

Symbiont choice in a fungus-growing ant (Attini, Formicidae)

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Cultivars of fungus-growing (attine) ants are vertically transmitted through inheritance from parent to offspring nest, but horizontal cultivar transfer between ant nests occurs occasionally, resulting in cultivar replacement within ant lineages. Two mechanisms could theoretically prevent the invasion of suboptimal cultivar strains and thus stabilize ant–cultivar coevolution: first, partner feedback inherent in vertical cultivar transmission and second, partner (symbiont) choice if the ants differentiate between productive and inferior cultivars during replacements. To elucidate the nature of symbiont choice, we presented workers of *Cyphomyrmex muelleri* with novel cultivars representing a phylogenetic cline of close and distant relatives of the native *C. muelleri* cultivar. Workers invariably preferred their native cultivar, discriminating against even very close relatives of the native cultivar. When given a choice between two non-native cultivar strains, workers accepted the strain most closely related to their native cultivar. Two conclusions emerge. First, colony switches to distantly related cultivars are behaviorally unlikely and may not be preference-based; rather, distant switches may occur under constrained choice, such as pathogen-related garden losses that force colonies to import novel cultivars. Second, the ability of attine ants to differentiate between closely related cultivar strains suggests that the ant–fungus mutualism is stabilized evolutionarily not only by partner feedback inherent in vertical cultivar transmission, but possibly also by symbiont choice through which the ants select against unwanted, presumably inferior, cultivars. The efficacy of symbiont choice now needs to be tested experimentally. Such research may benefit from application of theory and experimental paradigms that have been developed within the areas of mate choice and sexual selection. *Key words*: Attini, *Cyphomyrmex*, fungus-growing ant, mutualism, symbiont choice, symbiosis. [*Behav Ecol* 15:357–364 (2004)]

All of the 210 known fungus-growing ant species in the tribe Attini are obligate fungivores, depending on fungal gardens to grow an exclusive diet for larvae and a partial diet for adult ants (Mueller, 2002; Mueller et al., 2001; Stradling, 1978; Weber, 1972). The ants in turn provide the fungus with vegetable material, fecal manure, shelter, and protection from fungivores and pathogens (Currie et al., 1999, 2003; Martin, 1987). Attine cultivars have long been thought to be vegetatively propagated (as asexual clones) by the ants within the nest and also from parent to offspring nest (Weber, 1972), and genotyping analyses have indeed confirmed a clonal genetic signature within cultivar populations (Green et al., 2002; Mueller et al., 1996, 1998). Clonal propagation between ant generations is based on the nest-founding behavior typical for attine foundress queens, which carry a small piece of natal fungus with them on their nuptial flight and use this inoculum to start their own gardens (Bruch, 1923; Huber, 1905a,b; Ihering, 1898; Mueller, 2002; Mueller et al., 2001).

Observations of cultivar transmission from maternal to offspring nest during nest-founding led to the traditional view that cultivar propagation is strictly vertical within ant lineages (Weber, 1972), which in turn generated two widespread preconceptions in the literature: first, that cultivars are strictly vertically transmitted within ant lineages over millions of year and, second, that the observed vertical inheritance of cultivars closely aligns evolutionary interests between ant farmers and their cultivars (sensu Herre et al., 1999). Both of these

preconceptions have recently been challenged (Bot et al., 2001; Green et al., 2002; Mueller et al., 1998), prompting a revision of the complexities of ant–cultivar coevolution (Mueller, 2002).

The first traditional preconception claims that cultivars have been vertically propagated as ancient clones for millions of years, potentially dating back to the origin of attine fungiculture 50–60 million years ago (Chapela et al., 1994; Weber, 1972). However, recent phylogenetic and population genetic analyses invalidated this view by refuting the rather simplistic scenario of a single domestication event followed by millions of years of tight coevolution. Specifically, population genetic patterns and field observations indicated that cultivars are occasionally transferred between ant lineages (Autuori, 1950; Bot et al., 2001; Green et al., 2002; Mueller, 2002; Mueller et al., 1998; Rissing et al., 1989). Horizontal transfer of cultivars is generally constrained in the sense that entire ant clades associate with only specific fungal clades, but ants appear to switch relatively easy between cultivars from within their respective cultivar clade (Bot et al., 2001; Mueller, 2002; Mueller and Gerardo, 2002; Mueller et al., 1998). For example, in the case studied in greatest detail so far, the two sympatric ant species *Cyphomyrmex costatus* and *C. muelleri* regularly exchange cultivars between each other, and even higher levels of cultivar exchange probably exist within each of these two ant species (Green et al., 2002). Neither of the two *Cyphomyrmex* species cultivates any fungus outside their phylogenetically narrow clade of cultivars (possibly a single cultivar species on which the two ant species appear fully specialized), but widespread cultivar exchange between the two ant species clearly violates the traditional preconception of strict vertical transmission within single ant lineages.

The second traditional preconception claims that vertical inheritance of cultivars closely aligns evolutionary interests

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between ant farmers and their cultivars: What is evolutionarily good for the ants must also be good for the fungi (and vice versa), and the ant–fungus mutualism therefore should exhibit highly integrated features of cooperation through reciprocated exchanges of benefits (i.e., the ant–fungus mutualism should be largely free of conflict of interest between the two mutualistic partners). This preconception is invalid in that, first, ant–cultivar conflict can exist even under strict vertical cultivar transmission (e.g., over the ant sex ratio, because cultivars are exclusively maternally transmitted, and males are therefore “useless” to the fungus; Mueller, 2002); and second, the documented horizontal transfer of cultivars between ant lineages should further erode the alignment of evolutionary interest between the ant and cultivar partners (Mueller, 2002).

