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ORIGINAL PAPER

# Construction of chimaeric gardens through fungal intercropping: a symbiont choice experiment in the leafcutter ant *Atta texana* (Attini, Formicidae)

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**Abstract** Interspecies or intraspecies cooperation can be stabilized evolutionarily if choosing partners favor beneficial partners and discriminate against non-beneficial partners. We quantified such partner choice (symbiont choice) in the leafcutter ant *Atta texana* (Attini, Formicidae) by presenting the ants in a cafeteria-style preference assay with genotypically distinct fungal cultivars from *A. texana* and *Acromyrmex versicolor*. Symbiont choice was measured as the ants' tendency to choose one or more cultivar(s) from several pure (axenic) cultivar fragments and convert a given fungal fragment into a garden. Microsatellite DNA fingerprinting enabled us to identify the cultivars chosen by the ants for their gardens. In 91% of the choice tests, *A. texana* workers combined multiple cultivars into a single intercropped, chimaeric garden, and the cultivars coexisted in such chimaeric gardens for as long as 4 months. Coexistence of distinct fungal genotypes in chimaeric gardens appears to contradict a recent model of cultivar competition postulating that each cultivar secretes incompatibility compounds harming other cultivars, which presumably would preclude the intercropped polyculture observed in our experiments. Although we found no clear evidence of novel, recombinant genotypes in the experimental chimaeric gardens, the intercropping of cultivar genotypes may occasionally lead under natural conditions to exchange of genetic material between coexisting cultivars, thus introducing novel cultivar genotypes into the leafcutter symbiosis. Symbiont choice by ants and any competition between coexisting cultivar strains in chimaeric gardens do not appear to operate fast enough in our laboratory assay to convert chimaeric gardens into the monocultures observed for *A. texana* under natural conditions.

**Keywords** *Atta texana* · Attini · Leafcutter ant · Ant-fungus mutualism · Symbiont choice

## Introduction

Cooperation between partners, either within species or between species, can be evolutionarily stabilized by several mechanisms of partner choice whenever a choosing partner favors beneficial partners over non-beneficial partners (Bull and Rice 1991; Noë and Hammerstein 1994; Noë 2001; Sachs et al. 2004). As a first mechanism, beneficial partners can be preferentially chosen and thus become rewarded by the choice to participate in a mutualism, whereas non-chosen partners remain unrewarded. Second, interactions can be prolonged or intensified with beneficial partners, but terminated or weakened with poor or non-beneficial partners. Finally, non-cooperative partners can be punished, thus indirectly favoring beneficial partners (Foster and Wenseleers 2006; Sachs et al. 2004; West et al. 2002). These mechanisms underlying partner choice apply widely to vertebrate, invertebrate, and microbial cooperation (Douglas 2008; Kiers and Denison 2008; Lehmann and Keller 2006; Sachs et al. 2004), including the diverse mutualisms between fungus-growing insects and their cultivated fungi (Korb and Aanen 2003; Mueller 2002; Mueller et al. 2004, 2005). Despite the recognized

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theoretical relevance, only a few studies have empirically tested the efficacy of partner choice in carefully controlled experiments (Bshary and Grutter 2002; Grutter and Bshary 2003; Mueller et al. 2004; Simms et al. 2006).

The ability to cultivate fungi for food evolved about 50 million years ago in the ancestors of the fungus-growing ants (Attini, Formicidae), (Mueller et al. 2005; Schultz and Brady 2008). Attine ants provide nutrition, shelter, and disease protection to the cultivated fungi, while the fungi serve as the main source of food for the ants. In this mutualistic relationship, fungus-growing ants are obligatorily dependent on their cultivars for food. Each attine virgin queen carries a clonal inoculum of the cultivar from her maternal nest and uses it as a starter culture for the garden of her newly founded nest. The fungus is therefore vertically transmitted between generations (Hölldobler and Wilson 1990; Mueller 2002; Weber 1972; Wheeler 1907), and a single fungal lineage is transmitted from parental to offspring colonies. However, horizontal transfer of cultivar clones has been inferred from population-genetic analyses of cultivated fungi (Green et al. 2002; Mikheyev et al. 2006; Mueller et al. 1998; Mueller 2002; Mueller et al. unpublished data). Transfer of fragments of fungal garden between nests has been observed in the field (e.g., raiding of small colonies by mature colonies; Autuori 1950) and in laboratory experiments (Adams et al. 2000; Higgins 1988; Poulsen et al. 2009; Rissing et al. 1989). However, the proximate mechanisms underlying horizontal cultivar transfer in attine ants are largely unknown, except that larger workers (majors), but not smaller workers, of the ant *Acromyrmex echinatior* appear to discriminate against novel cultivar genotypes that may be accidentally imported into a native garden (Ivens et al. 2009). Effective transfer of cultivar lineages between attine nests could in principle be influenced by several factors, including (1) ant preferences for specific fungal genotypes (symbiont choice); (2) within-nest growth-competition between cultivar genotypes coexisting in the same garden (symbiont competition); or (3) mycelial compatibility interactions permitting or precluding the formation of cultivar recombinants (symbiont compatibility).

