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Research article

A preference assay for quantifying symbiont choice in fungus-growing ants (Attini, Formicidae)

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Abstract. We describe a bioassay for the quantification of cultivar preference (symbiont choice) of fungus-growing ants. The bioassay simultaneously presents mycelium of multiple pure cultivar genotypes to worker ants in a cafeteria-style test arena, and preferred versus non-preferred cultivar genotypes can then be identified based on the ants' quantifiable behavioral tendencies to convert any of the offered mycelium into a fungus garden. Under natural conditions, fungus-growing ants are likely to express such cultivar preferences when mutant cultivars arise in a garden, or when colonies acquire a novel cultivar from a neighboring colony to replace their resident cultivar. We show that workers from different nests of the fungus-growing ant *Cyphomyrmex costatus* exhibit repeatable preferences vis-à-vis specific cultivar genotypes. The identified preferred and rejected cultivars can then be used in a performance assay to test whether the ants prefer cultivar genotypes that are superior in enhancing colony fitness (measured, for example, as garden productivity or colony growth), as predicted by symbiont-choice theory.

Keywords: Cyphomyrmex costatus, mutualism, partner choice, symbiont choice, symbiosis.

Introduction

Partner choice is one of the main evolutionary mechanisms stabilizing cooperative interactions, particularly mutualisms between species (Bull and Rice, 1991; Noë, 2001; Sachs et al., 2004). Under partner choice, at least one of the partners engaged in a cooperative interaction is able to discriminate between superior versus inferior partners by preferentially entering interactions with valuable partners (so-called "cooperative" partners) or by terminating interactions with poor-performance partners (so-called "non-cooperative" or "cheater" partners; Herre et al., 1999; Sachs et al., 2004). This control over engagement or disengagement by one partner imposes selection on the other partner, favoring cooperative types while curbing (or even precluding) the invasion and spread of poor-performance or cheater partner-types.

In the case of between-species mutualism, partner choice has been called more descriptively "symbiont choice" (Mueller, 2002; Mueller et al., 2005), and empirical studies have shown that such choice operates in a number of mutualistic systems, including several plant-rhizobium mutualisms (Denison, 2000; Denison et al., 2003; Kiers et al., 2003; Simms and Taylor, 2002; West et al., 2002; Simms et al., 2006), the attine ant-fungus symbiosis (Mueller et al., 2004), and possibly the squid-*Vibrio* mutualism (Nishiguchi et al., 1998; Nishiguchi, 2002). For example, the primitive fungus-growing ant *Cyphomyrmex muelleri* (Attini, Formicidae) has recently been shown to possess the sensory acuity to prefer its "native" cultivar (the cultivar with which this ant species is naturally associated) over very closely related cultivars that are propagated by other sympatric attine ant species (Mueller et al., 2004). The operation of efficient cultivar-choice sensory mechanisms, predisposing single ant species to favor specific cultivar types, therefore explains why single ant lineages associate in the wild only with a phylogenetically narrow range of cultivar types, despite occasional cultivar exchange between different ant colonies, sometimes even between different ant species and

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ant genera (Bot et al., 2001; Green et al., 2002; Mueller, 2002; Silva-Pinhati et al., 2004). The observation of symbiont choice in attine ants furthermore suggests that cultivar preferences could bias the transfer rate of particular cultivars between different ant colonies (Mueller et al., 2004), possibly favoring the exchange and spread of preferred, beneficial cultivar genotypes over inferior genotypes within a sympatric ant community.

The level of symbiont discrimination documented by Mueller et al. (2004) for the fungus-growing ant *C. muelleri* was rather crude, because the ants were tested in this study merely for their sensory abilities to discriminate between phylogenetically distinct lineages of cultivars, but not between minimally differentiated genotypes of the same cultivar lineage. For symbiont choice to select efficiently for beneficial symbionts, a discriminatory ability of higher resolution is needed, such that beneficial ("cooperative") symbiont genotypes can be differentiated from inferior mutant genotypes that may arise within a lineage (Sachs et al., 2004). Such acute differentiation between symbiont genotypes appears to exist in some plant-bacterial mutualisms (Kiers et al., 2003; Simms and Taylor, 2002), but has not yet been shown for any attine ant-fungus mutualism.

We develop here a choice bioassay that assesses cultivar preferences in fungus-growing ants and permits identification of preferred versus non-preferred genotypes of a cultivar lineage. Choice experiments were conducted with workers of the lower attine ant *Cyphomyrmex costatus*, a fungus-growing ant species that, under natural conditions in central Panamá, frequently exchanges cultivar clones with the sympatric species *C. muelleri* (Green et al., 2002; Mueller and Wcislo, 1998; Schultz et al., 2002). Exchange of cultivar clones between different species of attine ants appears to be a universal phenomenon of probably all fungus-growing ant species (Autuori, 1950; Adams et al., 2000; Bot et al., 2001; Green et al., 2002; Mueller, 2002; Mueller and Gerardo, 2002; Villesen et al., 2004; Silva-Pinhati et al., 2004; Poulsen and Boomsma, 2005; Mueller et al., 2005), but these exchanges appear to be constrained in that each ant species is specialized on a phylogenetically narrow clade of cultivars (possibly a single species of fungus, though exact species boundaries of the cultivated fungi remain unclear; Mueller, 2002). In other words, each fungus-growing ant is specialized on a narrow clade of cultivars, but one or several other, sympatric fungus-growing ant species (some of them distantly related) may be specialized on the same clade of cultivars. Because of these frequent exchanges of cultivar clones within a local community of attine ants, Mueller et al. (1998; see also Mueller, 2002) speculated that beneficial clones arising during cultivation in one ant species (for example through occasional recombination, or through mutation) may be preferentially acquired by colonies of other ant species if workers from these acquiring colonies express symbiont choice.

