

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

A. M. GREEN, U. G. MUELLER and R. M. M. ADAMS

Section of Integrative Biology, University of Texas, Austin TX 78712, USA

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

Correspondence: Ulrich G. Mueller. Fax: 512 4713878; E-mail: umueller@mail.utexas.edu

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°C until DNA extraction. Genomic DNA was extracted from 25 mg of powdered mycelium using Qiagen DneasyTM columns, then diluted to 9 ng/ μL .

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 AFLPTM 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

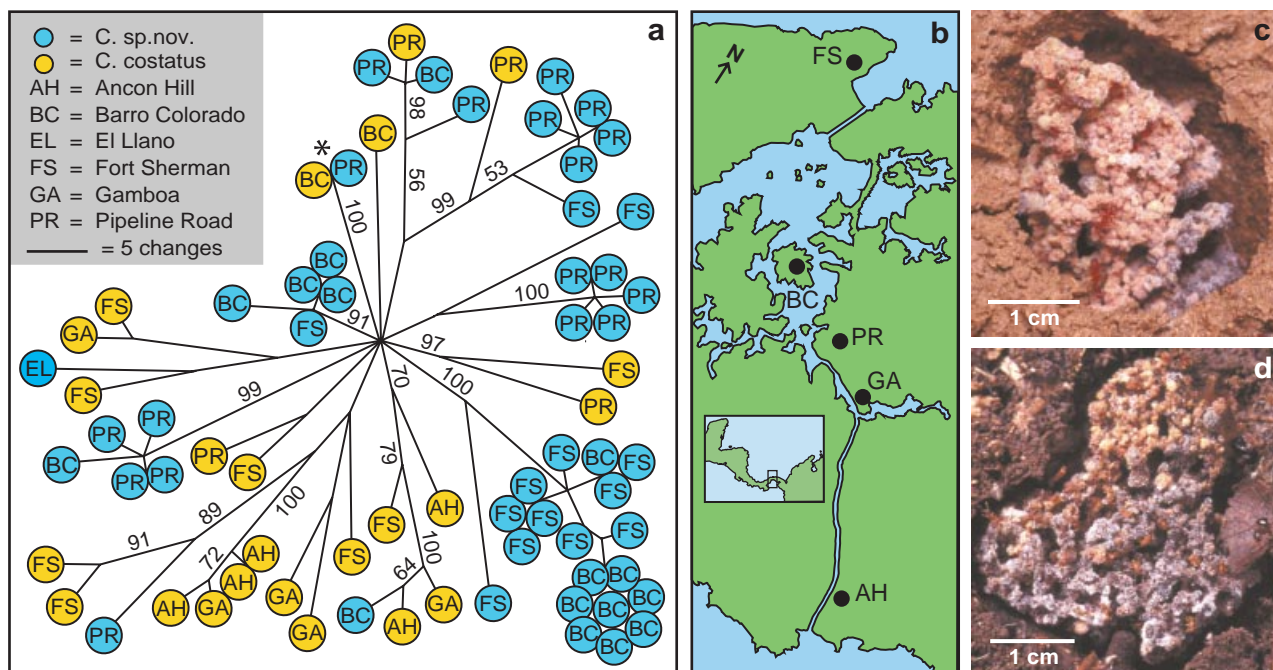


Fig. 1 Exchange of fungal cultivars between the two sympatric fungus-growing ant species *Cyphomyrmex costatus* and *C. sp.nov.* (a) Phylogenetic relationships between 72 cultivars propagated by *C. costatus* (yellow) and *C. sp.nov.* (blue) indicate at least six cases of recent cultivar transfers (grouping of cultivars from both ant species into the same clade); five of these transfers have bootstrap support of about 90% or higher. The asterisk marks two cultivars, one each from the two ant species, that are identical across all 127 AFLP markers (see text). (b) Collecting sites along the Panama Canal across the Isthmus of Panama (insert); El Llano (EL) is located about 80 km east of the Panama Canal and is therefore not indicated on the map. (c) Garden of *C. sp.nov.* in a shallow cavity in a clay embankment. (d) Garden of *C. costatus* under a log on the rainforest floor.

these *C. costatus/sp.nov.* groupings have bootstrap support of about 90% or higher. These sister-group relationships and the interdigitated phylogenetic patterns reveal the multiple cultivar transfers from one species to the other that must have occurred during recent cultivar evolution. The transfers appear to be bidirectional between the ant species, because single *C. costatus/sp.nov.* cultivars are sometimes nested within larger clades of the other ant species (Fig. 1a). In one case, a *C. costatus* cultivar was identical to a *C. sp.nov.* cultivar across all 127 AFLP markers scored; these identical AFLP 'fingerprint' profiles document a very recent transfer of a cultivar clone from *C. costatus* to *C. sp.nov.* (or vice versa), so recent that the two cultivar clones have yet to diverge across the fast-evolving AFLP markers. Cultivar exchange between the two sympatric ant species therefore emerges as an ongoing and frequent process, and more comprehensive sampling of cultivars is likely to reveal additional cases of cultivar sharing between the two ant species.

The surprisingly high level of cultivar exchange implies that a single ant lineage may interact with different cultivar clones in short evolutionary succession (or, vice versa, a single cultivar lineage may be propagated in succession by several ant lineages). Single ant lineages are therefore not

locked in a tight coevolutionary process with single cultivar lineages over long evolutionary time. Rather, because cultivar clones are passed around on a local level between different attine species, ant-cultivar coevolution appears substantially more diffuse (Futuyma & Slatkin 1983) than previously thought (Weber 1972; Hölldobler & Wilson 1990; Chapela *et al.* 1994; Mueller *et al.* 1998).