Symbiont choice in the form of behavioral preference by attine ants for specific cultivar genotypes has been hypothesized to influence the persistence and spread of cultivars in attine populations (Green et al. 2002; Mueller et al. 1998; Mueller 2002). Under this view of partner choice, natural selection should favor both the choosing partner (ants) and the chosen partner (fungus) whenever a more rewarding partner is chosen (Sachs et al. 2004). For example, among several coexisting fungal cultivars, a secondarily acquired fungus or a mutant strain with novel beneficial properties arising in a garden could be a more rewarding partner. The importance of partner choice under horizontal cultivar transfer under natural conditions in the field is difficult to study in attine ants, but partner choice by ants for cultivars can be readily assessed in the laboratory (Advani and Mueller 2006; Ivens et al. 2009; Mueller et al. 2004). For example, workers of the lower-attine ant *Cyphomyrmex muelleri* prefer their own or closely related cultivars over a novel cultivar (Mueller et al. 2004), and *Cyphomyrmex costatus* workers show repeatable preferences to a particular set of fungi when an array of fungi from *C. costatus* is offered to them (Advani and Mueller 2006). Here, we investigate the symbiont choice behavior in the leafcutter ant, *Atta texana*, by testing whether worker ants choose any particular cultivar from among an array of cultivars. Specifically, we adapt a cafeteria-style preference assay developed previously for the lower-attine *C. costatus* (Advani and Mueller 2006) to assess cultivar preference in *A. texana*, then verify the choice by DNA fingerprinting of fungal cultivars. Our choice experiments indicate that *A. texana* workers occasionally select a single strain of fungus to construct a monoculture garden, but they often combine more than one fungus to generate a chimaeric garden (polyculture) with several intercropped fungi.

## Materials and methods

### Experimental setup

We conducted the symbiont choice experiment with two types of ants (fresh and purged; see below). To each type of ant, two sets of cultivars were offered. Each cultivar set was offered to ants from six different colonies (i.e., six replicates were conducted with each set of cultivar; Fig. 1 summarizes the number of replicates per treatment). In each replicate with fresh ants, we used 20 workers, collected from healthy, queenright colonies of *A. texana* (Supplementary Table S1) immediately before the start of the choice tests. At the time of testing, the colonies appeared

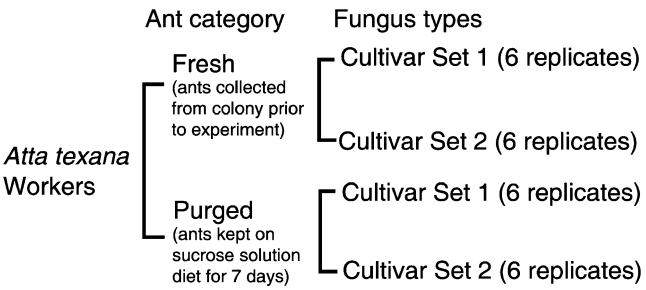


**Fig. 1** Experimental design of the symbiont-choice assay and number of tests with purged and fresh ants tested in two experimental series with two sets of cultivars (cultivar sets 1 and 2). *Fresh* or *purged* indicate whether the tested workers were used directly (*fresh*) from a colony or purged first of gut contents by maintaining them on sucrose solution for 7 days (see Materials and methods)

vigorous and had been maintained under laboratory conditions for more than 1 year. Poulsen and Boomsma (2005) showed that the fecal matter of *Acromyrmex* leafcutter ants living on a cultivar diet can induce incompatibility towards fungi of different genetic composition. To prevent such incompatibility towards one or more of the offered fungal inocula, we conducted 12 replicates with "purged" ants (from the same set of experimental nests as fresh ants). We purged the ants of the putative incompatibility factors by keeping them in moist plaster-bottom plastic boxes on an ad libitum water and 10% sucrose solution diet for 7 days before starting the behavioral testing. Twelve parallel replicates were conducted with unpurged ants (here called "fresh" ants) that were taken directly from the laboratory nests. Overall, we conducted a total of 24 replicates (12 with fresh ants and 12 with purged ants). Each worker ant was used only once.

We prepared experimental arenas with round plastic trays (14 cm diameter, 2 cm height). We poured a 1 cm thick layer of plaster-of-Paris into each plate and immediately inserted eight rectangular plastic sheets (5.5 cm× 2 cm) to divide the arena into eight equal chambers, leaving a circular space of 3 cm diameter in the center (Fig. 2). As in Advani and Mueller (2006), we placed individual 1 cm× 1 cm×0.5 cm pieces of inoculum growing on oat flakes on small plastic trays (1.5 cm×1.5 cm) in each chamber of the arena. Six larvae and/or pupae collected from the same colony as the test ants and four to five whole oat flakes (substrate given to the ants to nourish a garden) were placed in the central space. We placed either 20 fresh or 20 purged ants at the center, covered the arena, and then sealed the entire arena with white masking tape to prevent loss of humidity.

