C1 *Mycologia,* 101(2), 2009, pp. 000–000. DOI:

Free-living fungal symbionts (Lepiotaceae) of fungus-growing ants (Attini: Formicidae)

Tanya L. Vo

Section of Integrative Biology, Patterson Laboratories, 1 University Station C0930, University of Texas at Austin, Austin, Texas 78712

Ulrich G. Mueller

Section of Integrative Biology, Patterson Laboratories, 1 University Station C0930, University of Texas at Austin, Austin, Texas 78712, and Smithsonian Tropical Research Institute, Apartado 2072, Balboa, Republic of Panama

Alexander S. Mikheyev[1]

Section of Integrative Biology, Patterson Laboratories, 1 University Station C0930, University of Texas at Austin, Austin, Texas 78712

Abstract: Surveys of leucocoprinaceous fungi (Lepiotaceae, Agaricales, Basidiomycota) in the rainforests of Panama and Brazil revealed several free-living counterparts of fungi cultivated by primitive attine ants (the lower Attini, Formicidae, Hymenoptera), adding to two such collections identified in a survey by Mueller et al (1998). The accumulated evidence supports the hypothesis that perhaps all fungi of lower attine ants have close free-living relatives. Free-living counterparts of ant-cultivated fungi are collected most readily during the early rainy season; in particular these are free-living mushrooms of fungal counterparts that are cultivated as yeasts in gardens of ants in the *Cyphomyrmex rimosus* group. Free-living and symbiotic fungi of these yeast-cultivating ant species might represent a promising study system to compare the biology of sympatric, conspecific fungi existing outside versus inside the attine symbiosis.

Key words: basidiocarp, coevolution, fungus gardening, mutualism, symbiosis

INTRODUCTION

Fungus-growing ants in the Neotropical tribe Attini (Formicidae) obligately depend on the cultivation of fungi for food (Weber 1972, Mueller et al 2005). Their cultivated fungi are the sole source of food for the larvae and an important source for the adults (Weber 1972). Primitive attine lineages (the so-called ''lower attines'') use flower parts, arthropod frass, seeds, wood fragments or other similar plant debris as substrate on which they grow their fungi, whereas the leaf-cutting genera *Atta* and *Acromyrmex* primarily use freshly cut leaves and flowers (Weber 1972, Schultz and Meier 1995). Fungus-growing ants reach their greatest diversity in the rainforests of Amazonian South America, the region of their presumed evolutionary origin (Mueller et al 2001).

A subset of species of fungus-growing ants in the ''*ramosus*'' group of the lower-attine genus *Cyphomyrmex* are particularly unusual because they culture fungus as a yeast (a single-celled growth form) instead of mycelium (the hyphal, filamentous form typical of all other attines). In artificial culture however *Cyphomyrmex* yeast fungi revert to a filamentous phase, indicating that the *Cyphomyrmex* yeast cultivars are pleomorphs (Weber 1972). The most basal attine lineages all cultivate hyphal fungi, indicating that the ancestral attine ant cultivated hyphal instead of yeast gardens (Schultz and Meier 1995, Mueller 2002).

The great majority of attine fungi, including the yeast cultivars, belong to two genera, *Leucoagaricus* and *Leucocoprinus*, which together comprise tribe Leucocoprineae in family Lepiotaceae (Agaricales: Basidiomycota), a group of saprobic litter specialists (Johnson and Vilgalys 1998; Mueller et al 1998; Vellinga 2003, 2004). Because the most basal attine lineages cultivate leucocoprineous mutualists attine fungiculture likely originated with the cultivation of leucocoprineous fungi. Domestication of these cultivars probably was aided by the abundance of the Leucocoprineae in the tropics (Vellinga 2003, 2004), specialized as litter decomposers and occurring in the same microhabitats as leaf-litter-dwelling ants. In a unique event in attine ant evolution, one group in genus *Apterostigma* had switched secondarily to non-lepiotaceous fungi that are distantly related to lepiotaceous cultivars and that belong to a phylogenetically narrow group within the agaric family Pterulaceae (Munkacsi et al 2004, Villesen et al 2004). Apart from these pterulaceous mutualists, attine ants appear to be entirely specialized on a closely related group of leucocoprineous fungi (Chapela et al 1994).