In the present study, we assess the plausibility of this hypothesis of selective spread of cultivar clones through a sympatric ant community by testing whether an acquiring ant species (*C. costatus* in the present study) is able to discriminate between different cultivar clones propagated by the donor ant species *C. muelleri*, from which *C. costatus* is known to acquire cultivars occasionally through horizontal transfer (Green et al., 2002). The exact mechanisms of horizontal transfer in the field are not known. However, in lab experiments, between-colony transfers of cultivar occur when garden-deprived colonies steal garden from or usurp neighboring colonies (Adams et al., 2000). To explore ultimately the role of symbiont choice in the evolutionary maintenance of the attine ant-microbe symbiosis, we develop here the requisite methodology to quantify cultivar preferences in attine ants.

Methods

Ant and cultivar study systems

The workers used in the choice experiments were taken from six queenright *Cyphomyrmex costatus* colonies collected in Oct-Dec 2001 in Parque Soberanía in the Republic of Panamá. Live colonies with their gardens were maintained at room temperature (20–22°C) in artificial colony chambers as described by Schultz (1993). The cultivars tested were isolated from ten *C. muelleri* colonies, five of them also from Parque Soberanía, but the other five colonies were collected at three other sites in Panamá at least 40 km distant from Parque Soberanía (two colonies from the Fort Sherman Military Reservation, two colonies from El Llano, and one colony from near Coclesito; see Green et al., 2002 for a map showing the locations of the collecting sites).

Mycolocgical methods

Mycological methods for the isolation and propagation of the cultivars essentially followed the procedures described in Mueller et al. (1996) and Mueller et al. (2004), with some important improvements, as described below. All isolations, subculturing, and inoculations were performed at the same time, to ensure that the tested cultivars had undergone identical subculturing histories in the laboratory prior to testing.

Isolation of Cultivars: A small (1mm diameter) tuft of healthy fungal garden of *C. muelleri* was plucked with sterile forceps from a garden of *C. muelleri* and placed on potato dextrose agar (PDA; Difco, Michigan) supplemented with 10% (mg/ml) streptomycin and penicillin antibiotics to suppress bacterial contaminants. Four pieces of garden were isolated from each *C. muelleri* colony, but only one uncontaminated cultivar was chosen from among these four for subculturing. Plates were monitored daily and any contaminants or garden pathogens were immediately removed. Once the tufts from the initial isolation had grown to approximately 10mm in diameter, a small plug (5mm × 5mm) of agar was cut from the growth front and moved to a new PDA plate. This process of successive subculturing was repeated two more times to ensure that the cultivar isolate was free of contaminant microbes. The time from initial plating to obtaining a pure culture after three rounds of subculturing lasted approximately six weeks.

Inoculation: Direct presentation to ants of agar plugs suffused with cultivar invariably results in growth of contaminants on the agar, and cultivar cultures therefore need to be transferred to a different growth medium (oat flakes) before presentation to the ants (oat flakes are