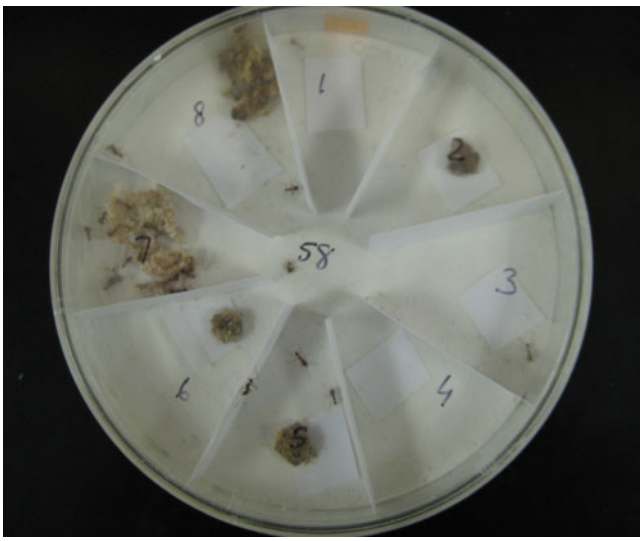


**Fig. 2** Experimental arena 6 days after the start of replicate FPA-58 in which *A. texana* workers were presented with eight pure (axenic) cultivar strains. Workers built a garden in chamber 7 (*9 o'clock position*) and accumulated a trash pile in chamber 8. Inocula in chambers *1*, *3*, and *4* were dismantled by the ants and transported to the trash pile in chamber *8*. The inocula in chambers *2*, *5*, and *6* were ignored by the ants after partial dismantling. Workers therefore preferred cultivation of the fungus in chamber *7* and rejected all other fungi

## Cultivar strain selection

Fungal cultures for the choice experiments were selected from a large collection of about 150 live cultivars isolated from *A. texana* gardens collected in 2006 and 2007 from throughout the US range of *A. texana* (Mueller et al. unpublished data). In addition, one strain from the Arizona leafcutter ant, *Acromyrmex versicolor*, was included. All fungi had been genotyped at 12 microsatellite loci (A1132, A1030, A128, A1151, B12, B150, B319, B430, C101, C117, C126, C625; Scott et al. 2009), permitting identification of clonal lineages and assignment of clonal lineages to different sub-populations (Mueller et al. unpublished data). For each cultivar set, we selected fungi from distinct clonal lineages, thus ensuring that the ants were offered eight genetically distinct fungi with unique allele profiles in each assay. We cultured two sets of eight fungi (A-H and AA-AH, Supplementary Table S2) on sterilized, ground oat flakes to generate healthy mycelial growth that could be presented as fragments to the ants, following the mycological methods of Advani and Mueller (2006). These two sets of fungi were offered separately in a cafeteria-style arena to the fresh ants and purged ants from the above six ant colonies (Figs. 1 and 2).

## Behavioral observation

During pilot experiments, we identified and defined the following five interactions between the choosing ants and the chosen fungal cultivars. *Tending*: Some workers are always in close proximity to a particular inoculum. Workers protect the inoculum from desiccation and contamination, build the inoculum into a garden-like structure (with built-up ridges or cellulae as in typical leafcutter gardens), and add substrate to expand the garden. *Dismantling*: Workers break the inoculum into smaller pieces, which are either discarded near the inoculum or moved to a trash pile in a separate chamber. *Trashing*: Workers pile the dismantled pieces of one or more inocula onto or close to an inoculum. The trashed pile often becomes quickly overgrown with contaminant fungi (e.g., *Penicillium*). *Clearing*: Workers take out an inoculum bit-by-bit and eventually clear the chamber of the entire inoculum. *Ignoring*: Workers do not change the shape or add substrate to the inoculum. Workers occasionally inspect the inoculum and its chamber, but do not interact with the inoculum beyond inspection. In addition to these behavioral criteria, the visual appearance

(see below) of the gardens can be used to discriminate between chosen and rejected inocula.

The replicates were set up in the evening, and the positions of the ants and brood with respect to the chambers and inocula were recorded by scanning every half hour from 10 am to 6 pm on the following day. In each scan, we counted the number of ants and brood in each chamber, on each inoculum, and in the center of the test arena. Because *A. texana* ants were very mobile and moved extensively within the test chamber, ant associations with fungal inocula were assumed to be independent between successive observational scans. While counting the ants, we also recorded their behaviors to identify the number of ants showing preference (tending) and non-preference (dismantling, trashing, and clearing) behaviors towards the inocula. The status of each fungal inoculum was also recorded depending on whether it was tended, dismantled, trashed, or ignored. In some of the experiments, the ants actively moved, cleared, and/or trashed more than one inoculum within the first day; therefore, from the second day onwards, qualitative notes were taken to record the visual appearance of the inocula instead of quantitative measures of behavior. The chosen inoculum (or inocula) in each replicate was determined based on (1) inoculum appearance (i.e., largest and/or most perfected molding into the garden architecture typical for leafcutter ants), (2) addition of gardening substrate (oat flakes), (3) placement of brood on a garden, and/or (4) congregation of ants on an inoculum.

