

# Caste-specific symbiont policing by workers of *Acromyrmex* fungus-growing ants

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The interaction between leaf-cutting ants and their fungus garden mutualists is ideal for studying the evolutionary stability of interspecific cooperation. Although the mutualism has a long history of diffuse coevolution, there is ample potential for conflicts between the partners over the mixing and transmission of symbionts. Symbiont transmission is vertical by default, and both the ants and resident fungus actively protect the fungal monoculture growing in their nest against secondary introductions of genetically dissimilar symbionts from other colonies. An earlier study showed that mixtures of major and minor *Acromyrmex* workers eliminate alien fungus fragments even in subcolonies where their resident symbiont is not present. We hypothesize that the different tasks and behaviors performed by majors and minors are likely to select for differential responses to alien fungi. Major workers forage and cut new leaves and masticate them after delivery in the upper parts of the fungus garden and so are likely to more frequently encounter alien fungus than minor workers maintaining the established fungus garden and caring for the brood. We show that major workers of *Acromyrmex echinator* indeed express stronger incompatibility reactions toward alien fungus garden fragments than minor workers. This implies that only the major workers, through recognition and exclusion of foreign fungus clones at their point of entry to the nest, have a realistic possibility to eliminate alien fungal tissue before it gets incorporated in the fungus garden and starts competing with the resident fungal symbiont. *Key words*: evolutionary stability, leaf-cutting ants, mutualism, symbiont policing. [*Behav Ecol* 20:378–384 (2009)]

The obligate mutualism between the attine ants and their fungus garden symbionts is an ideal model system for studying the evolution of interspecific cooperation (Weber 1972; Hölldobler and Wilson 1990; Herre et al. 1999; Mueller 2002). The fungus is dependent on the ants for dispersal and substrate provisioning, whereas the ants and their brood rely on the food provided by the fungus. The fungus is clonally and vertically transmitted from generation to generation, although horizontal transmission is frequent enough to have obliterated most of the cocladogenesis that vertical transmission could have generated (Chapela et al. 1994; Mikheyev et al. 2006, 2007). A striking recent finding is that the ants grow their mutualistic fungus in a monoculture and actively resist mixing of the genetically variable symbionts that occur in the population as a whole (Bot et al. 2001; Poulsen and Boomsma 2005).

Vertical transmission and monocultures within nests are not necessarily in the interest of both mutualistic partners (Frank 1996). Even when the chance of survival is very low, at least some symbionts will always be selected to disperse horizontally, to escape competition with close relatives (Hamilton and May 1977). However, horizontal dispersal could increase symbiont diversity within nests and induce direct competition between strains of symbionts (Frank 1996). If this happens, a symbiont's increased investment in competition will automatically lead to a reduced investment in the mutualism, so that the productivity of the interaction as a whole decreases. It is therefore in the best interests of the host and resident

symbiont to discover and eliminate symbiont strains that disperse horizontally and mix with other strains (Frank 1995, 1996, 2003). As the original theory on this (Frank 1996) is about host–symbiont conflict over symbiont mixing, we term the discovery and elimination of mixing (i.e., intruding) strains “symbiont policing.” We use the term policing in this context because this behavior is analogous to policing behavior by workers or queens to regulate reproductive conflict between family members in insect societies (Wenseleers and Ratnieks 2006), thus enabling direct comparison of conflict resolution between interspecific and intraspecific cooperation. Two recent studies have indicated that this form of symbiont policing is likely to occur in *Acromyrmex* leaf-cutting ants. Bot et al. (2001) showed that isolated groups of workers tend to destroy fragments of alien fungus that are genetically different from their resident fungus, but to accept alien fragments that are genetically identical or very similar. This implies that the ants actively prevent their resident fungus from mixing with alien fungi. Apart from this behavioral incompatibility in the ants, there is also direct (mycelial) and indirect (fecal droplet) incompatibility between the resident and alien fungal fragments. Poulsen and Boomsma (2005) showed that agar-grown fungal clones become less somatically compatible with increasing genetic difference between them and that the resident fungus imprints the fecal droplets of ant workers to be incompatible with genetically different mycelia. This implies that somatic incompatibility between genetically different fungal strains, a common phenomenon in free-living Basidiomycete fungi (Worrall 1997), has been maintained despite a long-term association with default vertical transmission of the fungus by the ants.

