PARTIAL INCOMPATIBILITY BETWEEN ANTS AND SYMBIOTIC FUNGI IN TWO SYMPATRIC SPECIES OF *ACROMYRMEX* LEAF-CUTTING ANTS

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Abstract.—We investigate the nature and duration of incompatibility between certain combinations of *Acromyrmex* leaf-cutting ants and symbiotic fungi, taken from sympatric colonies of the same or a related species. Ant-fungus incompatibility appeared to be largely independent of the ant species involved, but could be explained partly by genetic differences among the fungus cultivars. Following current theoretical considerations, we develop a hypothesis, originally proposed by S. A. Frank, that the observed incompatibilities are ultimately due to competitive interactions between genetically different fungal lineages, and we predict that the ants should have evolved mechanisms to prevent such competition between cultivars within a single garden. This requires that the ants are able to recognize unfamiliar fungi, and we show that this is indeed the case. Amplified fragment length polymorphism genotyping further shows that the two sympatric *Acromyrmex* species share each other's major lineages of cultivar, confirming that horizontal transfer does occasionally take place. We argue and provide some evidence that chemical substances produced by the fungus garden may mediate recognition of alien fungi by the ants. We show that incompatibility between ants and transplanted, genetically different cultivars is indeed due to active killing of the novel cultivar by the ants. This incompatibility disappears when ants are force-fed the novel cultivar for about a week, a result that is consistent with our hypothesis of recognition induced by the resident fungus and eventual replacement of incompatibility compounds during force-feeding.

Key words.—Attini, compatibility, fungi, fungus-growing ants, mutualism, specificity, symbiosis.

Obligate mutualisms are shaped and maintained by a complex array of selection forces. On one hand, mutually beneficial traits are favored as long as they increase the efficiency of resource acquisition, metabolic performance, and/or defense. On the other hand, selfish traits in each partner may be maintained to some extent as long as they do not jeopardize the mutually beneficial symbiosis. Much of the evolutionary complexity shaping mutualistic symbioses can thus be traced back to Hamiltonian concepts of conflict and cooperation (Hamilton 1964). Lasting cooperation between independent genomes belonging to the same breeding population is normally maintained only when inclusive fitness benefits via kin are significant. In the absence of kinship in interactions between species, competition prevails over cooperation, unless the kinship incentives are replaced by reciprocity and mutual policing (Trivers 1971; Frank 1996b; Leigh 1999). When genomes belonging to different gene pools (i.e., taxa) evolve mutualistic cooperation, they usually obtain a joint niche that allows a consistently higher fitness than either of the single-species niches, for example, as in lichens, corals, and mycorrhizal symbioses (Thompson 1994). Although cooperation across taxa excludes Hamiltonian relatedness in a strict sense, many of the broader conceptual considerations regarding such associations are strongly reminiscent of inclusive fitness, with reciprocity and policing as stability-enhancing

mechanisms (Frank 1998). However, even in the most successful mutualistic partnerships there is no a priori reason why partial exploitation of one partner by the other would not yield even higher fitness than pure cooperation. This implies that mutualistic partners may occasionally pursue their own selfish reproductive interest (e.g., Herre and West 1997) and that the separation between parasitism and mutualism becomes blurred (Bronstein 1994; Thompson 1994; Herre 1999; Herre et al. 1999).

Obligate symbiosis provides special opportunities and constraints for mutualistic interactions. Symbionts living inside or in close association with a single host individual are normally either closely related among each other (when they belong to the same asexual clone) or unrelated (when they belong to different lineages that independently colonize a host). In the former case, the fitness interests of individual symbionts are largely or completely aligned with the fitness of the host (Herre et al. 1999), but this is not so in the latter case. As outlined by Frank (1996a) and previously by Eberhard (1980), Cosmides and Tooby (1981), Hoekstra (1987), and Hurst (1994), hosts and symbionts are in conflict over the mixing of symbiont lineages. Symbionts will always be under selection to realize some horizontal transmission to reduce competition with close relatives and colonize new hosts (Hamilton and May 1977; Frank 1997). However, the successful establishment of additional unrelated symbionts in an individual host tends to induce competition between symbiont lineages. The concomitant reduction in average fitness of all symbionts within a single host, and thus also of host fitness, can be referred to as virulence (Frank 1996a), in parallel with the negative effect of parasites or diseases on the fitness of their hosts (e.g., Lively 1999). Frank's theory

of host-symbiont conflict over mixing of symbiotic lineages predicts that hosts will evolve effective means to control symbiont transmission when a number of specific assumptions are met. This theory makes specific predictions regarding the low genetic variation expected among symbionts within a single host, but it does not allow inferences about the extent of genetic polymorphism that can be maintained in entire breeding populations of symbionts. The theory specifies that conditions for invasion of host modifier genes that restrict mixing of symbiont lineages are more lenient when frequency-dependent selection maintains high levels of symbiont polymorphism in the population (Frank 1996a). However, there are few specific models (Law 1985; Bever 1999; Parker 1999) and few empirical data (Rowan and Powers 1991; Martin 1992; Wilkinson et al. 1996; Parker 1999) addressing issues of populationwide polymorphism of mutualistic symbionts.

There may be a good reason for the absence of theoretical predictions for symbiont polymorphism. Negative frequency-dependent selection is of significant importance for maintaining high degrees of polymorphism in parasite-host interactions (Hamilton 1980; Seger 1988, 1992; Ebert and Hamilton 1996). A corollary of this hypothesis, that parasites are better adapted to and thus more successful with their local hosts than with hosts from other populations, has recently received considerable support from empirical studies (Lively 1989, 1999; Lively and Dybdahl 2000; Ebert 1994; for a review see Kaltz and Shykoff 1998). At the other extreme, pure mutualistic symbioses without any selfish tendencies have been hypothesized to be shaped by positive frequency-dependent selection favoring a single most productive lineage of mutualistic symbiont (Law 1985; Law and Koptur 1986). This implies that novel alleles arising in symbionts do not necessarily have an advantage and that, even in metapopulations, local dynamics and turnover should be less erratic than in antagonistic interactions, so that polymorphism should remain relatively low (Parker 1999). However, our current understanding of mutualistic interactions as arrangements for mutual net benefits despite reciprocal exploitation (Frank 1996a; Herre 1999) addresses intermediate situations where both positive frequency-dependence and negative frequency-dependence are likely to apply, consistent with the modeling approach by Bever (1999). In such a conceptual framework, mutualisms are shaped by evolutionary arms races between symbionts with variable tendencies of selfishness (virulence) and hosts with variable capacities to control symbiont transmission. The balance between positive and negative frequency dependence may thus differ from case to case, depending on specific biological properties and constraints of partners.

The issue of extant genetic variation in symbiont lineages is further complicated by geographic variation between populations of mutualists (Thompson 1994, 1999). At increasing geographical scales, populations may both be subjected to genetic drift of symbionts and they may adaptively converge on gradients of different mutualistic partners resulting in stable geographic mosaics (Parker 1999; Thompson 1999). Although firm generalizations are as yet impossible, there seems to be an emerging consensus that local coadaptation between mutualistic symbionts is unlikely as pronounced as in certain parasite-host interactions, where the host consistently evolves to minimize the interaction while imposing constant pressure for pathogen specialization (Thompson 1999). It has also become clear that not all combinations of hosts and mutualistic symbionts are equally fit (e.g., Bever 1999 and references therein). For example, in a recent study on *Bradyrhizobium* nitrogen-fixing bacteria and the legume host *Amphicarpaea bracteata,* Wilkinson et al. (1996) showed that all *Bradyrhizobium* strains enhance host fitness. However, the locally coexisting combinations performed better than the allopatric combinations, and the performance of the latter was related to the genetic distance from the experimental host to the native host.