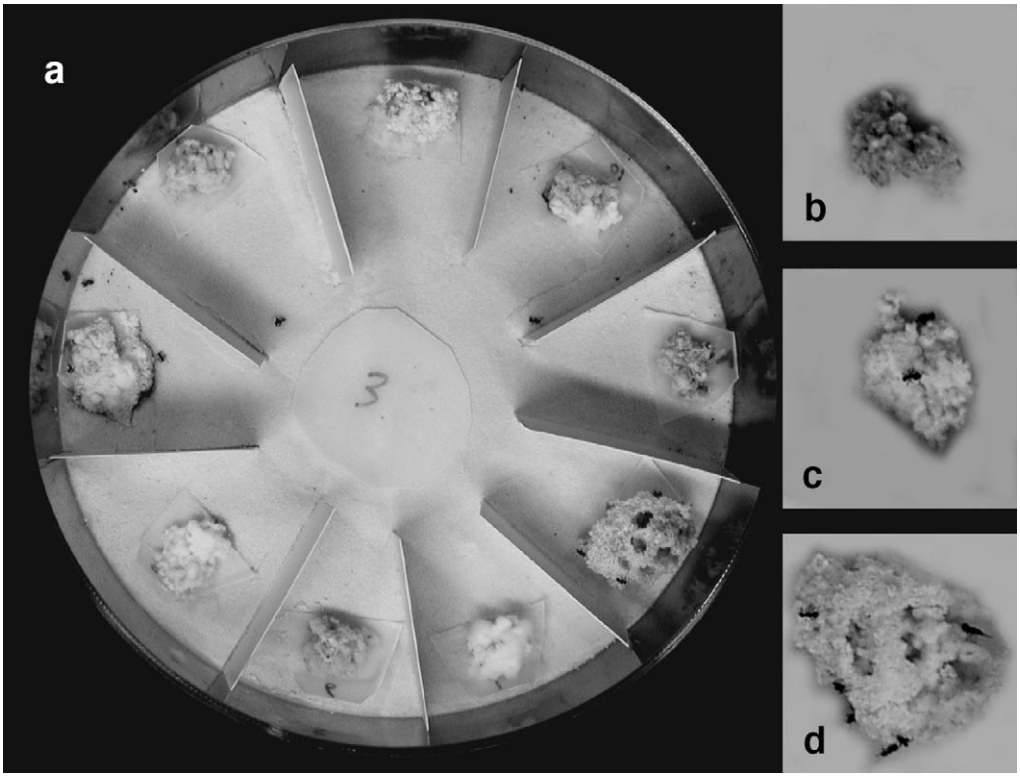

Figure 1. a. Experimental test chamber with the circular arrangement of the subcompartments, each presenting one of the cultivars in the cafeteria-style preference assay. The ants appear as small black dots on the fungus or on the chamber floor. In the particular experiment shown, most workers associate with the cultivars at the four and nine o'clock positions. **b.**–**d.** Appearance of cultivars when gardened to varying degrees by workers of *C. costatus*: **b.** An ungardened cultivar fragment is similar in size and texture to the fungal fragment initially presented to the ants, with noticeable brownish coloration developing over time. **c.** A cultivar fragment that is irregularly (but not continuously) gardened by workers is slightly larger than the initial fragment presented to the ants, with no or only minimal discoloration, but without the honeycombed (chambered) architecture of a typical garden of *C. costatus.* **d.** A well-gardened cultivar fragment resembles the honeycombed architecture of a typical garden, is larger in size than the original fragment presented (because of the addition of substrate by the workers), and shows no discoloration.

lower in nutrients than agar and thus do not readily permit germination of contaminant spores; in addition, cultivar grown on oat flakes resembles the growth form of the cultivated fungus in natural gardens). An agar plug (0.5cm × 1cm) with mycelium from near the growth front was cut from a pure culture and blended in a sterilized Waring blender (Eberbach Corporation, Ann Arbor, MI) with 15 ml of sterilized potato dextrose broth (PDB; Difco, Michigan). The blended liquid was then pipetted onto a layer (3–4 mm thick) of sterilized, ground oat flakes spread in glass Petri dishes. The dishes were then sealed with parafilm to prevent desiccation and contamination, and the oat cultures were allowed to grow in the dark at room temperature. All cultivars tested in a given trial were inoculated onto oats on the same day, and were thus of the same age at the time of testing. Oat cultures generally showed the first fine mycelial growth within a week, and the oat layer was completely overgrown with a thick mycelial coat at about two to three weeks. Small inocula (about 8 × 8 × 5 cubic mm) were cut from the mycelial coat three to four weeks after oat inoculation, and then presented to the ants in a choice arena.

Experimental paradigm

Experimental setup: The choice-bioassays developed in Mueller et al. (2004) presented simultaneously only two cultivars to ants and thus required a large number of individual tests for the comparison (in all pairwise combinations) of ant preferences. To improve the time-efficiency of testing, we modified the bioassay by simultaneously presenting multiple cultivars in a cafeteria-style choice arena (Fig. 1a).

Small pieces of fungal mycelium (of cultivars isolated originally from nests of the ant *C. muelleri*) were cut from oat cultures and placed on alcohol-sterilized, plastic trays (1 × 1 cm; cut from plastic weighing dishes), which were presented to worker ants of *C. costatus* in a test chamber. The test chamber consisted of a plastic dish (11 cm diameter) with a 1 cm deep layer of moistened plaster of Paris to provide stable humidity. The ten trays with fungi were spaced evenly in a circular arrangement within this test chamber (Fig. 1a). The order of the fungi in the circular array was randomized within each trial. To minimize the spread of possible contaminants from one mycelial fragment to the other, and to prevent the ants from combining cultivars from different trays onto one garden pile, plastic partitions were inserted into the bottom plaster (at the time when the plaster was poured), thus separating each piece of fungus into a small side chamber that opened towards the center of the test arena.

Cultivar fragments that became contaminated (visible as discoloration, pronounced aerial hyphal growth, or spore-bearing mycelium) were removed from the test arena with its tray, then replaced with a fresh piece cut from the respective oat-culture. If one of the ants died during the course of the trial, the ant was replaced with another ant from the same colony. Each individual ant was tested only once in the entire choice study.

In each test replicate, ten workers from the same *C. costatus* colony were given a choice between the ten different *C. muelleri* cultivars. A total of six *C. costatus* nests were used for testing. The chambers were kept in darkness during the entire experiment, except for very brief exposure to light for one-zero scans (Altmann, 1974) of ant behavior (see next).

Preference scoring: Starting 24 hours after presentation of the cultivars, each test arena was observed to determine the spatial association of workers with the mycelial fragments. *Ant association with the fungus* was the main criterion for fungal acceptance. An ant was scored as associating with a particular fungus if it was resting on or gardening the cultivar fragment at the time of one-zero scoring. Ants resting or walking on the plaster or on the side of the chamber, even if near a mycelial fragment, were conservatively scored as not associating with any fungus. Over the period of an experimental trial, the ants were scored daily for a continuous period of between 13 and 24 days (depending on the trial; see Table 1).

We used *substrate addition* as a second criterion for cultivar preference. Twice during the course of each trial (once mid-way through the trial and again towards the end), the ants were given ten pieces of polenta (a highly preferred gardening substrate made from corn) on a dish placed in the center of the testing arena. The day after substrate was given to the ants, each garden fragment was scored for whether any substrate had been added to it, and any unused substrate left in the tray was removed after scoring.

