Garden substrate preparation behaviours in fungus-growing ants

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Abstract—Fungus-growing ants (Hymenoptera: Formicidae: tribe Attini) engage in mutually beneficial symbioses with fungi (Basidiomycota) that serve as their main food source. The leaf-cutters (genera *Acromyrmex* Mayr and *Atta* Fabricius), the most derived attine ants, employ elaborate nest-hygiene behaviours, including substrate preparation. By preparing substrate prior to its incorporation into the fungus garden, workers facilitate the physical breakdown of leaf material while reducing the abundance of potentially harmful microbes that contact their fungal mutualist. Despite its importance in ant fungiculture, substrate preparation has not been investigated in other genera of fungus-growing ants. We examined substrate-preparation procedures used by five genera of fungus-growing ants (*Apterostigma* Mayr, *Cyphomyrmex* Mayr, *Trachymyrmex* Forel, *Acromyrmex*, and *Atta*) representing most of the phylogenetic range of the Attini. Behavioural observations revealed that all five genera engage in substrate-preparation behaviours. Furthermore, these behaviours vary by genus, with *Trachymyrmex*, *Acromyrmex*, and *Atta* engaging in more elaborate preparation behaviours than the other genera. Additionally, we found that during substrate preparation, leaf-cutting ants inoculate leaf fragments with actinomycetous bacteria. These filamentous bacteria are known to produce antibiotics that suppress fungal pathogens, which suggests that inoculation with the bacterial mutualist during substrate preparation helps protect the fungus gardens of leaf-cutter ants from these parasites. Our finding that substrate-preparation behaviours occur across the phylogenetic range of attine ants suggests that these behaviours are a critical component of successful fungiculture by ants.

Résumé—Les fourmis champignonistes (Hymenoptera: Formicidae: tribu Attini) établissent une symbiose mutuellement bénéfique avec les champignons (Basidiomycota) qui constituent leur principale source de nourriture. Les coupeuses de feuilles (des genres *Acromyrmex* Mayr et *Atta* Fabricius), les fourmis les plus évoluées des Attini, utilisent des comportements hygiéniques complexes, en particulier la préparation des substrats. En préparant le substrat avant son incorporation à la culture de champignons, les ouvrières facilitent la décomposition physique du matériel foliaire, tout en réduisant l'abondance des microorganismes potentiellement dangereux qui entrent en contact avec le champignon symbiotique. Malgré l'importance de la préparation des substrats dans la fongiculture des fourmis, personne ne l'a étudiée chez les autres genres de fourmis champignonistes. Nous examinons ici les procédures de préparation des substrats chez cinq genres de fourmis champignonistes (*Apterostigma* Mayr, *Cyphomyrmex* Mayr, *Trachymyrmex* Forel, *Acromyrmex* et *Atta*), qui représentent l'essentiel de la gamme de diversité phylogénétique chez les Attini. Des observations comportementales montrent que les cinq genres possèdent tous des comportements de préparation des substrats. De plus, ces comportements varient d'un genre à l'autre et ce sont *Trachymyrmex*, *Acromyrmex* et *Atta* qui ont les comportements de préparation les plus élaborés. Aussi, durant la préparation des substrats, nous avons observé les fourmis coupeuses de feuilles inoculer les fragments de feuilles de bactéries actinomycètes. Ces bactéries filamenteuses sont connues pour leur production d'antibiotiques qui inhibent les pathogènes des champignons, ce qui laisse croire que l'inoculation de cette bactérie symbiotique durant la préparation des substrats aide à protéger les cultures de champignons des fourmis coupeuses de feuilles de leurs parasites. Notre observation des comportements de préparation des

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substrats dans tout l'éventail phylogénétique des fourmis de la tribu Attini indique que ces comportements sont une composante essentielle d'une fongiculture réussie chez les fourmis.