Here we present an experimental study addressing questions of fungus cultivar diversity within and across their *Acromyrmex* leaf-cutting ant hosts. Using reciprocal transplant experiments, we investigate the specificity, short-term performance, and compatibility between fungus cultivars and ant colonies of two closely related sympatric species, *A. octospinosus* and *A. echinatior.* We combine the results of these transplant experiments with amplified fragment length polymorphism (AFLP) data estimating genetic variation of the entire population of fungus cultivars. We analyze and review some of the mechanisms responsible for partial incompatibility and interpret our results in the light of Frank's (1996a) theoretical concepts on host-symbiont conflict over the mixing of symbiotic lineages.

Natural History of the Model System

Attine ants cultivate fungus cultivars derived from five different lineages of mushrooms, four from within the Lepiotaceae and one from within the Tricholomataceae. Although the phylogenies of the ants and their fungus cultivars are congruent at the deepest levels, coevolutionary patterns between ants and their preferred cultivars are reticulate due to lateral transfer of cultivars and specificity across species is relatively low (Mueller et al. 1998; S. A. Rehner, U. G. Mueller, C. R. Currie, and T. M. Schultz, unpubl. ms.). The fungus cultivars of the evolutionary advanced leaf-cutting ants (the genera *Atta* and *Acromyrmex*) are derived from the Leucocoprineae (Lepiotaceae; Johnson 1999) and no closely related free-living species have yet been discovered, despite intensive sampling (Chapela et al. 1994). In contrast to the lower attines (see below), the symbiotic fungi of the higher attines have undergone both morphological and physiological adaptations in their association with ants (Weber 1966, 1972; Mueller et al. 2001), which explains why options for living outside the mutualism are severely restricted, if not completely absent. The ants are likewise fully dependent on their fungus cultivar for food.

In contrast to mycorrhizae (e.g., Bever et al. 1996; Sanders and Fitter 1992) and the legume-*Bradyrhizobium* mutualism (Wilkinson et al. 1996), where horizontal transmission is the rule, the fungal mutualist of leaf-cutting ants is vertically transmitted from one ant generation to the next (Weber 1972). This happens when gynes (winged, prospective queens) take a small fragment of the fungus garden from their natal colony along on their mating flight and use this to start their own fungus garden. Lateral transfers may occur, however, after

the loss of a fungus garden (Adams et al. 2001) or during the colony founding stage. Opportunities of the latter type may occur when colonies are initiated by multiple foundresses, as is the case in some *Acromyrmex* and *Atta* species (Rissing et al. 1989; Mintzer 1990; possibly also in one of our study species *A. echinatior,* Bekkevold et al. 1999). Alternatively (but not mutually exclusive), transfers of cultivars may be facilitated by close proximity of potentially competing founding colonies (Autuori 1950; Rissing et al. 1989), especially when they aggregate under the same log or stone (Rissing et al. 1989; observed for our other study species, *A. octospinosus,* in Panama, U. G. Mueller, pers. comm.).

In the terminology used by Frank (1996a), the natural history of the symbiosis between attine ants and their basidiomycete fungus can be characterized as follows. The symbiosis is an obligate mutualism and has a clear net beneficial effect on both partners. Dikaryotic mycelia of free-living basidiomycetes are known for their individualistic behaviors resulting from incompatibility reactions among nonrelated strains (see Discussion). The fungus cultivars of the lower attine ants have recently been shown to be closely related to (and sometimes indistinguishable from) free-living fungi (Mueller et al. 1998). This implies that incompatibility mechanisms and the potential for sexual reproduction probably have not been lost. It is therefore no surprise that vertical transmission of cultivars is already present in the lower attines and possibly represents a trait that predated the origin of fungus rearing in the attine tribe (Mueller et al. 2001). Even the mutualistic fungi of the higher attines are known to fruit on occasions (Muchovej et al. 1991; Dörfelt and Creutzburg 1994; Fisher et al. 1994 and references therein). This form of sexual reproduction sometimes occurs after horizontal transmission of, or contamination with, alien fungi. It is usually accompanied by severe worker mortality, often (but not always) followed by the complete collapse of the fungus garden and the death of the colony (Dörfelt and Creutzburg 1994; Fisher et al. 1994; A. N. M. Bot, pers. obs.). This implies that the attine fungus cultivars may have retained competitive traits that enhance the reproduction and/or transmission of cultivar lineages when mixing of lineages occurs. Because competition between cultivars leads to a reduction of the fitness of the ants, competitive traits of the fungus can be considered in the same light as virulence traits in parasitic symbioses.

It seems reasonable to assume that the ant hosts are able to recognize alien cultivars in the same way as they are able to recognize and weed out pathogens (Currie et al. 1999a; Currie and Stewart 2001; A. G. Hart, A. N. M. Bot, and M. J. Brown, unpubl. ms.). The resident fungus should also have retained mechanisms to recognize alien strains of fungus. However, instead of investing considerable resources in the expression of incompatibility virulence, the derived fungal lineages of the higher attines may suffice with producing signaling compounds that characterize a fungal clone and induce the ants to recognize and remove genetically different clones. With or without the help of their resident fungus, the ants are expected to have evolved efficient control mechanisms to retain their resident fungus in a genetically homogeneous state. This is because, as long as they have a well-functioning cultivar, the chance that they will reap long-term

benefits from acquiring a potentially superior cultivar is minor in comparison to the short-term risk of suffering immediate fitness loss or even death of the fungus garden due to competition between the resident and the introduced cultivar.

Both the ants and their resident fungus cultivar are thus expected to express hostile reactions to incoming unrelated strains of fungus, but to accept fungal strains from other colonies when they are genetically identical to the resident cultivar at specific recognition or compatibility loci. In the absence of knowledge on the identity of these compatibility loci, genetic similarity can be estimated by techniques that measure genomewide variation at mostly neutral loci. The latter type of variation can be used as a proxy for between-strain variation at incompatibility loci, although considerable noise on this predictor variable cannot be excluded (see Discussion).

Vertical transmission allows the ant hosts to control the movement and mixing of cultivars during mating flights, but this does not preclude confrontations with horizontally transmitted, alien strains later on (Autuori 1950). Cultivars, in contrast, have little power to oppose the interests of their hosts, except perhaps for rare sporulation events with the potential of horizontal infection of competing strains established as fungus gardens of other colonies. The effective control by the host ants over cultivar transmission may have significantly reduced the populationwide polymorphism for incompatibility genes (cf. Douglas 1994; Frank 1996a; see also Herre et al. 1999). However, it is unlikely to have eliminated genetic variation altogether, because control is not perfect and *Acromyrmex* populations are subdivided to at least a certain extent, so that local adaptations and population differentiation due to genetic drift are likely to occur (Parker 1999; Thompson 1999).