The two traditional preconceptions have prevented so far a proper and comprehensive analysis of the evolutionary mechanisms stabilizing the attine ant–fungus mutualism. In particular, of the two mechanisms that can stabilize cooperative interactions between species, partner choice and partner fidelity feedback (Bull and Rice, 1991; Noë, 2001; Sachs et al., 2004), only the latter has been emphasized in attine research. Partner choice (symbiont choice) operates via active discrimination against unproductive (“selfish” sensu Frank, 1996, 1997) partners, whereas partner fidelity is an automatic feedback mechanism in which uncooperative partners ultimately curtail their own fitness by harming the other partner’s fitness. Partner choice and partner fidelity often operate jointly, as, for example, under the tit-for-tat reciprocity (Axelrod and Hamilton, 1981) that is widely used to explain cooperation within and between species. However, partner choice and partner fidelity can act independently of each other, and therefore the two mechanisms need to be distinguished (Bull and Rice, 1991; Sachs et al., 2004). Partner choice is a particularly important mechanism in cases where the evolutionary rates differ between two cooperating partners; specifically, the slower evolving partner (e.g., the ant host) is predicted to exert the choice between variants of the faster evolving partner (e.g., the fungal cultivar), and thus the slower-evolving partner imposes selection favoring cooperative symbiont variants (e.g., productive cultivars) and prevents the invasion of noncooperative symbiont mutants (e.g., degenerate or suboptimal cultivars; Mueller, 2002; Sachs et al., 2004).

Traditional understanding of attine evolution holds that partner feedback inherent in vertical transmission is the main mechanism ensuring long-term evolutionary cooperation between ant and fungal partners. Partner choice, in contrast, has been largely ignored in both empirical and theoretical analysis, reflecting a general mindset in symbiosis research (but see Noë, 2001, or Simms and Taylor, 2002, for some recent work on symbiont choice). In hindsight, the traditional emphasis on partner fidelity feedback was a logical conclusion of the longstanding preconception of strict vertical cultivar transmission and absence of cultivar exchange; if this is true, ants are never faced with a choice between cultivars (except between strains arising through mutation within gardens), thus precluding symbiont choice. However, the recent realization that horizontal cultivar transfer appears to be an integral part of many, if not all, attine ant–cultivar systems (Bot et al., 2001; Mueller, 2002; Mueller and Gerardo, 2002; Mueller et al., 1998) implies that symbiont choice under horizontal cultivar transfer may help stabilize the mutualistic nature of the ant–fungus association, prompting a need for the first investigations into the ecological and sensory mechanisms underlying attine symbiont choice.

Little is known about the ecological and sensory mechanisms of horizontal cultivar transfers in attine ants. Mueller et al. (1998) hypothesized that cultivar transfers may result from

accidental mix-ups of disturbed gardens from neighboring colonies or from acquisition of a cultivar replacement from a neighboring colony after cultivar loss (e.g., due to garden pathogens; Currie et al., 1999, 2003). These two hypotheses are not mutually exclusive. Adams et al. (2000) provided support for the cultivar loss-replacement hypothesis in an experiment with laboratory colonies; by inference, pathogen-induced cultivar loss may also trigger cultivar transfer in natural populations.

Weber (1972, 1982) documented that attine laboratory colonies can be induced to accept substitute gardens taken from other attine species, but he also noted that workers sometimes reject gardens from other colonies of their own species. Because Weber was presenting garden pieces taken from active gardens of other colonies, acceptance and rejection of cultivars may have been influenced by the idiosyncrasies of the donor garden (age, substrate, ant secretions, pathogen and parasite prevalence, etc.). To control for cultivation peculiarities of donor colonies, Quinlan and Cherret (1978) therefore recommended that worker preferences for cultivars be tested with pure fungal cultures, rather than with fragments taken from active gardens.

Apart from the garden idiosyncrasies, acceptance and rejection of garden fragments in Weber’s experiments may also have been influenced by the phylogenetic proximity between the substituted (novel) cultivar and the native cultivar propagated by the ants before substitution. Weber lacked any knowledge of the phylogenetic relationships of the fungal cultivars used in his garden substitution experiments, so phylogenetic proximity between the cultivars propagated before and after experimental substitution remains an untested factor that could modulate cultivar acceptance behavior in attine ants.

To elucidate the sensory mechanisms of cultivar substitution within the wider phylogenetic context of the cultivars, we determined ant preferences for different novel fungi of known phylogenetic relationships. Tested ants exhibited an acute ability to differentiate between cultivar strains, suggesting that symbiont choice favoring productive cultivars and disfavoring unproductive cultivars may potentially stabilize the ant–fungus mutualism and prevent the spread of degenerate or “selfish” (sensu Frank, 1996, 1997) cultivar mutants.

MATERIALS AND METHODS

Ant and cultivar study systems

We conducted behavioral experiments with workers of the lower attine ant *Cyphomyrmex muelleri*, the recently described sister species of *C. longiscapus*. Both these species are sympatric in the Republic of Panama, but genetic fixation for different allozyme and microsatellite alleles (i.e., absence of heterozygotes) indicates reproductive isolation between these two sister species (Schultz et al., 2002). The two species show only minimal morphological differentiation (Schultz et al., 2002), suggesting very recent diversification. Despite the recent divergence, however, the cultivars propagated by each ant species are very distantly related (Mueller et al., 1998; Schultz et al., 2002; see Figure 1). Schultz et al. (2002) suggested the possibility that one of the two species came into existence coincident with a switch to a novel cultivar.

Cyphomyrmex muelleri cultivates a member of the so-called cultivar clade 2 (see Figure 1), a group of attine symbionts whose phylogenetic relations are well resolved. By exposing *C. muelleri* to representative cultivars from across the phylogenetic breadth of cultivar clade 2 and scoring the ant preferences for these cultivars, the resolved topology of this clade allows for a detailed analysis of the phylogenetic context underlying ant preferences for novel symbionts. The cultivar

