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**Ant genotype, not fungus genotype, predicts aggression in the asexual  
fungus-farming ant, *Mycocepurus smithii***

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**by**

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## **Abstract**

### **Ant genotype, not fungus genotype, predicts aggression in the asexual fungus-farming ant, *Mycocepurus smithii***

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Hamilton's rule specifies that the relatedness of two individuals will have a large bearing on whether an altruistic action is performed; however, it says little with regards to whether individuals are able to discern that relatedness. In this study we examine whether the fungus-farming ant *Mycocepurus smithii* uses genotypic information to decide whether to attack an introduced queen or if it utilizes environmental cues from the fungus that they cultivate. We performed 180 blind trials in which we introduced queens to queenless mesocosms and recorded and scored aggression behavior directed towards the queen. We find strong evidence that *M. smithii* uses genotype to mediate aggression, but find no support that fungal cultivar plays any role in recognition. These results serve to support Hamilton's notion that relatedness acts as a gateway to altruism.

## Table of Contents

List of Tables .....	vii
List of Figures .....	viii
Introduction.....	1
Methods.....	3
Study Organism .....	3
Study Design .....	5
Response Variables.....	6
Analysis.....	7
Results.....	8
Discussion .....	13
Conclusion .....	15
References.....	16

## **List of Tables**

Table 1: PC Loadings.....	7
Table 2: Importance of Components.....	7
Table 3: ANOVA of Principal Components.....	9

## List of Figures

Figure 1: Ant Phylogeny and Paired Fungus Cultivars .....	4
Figure 2: Results of Factorial MANOVA. ....	10
Figure 3: Results of ANOVAs .....	12

## Introduction

Altruistic behaviors in animals have long been of interest to behaviorists. Sacrificing one's personal fitness for the sake of others occurs repeatedly across the animal kingdom. However, no place is there such a concentration of altruism as there is in the Hymenoptera (ants, bees, wasps). The extreme altruism exhibited by ant and bee workers has made hymenopteran social insects attractive options for research into this topic (Fisher 1987; Michener and Brothers 1974; Ratnieks and Wenseleers 2008).

As explained by Hamilton, the evolutionary success of most altruistic actions are contingent on the relative benefits and costs of the action itself, as well as the relatedness between the altruistic actor and the recipient of the action (Hamilton 1964). Whether an individual engages in altruism can depend on the ability of an organism to discern the relatedness of the recipient individual. In hymenopteran species, recognition of nestmates provides one way to assess the relatedness of conspecifics: if an individual is accepted into a colony, it can reap the benefits of the altruistic actions performed by its nestmates. Nestmate recognition is the gateway to receiving the benefits of altruistic actions in eusocial species and helps to reduce the probability that an altruistic action will benefit a nest-parasite as opposed to a nestmate.

In ants, nestmate recognition is made possible by a combination of chemical and olfactory cues (Smith *et al.* 2009). When ants antennate one another, they can sense one another's cuticular hydrocarbons, which serve to function as a chemosensory fingerprint suitable for nestmate or kin recognition (Smith *et al.* 2009). Recognition cues such as cuticular hydrocarbons can be shared among individuals in ants nest, leading to a homogenization of hydrocarbon profiles throughout the colony and the development of a

“colony odor” (Dahbi *et al.* 1999; Soroker *et al.* 1998). Individuals who lack this odor profile are rejected or attacked by the colony.

The exact factors that influence a colony’s odor are the subject of some debate. Many studies have indicated that colony odor is determined, in large part, by its local environment (Heinze *et al.* 1996; Soroker *et al.* 1998), whereas others have indicated that it is the genotype of the ant that is the primary determinant (Ratnieks 1991). Still other studies suggest that the odor profile is determined by some combination of environment and genotype (Crozier and Dix 1979).