Ant-cultivated fungi traditionally have been assumed to be incapable of a free-living existence outside the symbiotic association with ants (Weber 1972). However recent phylogenetic analyses of 553

Accepted for publication 10 November 2008.

[1] Corresponding author. Phone: 512-471-7619 Fax: 512-471-3878 E-mail: mikheyev@mail.utexas.edu

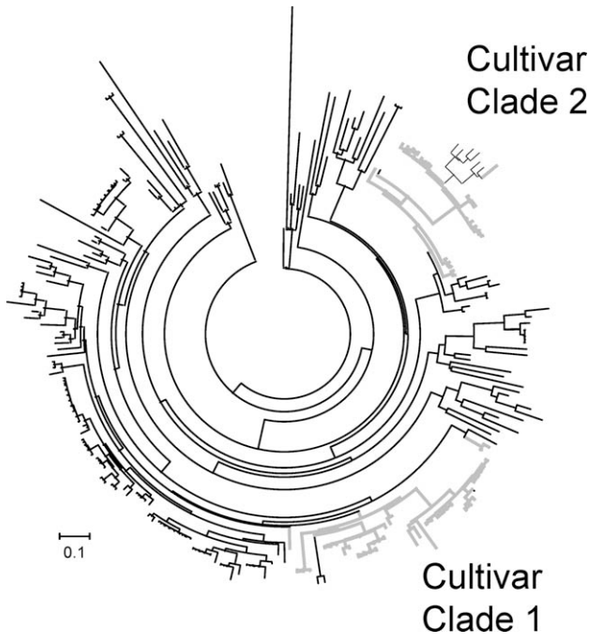

FIG. 1. Maximum likelihood phylogeny of the sampled fungi. As in Mueller et al 1998 there exist two large clades of cultivars (in gray), Clade 1 cultivars and Clade 2 cultivars. The scale bar corresponds to 0.1 substitutions/site.

fungi cultivated by primitive attine ants and 309 leucocoprinaceous mushrooms from Panama revealed two cultivated fungi that were more closely related to free-living leucocoprinaceous fungi than any other ant-associated fungus (Mueller et al [1998] includes photographs [FIG. 1] of these two free-living ant fungi). Each of these two free-living counterparts was sequence identical in the ITS r-DNA gene to a corresponding ant-cultivated fungus, indicating that these cultivated fungi were imported recently into the symbiosis or that the free-living counterparts recently escaped the symbiosis (Mueller et al 1998). In either case the discovery of free-living counterparts suggested that all cultivars of lower attine ants might have close links to free-living fungal populations.

To further test this hypothesized close links between symbiotic and free-living fungal populations we continued the survey of free-living leucocoprinaceous fungi from Panama in two subsequent years. These surveys concentrated on the collections of fungi fruiting during the early rainy season, a period when lepiotaceous fungi fruit most abundantly in Panamanian rainforests (UGM unpubl) and when the two known free-living counterparts had been collected previously.

MATERIALS AND METHODS

Collecting.—As in the 1996 survey by Mueller et al (1998), leucocoprinaceous fungi were collected in Parque Soberanía in central Panama, primarily in the forests near km 6 of Pipeline Road and the surrounds of the village of Gamboa. Leucocoprinaceous fungi were collected 1–27 May 1997 (13 collections) and 22 May–19 Jun 2002 (53 collections). Two leucocoprinaceous fungi from Texas (TX006, TX008) were collected in Austin in Apr 2002. Two additional leucocoprinaceous fungi (ASM1 and ASM2) were collected in Basse Terre, Guadeloupe, in Dec 2003. Three more leucocoprinaceous fungi had been collected during the 1996 survey but had not been part of the original Mueller et al (1998) analysis because the initial sequencing was unsuccessful. The Brazilian collection was surveyed during the rainy season, 20 Jan–25 Mar 2004, with 41 sequenced specimens. A total of 114 specimens therefore were part of this survey. Further details of the collection information are summarized along with corresponding GenBank records (see below). Mushroom collections were taken to the lab for morphological description or in the case of Brazilian fungi photographed in the field (descriptions and photographs available from the authors). Tissues from the stipe and cap were preserved in 100% ethanol and stored in the refrigerator until DNA extraction.