Although we could measure preferential association based on the number of ants associating with an inoculum (tending or sitting on an inoculum), we could not unambiguously quantify rejection of an inoculum because rejection behaviors (trashing, dismantling, and clearing) were infrequent and were rarely observed during the instantaneous scans. The absence of ants on an inoculum due to ignoring or not interacting with the inoculum also was not an accurate indicator of whether the ants rejected or preferred that inoculum. Consequently, we could not use the number of ants to analyze rejection tendencies statistically. However, even in the absence of direct observation of rejection behaviors, we could assess rejection of an inoculum indirectly by its deteriorating appearance. A rejected inoculum was either dismantled, was converted by the ants into a trash pile, or was blackened by bacterial contamination. All observations were blind with respect to the identity of the fungi and the ants.

## Statistical assessment

To test statistically whether the ants had any preferential association with one or more fungi, we calculated a skew statistic using the total number of ants present on each inoculum, summed across all scans (skew calculator, developed by Peter Nonacs, University of California Los Angeles, http://www.eeb.ucla.edu/Faculty/Nonacs/, Nonacs 2000). We analyzed the data separately for each of the following treatments: (1) fresh ants on cultivar set 1, (2) purged ants on cultivar set 1, (3) fresh ants on cultivar set 2, and (4) purged ants on cultivar set 2. Each treatment had six replicates (Fig. 1), and the skew program calculated the skew statistic for each replicate. We used Fisher's exact test to compare the proportions of preference of the most preferred fungal lineage in each cultivar set with the proportion for the same lineage in the corresponding cultivar set (Supplementary Table S4).

## Molecular analysis

The chosen inoculum was identified when an amorphous inoculum given to the ants was converted by the ants into a garden-like structure (through tending; Fig. 2, leftmost garden) or when the ants placed brood on a particular inoculum. To verify fungal choices with molecular methods, small parts of the ant-tended (chosen) gardens were preserved in 100% ethanol for microsatellite genoptying. Small (~1 mm×1 mm) mycelial tufts were separated from the ethanol-preserved inocula with sterilized forceps and genotyped individually by multiplexed microsatellite DNA fingerprinting using a set of 13 loci (A1132, A1030, A128, A1151, A435, B12, B150, B319, C101, C117, C126, C606, D115; Scott et al. 2009; Ishak et al. unpublished data; Supplementary Table S3). The fungal genotypes of the chosen inocula were then compared with the genotypes of each of the eight original fungi presented to the ants.

Because repeat genotyping of the same pure fungal strain can occasionally yield minimally different microsatellite allele profiles (due to quality or quantity differences between DNA templates or due to minor PCR artifacts during the multiplex amplification; Ishak et al. unpublished data), we devised the following rule to match the preferred fungal genotype to one of the original eight fungi presented to the ants. Each preferred fungal inoculum was compared with each of the original fungi to calculate the total number of discrepancies in presence/absence of alleles (total of exactly 100 alleles scored per fungus). If the match was not perfect, the preferred fungus was considered to derive from one of the original fungi if it shared 97% or more alleles with one of the original test fungi (i.e., allowing for a 3% artifactual presence/absence differences). This 3%-difference decision rule is conservative because, in blind repeat genotyping (starting from DNA extraction from tissue of 88 samples), we observed an error rate of only 0.9% incorrectly scored alleles in a total of 5,130 rescored alleles (Scott et al. 2009). To assess whether a resulting garden contained a mixture of two fungi, each preferred fungal genotype was also compared with every

possible combination of two cultivars (Supplementary Table S3). The combinations of any two original fungi with the least number of unexplained alleles (alleles that could not be matched to the two putative donor strains within each combination) were considered to be the possible donors for the resulting microsatellite allele profile.

## Results

### Behavioral assessment

In ten out of 24 replicates (42%; FPA 58, 59, 62, 63, 64, 65, 66, 67, 68, and 69), workers built garden on the location of a particular inoculum and in five replicates (21%; FPA 60, 78, 84, 87, and 88), workers built a new garden away from any inoculum (i.e., the workers moved one particular inoculum or more to a new location). In eight replicates (33%), the ants did not construct a garden, but the chosen inocula were determined based on the placement of brood and congregation of the ants around a particular inoculum (Table 1, Supplementary Table S4). In one replicate (FPA 61), the ants neither built a garden nor did they place brood on any inoculum. The rejected cultivars differed between replicates, and each fungal lineage was rejected in 20–33% of the replicates (Table 1, Supplementary Table S5), i.e., every fungal lineage was rejected in multiple replicates, so we did not find any lineage that was never rejected in any replicate.