Using the fungus-farming ant, *Mycocepurus smithii* and its cultivated fungi, we developed an assay of aggression directed towards an introduced queen as an indicator of nestmate recognition and tested ant genotype or environmental odors acquired from the fungal garden best predicted ant aggression and queen-acceptance. *M. smithii* is unique among fungus-growing ants. It is asexual through most of its geographic distribution ranging from Argentina to Mexico (no males of the species have ever been found; Himler *et al.* 2009; Rabeling *et al.* 2009, 2011; Kellner *et al.* 2013], though some sexual populations appear to exist in Amazonian Brazil (Rabeling *et al.* 2011). Queens produce diploid offspring through thelytokus parthenogenesis (Rabeling *et al.* 2009, 2011). *M. smithii* is also special in that it frequently switches between asexually-propagated fungal lineages cultivated by the ants (Mueller *et al.* 1998; Kellner *et al.* 2013), whereas most fungus-farming ants are obligate mutualists on a single fungal strain (Mueller *et al.* 1998, 2004; Mehdiabadi *et al.* 2006, 2012; Mikheyev *et al.* 2010; Seal and Mueller 2013). In the laboratory, asexual clones of *M. smithii* ants can therefore be experimentally cross-fostered with different fungal clones to generate colonies of ant-fungus combinations that allow experimental disentangling of ant-associated and fungus-associated factors in

determining colony features (Kellner *et al.* 2013, 2015), such as the nestmate acceptance behaviors investigated in our study.

In our study, we introduced queens from differing ant-clone and different fungus-clone lineages into established, queenless colonies of workers to test whether ant-clone-type or fungus-clone-type, or an interaction between both these factors, influences aggressive interactions directed at the introduced queen. If nestmate recognition serves as a way to limit movement of queens between ant colonies, then proper recognition of queens should be of vital importance because they are the recipients of much of the colony's altruistic actions. If environmental cues are the primary determinants for nestmate recognition, then the identity of the introduced queen's native fungus-genotype could be a predictor of aggression. However, if ant genotype is the primary determinant, then the introduced queen's genotype-lineage should play a key role. We expected to see that ant-genotype would be the major factor in mediating aggressiveness and that ants that were more genetically similar to each other would be less aggressive towards each other than ants that were more genetically disparate.

## **Methods**

### **STUDY ORGANISM**

We used three lineages (A, G, J) of lab-reared colonies of *M. smithii* and two fungal lineages (lineage 5, lineage 7; as defined by Kellner *et al.* 2013). All six combinations of ant and fungal lineages were created, for a total of six source colonies. We refer to each combination using the ant and fungal lineage designations such that a colony of ants of the G lineage entrained on fungus of the lineage 7 would be denoted G7. Using the phylogeny established by Kellner *et al.* (2013) (Figure 1), we selected one distantly related lineage (lineage G) and two relatively closely related lineages (lineages

A and J) as well as one fungus from each branch of the phylogeny. In the native habitat in Central Panamá, lineage G ants was most frequently collected in association with lineage 7 fungi and very rarely with lineage 5 (Kellner *et al.* 2013). Contrarily, lineages A and J are frequently associated with lineage 5 fungi and almost never with lineage 7 (Figure 1). In a subsequent experiment testing for ant-fungus synergisms (Kellner *et al.*, in prep), ants were host-switched and ants and gardens some of these ant-fungus combinations (i.e., A5, A7, G5, G7, J5, J7) were available in sufficient numbers for experimentation in our nestmate-recognition study. Because colonies A7, G5, and J7 are not particularly robust in laboratory colonies, only workers were drawn from these colonies. Queens and worker ants from A5, G7, and J5 were used. The source colonies were all housed in the same room, given identical diets of polenta and oats, and placed in identical nest boxes, as described by Kellner *et al.*, (2015, in prep). The only difference between the source colonies was the particular ant-fungus combination.