A third criterion for cultivar preference, which emerged from the observations during Trials 1–4 (see below) and was measured in Trials 5 and 6, was *how well the ants had attended to a mycelial fragment and worked it into a garden*. Typically, an unworked piece of cultivar remained similar in size and texture to the fragment of fungus initially presented to the ants, with noticeable brownish coloration developing with time (Fig. 1b). In contrast, a well-gardened mycelial fragment showed the "honeycombed" texture of a garden in a healthy ant colony, was noticeably larger in size than the original fragment presented (because of the addition of substrate by the workers), and was not discolored (Fig. 1d). A mycelial fragment that is irregularly (but not continuously) attended was slightly larger than the initial mycelial fragment presented to the ants, with no or only minimal discoloration, but without the honeycombed texture of a garden in a healthy ant colony (Fig. 1c). These three states (Figs 1b-d) demarcated a continuum of five fragment classifications along which each mycelial fragment was evaluated at the mid-way point and at the end of Trials 5 and 6, with scores of 1 (Fig. 1b), 3 (Fig. 1c), and 5 (Fig. 1d) assigned as explained above, and scores of 2 and 4 assigned when the garden states appeared intermediate between these.

Experimental replication: A total of six replicate trials were conducted (Table 1), the first two after the first inoculation (Trials 1&2), the next two after a second inoculation (Trials 3&4), and the last two after a third inoculation (Trials 5&6) (Table 1). This replication allowed assessment of preference variation between separate trials and between separate inoculations. Table 1 lists the inoculation dates, the observation periods over which ant choices were scored (Trials 1–6), and the number of actual days that ants were observed during each trial. Trials 1–4 involved all six *C. costatus* colonies and all ten *C. muelleri* cultivars (named Cultivars A through J). Because of loss of Cultivar D due to accidental contamination, and because of eventual worker depletion in two *C. costatus* colonies during the latter trials, Trial 5 involved five ant colonies and nine cultivars (excluding the lost Cultivar D), and Trial 6 involved four ant colonies and nine cultivars (Table 1).

Subanalysis of restricted dataset: Ant preference scores were most variable during the initial few days during each period of testing, but preferences were usually expressed consistently by about the third or fourth experimental day and rarely seemed to change thereafter. We therefore investigated whether a shorter observation period (three days only), starting on Day 5 after presentation of the fungi (i.e., thus ignoring behavioral observations during the initial period when ant preference scores seemed to fluctuate) would suffice to determine consistent cultivar preferences and would yield results comparable to those obtained when analyzing the complete dataset.

Results

Overall preferences: Workers of *C. costatus* exhibited significantly different preferences when choosing between the 10 different pure cultivars for potential cultivation.

In a Kruskall-Wallis analysis of variance, nest (χ^2 approximation = 1.95; df = 5; P = 0.856), trial (χ^2 approximation = 1.80; df = 5; P = 0.877), and inoculation (χ^2 approximation = 1.37; df = 2; P = 0.503) were not significant predictors of the number of ants that associated with a cultivar (first criterion of cultivar preference), whereas the type of cultivar presented to the ants was a significant predictor of ant association behavior (χ^2 approximation = 83.66; df = 9; $P < 0.0001$).

Most noticeably, workers consistently disfavored three cultivars (G, I, and J; Figs 2–4, Table 1). A Principal Components (PC) analysis of the average ant association with a cultivar reflected this consistent treatment of Cultivars G, I, and J across all trials and nests, grouping these cultivars closely together in PC-space (Fig. 5). In contrast, the set of favored cultivars was less clearly defined and included probably five cultivars, Cultivars B, C, D, E, and H, but clearly excluded the intermediately-ranked Cultivar F (Figs 2–4, Table 1). The substantially lower *median* preference score compared to the *mean* score for Cultivars D, E, and H indicates that the preference averages for these three cultivars were influenced by outlier high-scores, thus leaving Cultivars B and C as the two cultivars that were most consistently favored across all six trials (i.e., their respective preference mean and median nearly coincided on the same value). Of the favored cultivars, Cultivar B showed the least preference variation (compare box-plots for the most-preferred Cultivars B, C, D, E and H in Fig. 2a), underscoring the greatest consistency of the choice favoring Cultivar B.

Preference variation between trials: As already apparent from the overall averages, Cultivars G, I, and J were consistently disfavored in all trials, but the most-preferred cultivars varied between trials (Fig. 3; Table 1). For example, Cultivars A, B, C, and H were most-preferred in the first two trials (first inoculation), and these results correlated with those of the last two trials (third inoculation) in which Cultivars B, C, and H were favored. However, the most-preferred cultivars in Trials 3 and 4 (second inoculation) were different and included Cultivars D, E, and to a lesser extent also A (Fig. 3). The between-trial variation in average choices suggests that, using this behavioral assay, it seems easier to identify disfavored cultivars rather than favored cultivars, and that the unambiguous identification of favored cultivars requires several replicate trials.

Preference variation between nests: Even though ant nests agreed consistently on the choice of the three least-preferred Cultivars (G, I, and J), nests differed in their

