii

31. Pagnocca FC, Masutisone VE, Rodrigues A (2012) Specialized fungal parasites and opportunistic fungi in gardens of attine ants. Psyche 2012, Article ID 905109, doi: 10.1153/905109.

32. Vo TL, Mueller UG, Mikheyev AS (2009) Free-living fungal symbionts (Fungi: Ascomycota) of fungus-growing ants (Formicidae: Mycetini). Mycologia 101: 206-210.

33. Holldobler B, Wilson EO (1990) The ants. Cambridge: Harvard University Press. 737 p.

34. Holldobler B, Wilson EO (2016) The Leafcutter Ants: Civilization by Instinct. New York: Norton WW&Co. 160 p.

35. Mueller UG, Schultz TR, Currie CR, Adams RM, Malloch D (2001) The origin of the ant-fungus mutualism. Q Rev Biol 76: 169-197.

36. Rabeling C, Lino-Neto J, Cappellari SC, Dos-Santos IA, Mueller UG, et al. (2009) Natural history and phylogeny of the fungus-farming ants (Hymenoptera: Formicidae: Mycetini). Myrmecol News 13: 37-53.

37. Solomon SE, Lopes CT, Mueller UG, Rodrigues A, Soza-Calvo J, et al. (2011) N. Physiol. Entomol 36: 13-15.

38. Weber NA (1957) Dry season adaptations of fungus-growing ants and their fungi. Ann Entomol Soc Am 52: 735-740.

39. Lattke W, M. Rabeling J. M., (1976) Direct ingestion of plant sap from cut leaves by the leaf-cutting ants Atta cephalotes (L.) and Acromyrmex octospinosus Reich (Formicidae: Attini). Bull Entomol Res 66: 205-217.

40. Qunitan RJ, Rabeling J.M. (1975) Aspects of the symbiosis of the leaf-cutting ant Acromyrmex octospinosus Reich and its food fungus. Ecol Entomol 3: 221-230.

41. Angulo-Papa J, Eymé J (1982) Les champignons cultivés par les fourmis Attinées. Ann Sci Nat Bot Biol 7: 103-129.

42. M. Bass M, Rabeling J.M. (1993) Fungal hyphae as source of nutrients for the leaf-cutting ant Atta cephalotes. Physiol Entomol 20: 1-6.

43. Murakami T, Higashi S. (1997) Social organization in two primitive attine ants, Cyphomyrmex rimosus and Myrmecocystus emiliae, with reference to their fungus substrates and food sources. J Ethol 15: 17-23.

44. Silva A, Bacci M, Bueno OC, Pagnocca FC, Holldobler MHA (2003) Survival of Atta sexdens workers on different food sources. J Insect Physiol 49: 307-313.

45. Martin MM, Carman RM, Mac Connell JG (1969) Nutrients derived from the fungus to increase and direct its productivity. Funct Ecol 10: 55-61.

46. Monaco Furletti ME, Serzedello A (1983) Determinação de carboidratos em Rostro de Mycetagroicus. Ann Entomol Soc Am 62(9): 11-13.

47. De Fine Licht HH, Schmitt M, Rogowska-Wrzesinska A, Nygaard S, Roepstorff P, et al. (2015) Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts. PNAS 110: 583-587.

48. Mueller UG (2002) Ant versus fungus versus mutualism: ant-cultivar conflict and symbiont choice in a fungus-farming ant Mycocepurus crenulatus: new light on the origin of higher attine agriculture. J Insect Sci 11: 12.

49. Weber NA (1957) Dry season adaptations of fungus-growing ants and their fungi. Ann Entomol Soc Am 52: 735-740.

50. Holldobler B, Wilson EO (1990) The ants. Cambridge: Harvard University Press. 737 p.

51. Rabeling C, Schulte TR, Currie CR, Adams RM, Malloch D (2001) The origin of the ant-fungus mutualism. Q Rev Biol 76: 169-197.

52. Rabeling C, Verhaagh M, Engel W (2007) Comparative study of nest architecture and colony structure of the fungus-growing ants, Mycocepurus goeldii and M. smithii. J Insect Sci 7: 40.

53. Holldobler B, Wilson EO (2016) The Leafcutter Ants: Civilization by Instinct. New York: Norton WW&Co. 160 p.

54. Mueller UG, Schultz TR, Currie CR, Adams RM, Malloch D (2001) The origin of the ant-fungus mutualism. Q Rev Biol 76: 169-197.