MATERIALS AND METHODS

The Fungus Exchange Experiment

Three colonies of *A. octospinosus* (nos. 31, 38, 41) and three colonies of *A. echinatior* (33, 47, 48) were used. These queenright colonies were part of a larger sample collected in January and February 1996 from a remnant of secondary growth forest in Gamboa, Republic of Panama. The total area of the collection site was about 0.3 km². Within the area the typical ranges of the two species overlap, although *A. octospinosus* tends to be restricted to shaded habitats within the forest, whereas *A. echinatior* is more common in partly sun-exposed habitats. The mature colonies sampled were quite spaced out and never within foraging distance of each other. For each colony, part of the fungus garden, worker ants, and the queen were transported to Denmark and set up as laboratory colonies in a climate room with a constant temperature of 25°C and a relative humidity of 60–70%. *Acromyrmex echinatior* used to be known as a local form of *A. octospinosus,* but was recently elevated to full species status on the basis of morphological and allozyme evidence (Schultz et al. 1998). The *Acromyrmex* colonies were maintained on a diet of bramble leaves (*Rubus* sp.; cf. Bot and Boomsma 1996) and had maintained a stable fungus garden size of circa 1 L for at least two months prior to the start of the experiment.

The experimental set-up consisted of small round plastic vials (diameter 2.5 cm, height 4 cm) with moist tissue paper at the bottom to provide humidity. A fresh green bramble leaf was folded into the vial and a fungus fragment of approximately 80 mg was placed on top of the leaf. The fungus fragments used were always collected from the top of the fungus garden, because active mycelial growth is restricted to this upper region, which therefore tends to be more vigorous and homogeneous than the older, lower part of the fungus garden. By choosing fungus from the upper part of the garden, any differences in fungus quality between replicates was minimized. One pupa was added to each vial, to minimize the possibility of abnormal worker behavior resulting from the absence of brood. The experimental subcolonies were completed by adding a total of eight worker ants (four minors, two media, and two majors) per vial.

Fungus garden fragments were combined with ants from either their original colony or from one of the five other colonies, so that all pairwise combinations of ants and fungus were tested with two replicates each. The fungus was weighed on days 0, 2, and 4 of the experiment, and on day 2 the subcolonies were given clean vials with fresh leaves. The experiment was repeated six times over a period of five weeks in January and February 1998, so that a total of 12 replicates per treatment were available for analysis. In the entire series of experiments, only 12 of 432 (3%) pupa died and only 19 of 3456 (0.5%) workers died. There was never more than one mortality case per vial, so that these low mortality rates were assumed not to have affected the condition of fungus fragments.

Genetic Comparison of Fungus Clones

Genetic relatedness among fungus clones originating from different experimental colonies was inferred from analysis of AFLP markers. Fungus cultivars from 40 colonies of *A. octospinosus* and *A. echinatior,* collected from the Gamboa population between 1993 and 1998, were isolated into pure culture. Isolation was accomplished by transferring small fragments of fungus garden onto petri dishes containing potato dextrose agar (PDA; Difco, Detroit, MI) adjusted to 50 μg/ml streptomycin sulfate and penicillin G. Cultivar outgrowths from the garden explants were serially subcultured to fresh plates until they were free of contaminant fungi and bacteria. Mycelium for DNA extractions was produced by transferring cultivar strains into liquid potato broth (PDB; Difco) and shaking at 100 rpm for approximately 20 days. DNA extractions followed Rehner and Samuels (1994), and AFLP reactions and data acquisition were performed as described by Vos et al. (1995). AFLP data were generated for two selective primer combinations, Eco-CC × Mse CAT and Eco-CC × Mse-CAC. AFLP bands were treated as binary characters and scored as present or absent for each cultivar strain and entered into a data matrix. The AFLP data were analyzed under the parsimony criterion with PAUP* (Swofford 1998).

Phylogenetic trees were inferred from the AFLP data using a heuristic search with random input order and TBR branch swapping. Node support was assessed by 2500 bootstrap pseudoreplicates using input and swapping parameters. The tree presented in this paper represents a monophyletic group

of cultivars, which contains the six colonies that were used for the experiments and is nested within a larger tree (not shown) that also includes the cultivars of three Panamanian species of leaf-cutting ants classified in the genus *Atta* (S. A. Rehner, U. G. Mueller, C. R. Currie, and T. M. Schultz, unpubl. ms.). The overall phylogenetic relationships of the fungi were not known at the time when the exchange experiments were performed.

Experiments on the Background of Incompatibility

Two additional experiments were performed to investigate whether the ant-fungus incompatibility observed in one of the reciprocal transplants was due to antagonistic behavior of the ants or their resident fungus. Attine ants are known to transfer fungal enzymes acquired from eating mycelium and gongylidia in the older (lower) parts of the fungus garden to the freshly incorporated vegetable material and newly grown fungus in the upper part of the garden (Boyd and Martin 1975; Martin et al. 1975). These enzymes are essential for the predigestion of leaf material. They are ingested by the ants and are subsequently deposited as fecal droplets on freshly harvested leaf material, so that new inoculates of the fungus cultivar will be able to grow faster (Boyd and Martin 1975; Martin et al. 1975). If the cultivar fungi produce chemical compounds that inhibit the growth of alien strains, it seems not impossible that these compounds could also hitchhike through the ant gut together with the growth-enhancing enzymes, because: they can then be produced in the central part of the garden, which is most likely to be genetically pure; they will be targeted in the best possible way, because the ants will deposit fecal droplets on any piece of newly inoculated fungus at the top of the garden that has not yet been recognized and rejected as alien; and they only need to produce relatively subtle incompatibility reactions with fragments of alien fungus, as long as these reactions are sufficient to inform the tending ants that some fragments are genetically different from the resident fungus cultivar.

First, we have tested the effect of ant fecal droplets on isolated, axenic fungus in a reciprocal-cross experiment involving the two most incompatible colonies, *A. echinatior* colony 48 and *A. octospinosus* colony 38, which have genetically distinct fungus cultivars (see Results). Fecal droplets of the ants were obtained by holding individual ant workers by the head. This stressful posture usually induced release of a fecal droplet, which could then be collected in a capillary and applied directly on small tufts of fungus that had been subcultured onto fresh medium (PDA, Difco) several days in advance. Each of the four combinations of fungus and fecal droplets was replicated eight times. After 24 h, we assessed whether the fungus had absorbed the fecal droplets.

Second, we tested whether the incompatibility between ants and fungi was transient. If fungal incompatibility substances are transferred through the gut of ant workers, prolonged enforced exposure to a novel fungus should make the incompatibility effect gradually disappear, as the ants acquire enzymes from the new fungus. This could imply that this proximate mechanism is in fact adaptive. Although the ants are likely to have a direct fitness interest (sensu Frank 1996a) in a genetically homogenous fungus garden, this does not

imply that their hostile responses against alien strains of fungus should last forever. Antagonistic reactions during the first few days are probably quite sufficient to cull any small quantity of introduced alien cultivar. However, retaining this behavior indefinitely may not be selected for, because it will preclude rare opportunities of obtaining a new fungus garden by raiding a neighboring colony after an irreversible loss of the resident fungus garden. The expected parallel interests of both mutualistic partners in a gradual decrease of incompatibility between ants and alien fungi may thus imply that the concentration of compounds of the old fungus in the gut of the ants serves as the proximate cue to regulate the extent of behavioral aggression against alien fungi.

