

# Extensive exchange of fungal cultivars between sympatric species of fungus-growing ants

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## Abstract

Fungal cultivars of fungus-growing ants (Attini, Formicidae) are carried by dispersing queens from parent to offspring nest. This vertical cultivar transmission between generations is thought to result in long-term ant-fungus coevolution and selection for beneficial cultivar traits that maximize harvests and thus colony productivity. In contrast to this traditional view of vertical cultivar transmission, frequent horizontal cultivar transmission between ant species is implicated by a phylogenetic analysis of 72 cultivars propagated by two fungus-growing ant species coexisting sympatrically in central Panama. The two ant species are specialized on the same group of closely related cultivars, but in six of 12 cultivar clades identifiable within this group, cultivars from both ant species were united in the same clade. Five of these 'mixed' clades were supported by bootstrap values of about 90% or higher. In one instance, colonies from the two ant species cultivated the same, genetically identical, cultivar clone. These phylogenetic patterns indicate that: (i) cultivar exchanges between the two ant species occur routinely throughout ecological time; and that (ii) coevolutionary processes between ants and their fungi are more diffuse than previously assumed. Because the two ant species are specialized on a narrow group of closely related cultivars that they regularly exchange among each other, but not with other sympatric ant species, cultivar exchanges are constrained, most likely, by ant preferences for their own cultivar group or by stringent selection against transitions of ant lineages to distantly related cultivars.

*Keywords:* Attini, coevolution, *Cyphomyrmex*, fungus-growing ants, horizontal transmission, specialization

Received 13 July 2001; revision received 22 October 2001; accepted 22 October 2001

## Introduction

The ability to cultivate fungi for food evolved about 50–60 million years ago in the ancestor of fungus-growing ants (tribe Attini, Formicidae) as part of a transition from hunter-gatherer to fungus-farmer ant (Hölldobler & Wilson 1990; Mueller *et al.* 2001). All of the ~200 extant attine ant species are obligately dependent on cultivated fungi for food. As far as is known, all attine ants also propagate their cultivars clonally (asexually) within nests and during the transfer of starter inocula by dispersing queens from parent to offspring nest (von Ihering 1898; Huber 1905; Weber 1972; Mueller *et al.* 1996). Recent phylogenetic analyses of the higher-level relationships between cultivated fungi have implicated evolutionarily

unique switches of ant lineages to novel, distantly related cultivars (Chapela *et al.* 1994; Mueller *et al.* 1998), but the resolution of these higher-level analyses was limited, and only a moderate level of switching was detected. Additional population genetic analyses revealed geographically widespread clonal lineages propagated by single species of fungus-growing ants (Mueller *et al.* 1996, 1998), consistent with the traditional view that transmission of attine cultivars occurs largely vertically through inheritance of vegetative cultivar clones from maternal to offspring nest (Wheeler 1907; Weber 1972; Hölldobler & Wilson 1990). Taken together, phylogenetic and population genetic patterns seemed to indicate that ant-fungus coevolution is tight within ant species, but that coevolution is punctuated occasionally by lateral cultivar transfers between distantly related ant lineages (Chapela *et al.* 1994; Mueller *et al.* 1998).

The low-resolution analyses of the previous higher-level phylogenetic reconstructions (Chapela *et al.* 1994; Mueller

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*et al.* 1998) do not rule out the possibility of frequent cultivar transfers between nests of the same ant species, or between nests of different ant species that cultivate closely related cultivars. Indeed, in a laboratory experiment testing for horizontal transfer of cultivars as replacements after pathogen-induced garden loss, intraspecific exchanges involving nests with closely related cultivars were almost four times more likely than interspecific exchanges involving nests with distantly related cultivars (Adams *et al.* 2000). Under natural conditions therefore cultivar transfers may be common if newly acquired and substituted (lost) cultivars are closely related.

We tested this hypothesis by examining the phylogenetic relationships between cultivars propagated by the two sympatric fungus-growing ant species *Cyphomyrmex costatus* and *Cyphomyrmex sp.nov.* [formerly a variant of *C. longiscapus* (Schultz *et al.* in preparation); see below] in central Panama. Although *C. costatus* and *C. sp.nov.* are not closely related (Snelling & Longino 1992; Schultz *et al.* in preparation), their cultivars are sequence-identical in two fast-evolving gene regions (ITS1 and ITS2) (Mueller *et al.* 1998), indicating the phylogenetic proximity of the cultivars. The phylogenetically primitive fungus-growers *C. costatus* and *C. sp.nov.* are abundant in the rainforests of Panama, but the two species occupy different microhabitats with little niche overlap: *C. costatus* constructs nests under logs or rocks on the rainforest floor (Weber 1957), whereas *C. sp.nov.* constructs shallow nests in the clay of steep embankments along streams (Mueller & Weislo 1998).