In spite of these consistent and highly significant correlations between incompatibility and genetic distance, a large

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part of the variance in incompatibility reactions in the studies by Bot et al. (2001) and Poulsen and Boomsma (2005) remained unexplained. We hypothesize that a significant part of this may be due to these studies only considering average incompatibility responses across different worker castes and not investigating whether the worker castes themselves may differ in their behavioral response to alien symbionts that are genetically different from the resident symbiont. Such differences would seem likely because the large (major) workers do all the foraging and most of the cutting and masticating of the fresh leaves and should thus be more frequently and directly exposed to accidentally collected fragments of alien symbionts than the small (minor) workers, who specialize on brood care and on maintaining the established parts of the fungus garden (Wetterer 1999; Hughes et al. 2003). Symbiont policing, as defined here, is therefore fundamentally different from weeding behavior toward pathogens. The latter presents a continuous threat to all parts of the fungus garden, so that the worker caste specialized on garden maintenance (minor workers) is likely to be most proficient in this form of prophylactic behavior. However, threats of symbiont competition are likely to be relatively rare but do require immediate attention by major workers during the initial processing of freshly cut leaves that might harbor alien symbionts. It is thus reasonable to hypothesize that the typical tasks of major and minor worker castes might have selected for differences in the acuteness of their symbiont-policing behavior. We therefore investigated whether and to what extent the major worker caste of *Acromyrmex echinator* showed a higher degree of behavioral incompatibility with fragments of alien fungal symbiont than the minor worker caste.

## MATERIALS AND METHODS

### Selection and maintenance of ant colonies

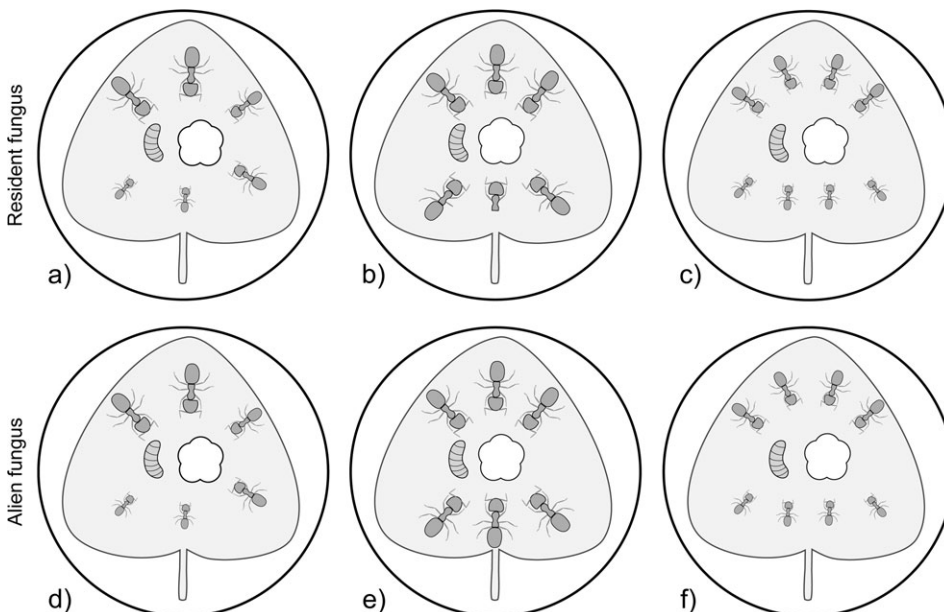
*Acromyrmex echinator* colonies were collected in May 2003 and 2004 in Gamboa, Panama, and were maintained in the laboratory in Fluon-coated (GP1, Van Appeldoorn Chemical logistics, Woudenberg, The Netherlands) boxes at 25 °C and 60–70% relative humidity. Colonies were fed with fresh bramble leaves (*Rubus fruticosus*), apple, and rice and cleaned twice a week (for further rearing details, see Bot and Boomsma

1996; Bot et al. 2001; Poulsen and Boomsma 2005). At the time of the experiments, the colonies had been kept in the laboratory for 2–3 years (after collection at an unknown age) and had an abundant worker force that maintained large and healthy fungus gardens.

### Experimental setup

We made a series of small subcolonies from 3 source colonies: Ae221, Ae263, and Ae266. Subcolonies consisted of a small plastic pot (height 3.5 cm, diameter 2 cm) with a lid with 15 pinholes to provide air. The bottom of the pot was covered with a folded piece of moist paper tissue (ca., 2.5 × 2.5 cm) onto which we placed a folded bramble leaf with a fragment of fungus (80 ± 2 mg) on top of the leaf (Figure 1). Subcolonies were provided with a work force consisting of different combinations of minor and major workers. Because body size variation among minor *Acromyrmex* workers is considerably larger than among major workers, large minors have sometimes been considered to be a medium-sized worker caste (Bot and Boomsma 1996). However, Wetterer (1999) showed that media workers are merely the tail end of a skewed size distribution of the minor caste. As media have never been observed to forage in the field, pooling them with minors was appropriate in the present study. We used equal mixtures of minors and media to have the entire size range of minors represented and to achieve a more constant ratio between ant mass and fungal mass across our different categories of subcolonies.