[Traduit par la Rédaction]

Introduction

Fungus-growing ants (Hymenoptera: Formicidae: tribe Attini) occur only in the New World, with a distribution from southern Argentina to New Jersey, United States of America (Weber 1972). Attine ants are most diverse in the Neotropics, where the leaf-cutters (genera *Acromyrmex* Mayr and *Atta* Fabricius) are dominant herbivores (Weber 1972; Hölldobler and Wilson 1990). The Attini is composed of 12 genera and more than 200 described species (Schultz and Meier 1995). Foraged substrate is brought into the nest, where it is used to grow basidiomycetous fungi — species in the family Lepiotaceae or Pterulaceae (Chapela *et al.* 1994; Mueller *et al.* 1998; Villesen *et al.* 2004) — which serve as the main food source for the ants. Attine ants grow their fungus gardens on diverse substrates. Leaf-cutting ants use fresh leaves and flowers as substrate, whereas *Apterostigma* Mayr, *Cyphomyrmex* Mayr, and *Trachymyrmex* Forel utilize primarily insect frass and dead plant material (Weber 1972). However, fresh material, such as fruit, flowers, leaves, and seeds, is also used by the latter genera (Leal and Oliveira 2000). *Acromyrmex* and *Atta* have large colonies, and workers show a high degree of polymorphism (Weber 1972; Hölldobler and Wilson 1990). *Trachymyrmex*, a phylogenetically intermediate attine genus, has smaller colonies and slight worker polymorphism. The phylogenetically basal genera, *Apterostigma* and *Cyphomyrmex*, have monomorphic workers and small colonies.

Attine ants and their fungal cultivars are only two components of a more complex symbiotic community. Microfungi in the genus *Escovopsis* Muchovej & Della Lucia (Ascomycota: Hypocreales) are specialized parasites of attine fungus gardens, consuming the cultivated fungi as their primary nutrient source (Currie *et al.* 1999*a*, 2003*b*; Reynolds and Currie 2004). A fourth participant in the symbiosis are actinomycetous bacteria, which grow on the ants' cuticle and produce antimicrobial compounds that protect the cultivar from *Escovopsis* spp. (Currie *et al.* 1999*b*, 2003*a*, 2006). The attine-associated actinomycetes also appear to have the potential to promote the cultivar's growth (Currie *et al.* 1999*b*). To deal with the parasitic *Escovopsis* spp., as well as other potentially detrimental microbes that could exploit the fungus garden, the ants engage in specific nest-hygiene behaviours (Weber 1957; Quinlan and Cherrett 1977; Fernandez-Marin *et al.* 2004; Little *et al.* 2006). Currie and Stuart (2001) showed that *Atta* workers groom the fungus garden by removing foreign microbes with their mouthparts. When a pathogen becomes fully established in a garden, workers remove infected pieces of substrate from the garden and dispose of them in the colony dump. Essential to nest hygiene are the infrabuccal pockets, oral cavities in workers' heads that are used to sequester potentially pathogenic microbial biomass collected during grooming (Eisner and Happ 1962; Quinlan and Cherrett 1978; Little *et al.* 2003, 2006). *Acromyrmex* and *Atta* deposit the resulting infrabuccal pellets in the dump; other attine genera maintain piles of pellets near or in the fungus garden (Quinlan and Cherrett 1978; Febvay and Kermarrec 1981; Currie and Stuart 2001; Little *et al.* 2003). In addition, the mutualistic actinomycetous bacterium is present within the infrabuccal pockets in the heads of fungus-growing ants, where it apparently plays a role in sterilizing pellets (Little *et al.* 2006).

The first line of fungus-garden defense involves reducing its exposure to foreign microbes (Poulsen and Currie 2006). One way the ants accomplish this is by employing specific substrate-preparation behaviours before incorporating foraged substrate into the fungus garden (Quinlan and Cherrett 1977; Andrade *et al.* 2002). *Acromyrmex* and *Atta* begin substrate preparation with an investigation of the substrate with their antennae. Workers then lick the leaf or flower material, cut it, chew it, and finally inoculate it with cultivar (Quinlan and Cherrett 1977). Licking consists of running the glossa in a stroking action across the surface of the substrate, which serves to reduce the substrate's wax layer and apparently helps to eliminate potentially harmful microbes. Through this preparation process, the ants apparently alter the substrate to facilitate fungal growth (Quinlan and Cherrett 1977). Despite the essential role of substrate-preparation behaviours, this process has only been examined in the leaf-cutting genera (Quinlan and Cherrett 1977; Andrade *et al.* 2002). We explored the substrate-preparation behaviours of three other fungus-growing ant genera:

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Apterostigma, *Cyphomyrmex*, and *Trachymyrmex*. Additionally, we investigated the possibility that during substrate preparation workers employ the mutualist actinomycetous bacteria to help promote fungus-garden hygiene. Poulsen *et al.* (2003) found that the mutualistic actinomycete could be isolated from the gardens of *Acromyrmex* spp., which suggests that the bacterium might be inoculated into the garden as part of substrate preparation. Considering the beneficial role that the actinomycetous bacteria would play in nest hygiene, we examined whether ants inoculate substrate with bacteria to provide antibiotic protection to their cultivar.

Methods

Study organisms

To examine garden-substrate preparation behaviours in fungus-growing ants, we studied queen-right colonies from five genera, *Apterostigma*, *Cyphomyrmex*, *Trachymyrmex*, *Acromyrmex*, and *Atta*. The species observed were *Apt. dentigerum* Wheeler, *Apt. cf. pilosum*, *C. longiscapus* Weber, *C. costatus* Mann, *T. zeteki* Weber, *T. bugnioni* (Forel), *T. cornetzi* (Forel), *Ac. octospinosus* (Reich), *Ac. laticeps* (Emery), *Ac. hispidus* Santschi, *Ac. echinatior* Forel, *At. sexdens* (L.), *At. colombica* Guérin-Méneville, and *At. cephalotes* (L.). The study genera represent most of the phylogenetic range observed in attine ants (see Schultz and Meier 1995). Colonies were collected in Panama from 2001 to 2003 and transported to the laboratory at the University of Kansas. *Apterostigma*, *Cyphomyrmex*, and *Trachymyrmex* colonies were housed in dual-chamber nest boxes (each 7.5 cm × 7.5 cm × 3 cm) connected by a 1 cm diameter plastic tube. Each colony's fungus garden was kept on a plaster of Paris base in one of the nest boxes. *Atta* and *Acromyrmex* colonies were kept in larger nest boxes (at least 16 cm × 13 cm × 6 cm), with the fungus garden housed in a smaller chamber within the nest box. All ant colonies were kept on islands in trays of soapy water, preventing the spread of mites and ants between nests.

Apterostigma, *Cyphomyrmex*, and *Trachymyrmex* colonies were provided with a variety of dry substrate for their fungus gardens, including oak catkins, polenta, ground orange peels, tea leaves, and oats. Substrate was placed in the box adjoining the garden chamber. To encourage foraging by *Apterostigma* and *Cyphomyrmex*, substrate was also placed within the garden chamber. Moisture was provided by watering the plaster biweekly through a tube in the box lid. Fresh leaves of *Euonymus fortunei* (Turcz.) Hand.-Maz. (Celastraceae) collected from two locations on the University of Kansas campus were presented every other day to *Atta* and *Acromyrmex* via the outer chamber of the nest box. To maintain higher humidity in the leaf-cutting ant nests, water-saturated cotton balls were placed in either the inner or outer nest-box chamber. Voucher specimens of the attine species used in the study have been deposited in the National Museum of Natural History, Washington, District of Columbia.