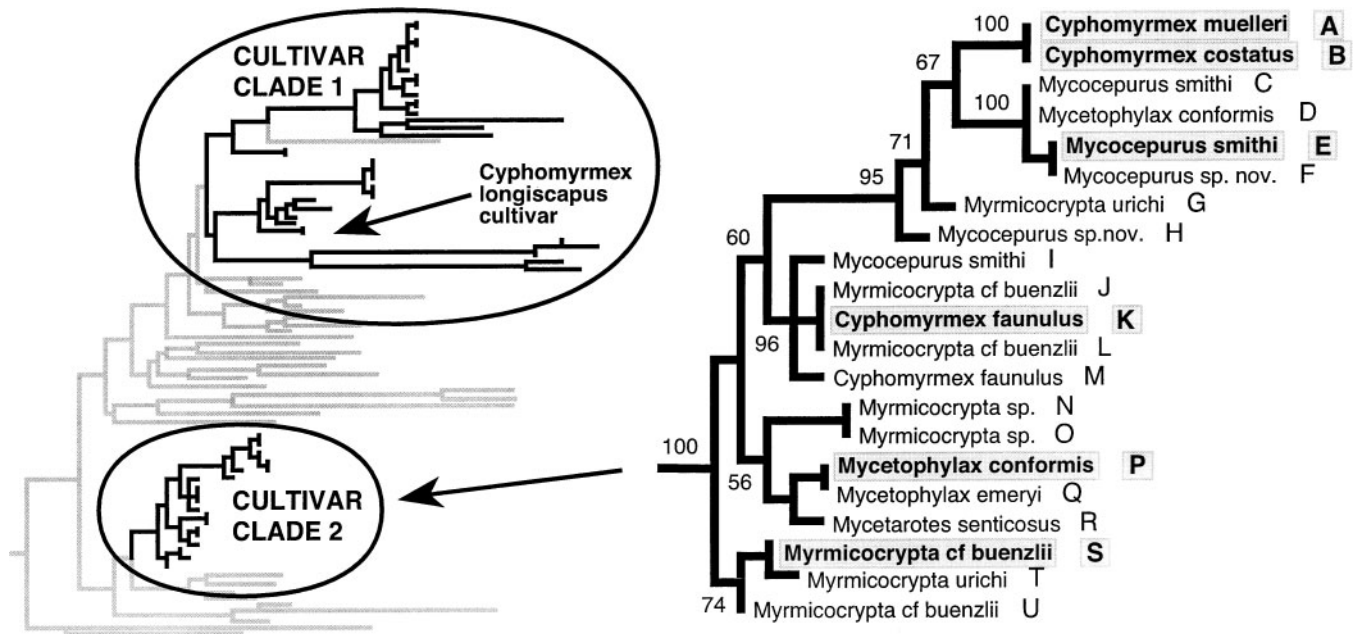


Figure 1

(Left) Broader phylogenetic relationships between leucocoprinaceous fungi (Leptotaceae, Agaricaceae, Basidiomycota) and the two main cultivar groups (cultivar clades 1 and 2) propagated by lower attine ants. The two main cultivar clades are distantly related within the larger framework of leucocoprinaceous fungi. (Right) Specific phylogenetic relationships of the clade 2 cultivars tested in the choice assays. Individual cultivars are identified by the names of the ants from which they had been isolated, and the cultivars are alphabetized from top to bottom (far right) to indicate the approximate phylogenetic distance to the native cultivar (A) of the focal ant species *C. muelleri*. Cultivars used in the preference tests are shaded in gray. Numbers at nodes indicate bootstrap supports of the branching pattern as reported in the original phylogenetic analysis by Mueller et al. (1998).

lineages propagated by the focal ant species *C. muelleri* (cultivar A, called the “native” cultivars here; Figure 1) are extremely closely related to the cultivar lineages propagated by the sympatric congener *C. costatus* (cultivar B; Figure 1), and high-resolution AFLP markers (Green et al., 2002) are needed to differentiate between these cultivars. As mentioned above, cultivars are occasionally exchanged between *C. muelleri* and *C. costatus*, and most certainly also within each ant species, suggesting that these two ant species are tapping into the same pool of shared cultivars that together may compose the same fungal “species” (Green et al., 2002).

Other members of cultivar clade 2 exhibit different degrees of divergence from the focal *costatus–muelleri* cultivars (Figure 1). This phylogenetic “cline,” composed of a series of cultivars that are increasingly more distantly related to the native cultivar of *C. muelleri*, provides an ideal system to test ant preferences within the phylogenetic context of closely and distantly related cultivar lineages.

Figure 1 labels each of the clade 2 cultivars in alphabetical order, to facilitate discussion of the experiments below. Because of the large number of cultivars in cultivar clade 2, only a representative subset was selected for the experiments (cultivars A, B, E, K, P, and S; shaded in Figure 1), but these cultivars were chosen to span the phylogenetic breadth of clade 2 fungi. Cultivars in clade 2 were part of an earlier phylogenetic study of attine cultivars (Mueller et al., 1998), and they had been kept alive on PDA medium (Difco, Detroit, Michigan) through regular subculturing every 3–6 months, following mycological methods described in Mueller et al. (1996).

Mycological methods

Workers of 39 queenright *C. muelleri* colonies were tested in July and August 1998. We had collected 28 of the colonies in June 1998 at Pipeline Road, Parque Soberanía, Republic of

Panama, following the methods of Mueller and Wcislo (1998), and collected the remaining 11 colonies at the same site in May 1997. Live colonies and their gardens were maintained at room temperature in artificial colony chambers (Schultz, 1993). All of the colonies contained at least eight workers and a queen at the start of the experiments.

Mycological methods

An agar plug ($0.5 \times 1 \text{ cm}^2$) with young mycelium was cut from a PDA (Difco) plate and blended with 10 ml of sterilized PDB (Difco) broth. The blended liquid was then pipetted onto a layer (about 3–4 mm thick) of sterilized, ground oat flakes spread in a glass Petri dish. The dishes were then sealed with parafilm to prevent desiccation, and the oat cultures were allowed to grow in the dark at room temperature. We inoculated all cultivars tested in a given experiment onto oats on the same day, and they were thus of the same age at the time of testing. Oat cultures generally showed the first fine mycelial growth within a week, and the oat layer was completely overgrown with a thick mycelial layer within 35 days. For the experiments, we cut small inocula from these oat cultures 7–21 days after oat inoculation. We used only inocula in the tests where the fungus had completely overgrown the oats because even small bits of oats not suffused with fungus become quickly overgrown with contaminant fungi or bacteria during the testing.