We used skew statistics to test if the ants visited the inocula preferentially. Skew statistics for each replicate in all treatments showed significant skew: (1) fresh ants on cultivar set 1, (average skew $Sc$=0.55; $P$<0.008), (2) purged ants on cultivar set 1 (average $Sc$=0.39; $P$<0.008), (3) fresh ants on cultivar set 2 (average $Sc$=0.62; $P$<0.008), and (4) purged ants on cultivar set 2 (average $Sc$=0.33; $P$< 0.008; $\alpha$ set in each of these four tests to 0.008 after Bonferroni correction due to six skew calculations in each test). All monopoly values were >0.8 (see Supplementary Tables S6–S9 for detailed skew statistics analysis). These values indicate that the ants preferentially associated with one or more fungi in each replicate, rather than interacting with all fungi randomly. Because there were more than two fungi tested in each replicate, a significant skew statistic in Nonacs' skew test does not reveal which particular inocula were chosen by the ants; rather, significant skew merely indicates that some fungi were attended to by the ants more often than other fungi.

We were able to identify preferred inocula in each treatment based on the visual appearance of the inocula (see Materials and Methods). In the treatments with cultivar set 1, D was the most tended inoculum, as it was tended in eight out of 12 replicates (Supplementary Table S4). The proportion of replicates in which fungus D was chosen from cultivar set 1 was significantly larger than the corresponding proportion of replicates for the fungus from the same lineage (AE) in cultivar set 2 (Fisher's exact test, $P$=0.001). In the treatments with cultivar set 2, AH was the most tended inoculum, as it was tended in seven out of 12 replicates (Supplementary Table S4). The proportion of replicates in which fungus AH lineage was chosen from cultivar set 2 was significantly larger than the corresponding proportion of replicates the fungus from the same lineage (F) in cultivar set 1 (Fisher's exact test, $P$=0.004).

The rejected inocula were not the same across all replicates. Out of eight tested fungal lineages, only one fungal lineage (G in experimental set 1 and AC in set 2) was never behaviorally preferred by the ants, i.e., the number of ants on those inocula were low in all replicates (Fig. 3). With one exception (FPA 61; this outlier raised the height of the bar and standard deviation for H fungus in panel 2 of Fig. 3), workers were never seen to perform any preferential behavior towards the inoculum with *Acromyrmex* fungus. However, in replicate FPA 76, the *Acromyrmex* fungus (AB) was found to be combined in the chimaeric garden (genotyping results in Table 1).

For both cultivar sets, the fresh and purged ants showed different preferential associations (Fig. 3). The fresh ants of the first experimental set showed preferential association with fungus E. In replicate FPA 61, the ants congregated around H on the first day, but no inoculum was molded into a garden. Purged ants of this set showed preferential association with five fungi (B, C, D, E, and F). In the replicates with the second cultivar set, the fresh ants showed preferential association with AH, and the purged ants showed preferential association with three fungi (AG, AD, and AH).

### Molecular verification of fungal choice

We were able to collect and genotype 40 mycelial tufts from the preserved gardens of ten replicates with fresh ants and 41 mycelial tufts from the preserved gardens of 12 replicates with purged ants. Among the total of 8,100 alleles scored, only two novel alleles were scored (alleles not present in any of the original fungi), and both were later judged to be likely stutter peaks or false peaks appearing next to actual peaks; the multiplex genotyping therefore created only a negligible fraction of artifactual alleles that could complicate the analysis. The allele profile of each genotyped mycelial tuft was first compared with the allele profiles of each original cultivar presented to the ants. In only two replicates, the ants built gardens each with a single fungal strain (FPA 59 and 62, Table 1), and in 20 replicates (91%), the gardens were built with more than one fungal

**Table 1** Preference and rejection of fungi by workers of *A. texana*, using behavioral criteria (gardening vs not-gardening) and molecular criteria (microsatellite genotyping)