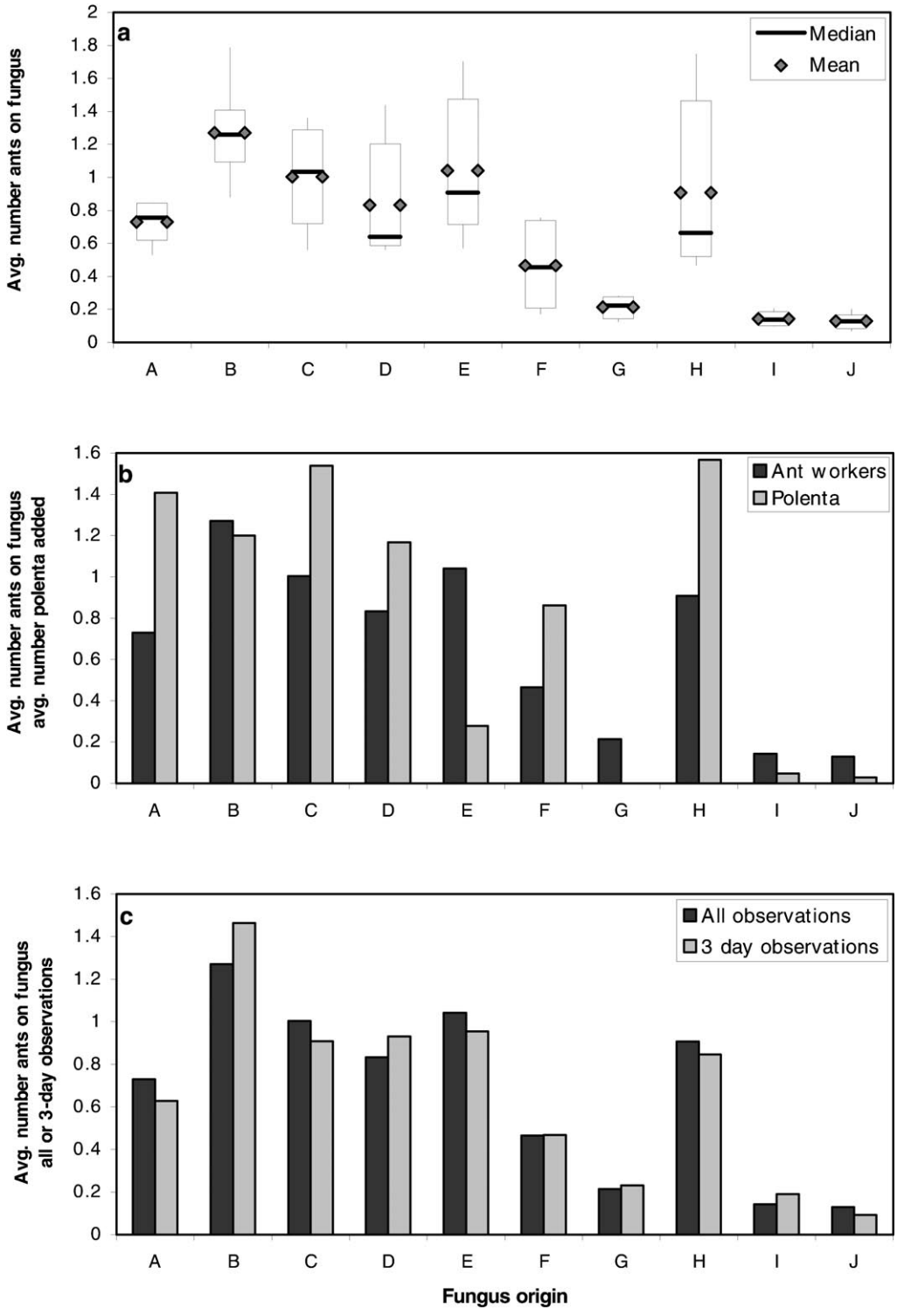

Figure 2. a. Average number of *C. costatus* workers observed during scans on the ten fungal cultivars (A-J) that were tested in the cafeteria-style choice assay. The box plots indicate the range (whiskers) and the 25–75 percentile intervals (box). Mean (diamond) and median (crossbar) are indicated inside each box plot. Cultivars G, I, and J were consistently disfavored by the ants, whereas Cultivars B and C were most consistently favored by the ants (see also text). **b.** Average number of workers observed on the ten tested cultivars, and average number of substrate (polenta) pieces added by the workers to the cultivars. **c.** Average number of workers observed in scans on the ten cultivars when analyzing all scans from the entire observational period, in comparison to observations from only a 3-day time window on Days 5–7.

preference for most-preferred cultivar (Fig. 4). For example, cultivars that were highly preferred by some nests were preferred at rank 4 or 5 by other nests, and vice versa. In addition, the PC analysis of choice behavior by nests (not shown) did not reveal any obvious patterns, and no nest exhibited an overall choice-profile that closely resembled the choice profile of any other nest. The picture emerging from all these analyses implicates either (a) the influence of significant "noise" in choice behavior towards the most-preferred cultivars (e.g., if the most-preferred cultivars were nearly indistinguishable for the ants), or (b) idiosyncratic choice-components of the different nests tested.