The persistence of incompatibility was analyzed in a reciprocal-cross experiment with the two most incompatible colonies, 38 and 48. For this experiment we used somewhat larger subcolonies, in which the fungus was replaced every day by fresh fungus from the source colony of the transplant. The treatments and controls had three replicates, each consisting of 40 ants (20 minors and 20 medium/large workers), four pupae, and 0.5 g of fungus. Each subcolony was maintained in a dish of 6-cm diameter, partly covered by a lid, which was placed inside a nest box of 11 cm by 17 cm. A moist cotton plug was added to each dish to maintain high humidity, and bramble leaves were supplied in the nest box outside the dish as forage for the ants. The weight of each fungus fragment was recorded just before transplanation and when replacing it each day, so that the extent of incompatibility could be inferred from the differences in weight from one day to the next. This experiment also tested whether the ants were able to recognize new fungus cultivars and whether they expressed direct hostile behavior toward new fungus cultivars. To this end, the behavior of the ants was observed for about 15 min directly after introducing a fresh fragment of fungus.

Statistical Analysis

Because not all replicates in the fungus exchange experiment were set up on the same day, we first tested for the effect of starting date on fungus mortality. We used a χ^2-analysis to test the observed distribution of fungus mortality against the expected distribution for each of the 16 (of 36) series of ant-fungus replicates where any fungus mortality took place. The null hypothesis was that mortality was spread evenly over all replicates, and the alternative hypothesis was that mortality occurred more or less often within replicates that were set up on the same day. The relative changes in fungal weight from day 0 to day 4 (see Fig. 1) were analyzed by means of a two-way mixed analysis of variance (ANOVA) using PROC GLM in SAS for Windows (ver. 6.12, SAS Institute 1994). To achieve normally distributed residuals with homogenous variance, the values of the dependent variable ($y = W/W_0 = [W_4 - W_0]/W_0$, where W_0 and W_4 are fungal weights at the beginning and at the end of the experiment respectively) were subjected to a square root transformation: $y^* = [(W_4 - W_0)/W_0]^{1/2}$ if $W_4 > W_0$ and $y^* = -[(W_0 - W_4)/W_0]^{1/2}$ if $W_0 > W_4$. Ant species and fungus ''species'' (''species'' for fungus because it was unknown at the time of the experiment whether cultivars from the two ant species

were distinct) were included in the model as main effects, and colonies were nested within species (random effects). The full model also included interaction terms between these variables. Statistical tests were based on Type III expected mean squares when random effects were included in the model, otherwise on Type III sums of squares. The residuals were tested for normality by means of Shapiro-Wilk's test (W) using PROC UNIVARIATE in SAS, and variance homoscedasticity was assessed visually from plotting the residuals against the predicted values.

We used correspondence analysis in JMP (SAS Inst. 1995) to quantify the degree of similarity in the pattern of mortality between the transplanted fungus cultivars of the six *Acromyrmex* colonies and pooled the data of all 12 replicates also for this analysis. The obtained correspondence scores were subsequently compared with the phylogenetic distance of the fungus cultivars. Correspondence analysis is similar to principal component analysis, but uses binary data, which may be, but are not necessarily, symmetrical. Our hypothesis that incompatibility is ultimately due to fungus-fungus interactions and/or to selection on ants to cull novel fragments of cultivar predicts that patterns of incompatibility should be symmetrical. This means that any degree of incompatibility of ants from colony A toward fungus from colony B should be expressed to the same extent as the reciprocal incompatibility between ants from colony B and fungus from colony A. Correspondence analysis provides a quantitative analysis of the (a)symmetry of interactions by producing two plots, one describing the fungus-ant (in)compatibility and another describing the ant-fungus (in)compatibility. Any difference between these two plots thus indicates asymmetry of the observed incompatibility interactions. This analysis incorporates both the direct incompatibility between two colonies and the similarity in incompatibility patterns toward other colonies.

RESULTS

Variable Compatibility between Ants and Fungi

None of the controls where ants tended their own fungus showed any fungus mortality. Both in these controls and in a number of transplants involving different colonies, the fungal fragments maintained an almost constant weight over the four days of the experiment (see Fig. 1), with a slight increase in variance over time. However, in other combinations involving different colonies, fungal fragments died at varying rates, and in some combinations most or all of the 12 replicate fungal fragments died. The 16 *P*-values from the χ^2-tests for independence of fungus mortality of starting date of the experiment ranged from 0.049 to 0.998. Only one of these *P*-values was significant at the 5% level prior to a Bonferroni adjustment, so that we decided to ignore the effect of starting date and to pool all data in the subsequent analyses.

Figure 1 shows that if the ants of one colony were incompatible with the fungus of another colony, the reverse was also the case. *Acromyrmex octospinosus* colony 38 is the main source of fungus mortality. It is incompatible with all other colonies, irrespective of the species to which they belong. *Acromyrmex octospinosus* colony 31, on the other hand is compatible with two other colonies of the same species and
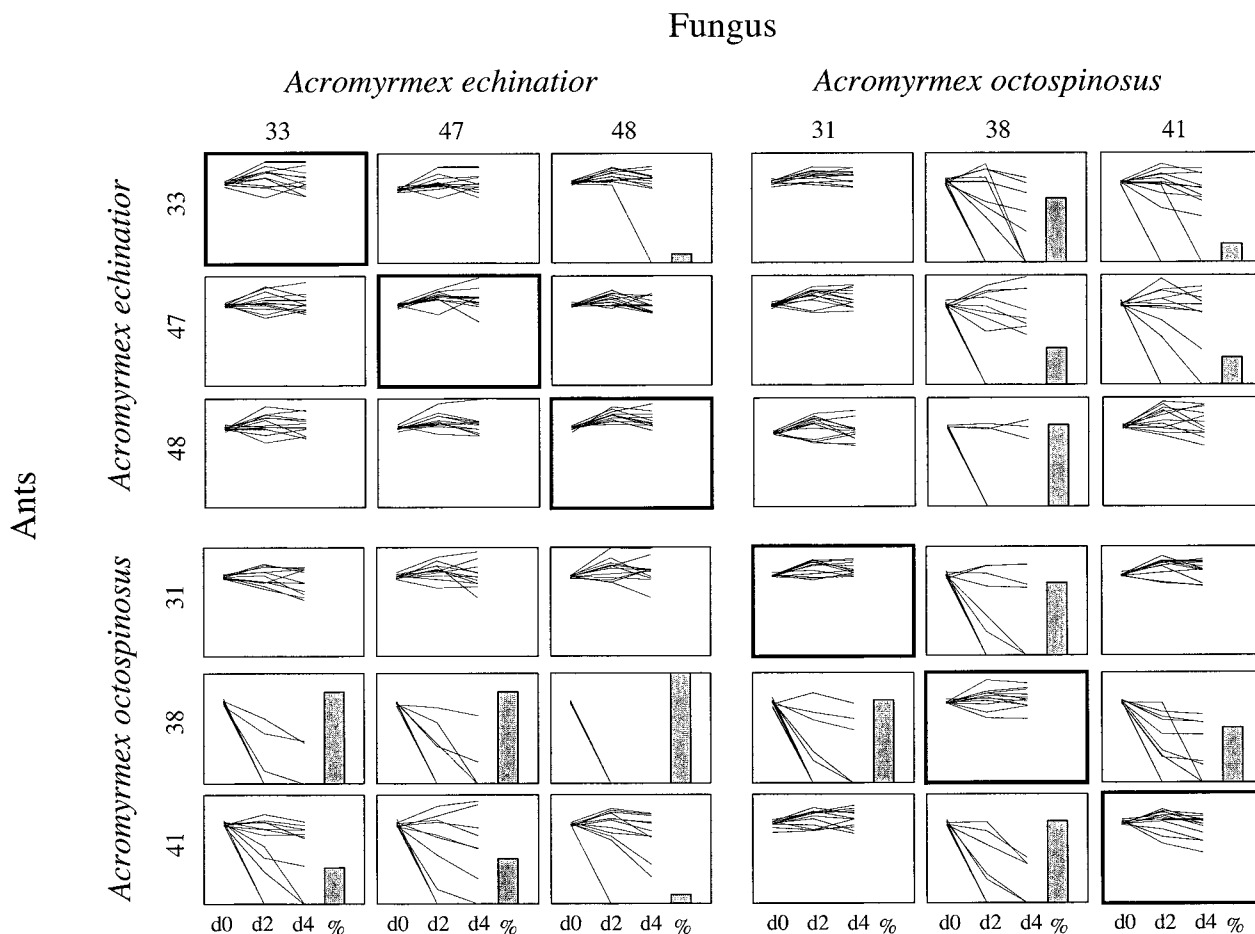
Fungus