| Offered fungal lineages | Ant colony | Fresh/ purged | Replicate code | Brood position | Behavioral evidence | | Molecular evidence | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Chosen inoculum (based on appearance and number ants/brood on the inocula) | Rejected inoculum (based on appearance of the inocula) | Microsatellite identity of fungal tufts from preferred inoculum | |
| | | | | | | | Pure genotype | Mixed or combined genotype |
| A-H | AT-10 | Fresh | FPA-63 | CENTER, C, G | C, E, D | A | 1C, 4 E, | 6 combined |
| A-H | AT-10 | Purged | FPA-69 | D | D | B, C, H | None | 4 combined |
| A-H | AT-11 | Fresh | FPA-62 | CENTER, E | E | C, D, G, H | 4 E, | None |
| A-H | AT-11 | Purged | FPA-65 | D | D | F, H | 2 C, | 1 combined |
| A-H | AT-17 | Fresh | FPA-61 | Not applicable | H | Not applicable | Fungus could not be preserved | |
| A-H | AT-17 | Purged | FPA-64 | D | D | H | None | 4 combined |
| A-H | AT-18 | Fresh | FPA-58 | D | D | C, G | 3 E | 5 combined |
| A-H | AT-18 | Purged | FPA-68 | E, D | D | H | 2 C, 2 F, | 3 combined |
| A-H | AT-22 | Fresh | FPA-60 | CENTER (New) | New | B, F | 3 E, | 1 combined |
| A-H | AT-22 | Purged | FPA-67 | H, D | D | E, F | None | 6 combined |
| A-H | AT-25 | Fresh | FPA-59 | CENTER, E | E | C, G, F, A | 4 E | None |
| A-H | AT-25 | Purged | FPA-66 | D | D | A, C, H | 1 C | 2 combined |
| AA-AH | AT-10 | Fresh | FPA-86 | AE | None | AH, AA, AD, AG, AB | Not applicable | |
| AA-AH | AT-10 | Purged | FPA-88 | CENTER (New) | New | AB, AF | None | 4 combined |
| AA-AH | AT-11 | Fresh | FPA-83 | AH | AH | AA, AC | 1 AH, | 1 combined |
| AA-AH | AT-11 | Purged | FPA-78 | CENTER (New) | New | AG, AE, AH | None | 3 combined |
| AA-AH | AT-17 | Fresh | FPA-76 | CENTER | AH | AF, AA, AG | 1 AB, 1 AH, | 1 combined |
| AA-AH | AT-17 | Purged | FPA-87 | AF | New | AA | None | 5 combined |
| AA-AH | AT-18 | Fresh | FPA-79 | AH | AH | AD, AA, AC | 1 AG | 1 combined |
| AA-AH | AT-18 | Purged | FPA-81 | CENTER | AH | AF | 1 AH, | 1 combined |
| AA-AH | AT-22 | Fresh | FPA-82 | AH | AH | AF, AG | 1 AH, | 2 combined |
| AA-AH | AT-22 | Purged | FPA-77 | AH | AH | AD | None | 1 combined |
| AA-AH | AT-25 | Fresh | FPA-85 | AE | AH, AG | AA | 4 AG, 2 AH, | 1 combined |
| AA-AH | AT-25 | Purged | FPA-84 | CENTER (New) | New | AC | None | 3 combined |

In the third column, fresh or purged indicates whether the tested workers were used directly (fresh) from a colony or purged first of gut contents by maintaining them on sucrose solution for 7 days (see Materials and methods). *Letters* in the 5th, 6th, 7th and 8th columns indicate the identities of the inocula. In the 5th and 6th column, "*New*" refers to a newly built garden away from the location of any original inocula. In the 5th column, "*CENTER*" refers to the brood placement in the center of the experimental arena. The *rightmost column* contains the number of fungal tufts that were a combination of at least two different fungi

strain. In the chimaeric gardens with multiple coexisting strains, we could explain 34.4% of the allele profiles as derived from one of the original eight cultivars used in a particular choice assay. The remaining 65.8% of the preferred fungal genotypes differed from all of the original eight cultivars, suggesting that they could represent two or more physically intermixed fungi growing in close spatial proximity in the same tuft (Table 1). In the absence of allele dropout due to PCR artifacts of the multiplex genotyping, a simple physical blend of different fungi should yield allele profile that combines all alleles present in the contributing fungal strains. However, in the profiles to which more than one fungus contributed, we found the presence of only subsets of alleles from the different donor strains (i.e., the profile clearly indicated the presence of two or more fungi in a single mycelial tuft, but some alleles present in the