Experimental paradigm

At the day of testing, small pieces of fungus (20–30 mm³) were cut from the oats and placed on alcohol-sterilized, labeled plastic trays (about $6 \times 6 \text{ mm}^2$), which were presented to workers in a test chamber. The test chamber consisted of a small plastic Petri dish (60 mm diameter) with a bottom of moistened plaster of Paris to provide stable humidity, and the

Table 1
Preferences of *Cyphomyrmex muelleri* ants in choice assays between fungal cultivars from different ant species

Choices in experiments	<i>n</i>	Preference (%) for			<i>p</i> values	
		1st cultivar	2nd cultivar	Both cultivars	χ^2 test	<i>G</i> test
Experiment 1.1						
Cultivar A (<i>C. muelleri</i>) vs. cultivar B (<i>C. costatus</i>)	10	70	0	30	.023	<.01
Experiment 1.2						
Cultivar A (<i>C. muelleri</i>) vs. cultivar E (<i>Myco. smithi</i>)	5	100	0	0		
Cultivar A (<i>C. muelleri</i>) vs. cultivar K (<i>C. faunulus</i>)	5	80	0	20		
Cultivar A (<i>C. muelleri</i>) vs. cultivar P (<i>Myce. conformis</i>)	5	80	0	20		
Cultivar A (<i>C. muelleri</i>) vs. cultivar S (<i>Myrm. cf. buenzlii</i>)	5	60	0	40		
Average	20	80	0	20	<.0001	<.0001
Experiment 2.1						
Cultivar B (<i>C. costatus</i>) vs. cultivar E (<i>Myco. smithi</i>)	10	30	60	10	.147	n/a
Experiment 2.2						
Cultivar B (<i>C. costatus</i>) vs. cultivar K (<i>C. faunulus</i>)	5	100	0	0		
Cultivar B (<i>C. costatus</i>) vs. cultivar P (<i>Myce. conformis</i>)	5	60	0	40		
Cultivar B (<i>C. costatus</i>) vs. cultivar S (<i>Myrm. cf. buenzlii</i>)	5	80	0	20		
Average	15	80	0	20	<.001	<.0001
Experiment 3						
Cultivar E (<i>M. smithi</i>) vs. cultivar K (<i>C. faunulus</i>)	10	100	0	0	<.0001	<.0001
Experiment 4						
Cultivar A (<i>C. muelleri</i>) vs. clade 1 cultivar (<i>C. longiscapus</i>)	10	100	0	0	<.0001	<.0001

See Figure 1 for the phylogenetic relationships between tested cultivars (A, B, E, K, P, and S). Ant genera from where cultivars were isolated are abbreviated as *C.* = *Cyphomyrmex*; *Myco.* = *Mycocepurus*; *Myce.* = *Mycetophylax*; *Myrm.* = *Myrmicocrypta*. *n* = number of replicates per choice test; n/a = test not applicable (see Materials and Methods).

two trays with fungi were spaced about 3 cm apart within the test chamber. In each test replicate, we gave three workers from the same *C. muelleri* nest a choice between a pair of cultivars (i.e., confronting only two cultivars at a time against one another). We randomized the position of the two fungal choices in the test arena (left vs. right), and tray/cultivar labels were coded to ensure blind observation.

Twenty-four hours after presentation of the pure fungal pieces, the ants were given oat catkins, a highly preferred substrate (Weber, 1972; R.M.A., personal observation), to add as substrate to the fungal pieces. For the next 6 days, we removed old catkins and provided fresh catkins daily, and the ants were observed to determine whether any substrate had been added to any of the fungal pieces (i.e., whether the ants had accepted a fungal piece and converted it into a small garden). While previous studies simply used apparent consumption of mycelium as an indicator of acceptance by the ants (Martin, 1987; Stradling and Powell, 1992; Weber, 1945, 1972), consumption (true ingestion) cannot be scored unambiguously because the ants use behaviors that resemble feeding to weed out contaminant fungi or to merely investigate substrate (Currie and Stuart, 2001; U.G.M., personal observation). Without observing actual fungiculture, it is thus unclear whether the ants truly accept a fungus as a suitable cultivar. We therefore used the more conservative observation of substrate addition (fungiculture) as the definitive criterion for fungal acceptance.

On the seventh experimental day, we scored ant preferences either as (1) preference (one or the other of the two inocula was converted into a garden through the addition of catkins) or (2) dual preference (substrate was added to both inocula). Workers always added substrate to at least one fungal inoculum. Experiments were scored on day 7, but eight experiments were continued for 14 days to test whether ant preferences changed over a longer time period. Preferences

were usually expressed (i.e., substrate was added) by the third or fourth experimental day, and preferences did not change after the seventh day in any of the eight 2-week trials.

We discontinued experiments when at least one of the inocula became contaminated (visible as discoloration or as aerial hyphae bearing spores). Experiments were continued if one of the three ants died in the course of the experiment but were terminated once two ants died. We repeated prematurely terminated experiments with novel ants and fresh inocula. After the experiment, the ants were marked with white acrylic paint and returned to their colonies. Although ants from the same colonies were used in successive experiments, the markings ensured that each individual ant was tested only once in the entire choice study. Replicate trials within each of the six separate experiments always used ants from different colonies to ensure statistical independence.

Experiments

Sample sizes for each of the cultivar choice experiments are given in Table 1. Letter IDs for the cultivars (e.g., "cultivar A" or "cultivar P") are given in Figure 1 for the various clade 2 cultivars tested. The cultivars in clade 2 are alphabetized to indicate the approximate phylogenetic distance to native *C. muelleri* cultivar (denoted cultivar A), with phylogenetic distance increasing with alphabetic position.

Experiment 1.1 tested the choice between cultivar A (isolated from *C. muelleri*) and cultivar B (isolated from the distantly related congeneric ant species *C. costatus*). Experiment 1.1 tested whether the *C. muelleri* workers were capable of discriminating between these very closely related cultivars and whether *C. muelleri* workers expressed a preference for the cultivar that had been isolated from a colony of their own species.

Experiment 1.2 tested the choice between the native cultivar A (isolated from *C. muelleri*) and a set of four

representative, more distantly related clade 2 cultivars. Because of time constraints, only four clade 2 cultivars could be tested against the native cultivar, but these cultivars (cultivars E, K, P, and S; Figure 1) were chosen to compose a phylogenetic cline of cultivars that were gradually more distantly related to the *C. muelleri* cultivar.