Naming convention.—Following Mueller et al (1998), cultivar samples are referred to by host ant species name (where applicable) and their collection code, as listed in the title of the corresponding GenBank record (e.g. ''Cyphomyrmex minutus S57'' is cultivar isolate S57 isolated from a nest of the ant species *Cyphomyrmex minutus*).

Molecular analyses.—Fungal tissue samples taken from the field were preserved in 100% molecular-grade ethanol for later extraction in the lab. A modified CTAB extraction was used to isolate DNA from fungal tissue (Mueller et al 1998). A small fragment of fungal tissue was removed from the ethanol vial and patted with porous paper to remove ethanol. The tissue was ground in liquid nitrogen with a mortar and pestle. A 10% CTAB solution was added and incubated at 65 C for 10 min. A chloroform isoamyl-alcohol treatment was performed, followed by two alcohol washes. Approximately 30 ng of template was amplified in 10 μL reaction-volume containing 0.65 mM of each primer (ITS4 and ITS5 [White et al 1990]), 0.31 mM of each dNTP, 1.85 mM $MgCl_2$, 0.031 U Biolase Taq, and 1.25 μL 10 × buffer (Bioline). PCR reaction was carried out on a MJ Research thermal cycler with an initial denaturation of 93 C for 3 min, followed by 35 cycles of 93 C for 1 min, 55 C for 1 min, 72 C for 2 min. The reaction was concluded by a final extension of 72 C for 10 min. PCR products were purified with polyethylene-glycol (PEG) precipitation, incubating a 1:1 mixture of PCR product with 2.5 M NaCl and 25 mM PEG 8000 for 15 min at 37 C, followed by two ethanol washes. Sequencing of the products was performed on an ABI 3100 Genetic Analyzer in accordance with the manufacturer's instructions. Forward and reverse sequences for ITS were analyzed with Seqman II and Sequencher 4.5 for the entire ITS region. The sequence data and alignment were deposited in GenBank under accession numbers EF527280-EF527400.

Phylogenetic analyses.—New sequences from the Panama-

nian and Brazilian collections were added to the original ITS alignment of Mueller et al (1998) with the profile alignment mode of Clustal (Thompson et al 1994). The addition of the new sequences made only slight changes to the alignment used in the Mueller et al (1998) analysis. The final alignment reconstructed the general features of the Mueller et al (1998) tree by generating an overall maximum likelihood tree with the default settings of GARLI (v. 0.95) (Zwickl 2006). Cultivars, as well as any free-living fungi nesting within two distinct cultivar clades, referred to as Clades 1 and 2 by Mueller et al (1998), were re-aligned and each clade was analyzed further separately. This step restricted further analyses to closely related fungi and corresponding ITS-sequences could be aligned unambiguously for two subanalyses (one analysis each for on Cultivar Clade 1 and 2 respectively). For each subanalysis some members of the other cultivar clade were included and used to root the phylogenies. For Clade 1 we used *Mycocepurus curvispinosus* CR10 and *Mycocepurus smithii* S60 for rooting. For Clade 2 we used *Myrmicocrypta infuscata* G11, *Cyphomyrmex minutus* FL02 and free-living fungus PA234 for rooting.

A DNA substitution model for each of the clades was determined with Modeltest (Posada and Crandall 1998). The most likely trees as well as bootstrap support values subsequently were computed with GARLI default settings and 100 pseudo-replicates. In addition Bayesian trees were computed with MrBayes with an appropriate DNA substitution model a temperature value of 0.1 (v. 3.1.1) (Huelsenbeck and Ronquist 2001). Each computation was continued until the average standard deviation of split frequencies between the two runs dropped below 0.01. Afterward the first 75% of the generations were discarded as burn-in and a majority rule consensus tree was computed.

RESULTS

The phylogenetic tree with all taxa included had the same overall topology as that of Mueller et al (1998) with two distinct clades of cultivar fungi (FIG. 1).