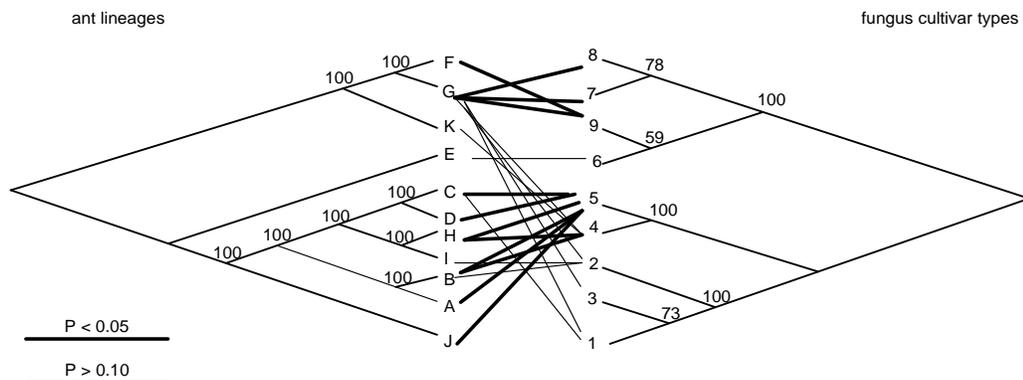


Figure 1: Ant Phylogeny and Associated Fungus Cultivars of *Mycocepurus smithii*. This figure depicts the tanglegram phylogeny of both the ants and their fungal cultivars reported by Kellner *et al.* (2013). The lines between the phylogenies show the ant-fungus associations observed in natural nests; thickness of lines indicates the statistically significant ant-fungus associations inferred via ParaFit Kellner *et al.* (2013).

## STUDY DESIGN

From each of the six source colonies, thirty mesocosms were established in laboratory nests (see description in Mehidabadi *et al.* 2006), for a total of 180 mesocosms, each containing ten workers and an approximately 1cm<sup>3</sup> fragment of fungus. Each cohort of ten workers had developed as larvae on the fungus-type they were cultivating, and they had never been exposed to a different fungus or ants from different ant-clone-lineages. Each mesocosm was given 14 days to habituate workers to their new nest-environment. To ensure blind experimentation (Kardisch *et al.* 2015), each colony was given a computer-generated random number and each mesocosm was labeled by a third party such that the observer would have no knowledge of which ant-fungus combination was in each mesocosm. For each observation, a queen from the A5, G7, or J5 colony was introduced into each mesocosm. The randomization was performed such that there were ten replicate introductions of each of the eighteen permutations of queens and workers. All tests were performed in a randomly generated order to control for test-order effects and to further obscure any information on the ant-fungus combination tested to the observer.

The first five minutes after introduction of the queen were video recorded and later scored by a naïve observer for queen-directed aggression by the workers. Each five-minute video was broken into 30 ten-second intervals. Every queen-worker interaction inside of each 10-sec interval was scored according to the following aggression scale modified from Carlin and Hölldobler (1986):

- -2: The queen was not visible for at least 75% of the interval.
- 0: There were no worker-queen interactions during the interval, but the queen was visible for at least 25% of the time interval.
- 1: The worker briefly antennated the queen (lasting less than 3 seconds).

- 2: The worker antennated the queen for a prolonged period (great than three seconds).
- 3: The worker placed two or more legs on top of the queen.
- 4: The worker bit the queen, but did not attempt to move her.
- 5: The worker bit the queen and dragged her or otherwise moved her.
- 6: The worker dismembered the queen (i.e., removal of antennae, wing, leg, etc.).
- 7: The worker killed the queen.

The most aggressive interaction exhibited by each worker in a given time interval was reported such that there was an aggression score for every worker that had some interaction with the queen. If there was any doubt about how to score a particular time interval, that time interval was noted and then blindly rescored by a second observer.

#### **RESPONSE VARIABLES**

We calculated five metrics for quantifying aggression for each trial: maximum aggression over the course of each trial, average aggression, latency to aggression, duration of aggression, and the maximum number of workers interacting with the queen during a 10-second time interval. Average aggression was calculated as the sum of all worker-queen interactions divided by the number of time intervals the queen was visible. If the queen was not visible for the entirety of the trial, this value was set to 0. Latency to aggression was calculated as the number of time intervals before the first interaction with an aggression score of at least 3. If the queen was not visible during the trial, the latency to aggression for that trial was set to 300 seconds, the maximum length of the recording. Duration of aggression was calculated as the number of time intervals with an aggression score of at least 3, divided by the number of time intervals the queen was visible. If the queen was not visible for the entirety of the trial, this value was set to zero.