FIG. 1. The fungus weight measurements at days 0, 2, and 4 for 12 replicates in the different combinations of ants and fungus from three colonies of *Acromyrmex octospinosus* and three colonies of *A. echinatior*. Control combinations, in which fungus and ants came from the same colony, run the diagonal of the figure and are accentuated with bold frames. Y-axes range from 0 mg to 110 mg in all figures and the fungus weight at day 0 is approximately 80 mg in all cases. Bars show the proportion (ranging from zero to one) of the 12 replicates in which the fungus had died completely by day 4 of the experiment.

with all *A. echinatior* colonies. Barring the single and probably accidental death in the 33-48 combination, the only other cases where mortality of part of the fragments occurred where combinations involving *A. octospinosus* colony 41, on one hand, and *A. echinatior* colonies 33, 47, and 48 on the other.

The full model analyzing the data of Figure 1 explains 60% of the change in weight of the fungal fragments from day 0 to day 4 (see Table 1). None of the species-level main factors are significant, but both the colony-level effects and the interactions are ($P < 0.0001$ in all cases). This indicates that interactions between ants from one colony and a fungal fragment from another colony cannot be predicted from knowledge about the species of ant, without simultaneously taking the source of the fungus into account. However, gradually reducing the model to fewer factors showed that ant species alone can explain almost 10% of the variation, which is the simplest possible significant model ($P < 0.0001$), whereas fungus ''species'' never has any significant effect (see Table 1). W-values of the Shapiro-Wilk tests are close to the expected value of one. This indicates that deviations from normality are small, although the large sample size makes these deviations significant, particularly in the reduced

models. Residual plots showed no indication of variance heteroscedasticity. Overall, the analyses of Table 1 show that the major patterns visible in Figure 1 are highly significant: That material sampled from colonies of *A. octospinosus* is equally likely to be compatible or incompatible with material from its own species or the other species *A. echinatior*, and *A. echinatior* shows a striking difference between interactions with material from its own species and material from the other species, *A. octospinosus*.

Genetic Similarity of Fungus Cultivars and Ant-Fungus Incompatibility

We compared the experimentally obtained fungus mortality data with the genetic distances of the fungal clones in the AFLP phylogeny of a much larger sample of *Acromyrmex* cultivars in the Gamboa area (see Fig. 2A). AFLP comparisons among the *Acromyrmex* fungus cultivars yielded a total of 56 bands that were polymorphic within the sample of isolates examined. Parsimony analysis of these data gave 896 equally most parsimonious trees of length 105 steps, CI = 0.5243 (excluding uninformative characters), and RI =

TABLE 1. Summary of the statistical analysis of the data of the transplant experiment illustrated in Figure 1. The first column (source) gives the independent variables and their interaction terms. The following columns give the results (F-values with P-values in parentheses) of various general-linear-model analyses of the weight change of the fungal fragments between day 0 and 4. The Shapiro-Wilk test statistic for normality of residuals (W, with P-value in parentheses), the fit of the entire model (F-values with P-values in parentheses), and the percentage of the total variance of the dependent variable explained by the model R^2) are given at the bottom.

Source	Main effects only	Plus first-order interaction	Plus nested effects	Full model
Fungus ''species''	$2.94_{1,429}$ (0.0873)	$3.11_{1,428}$ (0.0785)	$0.25_{1,4}$ (0.6437)	$0.25_{1,4}$ (0.6437)
Ant species	$42.27_{1,429}$ (<0.0001)	$44.76_{1,428}$ (<0.0001)	$2.47_{1,4}$ (0.1909)	$2.47_{1,4}$ (0.1909)
Fungus ''species'' $\times$ ant species		$26.30_{1,428}$ (<0.001)	$36.13_{1,420}$ (<0.0001)	$52.26_{1,396}$ (<0.0001)
Fungus colony within fungus ''species''			$17.13_{4,420}$ (<0.0001)	$24.78_{4,396}$ (<0.0001)
Ant colony within ant species			$24.86_{4,420}$ (<0.0001)	$35.96_{4,396}$ (<0.0001)
Fungus colony within fungus ''species'' $\times$ ant colony within ant species				$8.82_{24,396}$ (<0.0001)
W (Shapiro-Wilk test)	0.930 (<0.0001)	0.934 (<0.0001)	0.970 (<0.0001)	0.978 (0.0267)
Fit of model (F)	$22.60_{2,429}$ (<0.0001)	$24.72_{3,429}$ (<0.0001)	$24.53_{11,420}$ (<0.0001)	$17.20_{35,396}$ (<0.0001)
R^2	0.0953	0.1477	0.3912	0.6032

0.9030 (see Fig. 2A). The cultivars of the two sympatric species of *Acromyrmex* leaf-cutting ants are represented on all major branches of the cultivar phylogeny, and the two ant species thus share all major fungal clones. The two plots that resulted from the correspondence analysis (see Fig. 2B, C) are almost each other's mirror images, confirming the apparent symmetry in the mortality results illustrated in Figure 1. The distances between colonies in the correspondence plots indicate how similar or different the observed incompatibility patterns were. Compatible colonies with similar patterns of incompatibility toward other colonies are close together in the correspondence plots. Colonies 48 and 31, for example, are the only colonies that are incompatible to colony 38 but at least partially compatible to all other colonies. In the ant-fungus incompatibility plot (see Fig. 2B), these two colonies are identical in value, whereas there is a minor difference between them in the fungus-ant incompatibility plot (see Fig. 2C), due to a single case of possibly accidental mortality in combinations between the fungus from colony 48 with the ants from the colonies 33 and 41 (see Fig. 1).