Three categories of subcolonies were set up: 1) mixed caste (2 minors, 2 media, and 2 majors); 2) major caste (6 majors); and 3) minor caste (4 minors and 4 media) (Figure 1). We used noncallow workers sampled from the top of the fungus gardens of the 3 test colonies and added a single larva from the same colony to maintain normal motivational stimuli for feeding, gardening, and nursing (Bot et al. 2001). The fragment of fungus placed in each subcolony came either from one of the other 2 source colonies (alien fungus) or, in the controls, from the same colony as the workers (resident fungus) (Figure 1). Whereas the subcolonies with resident fungus served as controls for symbiont compatibility, the mixed-caste subcolonies served as controls for standard worker nursing, so that the major-caste and minor-caste subcolonies could test for differences in fungus rearing with either of the single



**Figure 1**

Experimental setup: Top view of the different subcolony categories: mixed-worker subcolonies (a, d), major-worker subcolonies (b, e), and minor-worker subcolonies (c, f) with a fragment of their resident fungus (controls: a, b, c) or a fragment of alien fungus (d, e, f) placed on top of a fresh bramble leaf. All subcolonies received 1 larva.

worker caste categories of subcolonies. All possible combinations between source colonies of fungi and ants ( $3 \times 3$ ) were tested, and the experiment was repeated 3 times during 3 subsequent weeks, with 2 replicates every week. To control for minor workers possibly being less capable of cutting leaves, we provided each subcolony without majors with leaf fragments that had been freshly cut by majors from their own source colony, as soon as cut pieces of leaf were observed (normally after 6 h) in the equivalent subcolonies with majors. After this, minors were equally capable of processing the leaves because the growth and maintenance of fungus fragments in the minor-caste subcolonies with resident fungus (Figure 2d, diagonals) was similar to that in the subcolonies with major workers (Figure 2b,c; see Results).