Experiment 2.1 tested whether *C. muelleri* workers preferred a closely related cultivar (cultivar B) that had been isolated from a different, congeneric ant species (*C. costatus*) over a slightly more distantly related cultivar (cultivar E) isolated from a non-congeneric ant species (*Mycocepurus smithi*).

Experiment 2.2 tested the choice between cultivar B (isolated from *C. costatus*) and a set of three representative, more distantly related clade 2 cultivars (cultivars K, P, S). The three more distantly related cultivars spanned the breadth of cultivar clade 2 and were tested already in experiment 1.2. Experiment 2.2 tested whether the *C. muelleri* workers preferred the *C. costatus* cultivar, the closest known relative of the *C. muelleri* cultivar, over three more distantly related clade 2 representatives.

Experiments 2.1 and 2.2 established (see Results) a general preference of *C. muelleri* workers for the closely related *C. costatus* cultivar over more distantly related clade 2 cultivars. The preference for the *C. costatus* cultivar could have been due to the close phylogenetic proximity to the native fungus, or to the recent history of cultivation by a congeneric ant species that created or selected for fungal traits preferred by *Cyphomyrmex* species. To differentiate between these two scenarios, experiment 3 tested a closely related fungus isolated from the non-congeneric ant *Mycocepurus smithi* (cultivar E) against a distantly related cultivar isolated from the congeneric ant *C. faunulus* (cultivar K).

Experiment 4 tested the choice between the native cultivar 1 (isolated from *C. muelleri*) and a cultivar isolated from *C. longiscapus*, the sister species of *C. muelleri* (Schultz et al. 2002). *C. longiscapus* cultivates a cultivar clade 1 fungus, distantly related to cultivar clade 2 fungi (Figure 1, left). As explained above, these two ant species are cryptic sister species, yet they cultivate widely different fungi. Experiment 4 thus tested whether *C. muelleri* workers preferred a fungus isolated from its own species over a cultivar isolated from its sibling species *C. longiscapus*.

Statistical analyses

Worker preferences were analyzed with chi-square analysis with Yates correction for continuity (Zar, 1999). In an initial chi-square analysis of each experiment, counts were compared between all three preference categories (unambiguous preference for first cultivar, unambiguous preference for second cultivar, preference for both cultivars = dual preference; see above and Table 1). The null expectation was equal observation of each of these three outcomes.

Within each of experiment 1.2 and 2.2, counts were combined for a pooled chi-square analysis (Zar, 1999), combining counts from the multiple subtests with different cultivars tested within each of these two experiments (Table 1). Pooling was justified because of the absence of significant differences within experiment 1.2 (heterogeneity $\chi^2 = 3.233$, $df = 3$, $p > .05$) and within experiment 2.2 (heterogeneity $\chi^2 = 5.893$, $df = 2$, $p > .05$).

For comparisons that yielded significance in the initial chi-square analysis, a subdivided *G* test (Zar, 1999) was performed that compared counts between only the two unambiguous preference categories (i.e., preference for one or the other cultivar, but ignoring the ambiguous dual preference category; thus, the null expectation was equal observation of two outcomes). This second analysis tested for significance of

a preference favoring one of the two tested cultivars, rather than testing for the overall differences (heterogeneity) between three categories in the initial chi-square analysis that included the uninformative dual preference category.

RESULTS

In experiment 1.1 (native *C. muelleri* cultivar vs. *C. costatus* cultivar), *C. muelleri* workers were capable of discriminating between two very closely related cultivars presented as pure cultures, one cultivar isolated from a colony of their own species (cultivar A), the other cultivar isolated from a *C. costatus* colony (cultivar B) (Table 1). In none of the 10 replicates did the *C. muelleri* workers prefer the *C. costatus* cultivar over the *C. muelleri* cultivar. *C. muelleri* workers exhibited an unambiguous preference in favor of the *C. muelleri* cultivar in 7 of 10 replicates, while the remaining three replicates were inconclusive (dual preference = workers converted both inocula into gardens). This reveals a decisive preference for the *C. muelleri* cultivar over the most closely related cultivar among the clade 2 cultivars (chi-square test, $p = .023$; *G* test, $p < .01$; Table 1).

In experiment 1.2 (native *C. muelleri* cultivar vs. distant clade 2 cultivars), *C. muelleri* workers preferred the *C. muelleri* cultivar over the representative four novel clade 2 cultivars (cultivars E, K, P, S; see Figure 1; chi-square test, $p < .0001$; *G* test, $p < .0001$). Seven of the 20 tests were inconclusive because of dual choice (Table 1), but in the remaining 13 cases the *C. muelleri* workers always preferred the *C. muelleri* cultivar. *C. muelleri* workers therefore are capable of differentiating other representative clade 2 cultivars from the *C. muelleri* cultivar and express a preference for the cultivar that had been isolated from a colony of their own species.

In experiment 2.1 (*C. costatus* cultivar vs. *Mycocepurus smithi* cultivar), when choosing between the very closely related *C. costatus* cultivar (cultivar B) and the slightly more distantly related *M. smithi* cultivar (cultivar E; Figure 1), *C. muelleri* workers failed to discriminate between these two cultivars, and both cultivars appear to be about equally attractive (chi-square test, $p = .0147$; *G* test, $p > .5$; Table 1). The absence of any preference occurred despite the fact that the phylogenetic distance between the *C. costatus* cultivar and the *M. smithi* cultivar is greater than the phylogenetic distance between the *C. costatus* and the *C. muelleri* cultivars tested in experiment 1.1 (see Figure 1).

In experiment 2.2 (*C. costatus* cultivar vs. distant clade 2 cultivars), *C. muelleri* preferred the *C. costatus* cultivar over the three representative clade 2 cultivars (cultivars K, P, S; see Figure 1; chi-square test, $p < .001$; *G* test, $p < .0001$). Three of the 15 replicates were inconclusive because of dual preference (Table 1), but in the remaining 12 cases the *C. muelleri* workers preferred the *C. costatus* cultivar. *C. muelleri* workers therefore are capable of differentiating clade 2 cultivars from the *C. costatus* cultivar (the closest relative to the *C. muelleri* cultivar) if the clade 2 cultivars are phylogenetically more distant than the distance tested in experiment 2.1 (between cultivars B and E).