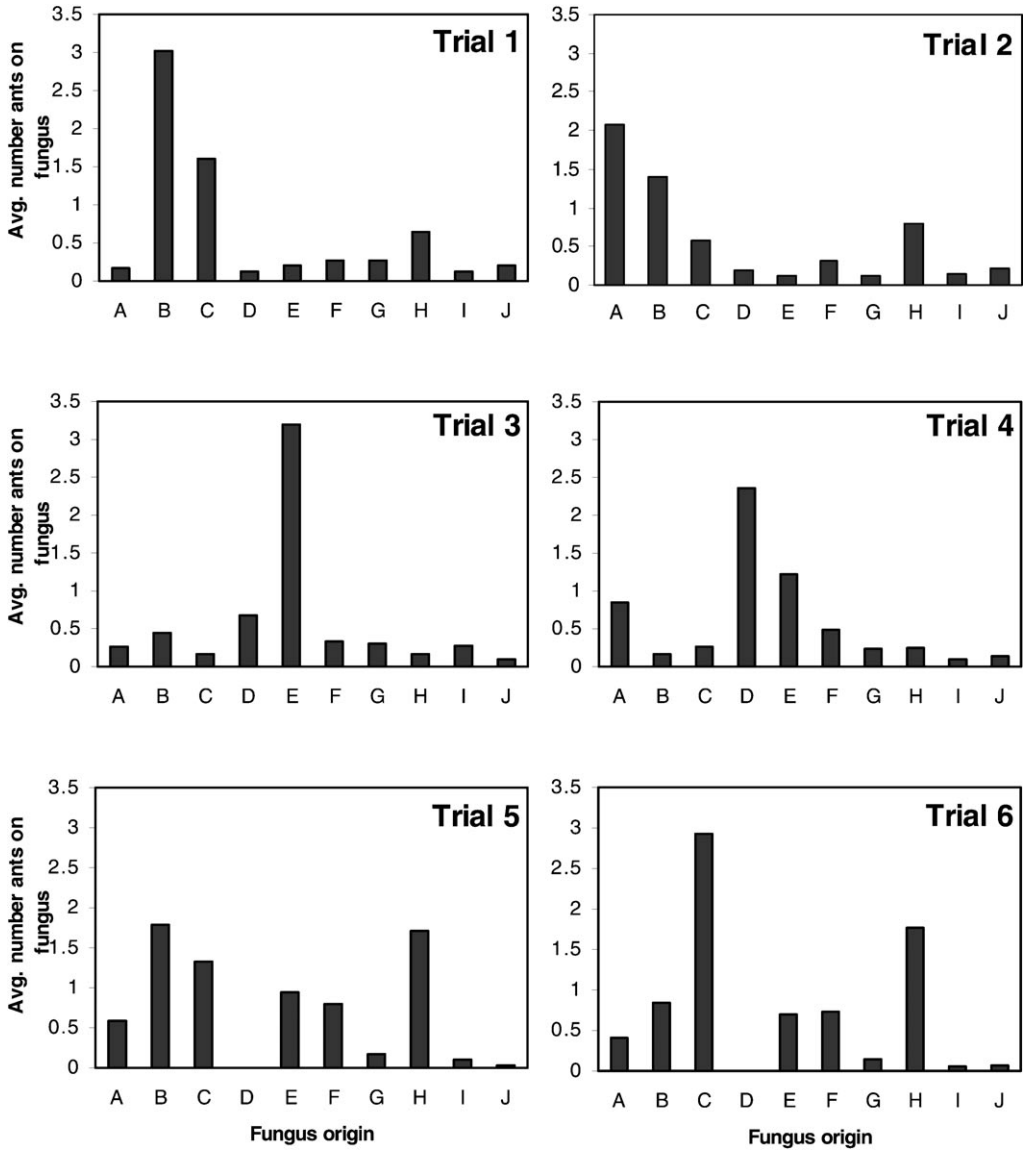

Figure 3. Average number of workers observed on the ten cultivars (A-J), graphed separately for each of the six trials conducted (averaged across the six nests for each separate trial). Cultivar D was lost during the experiment due to contamination and therefore could not be tested in Trials 5 and 6.

Table 1. Methodological details of the six experimental trials, the four most-preferred cultivars, and the four least-preferred cultivars for each of the six trials.

	Inoculation date	Observation period	# of days ant choices were scored	4 most-preferred cultivars*	4 least-preferred cultivars*
Trial 1	20 Jan 2003	20 Feb – 11 Mar 2003	8	B, C, H, F	J, A, D, I
Trial 2	20 Jan 2003	28 Mar – 21 Apr 2003	12	A, B, H, C	J, E, G, I
Trial 3	20 Aug 2003	16 Sep – 30 Sep 2003	12	E, D, B, F	I, C, H, J
Trial 4	20 Aug 2003	6 Nov – 20 Nov 2003	12	D, E, A, F	G, B, I, J
Trial 5	5 Apr 2004	29 Apr – 12 May 2004	14	B, H, C, E*	A, G, I, J*
Trial 6	5 Apr 2004	29 Apr – 12 May 2004	14	C, H, B, F*	A, G, J, I*

* Cultivar D, which was one of the least-preferred cultivars in Trials 1 and 2, but one of the most-preferred cultivars in Trials 3 and 4, was lost due to contamination in early 2004, and therefore could not be tested in Trials 5 and 6.

Comparison of the three choice criteria: The two main preference criteria, the number of workers associated with a cultivar in one-zero scans and the number of polenta pieces added to a cultivar, were significantly correlated (Pearson correlation = 0.799; $p = 0.01$). Most notably, cultivars with which few workers associated also