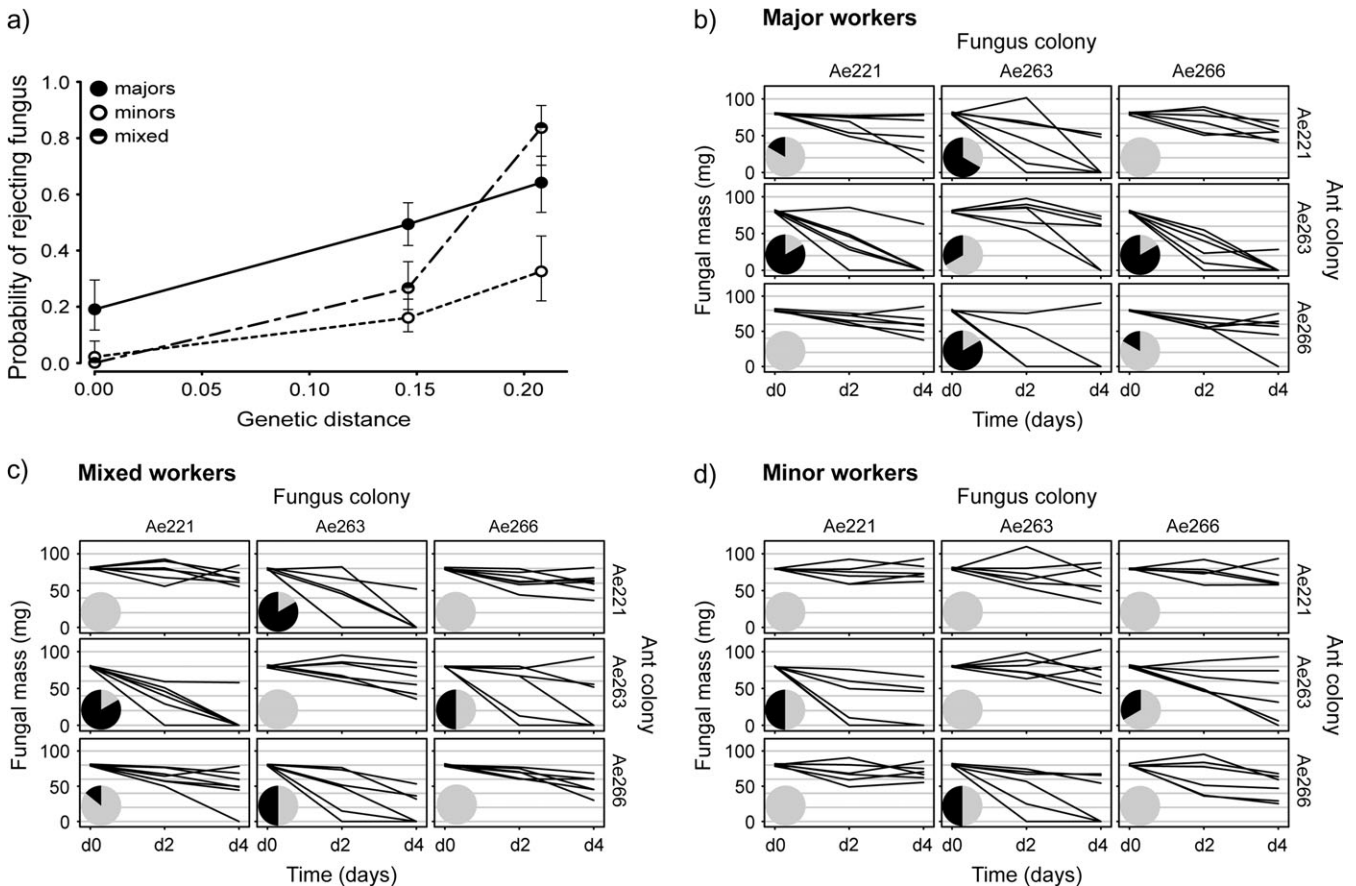
A total of 164 subcolonies were tested. Overall worker mortality was only 1.4%, but larval mortality was 7.0%, possibly because fungal fragments often died when tended by new ants. Another 7.0% of the larvae pupated during the experiment. As these mortality and pupation events were spread evenly over colony combinations and subcolony categories (general linear model with binomial errors; larval mortality: effect of colony combination  $\chi^2 = 0.056$ ,  $P = 0.812$ , and effect of subcolony category  $\chi^2 = 1.393$ ,  $P = 0.498$ ; larval pupation: effect of colony combination  $\chi^2 = 0.204$ ,  $P = 0.652$ , and effect of subcolony category  $\chi^2 = 1.592$ ,  $P = 0.45$ , respectively), we assumed that they did not affect the overall results.

Each experimental replicate lasted for 5 days in total. We determined the mass (milligrams) of the remaining healthy fungus (FM) on days 0, 2, and 4 as a direct measure of acceptance of the transplanted fungal fragment by the ants and the amount of garbage (GM) that had accumulated by day 2 and day 4 as an indirect measure of rejection of the fungus (similar to Bot et al. 2001, where further details are provided). For FM, we expressed the scores on day 2 (d2) and day 4 (d4) relative to the initial FM at day 0 (d0) to control for small differences in FM on d0 ( $80 \pm 2$  mg), so that  $FM_{d2}/FM_{d0}$  and  $FM_{d4}/FM_{d0}$  were the dependent variables in our statistical analyses. For GM, we analyzed the absolute amount produced between d0 and d2 ( $GM_{d2} - GM_{d0}$ ) and between d0 and d4 ( $GM_{d4} - GM_{d0}$ ).

### Culturing and genetic analysis of fungal symbionts

To infer genetic distances between the resident fungus and the alien fungus presented to a subcolony, we determined amplified fragment length polymorphism (AFLP) profiles of the fungal symbionts for the 3 colonies used in the experiment (Vos et al. 1995; Bot et al. 2001; Poulsen and Boomsma 2005).

First, we isolated the fungi of the source colonies and cultured them in vitro by inoculating approximately  $3 \text{ mm}^3$  of fungal tufts with visible gongylidia directly from the fungus garden on Petri dishes with potato dextrose agar ( $39 \text{ gL}^{-1}$ ;



**Figure 2**

Maintenance of healthy FM over time: The probability that fungal symbiont fragments will be rejected as a function of worker caste and genetic distance between the fungus of the donating and receiving colony (a) and plots showing the change in FM over time for major-worker subcolonies (b), mixed-worker subcolonies (c), and minor-worker subcolonies (d). Line plots depict changes in FM (range 0–110 mg) from day 0 to day 4. Control combinations are on the diagonals. Pie charts indicate the corresponding proportion of fungus mortality (black segments) across all replicates on day 4.