Under the distance or likelihood criteria two collections from Panama (PA607 and PA681) showed unambiguous, close affinities with ant-cultivated fungi. PA607 was nested firmly within the clade of ant-cultivated yeast fungi (FIG. 2A), and PA681 was nested within the Clade 2 cultivars (FIG. 2B). Jukes-Cantor differences between these free-living fungi and their closest cultivar relatives were zero between *Cyphomyrmex minutus* FL02 and PA607 and 0.44% between PA681 and *Mycocepurus curvispinosus* CR10. Likelihood bootstrap support for these placements is respectively 84% and 51%. Bayesian posterior probabilities for these placements are respectively 94% and 74%. In addition BR032 showed close affinity to some non-yeast Clade 1 cultivars, with 11% Jukes-Cantor-corrected divergence (FIG. 2A). All other free-living leucocoprinacous fungi collected in our survey fell outside the two known cultivar clades.

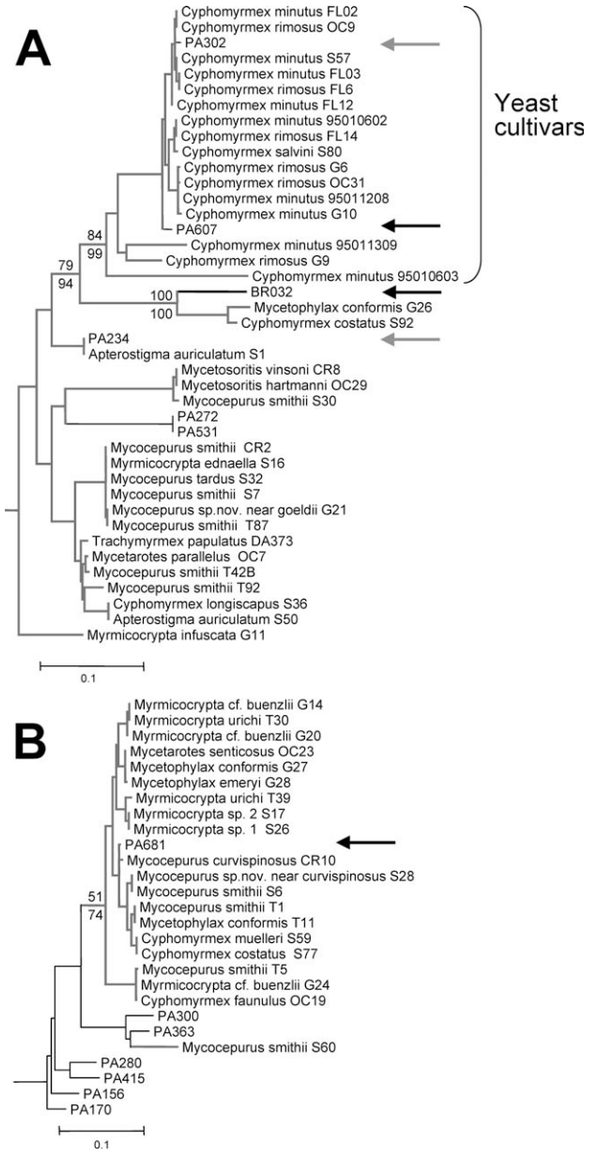

FIG. 2. Maximum likelihood (ML) phylogeny of the Clade 1 cultivars (A) and Clade 2 cultivars (B). Cultivars are highlighted in gray. Black arrows point to free-living fungi discovered in our survey as free-living cultivar counterparts. Gray arrows point to two free-living cultivar counterparts that were recognized by Mueller et al (1998). For selected nodes ML bootstrap support values and Bayesian posterior probabilities are provided above and below, respectively. The scale bar corresponds to 0.1 substitutions/site. The names of some of the ant species from which fungal cultivars were analyzed differ from the names used in Mueller et al (1998), conforming to recent taxonomic revisions of the exact position of some of the ant species (e.g. following Schultz et al 2002, *Cyphomyrmex muelleri* S59 was listed previously in Mueller et al 1998 as *Cyphomyrmex longiscapus* S59). Collections with labels starting with PA and BR represent respectively free-living fungi collected as fruiting bodies (mushrooms) in the Republic of Panama and Brazil.