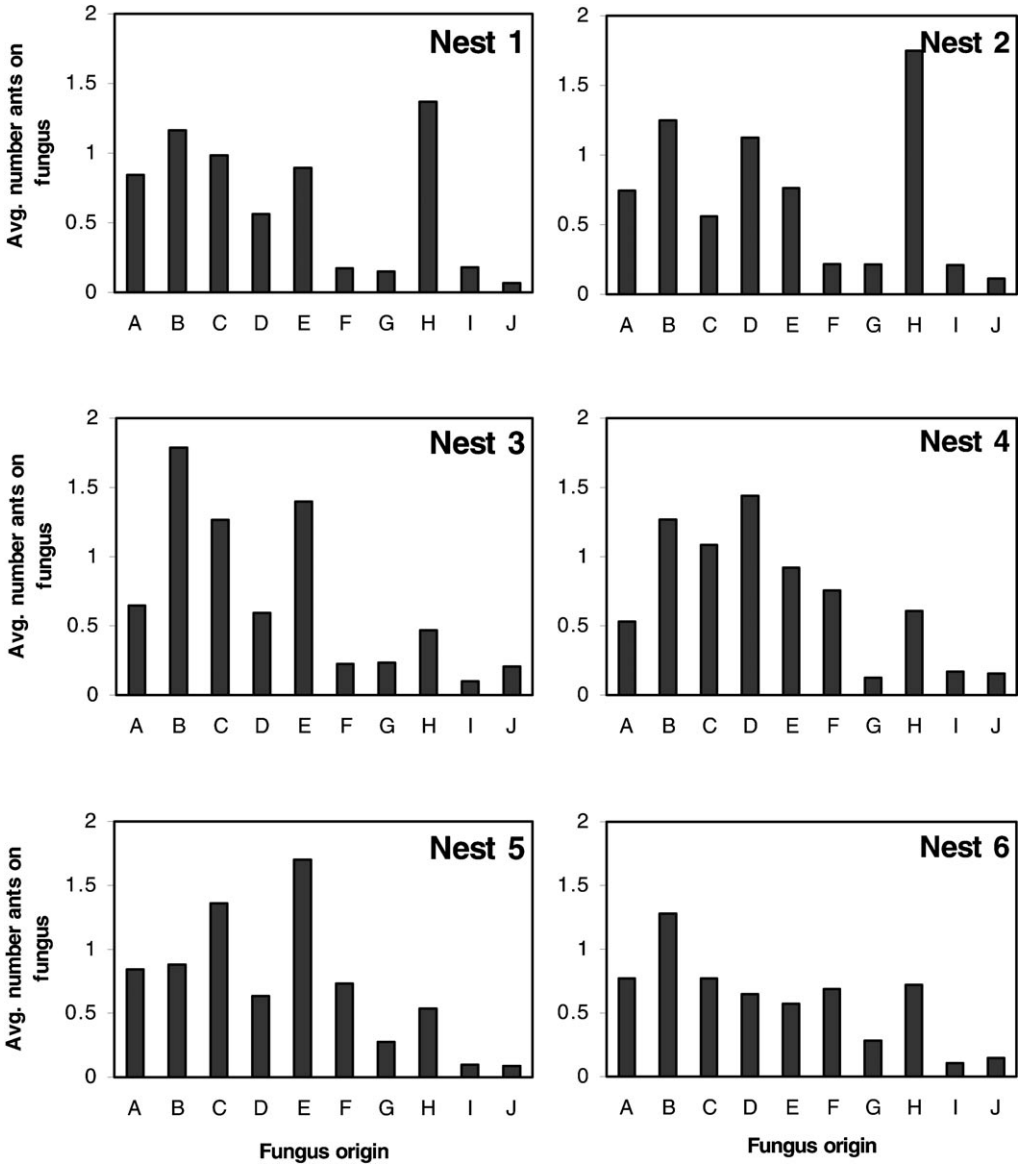

Figure 4. Average number of workers observed on the ten cultivars (A-J), graphed separately for each of the six nests tested (averaged across the six trials for each nest separately).

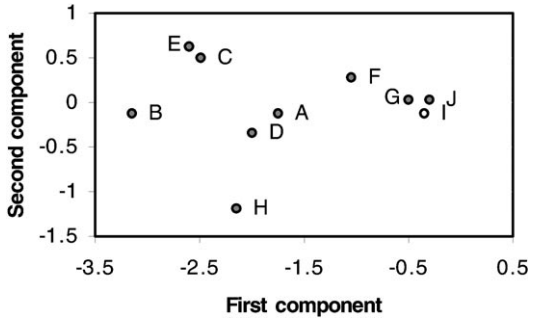

Figure 5. Analysis of principal components. The first principal component arranges cultivars along an axis from most preferred (left) to least preferred (right), as explained in the text. The biological interpretation of the second principal component is unclear.

received very little or no substrate (Cultivars G, I, and J), but the two preference measures were less correlated for the set of favored cultivars (see for example Cultivars E and H in Fig. 2b). On the other hand, Cultivars B and C, which emerged as the consistently most-preferred cultivars in the analyses of worker association, were also among the cultivars that received the highest average number of substrate additions (Fig. 2b). For Trials 5 and 6, where garden appearance (texture, color, honeycombing; see above) was judged as a third preference criterion, the number of workers associating with a cultivar was significantly correlated with garden appearance (Pearson correlation $= 0.771$; $p = 0.015$), but the number of substrate pieces added was not significantly correlated with garden appearance (Pearson correlation $= 0.408$; $p = 0.275$).

Subanalysis of restricted dataset: The main conclusions regarding cultivar preferences did not change when subanalyzing the overall dataset and restricting the

analysis only to the observations from Days 5–7 after initial presentation of the cultivars. Even in this limited dataset, Cultivars G, I, and J emerge as the unambiguously disfavored cultivars, whereas Cultivars B, C, D, and E emerge as preferred cultivars (Fig. 2c). This congruence in preferences between the entire and the restricted datasets suggests that it suffices to score preferences only for three days following an initial period of habituation of about four days (during which worker associations fluctuate, see above).

Discussion

Utility of the preference bioassay: The proposed symbiont-choice bioassay allows (a) the quantification of cultivar preferences, (b) categorization of cultivars into preferred and non-preferred cultivars, and (c) ranking of cultivars on a relative preference scale from most-preferred to least-preferred cultivar. For example, Cultivars G, I, and J were consistently rejected in all trials by workers from all nests, whereas Cultivars B and C not only emerged most consistently among the most-preferred cultivars, but also received the overall highest rankings averaged across all test trials (Table 1; Fig. 2a,b). The between-trial variation in average choices suggests that, using this behavioral assay, it seems easier to identify disfavored cultivars rather than favored cultivars, and that the unambiguous identification of favored cultivars requires several replicate trials.