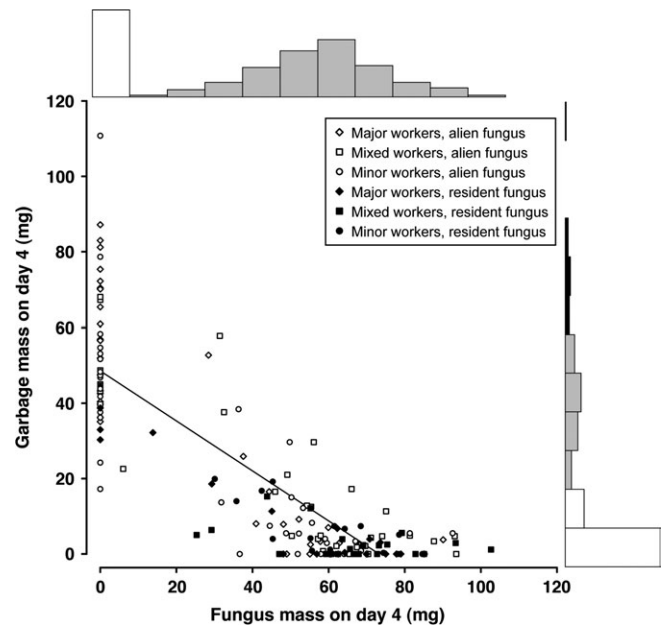
Sigma P2182-250G, Steinheim, Germany). Ampicillin (Sigma A-9518) was added to the medium to prevent bacterial growth. Subsequently, to obtain a quantity of fungal tissue sufficient for DNA extraction, 1 mm<sup>3</sup> of in vitro cultured fungus was transferred into a glass bottle with liquid medium (potato dextrose broth, 24 gL<sup>-1</sup>; Sigma-Aldrich P6685-250G). All cultures were kept in the dark at 25 °C, and liquid cultures were harvested for analysis as soon as the fungus balls had a diameter of 1.5 cm. Twenty milligrams of freeze-dried fungus were used to extract DNA using the plant DNA-Miniprep Kit (D5511-01, Omega Bio-Tek, Norcross, GA). After digestion of the DNA with *EcoRI*, *MseI*, and ligating adapters and pre-amplification, a selective amplification with 2 primer combinations was carried out (*Eco*-ACC × *Mse*-CAT and *Eco*-ACC × *MSe*-CAC) (Bot et al. 2001; Poulsen and Boomsma 2005). The product of this last amplification was then run on a 5% polyacrylamide gel, using an ABI-377 automatic sequencer. The gel image was processed using GeneScan Analysis 3.1 software and further analyzed in Genotyper (both Applied Biosystems, Foster City, CA). The threshold for scoring the peaks as present or absent in replicate runs of the same fungus clone was determined by Poulsen and Boomsma (2005), and we maintained the same criterion here. AFLP profiles obtained in the present study were similar to those obtained in previous studies (Poulsen and Boomsma 2005; Richard et al. 2007), confirming that this method yields consistent and reproducible results. Pairwise uncorrected genetic distances were then calculated in PAUP\*4.0b10 (<http://paup.csit.fsu.edu/>) as the proportion of unshared bands between a pair of fungal clones.

### Statistical analysis

FM and GM on day 2 and day 4 were examined using a logistic regression based on the distribution of these variables (see Results). Predictor variables included in the models were as follows: set (the set of replicates across the different weeks), fungus colony, ant colony, subcolony category (mixed caste, major caste, and minor caste), and all of their 2-way interactions except that between fungus colony and ant colony. We included the genetic distance between the alien and resident fungus (AFLP distance) instead of this last interaction, as these were completely confounded (i.e., there was a constant genetic distance between the fungi from each pair of colonies).