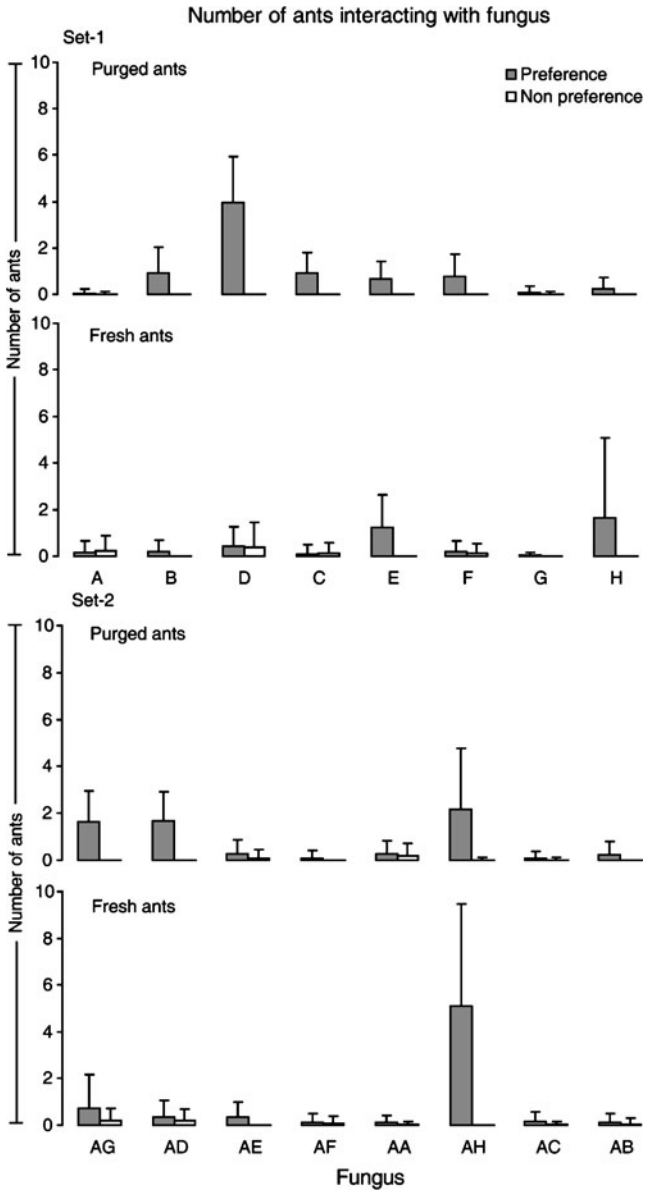


**Fig. 3** Average (±SD) number of ants showing preferential (*shaded bars*) and non-preferential (*not shaded bars*) interaction with fungal inocula in instantaneous scans carried out every half an hour for 8 h. In all four graphs, the order *from left to right* represents the same clonal fungus lineages. *H* and *AB* fungi were isolated from *A. versicolor* and all other fungi from *A. texana*

contributing fungi were missing). This indicates either (a) allele dropout due to PCR artifacts or (b) genetic recombination into a novel fungal genotype. Microsatellite screening, following the 10-day experiment, indicated that the intercropped fungi coexisted in chimaeric gardens for up to 4 months.

The proportion of samples where the ants combined inocula into a composite garden was significantly larger in choice experiments with purged ants (82%) compared with fresh ants (41%) (G test, $G_{adj}$=15.88, $P$<0.001). Most of the inocula chosen by fresh ants were similar in allele profile to cultivar E in the first set and cultivar AH in the second set (Table 1).

## Discussion

When confronted with a choice of distinct cultivar genotypes from an array of cultivars, *A. texana* workers show preference towards one fungus or several specific fungi, and the ants frequently (91%) build chimaeric gardens through intercropping of more than one fungus. Although we maximized genetic variation in our assay by presenting the ants with fungi from genetically distinct lineages (e.g., genetically diverse cultivars from throughout Texas, plus one cultivar from *A. versicolor*), DNA fingerprinting of the resulting gardens with microsatellite markers revealed that *A. texana* workers did not consistently favor any particular fungal strain under the test conditions. Compared with previous cultivar-choice experiments on the lower-attine ant *C. costatus*, the preferences or rejections of particular inocula could be scored much faster in *A. texana* (within 3–10 days) compared with *C. costatus* (13–24 days; Advani and Mueller 2006). The quicker assessment of fungal choice in *A. texana* permits testing of a larger number of cultivar strains in a short time span.

*A. texana* workers built new gardens by combining small pieces of substrate and mycelia from different inocula. Mycelia from different fungi coexisted for the duration of our experiment (up to 10 days) and the follow-up survey of chimaeric gardens lasting up to 4 months. The co-culturing of different fungi by ants in chimaeric gardens suggest that either the ants preferred more than one fungus in the cafeteria assay or that the ants perhaps cannot distinguish between some of the fungal genotypes. Although single *Atta* nests in nature appear to cultivate a single strain of cultivar (monoculture; Mueller et al. unpublished data), *A. texana* readily mixes more than one strain under laboratory conditions (polyculture). Under polyculture, strains coexisting in a single garden are predicted to compete with each other (Poulsen and Boomsma 2005), either competing directly by chemical inhibition or competing indirectly by differential utilization of resources (i.e., differential growth). Such competition should eventually lead to dominance of one fungus and elimination of competitively inferior fungi. Alternatively, competitively equivalent fungi may maintain their individual identities in an intercropped garden and perhaps even recombine genetically into novel, recombinant strains (e.g., by exchange of the haploid nuclei between the multinucleate fungal cultivars; Scott et al. 2009). Future research should monitor gardens for prolonged time to assess these possible fates of coexisting fungi.