Experiment 3 (phylogenetic distance between cultivars vs. distance between ants) confronted cultivar E (from *M. smithi*) and cultivar K (from *C. faunulus*) and tested whether the choice of novel cultivars by *C. muelleri* workers is based more on the phylogenetic distance to their native cultivar (cultivar E is more closely related to the native *C. muelleri* cultivar than cultivar K; Figure 1) or more on the phylogenetic distance to the ant hosts from which the cultivars had been isolated (the closely related congener ants *C. muelleri* vs. *C. faunulus*, and the non-congener ants *C. muelleri* vs. *M. smithi*). In all 10 of the cultivar choice tests (Table 1), the *C. muelleri* workers preferred to cultivate the fungus that was most closely related

to the *C. muelleri* cultivar (chi-square test, $p < .0001$; G test, $p < .0001$), even though the distantly related fungus had been cultivated most recently by the congener *C. faunulus*. This suggests for this single comparison that the recent history of cultivation by two closely related species (i.e., *C. muelleri* vs. *C. faunulus*) is less critical for choices than the phylogenetic proximity between novel and native cultivar.

In all of the 10 choice tests in experiment 4 (*C. muelleri* vs. *C. longiscapus* cultivars), *C. muelleri* workers added substrate only to the *C. muelleri* cultivar and started a garden but ignored the *C. longiscapus* clade 1 cultivar and never added substrate to this distantly related fungus (Table 1). Thus, *C. muelleri* ants strongly preferred their own clade 2 cultivar over the distantly related clade 1 cultivar propagated by the sister species *C. longiscapus* (chi-square test, $p < .0001$; G test, $p < .0001$).

DISCUSSION

Cyphomyrmex muelleri workers invariably preferred their native cultivar over any other cultivar tested (Figure 1, Table 1). Workers generally ignored the non-native cultivars, either because they did not recognize some of the non-native fungi as suitable cultivars, or because these cultivars represented a less attractive choice compared to their native fungus.

The preference for the *C. muelleri* cultivar is sensitive enough that the *C. muelleri* workers choose their native cultivar above the extremely closely related cultivar of *C. costatus* (Figure 1; Table 1; experiment 1.1). Based on amplified fragment length polymorphism (AFLP) fingerprints (Green et al., 2002), *C. costatus* cultivars are genetically very similar (sometimes even identical) to *C. muelleri* cultivars, and *C. costatus* and *C. muelleri* cultivars thus appear to be very recent descendents of the same, phylogenetically narrow set of clonal lineages (Green et al., 2002). The ability to differentiate between *C. muelleri* and *C. costatus* cultivars therefore indicates an acute sensitivity to minor genetic and physiological differences between cultivars. This implies that preferences of the ants for certain cultivars could (1) bias the likelihood of cultivar acceptance during cultivar transfers between attine nests and (2) modulate subculturing decisions of workers when deciding between cultivar strains that may coexist in the same ant nest and that are chosen for the planting of new gardens.

Symbiotic choice and ant–fungus coevolution

The acute ability of *C. muelleri* ants for cultivar differentiation supports the hypothesis that the ant–fungus mutualism may be stabilized evolutionarily by symbiotic choice through which the ants select against unwanted, presumably inferior cultivars (Bull and Rice, 1991; Mueller, 2002; Sachs et al., 2004). Symbiotic choice would complement the traditionally recognized partner feedback inherent in vertical cultivar transmission, and the joint operation of both mechanisms could greatly curtail or even prevent the invasion of suboptimal (“selfish” sensu Frank, 1997) cultivar strains into the symbiotic pool associated with an ant species. Such cultivars could arise in gardens through mutation, or they could become imported horizontally through transfer from another ant colony. Symbiotic choice thus would help maintain a genetically and physiologically efficient ant–fungus partnership (Bot et al., 2001; Martin, 1987) through ant-imposed selection against unpreferred, possibly unproductive or otherwise suboptimal cultivar types.

Symbiotic choice may be particularly important for fungus-growing ants because cultivar evolution could potentially progress at a much faster rate than the evolutionary rate of the ants. The cultivar undergoes 20–100 subculturing cycles

per year within an attine nest (Mueller, 2002), and suboptimal “cheater” cultivar mutants may well arise during a lifetime of an ant colony, then spread to fixation within gardens. Mueller (2002) therefore predicted that attine ants are under selection to monitor honest indicators such as nutrient levels that unambiguously reveal the requisite properties of a desirable cultivar. In contrast, the cultivars may be under selection to exploit the ants’ sensory biases operating during the subculturing process, such that relatively unproductive cultivar mutants may spread in a garden because they efficiently exploit the ants’ sensory predilections. The parallels with the evolutionary dynamics between chooser and chosen partner under intersexual selection (mate choice) is obvious. In fact, both symbiotic choice and mate choice can be understood as special cases of the process of partner choice (Bull and Rice, 1991; Sachs et al., 2004), expressed, respectively, in the context of either between-species or within-species reproductive cooperation. In both cases the chooser is expected to select a partner that enhances its own fitness, while the chosen partner may be selected to develop features that enhance the likelihood of being chosen. Future research on symbiotic choice in attine ants may benefit from application of theory and experimental paradigms (e.g., good genes, honest indicators, sensory drive, sensory exploitation) that have been developed within the areas of mate choice and sexual selection.