## RESULTS

The data were tested for normality using JMP 7.01 (SAS Institute). In contrast to the results of Bot et al. (2001), both the data on FM and GM were not normally distributed (after log transformation, Shapiro–Wilks,  $FM_{d2}$  0.556,  $P < 0.0001$ ;  $FM_{d4}$  0.675,  $P < 0.001$ ;  $GM_{d2}$  0.743,  $P < 0.0001$ ;  $GM_{d4}$  0.906,  $P < 0.0001$ ). FM data were bimodally distributed on both day 2 and day 4 (Figure 3), reflecting that fungal fragments essentially either survived ( $FM \approx FM_{d0}$ ) or disappeared ( $FM \approx 0$ ) over the 4 days of the experiment. GM data were trimodally distributed on both day 2 and day 4 (Figure 3), reflecting that the workers produced little garbage ( $GM \approx 0$ ), dismantled the fungus garden, and converted it into garbage ( $GM \approx 0.5FM_{d0}$ ) or in some cases dismantled the fungus garden and added additional leaf fragments to the garbage pile ( $0.75FM_{d0} < GM < 1.5FM_{d0}$ ). GM values close to zero were all found in pots with accepted fungal fragments, whereas the maximal garbage values came from pots with rejected fungus fragments (Figure 3). FM and GM on day 4 were highly negatively correlated (linear regression,  $F_{1,162} = 398.2$ ,  $P < 0.0001$ ,  $r^2 = 0.71$ ) showing that the workers had turned the healthy fungus that had disappeared by day 4 into garbage, rather than eating it.



**Figure 3**

Distributions of, and correlation between, GM and FM on day 4: Symbols refer to major-worker subcolonies (circles), minor-worker subcolonies (diamonds), and mixed-worker subcolonies (squares), which received their resident fungus (solid symbols: controls) or alien fungus (open symbols). The line is a fitted least square regression. The histograms above and to the right of the figure show the bi- and trimodal distribution of FM and GM, respectively, with the different modes shaded differently.

To overcome their high degree of nonnormality, the FM data were analyzed using logistic regression, dividing the replicates into those that survived and those that did not, using normal mixtures cluster analysis. GM data were also analyzed using logistic regression, combining the 2 classes in which large amounts of garbage were produced. The analyses were run separately with AFLP distance included as either a continuous or a categorical variable, but in all cases, the models treating AFLP distance as a categorical variable did not explain significantly more of the variance than those treating it as a continuous variable, demonstrating that the interaction between colony-of-fungus origin and colony-of-ant origin was a monotonic effect of the genetic distance between their fungal clones.

The full model for  $FM_{d4}$  explained 62% of the deviance and a reduced model with only the significant effects explained 56% of the deviance. The probability of fungus being rejected before day 4 was significantly affected by the identity of the colony that both received and donated the fungus (Figure 2, Table 1), with colony Ae263 consistently showing a greater probability of the fungus being rejected both as a donor and a recipient colony (Figure 2). There was a general increase in the probability of fungus being rejected with increasing genetic distance between a colony's own fungus and that of the fungus they were tending (Figure 2a, Table 1), but the pattern of this increase was different depending on the caste of the workers involved. The probability of rejection was always higher for subcolonies with only major workers than for colonies with only minor workers (leading to a significant effect of subcolony category), but mixed subcolonies behaved differently for different levels of genetic similarity (leading to a significant subcolony category × genetic distance interaction). At low genetic distances, mixed subcolonies behaved like minor-worker subcolonies, with a very low probability of

**Table 1**  
Sources of variation in FM and GM on days 2 and 4

Source	df	Fungus mass				Garbage mass			
		Day 2		Day 4		Day 2		Day 4	
		$\Delta d$	<i>P</i>	$\Delta d$	<i>P</i>	$\Delta d$	<i>P</i>	$\Delta d$	<i>P</i>
Subcolony category	2	2.79	0.248	<b>10.2</b>	<b>0.006</b>	3.32	0.189	<b>9.06</b>	<b>0.011</b>
AFLP distance	1	<b>30.8</b>	<b>&lt;0.001</b>	<b>41.8</b>	<b>&lt;0.001</b>	<b>10.3</b>	<b>0.001</b>	<b>21.3</b>	<b>&lt;0.001</b>
Ant colony	2	<b>17.6</b>	<b>&lt;0.001</b>	<b>12.1</b>	<b>0.002</b>	<b>6.56</b>	<b>0.038</b>	<b>10.1</b>	<b>0.006</b>
Fungus colony	2	<b>16.5</b>	<b>&lt;0.001</b>	<b>6.55</b>	<b>0.038</b>	1.88	0.391	<b>8.51</b>	<b>0.014</b>
AFLP distance $\times$ subcolony category	2	1.46	0.481	8.24	0.016	0.86	0.652	0.92	0.631
Set (weeks of replicated experiments)	2	1.05	0.590	1.93	0.381	<b>13.8</b>	<b>0.001</b>	<b>17.0</b>	<b>&lt;0.001</b>
Fungus colony $\times$ subcolony category	4	8.98	0.062	1.81	0.771	2.70	0.609	0.78	0.938
Ant colony $\times$ subcolony category	4	1.46	0.833	5.69	0.223	2.91	0.573	5.72	0.221

Results of logistic regression analysis. The table shows the reduced model (significant terms, in bold) and the full model (all factors, in plain text). Columns give the degrees of freedom (df), change of deviance ( $\Delta d$ ), and the approximate probability (*P*) associated with the removal of each term during model simplification.

rejecting the fungus, but at high genetic distances, mixed subcolonies had the highest probability of fungus rejection, slightly above that of major workers (Figure 2). A reduced model for FM<sub>d2</sub> gave similar but less pronounced results (Table 1), indicating that differences between subcolony categories take 4 days to become fully apparent.