## Materials and methods

### *Ant species and cultivar sampling*

Seventy-two fungal cultivars were isolated from 24 *Cyphomyrmex costatus* and 48 *C. sp.nov.* nests collected between 1995 and 1998 at the following sites in the Republic of Panama: Fort Sherman (*C. sp.nov.*  $n = 13$ ; *C. costatus*  $n = 8$ ), Pipeline Road (*C. sp.nov.*  $n = 18$ ; *C. costatus*  $n = 4$ ), Barro Colorado Island (*C. sp.nov.*  $n = 16$ ; *C. costatus*  $n = 2$ ), Ancon Hill (*C. costatus*  $n = 5$ ), Gamboa (*C. costatus*  $n = 5$ ), and El Llano (*C. sp.nov.*  $n = 1$ ). *C. costatus* occurs in forested areas throughout Panama. *C. sp.nov.* is known only from the Atlantic slope of the Panama Canal Zone and is the undescribed sister species to the more widely distributed *C. longiscapus*, from which it was separated recently based on molecular and morphological evidence (Schultz *et al.* in preparation). Thus *C. sp.nov.* is more closely related to *C. longiscapus* than to *C. costatus*, but *C. sp.nov.* and *C. longiscapus* cultivate very distantly related fungi (Mueller *et al.* 1998), whereas the more distantly related *C. sp.nov.* and *C. costatus* cultivate very closely related fungi (Mueller *et al.* 1998). Fungal cultivars were isolated from live gardens for liquid-culturing of axenic

isolates (Mueller *et al.* 1996), and the lyophilized mycelium was stored at  $-80^{\circ}\text{C}$  until DNA extraction. Genomic DNA was extracted from 25 mg of powdered mycelium using Qiagen Dneasy<sup>TM</sup> columns, then diluted to 9 ng/ $\mu\text{L}$ .

### *Amplified fragment length polymorphism (AFLP) marker development and phylogenetic analyses*

AFLP markers (Mueller & Wolfenbarger 1999) were generated on an ABI Prism 3100 Genetic Analyser and scored in GENESCAN 3.1 and GENOTYPER 2.5. AFLP restriction-ligation reactions, preselective and selective amplification reactions followed the AFLP<sup>TM</sup> protocol for small plant genomes (<http://www.pebiiodocs.com/pebiiodocs/00100509.pdf>). Of 24 *MseI*\**EcoRI* primer combinations screened in a preliminary analysis, the following seven primer combinations were chosen because they generated high levels of reliable polymorphisms: CAG/AC, CAA/AC, CAT/TC, CTC/TC, CAG/TC, CAT/TT and CTC/AT. AFLP markers were scored blindly by simultaneously comparing fragments of a given base-pair length across all 72 cultivars. Only markers (peaks) that could be scored as unambiguously present/absent across all 72 samples were used in the analysis. The final AFLP presence/absence matrix included 127 informative characters and was analysed under the parsimony criterion in PAUP\* 4.1 (Swofford 2000) using a heuristic search with 500 replicates (Maxtree set to 100 000). The parsimony bootstrap analysis included 1000 pseudo-replicates (10 random-addition replicates per pseudoreplicate; Maxtree = 100 000).

## Results

Parsimony analysis of the 127 informative AFLP markers from 72 cultivars identified 19 200 equally most-parsimonious trees (length = 533; CI = 0.235; RI = 0.681), the consensus of which is shown in Fig. 1(a). In six of 12 identifiable cultivar clades, cultivars from both *Cyphomyrmex sp.nov.* and *C. costatus* were united in the same clade, implicating six cultivar transfers between the two species. Five of these transfers are well-supported with bootstrap values of about 90% or higher (Fig. 1a). In one case, a *C. costatus* cultivar was identical to a *C. sp.nov.* cultivar across all 127 AFLP markers scored (see clade marked with an asterisk in Fig. 1a), uniting these two cultivars therefore with a bootstrap support of 100%.

## Discussion

### *Frequency and recency of cultivar transfers*

Phylogenetic analyses identified six instances where a clade of *Cyphomyrmex costatus* cultivars was most closely related to a clade of *C. sp.nov.* cultivars (Fig. 1a); five of



(Frank 1996a,b, 1997). These additional factors include the cultivar diversity within single ant colonies (cultivar competition; Frank 1996a,b; Bot *et al.* 2001), the rate of natural garden loss (cultivar clearance; Frank 1996a), and possibly also strong selection in the form of ant preferences for productive cultivars (symbiont choice; Mueller *et al.* 2002). Contrary to the assumptions of simple virulence models, for example, horizontal cultivar transfers among ant lineages can be triggered by garden losses followed by cultivar acquisition from neighbouring colonies (Adams *et al.* 2000). Productive ('nonvirulent') cultivars therefore experience positive selection during reacquisition by cultivar-deprived colonies, because such cultivars are the ones that produce healthy and stable gardens in neighbouring colonies and thus are most likely acquired as replacements. Future research should aim to estimate the key parameters of cultivar virulence/productivity evolution in attine cultivars, specifically the genetic cultivar diversity within single ant colonies, the rate of cultivar loss and replacement, and the strength of selection due to the preferential acquisition of productive over unproductive cultivars during cultivar transfers.