## ANALYSIS

We conducted a principal component analysis (PCA) based on the five response variables (Table 1). Principal component 1 (PC1) accounted for 68% of the variance in the data and correlated strongly with overall aggression (Table 2). Principal component 2 (PC2) accounted for 18% of the variance and was correlated with the number of workers interacting with the queen. We then ran ANOVAs on these principal components. We also conducted a factorial MANOVA on the response variables themselves. Because in some trials queens were hidden below the garden for more than 50% of the time intervals, we conducted the above two analyses also for the dataset excluding any trials where the queen was not visible for at least 50% of the time intervals.

	<b>PC1</b>	<b>PC2</b>
<b>Average Aggression</b>	-0.437	0.359
<b>Duration of Aggression</b>	-0.462	-0.180
<b>Maximum Aggression</b>	-0.390	-0.346
<b>Percentage of Duration Aggressed</b>	-0.468	-0.165
<b>Latency to Aggression</b>	0.403	0.161
<b>Max # of Workers Interacting w/Queen</b>	-0.250	0.816

Table 1: PC Loadings

	<b>PC1</b>	<b>PC2</b>	<b>PC3</b>	<b>PC4</b>	<b>PC5</b>	<b>PC6</b>
<b>Standard Deviation</b>	2.00	1.02	0.76	0.53	0.31	0.10
<b>Proportion of Variance</b>	0.67	0.17	0.10	0.05	0.02	< 0.01
<b>Total Proportion of Variance Explained</b>	0.67	0.84	0.94	0.98	1.00	1.00

Table 2: Importance of Components

## Results

Worker and queen genotypes and the interaction between worker and queen genotypes were the most important predictors of aggression (Table 3). Both PC1 and PC2 showed a significant effect of worker genotype ( $P < 0.015$ ) and of queen genotypes ( $P < 0.01$ ), and PC1 showed a significant interaction effect between worker and queen genotypes ( $P < 0.001$ ) (Table 3). These results remained consistent even when removing all time trials where the queen was obscured from view for at least 50% of the time intervals. Results from the MANOVA mirrored those found in the ANOVA of the principal components, with a consistent theme of worker genotype, queen genotype, and their interaction being significant (Figure 2).

	Df	Sum Sq	Mean Sq	F value	p-value
<b>PC1</b>					
WG	2	27.9	13.925	4.266	0.0159*
GWF	1	1.8	1.786	0.547	0.4607
QG	2	36.4	18.222	5.582	0.0046**
WG:GWF	2	8.6	4.313	1.321	0.2701
WG:QG	4	71.5	17.876	5.476	0.0004***
GWF:QG	2	11.2	5.588	1.712	0.1843
WG:GWF:QG	4	15.9	3.970	1.216	0.3066
Residuals	141	460.3	3.264		
<b>PC2</b>					
WG	2	7.75	3.875	4.885	0.0089**
GWF	1	0.66	0.656	0.826	0.3648
QG	2	27.78	13.891	17.512	<0.0001***
WG:GWF	2	3.27	1.635	2.062	0.1310
WG:QG	4	5.57	1.392	1.755	0.1413
GWF:QG	2	4.47	2.237	2.820	0.0630
WG:GWF:QG	4	1.98	0.495	0.624	0.6460
Residuals	141	111.84	0.793		
WG: Workers' genotype					
GWF: Genotype of worker's fungus					
QG: Queen's genotype					