55. Fernandez M-A, Bermudez J, Rabeling C, Holldobler B, Wilson EO (2007) Comparative study of nest architecture and demography of a basal fungus-growing ant, Mycocepurus smithii (Hymenoptera: Formicidae). J Nat Hist 39: 1735-1743.

56. Schultz TR (1993) Stalking the wild ant. Notes from Underground. Cambridge, Mass.: Museum of Comparative Zoology, Harvard University 8: 7-10.

57. Ayres M, Ayres M Jr, Ayres DL, dos Santos AS (2007) BioEstat 5.0. Imprensa Oficial do Estado do Pará. 325 p.

58. Martins Jr, J, Solomon SE, Mikheyev AS, Mueller UG, Ortiz A, et al. (2007) Nuclear mitochondrial-like sequences in ants: evidence from Atta cephalotes (Formicidae: Attini). Insect Molec Biol 16: 777-784.

59. Manter DR, Viveiros JM (2007) Use of the ITS, primers ITS 1 F and ITS 4, to characterize fungal abundance and diversity mixed-template samples by qPCR and length heterogeneity analysis. J Microbiol Meth 71: 7-14.

60. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR Protocols: a guide to methods and applications. New York: Academic Press. 315-322.

61. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Series 41: 95-98.

62. Thompson JD, Higgins DG, Gibson TJ (1994) ClustalW: improving the sensitivity of multiple sequence alignment through sequence weighting, position specific gaps penalties and weight matrix choice. Nucleic Acids Res 22: 4673-4680.

63. Kellner K, Fernandez-Marin H, Ishik H, Sen R, Linksvayer TA, et al. (2013) Co-evolutionary patterns and differentiation of ant-fungus associations in the asexual fungus-farming ant Mycocepurus smithii in Panama. J Evolution Biol 26: 1353-1362.

64. Rosquists F, Haeckelbeck J (2003) McBayes: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research 30: 3059-3066. doi: 10.1093/nar/gkf436.

65. Posada D (2008) jModelTest: phylogenetic model averaging. Mol Biol Evol 25: 1253-1256.

66. Zwickl DJ (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. PhD dissertation, The University of Texas at Austin, USA.

67. Maddison DR, Maddison WP (2000). MacClade v4.0. Sunderland: Sinauer Association.

68. Ronquist F, Huelsenbeck J (2003) MrBayes: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.

69. Brown JM, Heidke SM, Lemmon AR, Moriarty Lemmon E (2010) When trees grow too long: investigating the causes of highly inaccurate Bayesian branch-length estimates. Syst Biol 59: 145-161.

70. Ward PS, Brady SG, Fisher BL, Schultz TR (2010) Phylogeny and biogeography of dolicohedrine ants: effects of data partitioning and relict taxon on historical inference. Syst Biol 59: 342-362.

71. Rambaut A, Drummond AJ (2007) Tracer v1. 5. Available: http://beast.bio.ed.ac.uk/Tracer. Accessed 2012 October 29.

72. Newton MA, Rafferty AE (1994) Approximate Bayesian inference with the weighted likelihood bootstrap. J R Stat Soc, B, 56: 3-40.

73. Suchard MA, Weiss RE, Sinchheimer JS, White TJ, editors. PCR Protocols: a guide to methods and applications. New York: Academic Press. 315-322.

74. Speigazzi C (1922) Descripción de hongos micélicos. Ver Mias La Plata 26: 166-173.

75. Bass M, Rabeling J. M., (1996) Leaf-cutting ants (Formicidae: Attini) prune their fungus to increase and direct its productivity. Funct Ecol 10: 55-61.

76. Powell RJ, Stradling DJ (1986) Factors influencing the growth of Attomyces bromatipes, a symbiont of attine ants. Trans Br Mycol Soc 87: 205-213.

77. Himler AG, Caldera EJ, Bae RC, Fernandez-Marin H, Mueller UG (2009) No sex in fungus-farming ants or their crops. Proc R Soc B doi: 10.1098/ rspb.2009.0313.

78. Mueller UG, Paulin J, Adams RMM (2000) Symbiont choice in a fungus-growing ant (Attini, Formicidae). Behav Ecol 15: 357-364.

88. August 2014 | Volume 9 | Issue 8 | e103800