#### *Factors modulating cultivar exchanges*

Nest-proximity cannot explain the extensive cultivar exchange between the two fungus-growing ant species, because: (i) *C. sp.nov.* and *C. costatus* occupy different, minimally overlapping microhabitats (see above); and (ii) *C. sp.nov.* occurs in dense, mixed aggregations with its putative sister-species *C. longiscapus* [which propagates a cultivar that is distantly related to the *C. sp.nov./C. costatus* cultivar (see methods above; Mueller *et al.* 1998; Schultz *et al.* in preparation)], yet, despite the close physical proximity, no exchanges between the sister species *C. longiscapus* and *C. sp.nov.* have been observed in hundreds of nests collected from natural, mixed populations (Schultz *et al.* in preparation; U.G.M. personal observation). Similarly, *C. costatus* does not exchange cultivars with other attine species that co-occur with *C. costatus* under logs (Mueller *et al.* 1998), such as the primitive fungus-growers *C. minutus*, *C. rimosus* and *Apterostigma auriculatum* (Weber 1957, 1972; U.G.M. personal observation). Extensive cultivar transfers between *C. sp.nov.* and *C. costatus*, despite habitat separation of the two ant species, could indicate that some of the cultivar transfers may not be direct between nests, but may occasionally be indirect via an intermediate stage of a 'feralized' cultivar clone (Diamond 1998) that is reimported into the mutualism after an existence in a free-living cultivar population.

Like free-living mutualist populations known from other mutualisms (Douglas 1996; Herre *et al.* 1999), a hypothetical free-living cultivar population could consist of feralized cultivar clones that are embedded into a larger,

sexually recombining cultivar population. Two observations actually support this hypothesis: first, the ability of *C. costatus* cultivars to produce perfect sexual structures (mushrooms) and meiotic spores in artificial culture (Weber 1957), indicating recent selective maintenance of the capacity for sexual reproduction; and second, the starlike evolutionary relationships between the *C. sp.nov./costatus* cultivars (Fig. 1a), where the basal polytomy, unresolvable even with the large number of informative markers, could derive from a cohesive population of sexually recombining individuals, whereas the resolvable radii of the phylogenetic star derive from strictly clonal propagation and independent evolutionary histories of asexual cultivars under vertical and horizontal cultivar transmission. The possible existence of free-living cultivar populations suggests that the *C. sp.nov./costatus* ant-cultivar association may not be fundamentally different from other specialized mutualisms with connections to free-living populations, such as many lichens and endophytic fungi, the coral polyp-algae mutualism, or the diverse mutualistic associations between algae and aquatic invertebrates (Douglas 1996; Herre *et al.* 1999).

*C. costatus* and *C. sp.nov.* exchange cultivars only with each other, but not with other sympatric attine species, implicating constraints on cultivar transfers between fungus-growing ants. These constraints could include: (i) sensory preferences by the ant farmers for only a limited diversity of cultivars, thus largely precluding transitions to phylogenetically distant cultivars; (ii) physiological ant-cultivar matches (Martin 1987) that induce ant incompatibilities with unmatched, distantly related cultivars, as hypothesized by Bot *et al.* (2001); and (iii) antibiotic matches between specialized garden parasites and antibiotic-producing actinomycete bacteria grown by the ants on their cuticle for defence against these parasites (Currie *et al.* 1999a,b), where mismatched actinomycete antibiotics cannot prevent garden loss due to the invasion of parasites specialized to infest gardens of newly acquired, distantly related cultivars. The latter hypothesis makes the testable prediction that a successful transition to a novel, distantly related cultivar requires also the simultaneous acquisition of novel actinomycete defences. Experimentation on sensory preferences of the ant farmers for certain cultivars, as well as on the performance of experimental combinations of ant hosts, cultivars, parasites and actinomycete bacteria, are therefore promising routes to elucidate further the coevolutionary ecology of the attine ant-microbe symbiosis.

#### **Acknowledgements**

We thank the Autoridad Nacional del Ambiente of the Republic of Panama and the Smithsonian Tropical Research Institute for research permits; A. Herre, M. Leone and W. Wcislo for logistical support; M. O'Herron for liquid-culturing of fungi; D. Zwickl and

T. Schultz for advice on the phylogenetic analyses; and J. Boomsma, J. Bull, C. Currie, N. Gerardo, A. Himler, M. Kirkpatrick, M. Kweskin, N. Mehdiabadi, J. Sachs, S. Sanchez-Peña, T. Schultz, and two anonymous reviewer for comments on the manuscript. Ant and cultivar vouchers are deposited at the Museum of Natural History, Smithsonian Institution, Washington DC. Funding was provided by a NSF Research Experience for Undergraduates award to A.M.G. (supplement to NSF award DEB-9707209), an Undergraduate Research Award to A.M.G. from the University of Texas at Austin, and NSF CAREER award DEB-9983879 to U.G.M.

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