Table 3: ANOVA of Principal Components

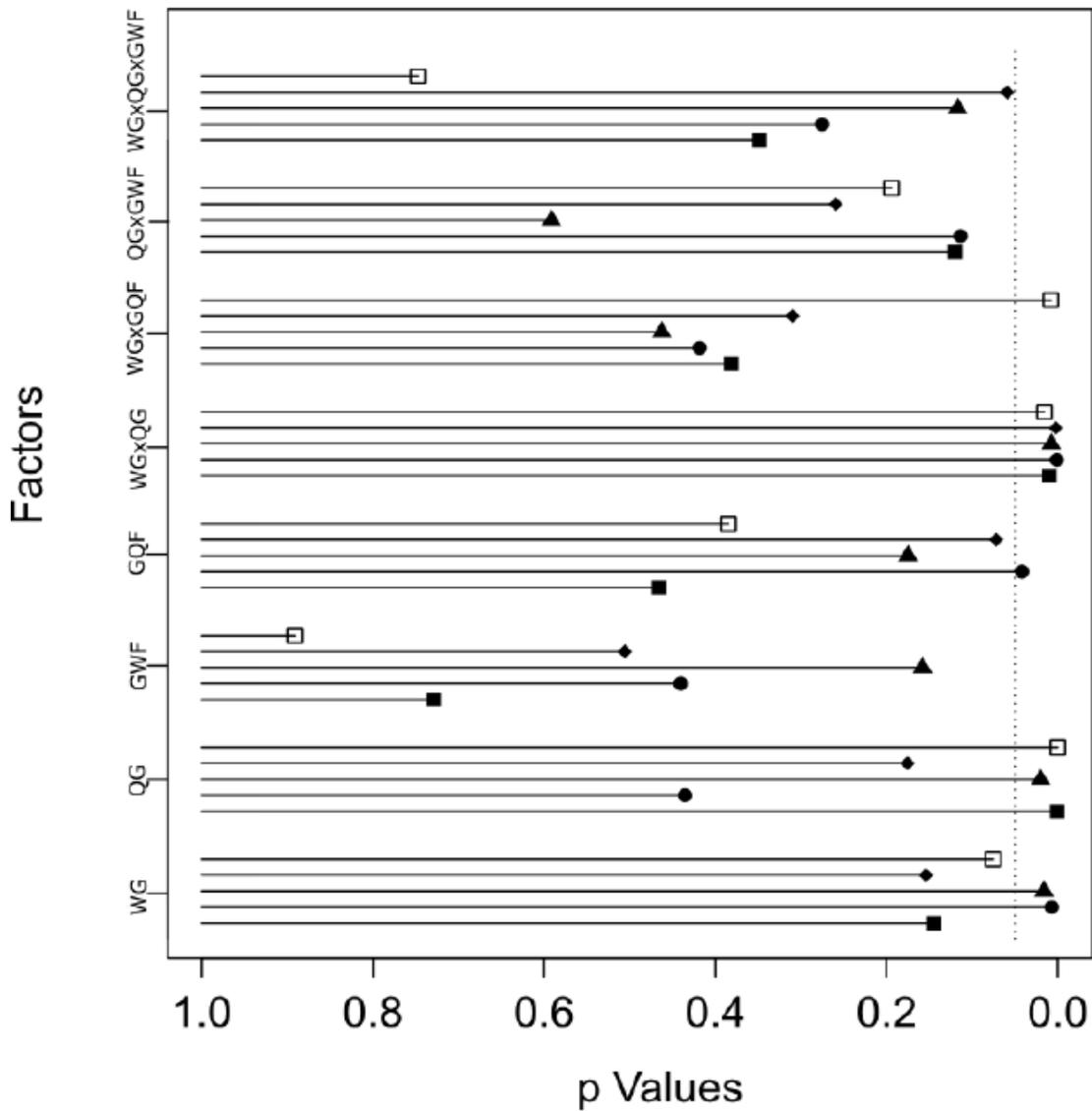


Figure 2: Results of Factorial MANOVA. Figure depicts the results of the factorial MANOVA. On the y-axis are the factors in the MANOVA. The dashed line indicates the threshold for  $p = 0.05$ . Lines that terminate to the right of the dashed line represent factors that are significant with respect to the five response variables. Filled squares represent average aggression; filled circles represent maximum aggression; filled triangles represent percent of duration of aggression; filled diamonds represent latency to aggression; open squares represent maximum number of workers interacting with the queen. WG = Worker's Genotype; QG = Queen's Genotype; GWF = Genotype of the Worker's Fungus; GQF = Genotype of the Queen's Fungus

The consistent finding that genotype is an important factor influencing aggressive behavior was driven, in large part, by two patterns. First, workers of genotype G were far less aggressive towards queens of genotype G than they were towards other queens (Figure 3). Second, queens of genotype A appear to received aggression more frequently from genotypes G and J than they did from genotype A (Figure 3).