Apart from the selective advantage of symbiotic choice, two additional selective pressures may help sharpen and fine-tune the ants’ ability to discriminate between cultivar types. First, the ants may be under selection to differentiate between cultivar strains that coexist in the same garden and that may compete with each other for garden resources. Acute differentiation may allow workers to eliminate all except one of the coexisting strains, thus maintaining gardens as monocultures that are free of between-cultivar competition. In essence, imposing monoculture prevents the within-nest evolution of selfish (virulent) cultivar strains that could easily be selected for under polyculture of multiple, intercropped cultivars (Bot et al., 2001; Frank 1996). Second, the ants may be under selection to detect parasitic fungi (“weeds”) that may be specialized to invade the garden by mimicking the cultivated fungi. Efficient detection of parasitic invaders within gardens has clear advantages because it allows workers to weed out infested parts before a parasite spreads throughout a garden, a behavior that has recently been described by Currie and Stuart (2001). One specialized fungal garden parasite, *Escovopsis*, appears to have been intimately associated with attine gardens since the origin of the ant–cultivar mutualism 50–60 million years ago (Currie et al., 2003), and it is quite possible that an ancient coevolutionary arms race has shaped the ants’ sensory systems to differentiate their desired cultivar from any undesired parasite that is under selection to evade the ants’ weeding activities by mimicking cultivar properties.

Cultivar transfers between conspecific colonies

The preference of *C. muelleri* workers for their native cultivar predicts that the most likely fungal transfers should occur between conspecific colonies with cultivars that are closely related and that between-ant-species transfers are most likely if the cultivars of the two ant species are closely related. Because many lower attines, including *C. muelleri*, occur in dense conspecific aggregations, cultivar transfers between colonies of the same species are thus facilitated by two factors, first by the close proximity between conspecific colonies and second by the close phylogenetic relatedness of the cultivars propagated by conspecific colonies (Bot et al., 2001; Mueller et al., 1996, 1998).

Mueller et al. (1998) and Green et al. (2002) tested for cultivar transfers between ant species and inferred several such cases from phylogenetic analyses. Because the number of between-species transfers revealed by these previous analyses is appreciable, the present preference study suggests that cultivar transfers between conspecific colonies may be much higher. For example, Green et al. (2002) inferred at least six recent between-species cultivar transfers between the sympatric *C. muelleri* and *C. costatus*, which probably grossly underestimates the community-wide extent of cultivar transfers, most of which are, based on the findings of this study, predicted to occur between conspecific ant colonies. In other words, under natural conditions, cultivars may be swapped regularly between colonies within each of these ant species. Regular cultivar exchange between and within ant species appears to be an integral part of the interlaced fungicultural systems of *C. muelleri* and *C. costatus*, possibly resulting in rapid sweeps of desirable varieties through ant communities (see additional discussion in Mueller, 2002, and Mueller et al., 1998).

Cultivar switches away from the native cultivar

When deprived of their native cultivar, *C. muelleri* workers selected the nearest relative of their native cultivar over more distantly related cultivars. Specifically, *C. muelleri* workers selected the closely related *C. costatus* cultivar over other clade 2 cultivar options. A similar preference for a closely related cultivar was revealed by experiment 3 that forced a choice between the fungus gardened by *Mycocetopus smithi* (the more closely related cultivar E) and *C. faunulus* (the more distantly related cultivar K), indicating that *C. muelleri* workers appear to base their choices on the phylogenetic distance between a novel cultivar and their native cultivar, rather than on their relatedness to the ants that gardened the novel cultivar most recently. Moreover, *C. muelleri* ants strongly rejected the clade 1 cultivar propagated by the sister species *C. longiscapus*, again showing a bias against a distantly related cultivar. When selecting between two non-native cultivars, workers seem to follow the rule of preferring a cultivar substitute that is most closely related to their native cultivar, while rejecting the more distantly related cultivar. The only possible exception to this rule was revealed in experiment 2.1; here workers did not exhibit any preference between the two cultivars presented, but it may be that these two cultivars (cultivars B and E) were not sufficiently distinct from each other (see Figure 1) to be discriminable by *C. muelleri* workers.

For novel cultivar acquisitions, the emerging picture thus suggests that switches to distantly related cultivars are unlikely if driven solely by ant preferences. Such distant switches therefore may represent evolutionary exceptions, prompted by inordinate circumstances, and the distant cultivar transfers between ant species that were revealed by previous phylogenetic analyses (Chapela et al., 1994; Mueller et al., 1998) may require explanations that are not based on preference for a newly acquired fungi. For example, ants may be forced to accept whatever cultivar they can obtain from neighboring colonies after accidental or pathogen-driven loss of their original cultivar, and these forced cultivar transitions may result in the acceptance of less attractive, novel cultivars. Subsequent evolution could lead to eventual coadaptation and improvement of the overall mutualism, but most forced associations with distant cultivars are probably short lived, either because the novel ant–cultivar combination becomes extinct or because the ants replace the unsuitable cultivar (e.g., by reverting to their native cultivar if such a strain can eventually be obtained).

Consistent with these expectations, experimental simulation of cultivar losses in conjoined laboratory colonies

revealed that garden-deprived ant colonies will try to obtain a replacement cultivar from almost any neighboring colony, usurping gardens and stealing cultivars that sometimes are distantly related to their native (lost) cultivar (Adams et al., 2000). Specifically, when deprived of a garden, *C. muelleri* workers occasionally usurped gardens from a neighboring *C. longiscapus* colony that cultivated a clade 1 cultivar like the one tested in experiment 4. This cultivar acceptance occurred despite the fact that the clade 1 cultivar is not preferred by *C. muelleri* workers (Table 1; experiment 4), but the absence of any other available substitute fungus in the garden deprivation experiments appeared to have forced the switch to the distantly related clade 1 cultivar.

Are symbiont preferences inborn or learned?

The symbiont choice experiments show that *C. muelleri* workers are very particular in their preference for their native fungus, but it remains unclear whether this preference is inborn or learned. Whether preferences are innate and fixed or modifiable through experience, these distinct mechanisms of preference formation do not alter the above conclusions regarding the likelihood of cultivar transfers between species; that is, regardless of whether the preference is inborn or learned, any such preference constrains potential cultivar exchanges. But if preferences are learned, rather than inborn, any accidental switch to a novel cultivar could imprint a switched colony to the novel cultivar and may preclude return (evolutionary reversal) to the original cultivar. Future research using Kaspar-Hauser techniques (rearing larvae and young workers on novel cultivars, then testing their preferences) should test for components of learning in the formation of symbiont choice. Such experiments will shed light onto possible cultural persistence of cultivar preferences within ant lineages, as well as the evolutionary persistence (non-reversal) of cultivar switches revealed by previous phylogenetic analyses (Mueller et al., 1998).