Consistency in preference between nests and trials can be evaluated in two ways, first by the overall statistical variance (see narrow width of box plot for Cultivar B in Fig. 2a, relative to the box plot widths of other preferred cultivars); and second by the congruence of *mean* score and *median* score of a particular cultivar, where a discrepancy between mean and median scores reveals the influence of outlier preference-scores (see box plots for Cultivars D, E, and H in Fig. 2a), whereas congruence of mean and median scores reveals a consistency of the preference that is less affected by outlier scores (see box plots for B and C in Fig. 2a). Interestingly, this average-based and consistency-based ranking of cultivars from the most consistently preferred Cultivar B, to the generally preferred cultivars (C, D, E, and H), to the intermediately preferred cultivar F, to the consistently rejected cultivars (G, I, and J) reflects the relative scores of the first component in the PC-analysis, suggesting that this component arranges cultivars along a preference-rejection axis (from left to right in Fig. 5).

The two main measures of behavioral preference (number of workers on mycelial fragment in one-zero scans; number of polenta added to fragment) were significantly correlated (Pearson correlations $= 0.799$; $p = 0.01$), but occasional discrepancies between these scores (e.g. cultivar C in Fig. 2b) suggest that a combined measure, incorporating information from several preference measures, may provide a more accurate estimator of cultivar preference than any single measure alone.

Of the three measures of behavioral preference used, garden appearance measured along the proposed 5-point "appearance scale" (see Methods) is undoubtedly the most subjective, but it provides an accurate *relative* measure of preference when comparing garden fragments in a given test arena against each other. Whereas the measure of worker number on a garden is probably influenced by stochastic fluctuations and by disturbances that cause workers to flee a garden during the one-zero scans, garden appearance (defined by garden size, texture, and coloration) is expected to provide a more accurate measure of continuous worker attendance over several days prior to scoring.

Implications for ant-cultivar coevolution: Workers of *C. costatus* are capable of differentiating between preferred and non-preferred cultivar genotypes and thus may well exert symbiont choice on their cultivars under natural conditions, as first hypothesized by Mueller et al. (1998; see also Mueller, 2002; Sachs et al., 2004). In addition, symbiont-choice biases operating against non-preferred cultivars may be more consistently expressed by the ants than biases favoring specific cultivars. If such biases operate in the field, preferences may bias the rate of between-colony transfer of specific cultivars, such that novel cultivar variants entering an ant community (e.g., as a novel domesticate, or more likely as a novel mutant or recombinant) may spread through an ant community driven in part by symbiont choice exerted by the ants. This would constitute a process analogous to artificial selection of crops in human farming (Mueller, 2002; Mueller et al., 2005; Schultz et al., 2005).

Recommendations for improvement of the cultivar-preference bioassay: In our experiments, several factors probably contributed to the observed preference variation between trials and nests. First, our sample sizes were small (e.g., only six nests were tested), and statistical noise can probably be reduced by testing more ant nests. Second, individual nests may differ inherently in their actual preferences. For example, workers may have innate or learned preferences for their native cultivar genotype (see discussion in Mueller et al., 2004) and thus select the cultivar genotype in the preference assay that most closely resembles their native cultivar genotype. Third, unknown and uncontrolled environmental variables (e.g., room temperature) may have induced physiological differences in the cultivars during growth, thus influencing ant preferences between the trials. Fourth, systematic differences between the three inoculations clearly contributed to between-trial variation in our experiments (see Fig. 3). This fourth source of variation should not be underestimated in future cultivar-choice studies, but can be controlled by conducting experiments with cultivars from several inoculations and with sufficient replication. Fifth, as recently documented for

leafcutter ants (Poulsen and Boomsma, 2005), ant-fungus incompatibility reactions mediated via the ants' feces during gardening could have influenced cultivar acceptance behaviors. Such incompatibility reactions can be controlled for by maintaining ants for a few days on a neutral diet prior to testing, allowing the ants to purge any ingested incompatibility factors from their digestive system. While incompatibility factors are currently unknown for lower attine ants (Poulsen and Boomsma, 2005), they may have modulated choice behaviors in this study on *C. costatus*; however, this potential complication does not invalidate the main methodological conclusions regarding the utility and reliability of the preference criteria developed here for the study of symbiont choice in attine ants.

To optimize the bioassay of cultivar-preference in fungus-growing ants, we therefore recommend the following precautions and methodological steps:

(1) Cultivars should be grown from at least two separate inoculations (and preferably more) to minimize confounding effects of uncontrolled inoculation-specific factors (Fig. 3).

(2) Workers from at least ten different colonies (and preferably more) should be tested to minimize the confounding effects of uncontrolled nest-specific factors (Fig. 4).

(3) To minimize observational effort, the period of about four days immediately following exposure of workers to the cultivars can probably be ignored because choices fluctuate markedly during this time and an overall preference pattern has yet to stabilize. Preference behaviors can be adequately assessed by observing the ants during a few days (e.g., Days 5 to 7) following these initial days of behavioral stabilization (Fig. 2c).

(4) Information of ant-cultivar association (counting the number of ants on a cultivar fragment) should ideally be supplemented with information from the two other preference criteria: (a) the addition of garden substrate (e.g. polenta) that the ants move onto the cultivars when converting a pure cultivar into a garden; and (b) the cultivar appearance rated on a relative garden-quality scale as the one proposed here. Incorporating information from all three preference scores in a multivariate analysis should provide the most accurate estimator of preference than any of the three measures alone.

(5) Prior to testing, ants should be separated from their native garden and maintained for five days on a neutral diet (e.g., sugar water). During this period, any ingested, fungus-derived incompatibility factors will be purged from the ants' digestive tract (Poulsen and Boomsma, 2005) and therefore will not confound preference behaviors towards the fungi during testing.

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