The results for GM largely reflected those for FM, although most effects were nonsignificant at day 2 (Table 1). On both day 2 and day 4, however, "set" also explained a significant proportion of the variation in GM, showing that the accumulation of garbage was somewhat different in the different replicates of the experiment. As expected from the FM results, combinations involving ant colony Ae263 produced more garbage in the higher mass class.

## DISCUSSION

### Caste-specific incompatibility

We hypothesized that incompatibility responses in the mutualistic interaction between workers of *A. echinator* and alien fungus garden fragments would be caste specific because the major-worker phenotype would have been under stronger selection to carry out symbiont-policing tasks than the minor-worker phenotype. Our results largely confirmed our expectations (Figure 2a). Subcolonies with major workers were more likely to reject alien fungal fragments than subcolonies with minor workers, and they also produced more garbage. The alternative interpretation that major workers are merely less efficient in garden maintenance is unlikely to be correct. If these were true, we should have seen a similar reduction in survival of fungus in the control subcolonies with resident fungus and major workers, whereas fungal fragments often increased in mass in these subcolonies. We therefore conclude that most of the significant differences in fungal acceptance and maintenance of FM were related to major workers being more sensitive to the risks of competition between resident and alien fungal symbionts when they are confronted with a novel symbiont.

The present findings suggest that symbiont-policing behaviors carry some costs and are therefore predominantly expressed in worker phenotypes where they are most relevant. The acquisition and processing of fresh leaf material in leaf-cutting ants follow conveyor belt principles, with a relatively fixed order of action. This implies that there is an optimal window for eliminating unwanted fungal fragments, namely, when new forage enters the colony. Later processing is then carried

out under the assumption that checking has already been done, so that minor workers primarily focus on efficiently rearing any fungus material available (Wetterer 1999; Hughes et al. 2003). However, minor workers did not rear fungal symbionts without any discrimination between "own" and "alien." They expressed incompatibility behavior less frequently and mainly in combinations with very strong incompatibility "signals" (i.e., large genetic distances among symbionts). For example, minor workers of colony Ae263 rejected fungus from colony Ae266 but later than their major-worker siblings. This could also reflect the ability of major workers to remove larger fragments of novel fungus per unit of time, so that FM is eliminated (and GM accumulated) faster than when minor workers handle the same task. When genetic distance between resident and alien fungus is relatively low, removal seems to be mostly done by major workers. However, the significant interaction effect of worker caste and genetic distance on FM (Table 1, Figure 2a) suggests that when removal is more urgent (i.e., when alien fungus is sufficiently genetically distinct from the workers' resident fungus, which also means it is likely to trigger stronger competitive interactions within the colony), the combined efforts of majors and minors are complementary, making the removal of alien strains most efficient.

### Symbiont-specific incompatibility

A general comparison of the statistical analyses from day 2 and day 4 shows that differences in fungus mortality between subcolonies with different worker castes take 4 days to become significant, whereas differences between specific combinations of ants and fungus are significant already after 2 days. The effects of the factors "ant colony" and "fungus colony" and of the AFLP-based whole-genome genetic distance (a factor that combined the 2) were indeed strong after 4 days. This may imply that particular ant colonies, such as Ae263, have more problems in accepting alien fungi than others, that particular fungi are more likely to be accepted than others, and that particular combinations of ants and fungal clones are more likely to be compatible than others. This confirms earlier findings by Bot et al. (2001) and Poulsen and Boomsma (2005) while adding a number of intriguing details that invite further explicit testing.

The logistics of the experimental procedure only allowed us to test 3 colony combinations. Adding more colonies may have made this pattern either more or less pronounced. For example, being more likely to be accepted as a new fungus by just 2

other colonies does not necessarily make this a general feature of that particular fungus (see also Poulsen and Boomsma 2005). However, we believe that our findings are likely to be robust because the effects of ant and fungus origin were similar to those found in an earlier study with only mixed-caste subcolonies (Bot et al. 2001), with the exception of the main effect of fungus colony, which was not significant in the study by Bot et al. (2001) but significant in the present study. The results of the study by Bot et al. (2001) based on 3 different colonies were thus highly repeatable in the present study.