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REFERENCES

- Adams RMM, Mueller UG, Holloway AK, Green AM, Narozniak J, 2000. Garden sharing and garden stealing in fungus-growing ants. *Naturwissenschaften* 87:491–493.
- Axelrod R, Hamilton WD, 1981. The evolution of cooperation. *Science* 211:1390–1396.
- Autuori M, 1950. Contribuição para o conhecimento da saúva (*Atta* spp. – Hymenoptera – Formicidae) V. Número de formas aladas e redução dos saúvas iniciais. *Arquiv Inst Biol Sao Paulo* 19:325–331.
- 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.
- Bruch C, 1923. Estudios mirmecológicos. *Rev Mus Plata* 27:172–220.
- Bull JJ, Rice WR, 1991. Distinguishing mechanisms for the evolution of co-operation. *J Theor Biol* 149:63–74.
- Chapela IH, Rehner SA, Schultz TR, Mueller UG, 1994. Evolutionary history of the symbiosis between fungus-growing ants and their fungi. *Science* 266:1991–1994.

- Currie C, Mueller UG, Malloch D, 1999. The agricultural pathology of ant fungus gardens. *Proc Natl Acad Sci* 96:7992–7995.
- Currie CR, Stuart AE, 2001. Weeding and grooming of pathogens in agriculture by ants. *Proc R Soc Lond* 263:339–344.
- Currie CR, Wong B, Stuart AE, Schultz TR, Rehner SA, Mueller UG, Sung G-H, Spatafora JW, Straus NA, 2003. Ancient tripartite coevolution in the attine ant–microbe symbiosis. *Science* 299:386–388.
- Frank SA, 1996. Host-symbiont conflict over the mixing of symbiotic lineages. *Proc R Soc Lond* 268:1033–1039.
- Frank SA, 1997. Models of symbiosis. *Am Nat* 150:S80–S99.
- Green AM, Adams RMM, Mueller UG, 2002. Extensive exchange of fungal cultivars between two sympatric species of fungus-growing ants. *Mol Ecol* 11:191–195.
- Herre EA, Knowlton N, Mueller UG, Rehner SA, 1999. The evolution of mutualisms: exploring the paths between conflict and cooperation. *Trends Ecol Evol* 14:49–53.
- Huber J, 1905a. Über die Koloniegründung bei *Atta sexdens*. *Biol Centralblatt* 25:606–619.
- Huber J, 1905b. Über die Koloniegründung bei *Atta sexdens*. *Biol Centralblatt* 25:625–635.
- Ihering R von, 1898. Die Anlagen neuer Colonien und Pizgärten bei *Atta sexdens*. *Zool Anz* 21:238–245.
- Martin MM, 1987. Invertebrate-microbial interactions: ingested fungal enzymes in arthropod biology. Ithaca, New York: Cornell University Press.
- 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.
- Mueller UG, Gerardo N, 2002. Fungus-farming insects: multiple origins and diverse evolutionary histories. *Proc Natl Acad Sci USA* 99:15247–15249.
- Mueller UG, Lipari SE, Milgroom MG, 1996. Amplified fragment length polymorphism (AFLP) fingerprinting of symbiotic fungi cultured by the fungus-growing ant *Cyphomyrmex minutus*. *Mol Ecol* 5:119–122.
- Mueller UG, Wcislo W, 1998. Nesting biology of the fungus-growing ant *Cyphomyrmex longiscapus* Weber (Attini, Formicidae). *Insect Soc* 45:181–189.
- Mueller UG, Rehner SA, Schultz TR, 1998. The evolution of agriculture in ants. *Science* 281:2034–2038.
- Mueller UG, Schultz TR, Currie CR, Adams RM, Malloch D, 2001. The origin of the attine ant-fungus symbiosis. *Q Rev Biol* 76:169–197.
- Noë R, 2001. Biological markets: partner choice as the driving force behind the evolution of mutualisms. In: *Economics in nature* (Noë R, van Hooff JA, Hammerstein P, eds). Cambridge: Cambridge University Press; pp. 93–118.
- Quinlan RJ, Cherrett JM, 1978. Aspects of the symbiosis of the leaf-cutting ant *Acromyrmex octospinosus* (Reich) and its food fungus. *Ecol Entomol* 3:221–230.
- Rissing SW, Pollock GB, Higgins MR, Hagen RH, Smith DR, 1989. Foraging specialization without relatedness or dominance among co-founding ant queens. *Nature* 338:420–422.
- Sachs JL, Mueller UG, Wilcox TP, Bull JJ, 2004. Evolution of cooperation. *Q Rev Biol* (in press).
- Simms E, Taylor DL, 2002. Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia. *Integrat Comp Biol* 42:369–380.
- Schultz TR, 1993. Stalking the wild attine. *Notes from the underground. Museum of Comparative Zoology, Harvard University* 8:7–10.
- Schultz TR, Solomon SA, Mueller UG, Villesen P, Boomsma JJ, Adams RMM, Norden B, 2002. Cryptic speciation in the fungus-growing ants *Cyphomyrmex longiscapus* Weber and *Cyphomyrmex muelleri* Schultz and Solomon, new species (Formicidae, Attini). *Insect Soc* 49:331–343.
- Stradling DJ, 1978. Food and feeding habits of ants. In: *Production ecology of ants and termites* (Brian MV, ed). Cambridge: Cambridge University Press; 81–106.
- Stradling DJ, Powell RJ, 1992. Fungiculture and the choice of substrates by attine ants. In: *Biology and evolution of social insects* (Billen J, ed). Leuven: Leuven University Press; 133–143.
- Weber NA, 1945. The biology of the fungus-growing ants. Part VIII. The Trinidad, B.W.I., species. *Rev Entomol* 16:1–88.
- Weber NA, 1972. Gardening ants, the Attines. Philadelphia: American Philosophical Society.
- Weber NA, 1982. Fungus ants. In: *Social insects, IV* (Hermann H, ed). New York: Academic Press; 255–363.
- Zar JH, 1999. *Biostatistical analysis*. Upper Saddle River, New Jersey: Prentice Hall.