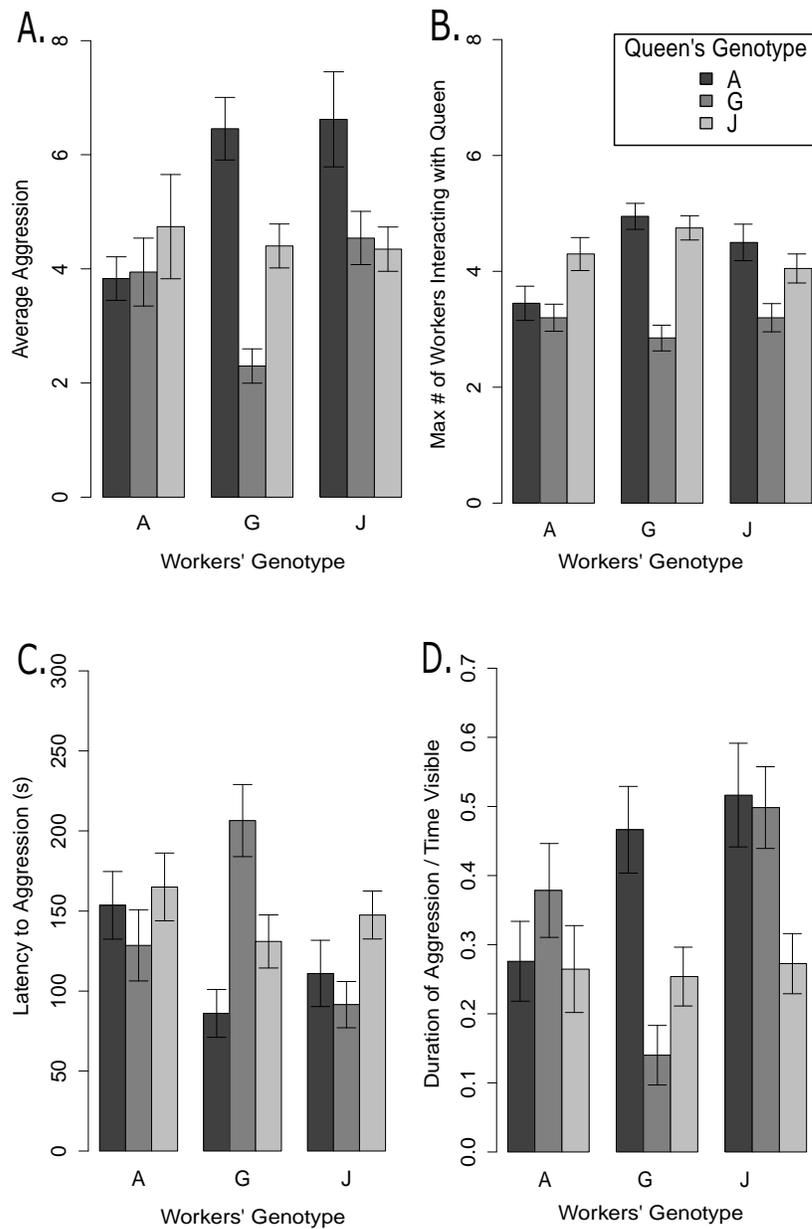


Figure 3: Results of ANOVAs: Panel A shows the average aggression of workers directed towards the queens. Panel B shows the maximum number of workers interacting with the queen in a single time interval. Panel C shows the latency to aggression. Panel D shows the percentage of time intervals the queen was visible and an aggressive interaction took place. In all cases, workers of genotype G were less aggressive towards queens of genotype G than they were towards other queens, and queens of genotype A received aggression more frequently from genotypes G and J than they did from A.