The two axes presented in the plots of Figures 2B and 2C explain 95% of the variance in the data. The first axis (C1) describes the variation between groups, which corresponds to differences between the major clades in the fungal phylogeny of Figure 2A. Colony 38, which is incompatible with all other colonies, has an opposite sign compared to the other five colonies along the C1 axis. The fungi of colony 31 and 48 are most distantly related to the fungus of colony 38, and they are most distant from this colony in the correspondence plots. Colonies 33, 41, and 47 belong to an intermediate branch in the phylogeny and also have an intermediate position in the correspondence plots. The second axis in Figures 2B and 2C describes the variation within clades of the fungal phylogeny and is only variable for the clade containing colony 33, 41, and 47. Colony 41 comes out with a sign opposite to colonies 33 and 47, which corresponds to the partial incompatibility between these colonies. The fungal clones of

these three colonies are almost identical, but 41 is an *A. octospinosus* colony, whereas 33 and 47 are *A. echinatior* colonies. The other two clades in the phylogeny do not have within-clade incompatibility and their colonies thus have values close to zero on the horizontal axis (C2).

Proximate Factors Contributing to Incompatibility

Incompatible ants and fungi became compatible after one to two weeks of repeated and enforced exposure to the initially incompatible cultivar (see Fig. 3). During the first days of exposure to a new cultivar, weight losses of the cultivar fragments amounted to 65–100%, which in the previous experiment implied complete incompatibility and rapid death of the transplanted fungal fragment. Gradually, however, incompatibility effects became less severe, and after approximately 10 days the daily weight losses of the introduced fungal fragments were no longer significantly different from the controls. During the experiment the ants were regularly observed to dissemble incompatible fungus in little pieces. They would turn these pieces around between their front legs and chew on them with their mandibles, possibly actively killing the incompatible fungus. This behavior was never observed in the controls.

Experimental application of fecal droplets to mycelia of nonresident fungi did not cause death of the artificial culture. However, cultures did appear to behave differently toward fecal droplets that originated from resident host ants and those that originated from the nonresident host ants (see Table 2). Fecal droplets could either be absorbed or rejected by the fungus. The fungus from colony 48 rejected most (75%) of the droplets from ants of colony 38 and absorbed most (88%) of the droplets of its host workers. This matches the 100% mortality observed for this (cultivar 48/ants 38) combination in the transplant experiment (see Fig. 1). However, the fungus of colony 38 rejected and accepted as many droplets from its own ants (50%) as from ants from colony 48 (50%). This
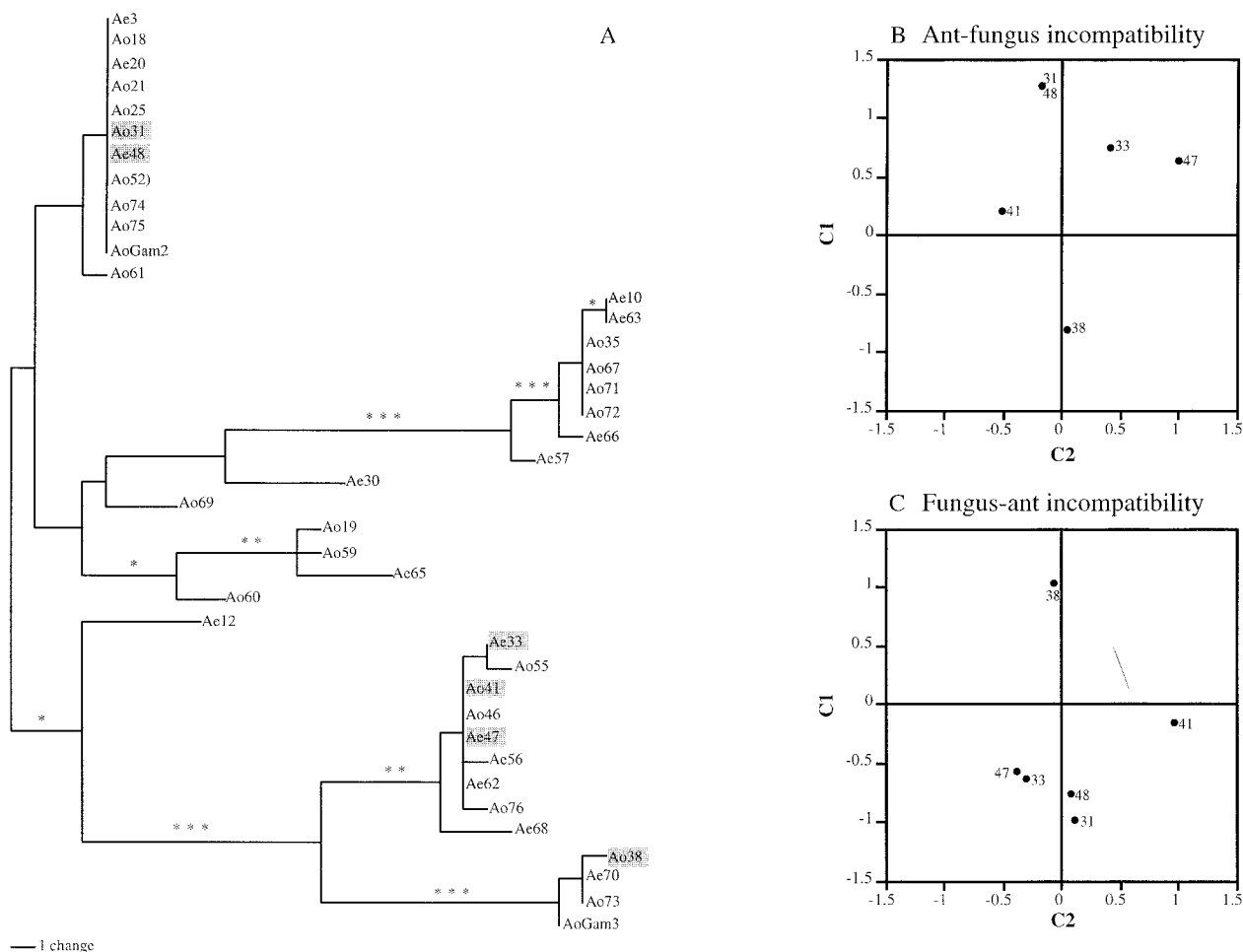
Fig. 2. The phylogeny of the fungus cultivars of 16 *Acromyrmex echinatior* and 24 *A. octospinosus* colonies collected in Gamboa, Panama, in 1993, 1994, and 1996. (A) The tree is a midpoint rooted phylogram. The branch lengths are proportional to the number of changes in the AFLP markers, and the asterisks indicate bootstrap support greater than 50% (*), greater than 70% (**), or greater than 90% (***). Shaded boxes around colony numbers indicate the six colonies that were included in the exchange experiment and in the correspondence analysis. (B) The results of the correspondence analysis for the reactions of ants to fungi. (C) The same results for the reactions of fungi to ants.

suggests that incompatibility is less pronounced in the reciprocal (cultivar 38/ants 48) combination, which corresponds again with the results given in Figure 1 (75% of the 12 transplanted fungal fragments died, but 25% maintained their original weight). The overall proportion of absorbed droplets (see Table 2) for within-colony interactions was 69%, whereas the corresponding figure for across-colony interactions was 37.5%. When tested by a logistic analysis (JMP) this overall difference was marginally significant (maximum-likelihood $\chi^2 = 3.7$; $P = 0.054$). The fungus quickly absorbed compatible droplets, but actively contained incompatible droplets. This was done by growing hyphal tips around the droplet, perpendicular to the surface, possibly to minimize contact at the interface. Contained droplets dried up after a few days without being absorbed. Occasional absorption of droplets from alien ants would sometimes lead to a dark red coloration of the PDA medium underneath the fungus. Such pigment accumulations often accompany incompatibility reactions of fungi both in vitro and in vivo, but the effect could not be quantified. This observation im-

plies, however, that the results of Table 2 would have been significant if the categories had been scored as ''normal absorption'' and ''abnormal absorption.''