#### Alien fungal symbiont: weed or pathogen?

Interestingly, the garbage piles in our experiment looked very similar to the piles of ant infrabuccal pellets described by Little et al. (2003). Moreover, the construction of these garbage piles, consisting of moist dark-colored clumps of processed rejected fungus hidden under the moist paper tissue in the small pots, was accompanied by active weeding behavior, which has been described as defensive behavior to control pathogens (Currie and Stuart 2001; Little et al. 2006). Weeding behavior and infrabuccal pellet production in our study were more often displayed in replicates with alien fungus than in the controls (presence/absence data: GLM with binomial error; weeding behavior:  $\chi^2 = 4.43$ ,  $P < 0.035$ , and infrabuccal pellets:  $\chi^2 = 20.37$ ,  $P < 0.0001$ ), which indicates that the ants display the same response toward alien fragments of mutualistic fungus as to infections with parasitic fungi, despite not having any fragment of resident fungus garden available to feed on in the experimental pots. Whereas the selective pressures to deal with incoming genetically different mutualistic fungi and parasites are likely to differ (cf., differential response toward *Trichoderma* and *Escovopsis* in a study by Currie and Stuart [2001]), the underlying mechanisms (recognition and exclusion) are likely to be the same.

The ants maintain a monoculture in their fungus garden not only through active weeding but also via manuring of gardens with their own fecal droplets. By this process, newly implanted fungal pieces gain access to useful enzymes to enhance processing of the leaf fragment substrate (Rønhede et al. 2004). However, at least in *Acromyrmex* leaf-cutting ants, these fecal droplets are “imprinted” with compounds from the resident fungus garden (on which the ants feed) that are hostile to genetically different fungi (Poulsen and Boomsma 2005). This process effectively precludes the ants from starting a different fungal culture even if it were isolated in a separate nest chamber. Based on this, and our present findings, we would predict that fecal droplets of major workers should show clearer antagonistic reactions with genetically different fungal symbionts than fecal droplets of minors. Future testing of this hypothesis would provide further insight into caste-specific incompatibilities and conflict resolution in the ant–fungus mutualism.

The data on garbage production mirrored the data on FM (Figure 3), indicating that the ants destroy rather than eat incompatible strains of fungus. This behavior may seem non-adaptive at first sight because the ants could gain nutrition from eating the fungus, but it is explicable because the fecal droplets of the ants also express incompatibility reactions with non-self-strains of symbionts (Poulsen and Boomsma 2005). This means that the ants would damage their resident symbiont by consuming mycelium of alien symbionts and that they have been selected to refrain from doing so even when they are temporarily away from their resident symbiont. However, Figure 3 also shows a large variation in GM on day 4 across the subcolonies in which all healthy fungus was destroyed. This variation can be explained, at least in part, by the ants’ behavior of adding variable amounts of small pieces of clipped

leaves to the garbage pile, which affects the uptake of moisture (and thus weight of the garbage) during the experiment.

#### CONCLUSION AND PERSPECTIVES

Our results add a novel layer of complexity to the sophistication of coadaptations in the attine ant–fungus symbiosis. They illustrate that mutualistic interactions need to be actively maintained to prevent potential conflicts, similar to the way that reproductive conflicts between queens and workers need regulation so that colony-level costs remain minimal (Ratnieks and Reeve 1992). This implies that conflict regulation and policing behavior are both likely to have been shaped by ergonomic efficiency, so that these behaviors are expressed in individuals (van Zweden et al. 2007) or castes (present study) when and where they are most appropriate.

Our results also indicate that there may be remarkable parallels between symbiont policing and hygienic reactions against pathogens. Although antagonisms of both types have completely different origins and are normally dealt with by different mechanisms (different worker castes in the present study), the principles of coping with them can apparently be very similar. However, there may be interesting differences in the degree to which multiple defenses are expressed (and traded off) among worker castes or even individual workers. Genetic variation for recognition efficiency of alien symbionts and different kinds of diseases can be relatively easily investigated by comparing patriline (i.e., groups of full sister workers) because *Acromyrmex* queens mate with multiple males (Hughes and Boomsma 2007, 2008). Addressing questions at this level may well allow at least some of the remaining variation in behavioral incompatibility to be explained.

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