For all three genotypes, workers were less aggressive towards queens of same genotype than they were towards queens of a different genotype. The trend is significant: the probability of this outcome occurring by chance is  $P = (1/3)^3 = 0.037$ . While we did find evidence that workers were less aggressive towards queens of same genotype than they were towards queens of alternate genotypes, we did not find consistent support that the extent of relatedness mediated aggression. Genotypes A and J were most closely related to one another, and yet genotype J was marginally significantly more aggressive towards A than it was towards G (Welch's two-sample t-test;  $t = 1.91$ ;  $P = 0.066$ ). Furthermore, for genotype J there was no significant difference between aggression directed towards their own genotype or that of genotype G (Welch's two-sample t-test;  $t = -0.28$ ;  $P = 0.78$ ).

We did not observe support for environmental cues mediating nestmate recognition. After a Bonferroni correction for multiple comparisons, there were no significant effects of fungus on any of the response variables. Fungal genotype was also insignificant when looking at principal components 1 and 2 ( $P > 0.25$ ).

## Discussion

Nestmate recognition and intraspecific aggression in the asexual ant *Mycocepurus smithii* is primarily determined by the combination of genotype of the experimentally introduced queens and the genotype of the resident worker ants (Table 3). We found no evidence in support of the hypothesis that ants use environmentally-acquired cues, such as factors derived from their cultivated fungal clone, in nestmate recognition. Because *M. smithii* are fungus-farmers participating in a mutualism with the fungus, the fungus dictates a substantial portion of the ants' nest environment. Because the nest environment has been shown to be an important determinant of nestmate acceptance across many ant

species (Heinze *et al.* 1996; Soroker *et al.* 1998), the lack of significant effect of fungus factors relative to ant factors in mediating nest-mate recognition in *M. smithii* is surprising.

While recognition may be correlated with ant genotype, aggression patterns do not appear to be correlated with genetic distance between clones (Figure 1). This may be an effect of the small genetic distance between the colonies used in our study: even the colony-pairs with the greatest genetic distance differed by only a few alleles at a few microsatellite loci (Rabeling *et al.* 2009; Figure 1, Kellner *et al.* 2013). It is possible that there is simply not enough resolution in the ants' sensory system to be able to distinguish between which colonies were more or less genetically distant in our study. It is also the case that there is simply not enough statistical power in our study to definitively identify such a pattern. A follow-up study with a greater assortment of colony genotypes is needed to adequately test whether gradations of relatedness modulate nestmate-recognition behaviors in *M. smithii*.

If the strong queen genotype x worker genotype interaction we observed was not correlated with relatedness, then it prompts the question of what was producing the response. One hypothesis is that certain ant genotypes — in our case genotype A — simply smell stronger and are thus more likely to elicit a vigorous response. However, given the small genetic distance between the colonies used, such a change would likely be the result of one or only a few mutations. In light of the size of the difference in aggression directed at queens of genotype A versus queens of other lineages, this seems unlikely.

Another possibility is that the ants are not using the actual genotypes as the basis of recognition cues, but rather that different genotypes play host to distinct microbiota (Kovacs *et al.* 2011; Kellner *et al.* 2015) and these microbiota in turn contribute to a

“colony odor”. If the assemblage of microbiota is in fact the source of the colony odor, we would not expect the relatedness of the ants to be the primary factor in aggression, rather it would be the similarity of the microbiome, which is only imperfectly correlated with the ants’ genotypes.

## **Conclusion**

This study demonstrates that genotype and not fungus plays a large role in determining nest-mate acceptance behaviors between resident workers and introduced queens of the fungus-farming ant *Mycocepurus smithii*. This finding supports the idea that nest-mate recognition is a gateway to altruistic actions, which in this case is shown through lack of aggression. Queens who are readily accepted by the colony (i.e., not aggressed against by workers) are able to reap the benefits of the colony’s labors without enduring a substantial physical hardship.

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