DISCUSSION

Our survey for free-living ant-cultivated fungi added two collections to the known two collections described in Mueller et al (1998). One of these (PA607) was placed into the clade of ants cultivating yeasts; this collection therefore confirms the finding of Mueller et al (1998) that the yeast ant cultivars have free-living counterparts (note the placement of the collection PA302 within the clade of free-living cultivars in FIG. 2A). More important is the placement of PA681 within the Clade 2 cultivars (FIG. 2B); this collection is the first free-living cultivar from this second major group, documenting now that fungi from both Clade 1 and Clade 2 cultivars can exist either as symbiotic or nonsymbiotic fungi. These data support the hypothesis of Mueller et al (1998) (see also Mueller 2002) that fungi of lower attine ants have close free-living relatives. The evidence suggests that some fungi associated with attine ants might have left the symbiosis and completed their life cycle as free-living fungi. Finally our results generalize those of Mueller et al (1998) by showing for the first time free-living fungi associated with both lower fungus-gardening ant clades.

Although we generally did not observe ant nests in the vicinity of fruiting bodies, our surveys cannot completely rule out the existence of cryptic hyphal connections between fruiting bodies and cultivated fungus gardens. The existence of such connections appears especially unlikely in the case of yeast cultivars, for which fruiting bodies were found repeatedly. However it is possible that, like the fungus-gardening termites and apparently of some the higher fungus-gardening ants, ant-tended gardens nonetheless might fruit occasionally (Johnson et al 1981, Pagnocca et al 2001, Katoh et al 2002). Whether connections exist, the existence of extensive horizontal cultivar transfer, probably due to fruiting events, appears to be a dominant feature across the attine-fungus symbiosis (Green et al 2002; Mikheyev et al 2006, 2007).

It is unclear how frequently fungi enter or leave the association with lower attine ants, and future research should focus on estimating this frequency, as well as the relative proportion of free-living individuals versus ant-associated individuals of the same fungal species. Both parameters have major implications for the nature of ant-fungus coevolution. If the frequency of moving between free-living and symbiotic populations is high and if the majority of individuals exist under a free-living instead of a symbiotic conditions, the properties of ant-cultivated fungi will be dictated largely by the selective forces operating on the fungi during the free-living stage; little ant-fungus co-adaptation may exist under such a scenario because selection within the symbiosis cannot easily overrule selection outside the symbiosis. In contrast if most individuals remain within the symbiosis for significant evolutionary time and free-living stages are transient the potential for ant-fungus co-adaptation is much higher.

The most promising system for determining the relative proportion of individuals inside and outside the symbiosis might be the yeast-fungi cultivated by species in the *Cyphomyrmex rimosus* group. Mueller et al (1998 unpubl) collected four free-living individuals (sequence-identical to PA302 across the entire ITS region) at different locations in Panama during the early rainy season of 1996, and the present survey adds one additional free-living counterpart (PA607) and one close relative (BR032) to the list of known free-living yeast-fungi of *Cyphomyrmex* ants (FIG. 2A). All these free-living fungi were collected during the early rainy season, suggesting this period is a productive one for any future survey of free-living *Cyphomyrmex* fungi. Moreover ant nests of species in the *Cyphomyrmex rimosus* group are easily collected under or inside rotting logs on the rainforest floor and a detailed survey of *Cyphomyrmex*-associated fungi therefore can be accomplished with comparatively little collecting effort. Such a survey, focusing intensively on a restricted area, might reveal the degree of genetic admixture between free-living versus ant-associated fungi of the same fungal species existing in sympatry. At the same time detailed spatial information on genotype distribution might inform hypotheses on ant-mediated versus ant-independent dispersal of the free-living counterparts.

ACKNOWLEDGMENTS

We thank two anonymous reviewers for their comments on the manuscript. The research was supported by an REU supplement to NSF CAREER award DEB-9983879 and an undergraduate research award to TV from the University of Texas at Austin. ASM was supported by a fellowship from the US Environmental Protection Agency and a US Fulbright grant. We thank the Autoridad Nacional del Ambiente of the Republic of Panamá and the Smithsonian Tropical Research Institute for collecting permits, and we thank M. Leone for logistical support.