We were able to improve the reliability of the preference scoring in *A. texana* by confirming choices by means of DNA fingerprinting of the chosen fungi. Although the behavioral observations give some indication of the preferences of the ants, the molecular information indicates that conclusions based solely on behavioral observation are sometimes not reliable, for two reasons. First, our criteria for preference (*tending*) were not stringent because they included molding the inoculum into a garden as well as sitting with no clear interaction with the fungus, and the latter may overestimate the preference. Second, although the ants can be observed to convert a particular inoculum into a fungal garden (and thus appear to choose a particular fungus), our molecular analyses reveal that the ants also incorporate mycelium from other inocula, leading to a chimaeric garden. Behavioral observations did not reliably indicate the cultivar mixing as indicated by the microsatellite genotyping.

In some cases, the genetic identity of the resultant garden was different from the original inoculum on which the ants built the garden, and consequently, the behavioral observations also did not reliably predict the genotype identity of the resultant garden constructed by the ants. Three reasons can explain the discrepancy between behaviorally and genetically scored choices. (1) An inoculum that is behaviorally scored as preferred may actually be used as the substrate to make a new garden. Several preferred fungi may be combined in a single garden on this substrate, and the "substrate cultivar" may not appear in the molecular screen. (2) Fungi in chimaeric, intercropped gardens may compete with each other, leading to dominance by one fungal genotype over other coexisting genotypes, and the most dominant fungal genotype prevails in the molecular analysis. Fungus–fungus competition, therefore, may produce biases that undo any choices imposed by the ants. (3) A resident fungus may recombine genetically with the fungi added by the ants, leading to eventual replacement of the resident fungus. This third possibility of genetic recombination might have occurred in eight of the 24 experiments (Table 1, FPA 58, 64, 65, 66, 67, 68, 69 and 79), but the genetic evidence is not conclusive because we cannot rule out with certainty PCR artifacts in the multiplex microsatellite loci analyses (e.g., we cannot rule out allele dropout artifacts due to template concentration biases).

Purged ants constructed chimaeric gardens more frequently than fresh ants. Specifically, the fungal genotypes of tended gardens in experiments with purged ants appeared more often as combinations of several strains than as one of the original (pure) strains (Table 1). This difference may be due to insufficient fungal recognition cues between test fungi for purged ants, motivational differences, or perhaps even fungal imprinting on ants of recently cultivated garden (i.e., fresh ants chose a fungus most similar to the one from their natal nest). Because purged ants were maintained on sucrose solution diet for 7 days, they might be less able to distinguish one strain from the other. Purged ants may also have lowered thresholds for fungal acceptance. Both these possibilities may lead to increased rates of fungal mixing in a single garden. As a second explanation for the different results between purged and fresh ants, purged ants may be more prone to fungal mixing because they may not carry any of the putative incompatibility factors in their feces (sensu Poulsen and Boomsma 2005). However, if incompatibility factors were operating in our experiments, it is surprising that in none of our experiments did the intercropped fungi show any visible sign of interfungal conflict such as browning or blackening (mycelial melanization indicative of vegetative incompatibility). Such signs of interfungal competition are expected based on the melanizing incompatibility reactions observed by Poulsen and Boomsma (2005). As a third explanation, workers in natural nests may have imprinted onto their native cultivar (from the colony they were born into), such that the imprinting effects extinguishes only after some disassociation with their native fungus (i.e., during our experimental purging period). Differentiating between these various explanations will be productive avenues for future studies on the biochemical and neuroethological mechanisms underlying symbiont choice in fungus-growing ants.

The frequent construction of chimaeric gardens constructed by *A. texana* workers in our assay was surprising, as this contradicts the observation of single-strain monocultures observed under natural conditions (Mueller et al. unpublished data). Coexistence of distinct fungal genotypes in chimaeric gardens is, furthermore surprising because it appears to contradict a recent model predicting cultivar competition in attine gardens. Poulsen and Boomsma (2005) postulated that each cultivar secretes incompatibility compounds harming other cultivars, which presumably should prevent the ants from growing cultivars in intercropped polyculture. Because this model was formulated for tropical leafcutter cultivars, it may not apply to the genetically less diverse leafcutter cultivars at the northern edge of the leafcutter distribution in the southern USA. It is also possible that, whenever chimaeric gardens are created under natural conditions, such intercropped gardens convert more quickly to monoculture or they perish quickly for unknown reasons (e.g., cultivar–cultivar competition in chimaeric gardens may make such gardens more susceptible to disease). In our laboratory assay, however, the combined effects of symbiont choice and any competition between coexisting cultivar strains in chimaeric gardens do not appear to operate fast enough to convert chimaeric gardens into the monocultures typical for *A. texana* under natural conditions. Future work should focus on understanding the relative efficacies of symbiont choice imposed

by the ants, cultivar–cultivar competition within intercropped gardens and possible selection on ant-fungus combinations to further understand the apparent prevalence of garden monoculture observed under natural conditions.

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**Ethical standards** The authors declare that the experiments comply with the current laws of the United States, where they were performed. The authors declare that they have no conflict of interest.

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