DISCUSSION

The results of our study show that there is substantial genetic variation among fungal cultivars reared by two sympatric species of leaf-cutting ants and that these two species share all major clades of cultivars, indicating that horizontal transfer does occasionally take place. Given the life-history characteristics of these basidiomycete symbionts, this variation implies that host-symbiont conflicts over symbiont mixing are unavoidable. We have extended a verbal model by Frank (1996a) to derive predictions as to how these conflicts might be expressed and have confirmed these predictions in a series of experiments. Our findings show that certain combinations of ants and cultivars are imcompatible. Genetic differences between cultivars can explain part of these incompatibily reactions, but incompatibility is mostly due to
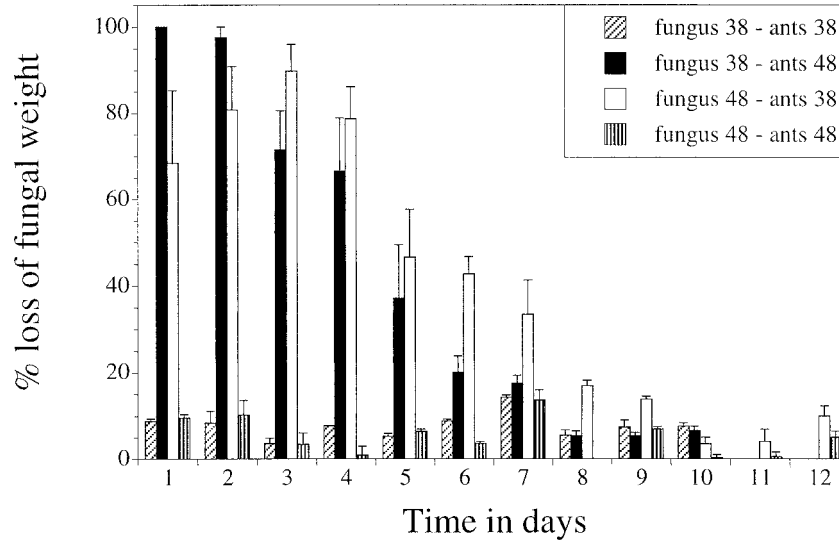
FIG. 3. The proportion of fungus weight that was lost in combinations of fungus and ants from two incompatible colonies (*Acromyrmex echinatior* colony 48 and *A. octospinosus* colony 38) versus the controls that contained ants and fungus from the same colony. The value 100% corresponds to 0.5 g of fungus. Error bars represent standard errors (*N* = 3).

hostile behavior of the ants toward introduced cultivars, although this behavior is possibly triggered by compounds from the fungus that are transferred via the alimentary tract of the ants. We also found that this imcompatibility is transient and disappears after about 10 days.

The experimentally observed incompatibility reactions suggest that the success of horizontal transmission is likely to be low under normal field conditions. However, we note that some transplants in Figure 1 were fairly successful, even though they crossed ant-species barriers and despite genotypic differences (e.g., colonies 41 and 48; 31 and 47). This would suggest that some horizontally transferred mycelial fragments could possibly survive in other fungus gardens without being recognized as a potential source of virulence by either the ants or the resident fungus. Applying an equivalent of the Hamilton and May (1977) model, it would thus seem that natural selection may maintain some horizontal transmission even in the higher leaf-cutting ants. This is because kin selection has both integrative and disruptive aspects, a phenomenon coined the paradox of kin selection by Frank's (1997): The higher the relatedness of symbionts within single hosts, the more they also compete with relatives so that some horizontal transmission is favored even if the success rate is very low. Because such horizontal transmission is normally detrimental for both the tending ants in a resident

colony (who have no interest in the fruiting bodies of their cultivar) and the recipient ants (whose fungus gardens may suffer from virulence expressed by their cultivar after infection with a novel strain), the ants are under constant selection to suppress these tendencies in their cultivars. Due to the extreme asymmetry in manipulative power between the host and cultivar (sensu Frank 1997), the leaf-cutting ants seem to have won the evolutionary conflict with their cultivars and seem to have obtained a high degree of control. However, full control has not been achieved, because the fungus cultivar is an ectosymbiont whose occasional spores or transported mycelial fragments can survive at least short periods of transfer from one host to the other. Given the continuing interest of fungus cultivars in normal, vertical transmission by ant gynes, this conceptual scenario would predict that rare events of horizontal transmission are most likely to occur when an ant colony or fungus garden for some reason is doomed. In other words the (small) benefits of horizontal dispersal by the fungus would no longer be opposed by a cost when future reproductive options via vertical transmission are lost but there is still the necessary mycelial mass to grow a fruiting body. This may happen for example when a rare and virulent disease kills most of the worker force in a matter of days, or when the mother queen becomes senescent or dies at a time when none of the diploid brood can be raised into reproductive gynes. Note that only gynes are vectors of vertical transmission of the cultivar, so that the presence of male brood is irrelevant from the perspective of the fungus.

TABLE 2. Rejection or absorption of fecal droplets by fungus of incompatible colonies grown on artificial medium. Eight droplets were applied and the number of visible (unabsorbed) or absorbed droplets were scored after 24 h.

Fungus origin	Ant-feces origin	Visible droplets	Absorbed droplets
Colony 38	colony 38	4	4
Colony 38	colony 48	4	4
Colony 48	colony 38	6	2
Colony 48	colony 48	1	7

Cultivar Polymorphism

Our results indicate that resident fungus cultivars have a significant direct and indirect (via hostile ant behavior) competitive advantage over nonrelated intruder cultivars. This implies that, although potential competition between strains of cultivars is common, actual cases of competition are probably rare. However, horizontal transmission is apparently still

common enough for related sympatric species to share a common pool of cultivars (see Fig. 2A). This result matches earlier findings for the lower attine ants, which share cultivar lineages across distantly related species and genera (Mueller et al. 1998). It is less clear, however, what maintains the substantial populationwide genetic variation among fungus cultivars. Law's (1985) conceptual scenario of conflict-free mutualism in a homogeneous environment inducing a single symbiont genotype through positive frequency-dependent selection is in fact the null hypothesis of the more modern pluralistic view of mutualistic interactions (e.g., Frank 1996a; Bever 1999; Herre et al. 1999; Parker 1999; Thompson 1999). It is clear that the observed genetic diversity of cultivars with at least three major clades in our study population (see Fig. 2A) rejects that null hypothesis. However, our present data from just one population do not allow us to distinguish between nonadaptive explanations invoking subdivided populations and genetic drift versus adaptive explanations emphasising negative frequency-dependent feedback mechanisms (Bever 1999) or geographic mosaics of locally coadapted host-symbiont complexes (Thompson 1994, 1999; Parker 1999).