LITERATURE CITED

Chapela IH, Rehner SA, Schultz TR, Mueller UG. 1994. Evolutionary history of the symbiosis between fungus-growing ants and their fungi. Science 266:1691–1694.

Green AM, Adams RMM, Mueller UG. 2002. Extensive exchange of fungal cultivars betweeen sympatric species of fungus-growing ants. Mol Ecol 11:191–195.

Huelsenbeck JP, Ronquist F. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755.

Johnson J, Vilgalys R. 1998. Phylogenetic systematics of Lepiota *sensu lato* based on nuclear large subunit rDNA evidence. Mycologia 90:971–979.

Johnson RA, Thomas RJ, Wood TG, Swift MJ. 1981. The inoculation of the fungus comb in newly founded colonies of some species of the Macrotermitinae (Isoptera) from Nigeria. J Nat His 15:751–756.

Katoh H, Miura T, Maekawa K, Shinzato N, Matsumoto T. 2002. Genetic variation of symbiotic fungi cultivated by the macrotermitine termite Odontotermes formosanus (Isoptera: Termitidae) in the Ryukyu Archipelago. Mol Ecol 11:1565–1572.

Mikheyev AS, Mueller UG, Abbot P. 2006. Cryptic sex and many-to-one coevolution in the fungus-growing ant symbiosis. Proc Natl Acad Sci USA, 10702–10706.

———, ———, Boomsma JJ. 2007. Population genetic signatures of diffuse co-evolution between leaf-cutting ants and their cultivar fungi. Mol Ecol 16.

Mueller UG. 2002. Ant versus fungus versus mutualism: ant-cultivar conflict and the deconstruction of the attine ant-fungus symbiosis. Am Nat 160:S67–S98.

———, Gerardo NM, Aanen DK, Six DL, Schultz TR. 2005. The evolution of agriculture in insects. Ann Rev Ecol Evol Sys 36:563–595.

———, Rehner SA, Schultz TR. 1998. The evolution of agriculture in ants. Science 281:2034–2038.

———, Schultz TR, Currie CR, Adams RMM. 2001. The origin of the attine-fungus symbiosis. Q Rev Biol 76: 169–197.

Munkacsi AB, Pan JJ, Villesen P, Mueller UG, Blackwell M, McLaughlin DJ. 2004. Convergent coevolution in the domestication of coral mushrooms by fungus-growing ants. Proc R Soc Lond B Biol Sci 271:1777–1782.

Pagnocca FC, Bacci M, Fungaro MH, Bueno OC, Hebling MJ, Sant'Anna A, Capelari M. 2001. RAPD analysis of the sexual state and sterile mycelium of the fungus cultivated by the leaf-cutting ant *Acromyrmex hispidus fallax*. Mycol Res 105:173–176.

Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818.

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.

Thompson JD, Higgins DG, Gibson TJ. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nuc Acid Res 22:4673–4680.

Vellinga EC. 2003. Phylogeny of Lepiota (Agaricaceae)—evidence from nrITS and nrLSU sequences. Mycol Progr 2:305–322.

———. 2004. Ecology and distribution of lepiotaceous fungi—a review. Nov Hedwig 78:273–299.

Villesen P, Mueller UG, Schultz TR, Adams RMM, Bouck AC. 2004. Evolution of ant-cultivar specialization and cultivar switching in *Apterostigma* fungus-growing ants. Evolution 58:2252–2265.

Weber NA. 1972. Gardening ants, the attines. Philadelphia: Memoirs of the American Philosophical Society.

Zwickl DJ. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion [Doctoral dissertation]. University of Texas at Austin

Query markers appear in boxes in the margins as a question mark followed by a number. Please respond to each query in the margin of the corresponding proof page. Comment markers are suggested corrections that will also appear in the margin, but as a "C" followed by a number. If you wish to implement the suggested correction, please transfer the correction to the margin and text of the corresponding proof page.

C1. Add "10.3852/07-055" after "DOI:"