Productive cultivars under frequent horizontal transmission

The theory of symbiotic interaction predicts that even low levels of horizontal transmission can intensify selection for host-fitness reducing, 'selfish' (in the sense of 'virulent' as defined by Frank 1996a,b, 1997) cultivar traits that promote horizontal cultivar dispersal at the expense of garden productivity, thus compromising the fitness of the ant hosts (and consequently reducing the likelihood of vertical cultivar transmission for the fungus). However, simple trade-off models based on the relative frequencies of vertical vs. horizontal transmission may not be able to predict trajectories of virulence evolution in the *C. costatus/sp.nov.* ant-cultivar system, because additional factors are likely to modulate virulence evolution and cultivar productivity

(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.

References

- Adams RMM, Mueller UG, Holloway AK, Green AM, Narozniak JM (2000) Garden sharing and garden stealing in fungus-growing ants. *Naturwissenschaften*, **87**, 491–493.
- Bot ANM, Rehner SA, Boomsma JJ (2001) Partial incompatibility between ants and symbiotic fungi in two sympatric species of *Acromyrmex* leaf-cutting ants. *Evolution*, **55**, 1980–1991.
- 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.
- Currie CR, Scott JA, Summerbell RC, Malloch D (1999a) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature*, **398**, 701–704.
- Currie CR, Mueller UG, Malloch D (1999b) The agricultural pathology of ant fungus gardens. *Proceedings of the National Academy of Sciences of the USA*, **96**, 7998–8002.
- Diamond J (1998) Ants, crops and history. *Science*, **281**, 1974–1975.
- Douglas AE (1996) The ecology of symbiotic micro-organisms. *Advances in Ecological Research*, **26**, 69–103.
- Frank SA (1996a) Models of parasite virulence. *Quarterly Review of Biology*, **71**, 37–78.
- Frank SA (1996b) Host-symbiont conflict over the mixing of symbiotic lineages. *Proceedings of the Royal Society of London*, **268**, 1033–1039.
- Frank SA (1997) Models of symbiosis. *American Naturalist*, **150**, S80–S99.
- Futuyma DJ, Slatkin M (1983) *Coevolution*. Sinauer Associates, Sunderland.
- Herre EA, Knowlton N, Mueller UG, Rehner SA (1999) The evolution of mutualisms: exploring the paths between conflict and cooperation. *Trends in Ecology and Evolution*, **14**, 49–53.
- Hölldobler B, Wilson EO (1990) *The Ants*. Harvard University Press, Cambridge.
- Huber J (1905) Über die Koloniegründung bei *Atta sexdens*. *Biologisches Centralblatt*, **25**, 606–619, 625–635.
- Martin MM (1987) *Invertebrate–Microbial Interactions*. Cornell University Press, Ithaca.
- Mueller UG, Wcislo WT (1998) Nesting biology of the fungus-growing ant *Cyphomyrmex longiscapus* Weber (Attini, Formicidae). *Insectes Sociaux*, **45**, 181–189.
- Mueller UG, Wolfenbarger LL (1999) AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution*, **14**, 389–394.
- Mueller UG, Lipari SE, Milgroom MG (1996) Amplified Fragment Length Polymorphism (AFLP) fingerprinting of fungi cultured by the fungus-growing ant *Cyphomyrmex minutus*. *Molecular Ecology*, **5**, 119–122.
- Mueller UG, Rehner SA, Schultz TR (1998) The evolution of agriculture in ants. *Science*, **281**, 2034–2038.
- Mueller UG, Schultz TR, Currie CR, Adams RMM, Malloch D (2001) The origin of the attine ant-fungus mutualism. *Quarterly Review of Biology*, **76**, 169–197.
- Mueller UG, Poulin J, Adams RMM (2002) Symbiont choice in a fungus-growing ant (Attini, Formicidae). *Behavioral Ecology*, in press.
- Snelling RR, Longino JT (1992) Revisionary notes on the fungus-growing ants in the genus *Cyphomyrmex*. In: *Insects of Panama and Mesoamerica* (eds Quintero D, Aniello A), pp. 481–494. Oxford University Press, New York.
- Swofford D (2000) *PAUP* 4.1: Phylogenetic Analysis Using Parsimony (*And Other Methods)*. Sinauer Associates, Sunderland.
- von Ihering H, (1898) Die Anlagen neuer Colonien und Pilzgärten bei *Atta sexdens*. *Zoologischer Anzeiger*, **21**, 238–245.
- Weber NA (1957) Fungus-growing ants and their fungi: *Cyphomyrmex costatus*. *Ecology*, **38**, 480–494.
- Weber NA (1972) *Gardening Ants: the Attines*. American Philosophical Society, Philadelphia.
- Wheeler WM (1907) The fungus-growing ants of North America. *Bulletin of the American Museum of Natural History*, **23**, 669–807.

The authors share an unabashed interest in fungus-growing ants. Abigail Green is an undergraduate student completing a dual degree in conservation ecology and English; her work was supported by a NSF Research Experience for Undergraduates award. Rachele Adams is a behavioural and molecular ecologist working in the laboratory of U.G.M. Ulrich Mueller is interested in behavioural ecology, molecular ecology, chemical ecology and evolution.