The extent of cultivar polymorphism obtained in our study qualitatively matches the patterns of polymorphism found in other recent studies of mutualistic interactions (e.g., Wilkinson et al. 1996; Rowan 1998; Parker 1999). Although variation is substantial, specificity is moderate at best, as the two *Acromyrmex* species share most if not all of the cultivar lineages. However, it is important to note that the *Acromyrmex* species do not share cultivar clones with sympatric or allopatric *Atta* species (S. A. Rehner, U. G. Mueller, C. R. Currie, and T. M. Schultz, unpubl. ms.), so that specificity at the genus level seems to be upheld. Even within the genus *Acromyrmex* some specificity may remain as the two species are somewhat differently represented in the three major clades of Figure 2A. The upper clade has a 3:1 ratio in favor of *A. octospinosus,* the middle clade has a 1.3:1 ratio, and the lower clade a 1:1 ratio. Furthermore, colonies sampled in 1993 and 1994 (numbers 30 and below) make up 5/12 of the upper clade of Figure 2A, 3/14 of the middle clade, and 1/14 of the lower clade. These differences are not significant, but do suggest that a larger-scale study of possible spatial and temporal segregation of cultivars may be rewarding.

Incompatibility in Free-living Basidiomycetes and Cultivar Fungi

Free-living fungi usually defend themselves against fusion with nonself mycelia by the mechanism of mycelial incompatibility (Leslie 1993; Anderson and Kohn 1995; Worrall 1997). Mycelial incompatibility is regulated by a multilocus, multiallele genetic system, where clones carrying identical alleles at incompatibility loci are compatible and can fuse to form a single physiologically and genetically integrated mycelium. In contrast, clones with different combinations of alleles at these loci will be incompatible so that physiological fusion will not take place, either because the mycelia show an avoidance reaction or because of programmed cell death of the hyphae in the contact zone. The intensity of incompatibility between mycelia of basidiomycete fungi increases

with an increasing number of such loci on which they carry different alleles (Hansen et al. 1993). Normally, mycelial incompatibility patterns are correlated with relatedness between fungi (Worrall 1997), so that stronger reactions occur between more distantly related fungi. However, genomewide similarity estimated by AFLP markers is not necessarily an accurate predictor of similarity at incompatibility loci. This may explain why the extent of incompatibility of the genetically most distant colony (38) with colonies on the other major clades of the phylogeny of Figure 2A reflects the genomewide genetic distances quite accurately, whereas the within-clade incompatibilities sometimes do not show such correlation. For example, the partial incompatibility pattern of colony 41 with colonies 47 and 33 is inconsistent with the absence of AFLP differences between the fungi involved, especially because colony 41 is mostly compatible with colonies 31 and 48, which belong to a different clade. An explanation for this inconsistency could be that colony 33, 41, and 47 were cultivated by different ant species (41 is *A. octospinosus* and 47 and 33 are *A. echinatior*). The slight incompatibility of the ants of colony 41 with the fungus of the third *A. echinatior* colony (48) corroborates the suggestion that some degree of ant-species specificity may exist, as is also suggested by the overall statistical analysis (see Table 1). However, the interaction between *A. octospinosus* colony 31 and *A. echinatior* colony 48, which have a genetically identical cultivar (as do colonies 41 and 47), is unaffected by species specificity.

Recognition and polymorphism are essential for genetic coevolution of host-parasite interactions (Frank 1994), but they seem equally important for mutualistic interactions as long as these include aspects of mutual exploitation and virulence (Frank 1996a; this study). Horizontally transmitted cultivars can only be eliminated by the host when they are properly recognized. When symbionts are asexual and transmitted vertically, the interests in accurate recognition of host and resident symbiont are virtually completely aligned, so that the resident symbiont is under selection to aid recognition, provided that the host has the power to cull unwanted symbionts. A possible mechanism serving this purpose in the attine ant-fungus mutualism has been identified in the present study and warrants further investigation to understand its details and to assess whether it also occurs in other symbioses with similar characteristics.

A recent experimental study on lower attines (Adams et al. 2001) has shown that *Cyphomyrmex* colonies whose fungus garden has been lost usurp neighboring colonies. The experiment was done with two sibling species that rear distantly related fungi. Cultivar transfers between colonies could be documented, corroborating inferences on occasional horizontal transmission based on molecular data (Mueller et al. 1998). However, the transfer success rate within ant species was almost four times higher than between ant species, suggesting that incompatibility reactions might have occurred here as well. Our results also provide an interesting alternative explanation to a result obtained by Rissing et al. (1989). In this study the desert leaf-cutting ant species *Acromyrmex versicolor* was shown to have unrelated multiple foundresses, where some foundresses seemingly voluntarily adopted more dangerous forager tasks. It would be interesting

to investigate whether it was predominantly or exclusively the foundress whose own fungus survived, who obtained the status of resident queen. Rissing et al. also observed that *A. versicolor* workers had a tendency to raid brood, leaves, and fungus from each others colonies, but the frequencies of transfers of these items across colonies was not reported. If raiding is indeed a universal phenomenon, the results of our study would make us expect that genetic variation among fungal clones of *A. versicolor* may have become low due to polygyny, so that incompatibility has become rare. However, if genetic variation among clones would prove to be comparable to what we found in Panamanian *Acromyrmex,* we would expect that raiding of fungus fragments should be facultative and conditional on compatibility or should be followed by distinct intranidal nursing behavior of workers to prevent mixing of cultivars.

Interactions with Other Symbionts

Interactions with other microorganisms associated with the ant-fungus mutualism, such as the fungal parasite *Escovopsis* (Currie et al. 1999b) and mutualistic *Streptomyces* bacteria (Currie et al. 1999a), may influence the viability of fungus fragments of different origin. The colonies that were used for the present study had been in the laboratory for two years prior to the experiments. They were thriving and continuously accumulating new fungus garden mass, characteristics that are generally incompatible with *Escovopsis* infections (Currie 2001). Leaf-cutting ant colonies are apparently able to suppress this parasite under optimal laboratory conditions and to remove it completely from their gardens after some time when reinfections do not take place (Currie et al. 1999b). The *Streptomyces* bacteria, however, are always present on the cuticle of *Acromyrmex* workers. Their direct effect on the fungus cultivar is not yet known, but at present it seems unlikely that these bacteria, which defend the fungus cultivar against *Escovopsis* infections (Currie et al. 1999a; C. R. Currie, A. N. M. Bot, and J. J. Boomsma, unpubl. ms.), will have a major effect on compatibility between strains of the fungus cultivar. However, surprises are possible, especially if it could be proven that the *Streptomyces* bacteria are also occasionally horizontally transferred, have ample genetic polymorphisms, and need to be controlled in ways that do not necessarily serve the interest of the fungus cultivar.

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