Behavioural observations

A total of over 60 observational hours of substrate-preparation behaviours of attine ants were conducted on 27 colonies, including 5 colonies each of *Apterostigma*, *Acromyrmex*, and *Atta* and 4 and 7 colonies of *Cyphomyrmex* and *Trachymyrmex*, respectively. Addition of new substrate into the fungus garden in colonies of *Apterostigma*, *Cyphomyrmex*, and *Trachymyrmex* is infrequent under normal laboratory conditions. Thus, to promote more active foraging, colonies of these genera were deprived of substrate for 4–7 days before observations were made. Observations were conducted using a Nikon SMZ 1500 stereomicroscope, recorded in narrative format, and used to generate a descriptive list of the behaviours involved in substrate preparation. Each genus was observed on a different day. Each colony was monitored for activity at least every hour throughout the 8 h observational sessions. Colonies were checked for 5–10 min at a time for any substrate-preparation behaviours, with the length of observations corresponding to the level of activity of the colonies. If a colony was inactive for several minutes, another colony was checked for activity. A colony that was found conducting substrate preparation was observed until the behaviours ceased or it was necessary to check the other colonies. The numbers of times behaviours integral to the substrate-preparation process were observed were tallied for *Apterostigma*, *Cyphomyrmex*, and *Trachymyrmex*. Tallies were not made for *Acromyrmex* and *Atta*; instead, observations were made to confirm existing behavioural descriptions of substrate preparation in these genera made by Quinlan and Cherrett (1977) and Andrade *et al.* (2002).

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Inoculation of substrate with actinomycetous bacteria

To examine whether leaf-cutting ants inoculate leaf material with actinomycetous bacteria during substrate preparation, we conducted microbiological isolations of pre- and post-treated substrate in 8 *Atta* and 15 *Acromyrmex* colonies. As above, fresh leaves of *E. fortunei* were used as substrate for this experiment. Fresh leaves were collected from two locations on the University of Kansas campus. We sampled leaf material for the presence of bacteria prior to any treatment (*i.e.*, fresh substrate used as a control) and at three different stages during substrate preparation, as follows: (1) leaf material cut and licked by ants, with no substrate-garden contact, (2) leaves chewed, with substrate-garden contact, and (3) substrate incorporated into the fungus garden and freshly inoculated with fungal mutualist.

Sampling was conducted aseptically using flame-sterilized forceps. To control for the cutting process, 4–6 mm diameter fragments were cut from leaves in the pretreated substrate isolations. Substrate accidentally coming into direct contact with an ant during sampling was discarded to eliminate contamination by actinomycetous bacteria from its cuticle.

In total, 50 samples were made from each of the four treatment stages. Two techniques were used for isolating any actinomycetous bacteria present. Collected material was placed in 1 mL of sterile water and agitated for approximately 10 s using a vortex, then the water was plated on chitin agar following the protocol for isolating actinomycetous bacteria described by Cafaro and Currie (2005). Other samples were particle plated on chitin agar, with five leaf fragments per plate. Plates were monitored biweekly and any colony-forming units (CFUs) of actinomycetous bacteria obtained were subcultured on malt yeast extract agar plates. We used a χ^2 test to look for differences in prevalence of actinomycetous bacteria between substrate-preparation stages.

Results

Behavioural observations

Behavioural observations revealed that all species of fungus-growing ants examined, spanning five genera, engage in substrate preparation and that these behaviours vary depending on ant genus (Tables 1, 2). In all genera, substrate was often treated by multiple ants either simultaneously or in succession, and was frequently

Table 1. Total number of times preparation behaviours were observed and recorded during all observational periods and for all substrate types.

Ant genus and individual colonies	Carried to garden without licking	Licking within garden	Cutting	Chewing	Fecal drop applied	Inoculation with fungus
Apterostigma	70	1	0	0	0	3
A. pilosum (n = 2)	59	1	0	0	0	0
A. dentigerum (n = 3)	11	0	0	0	0	3
Cyphomyrmex	21	16	0	0	0	4
C. longiscapus (n = 2)	16	12	0	0	0	2
C. costatus (n = 2)	5	4	0	0	0	2
Trachymyrmex	89	51	11	15	7	5
T. zeteki (n = 5)	89	50	7	15	7	5
T. bugnioni (n = 1)	0	0	4	0	0	0
T. cornetzi (n = 1)	0	1	0	0	0	0

Note: The data collected for each individual colony are also expressed as a grand total per genus. Cases where one piece of substrate was simultaneously treated by two ants were counted as one event.

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Table 2. A comparison of garden substrate preparation behaviours in five genera of attine ants.

Behaviour	*Apterostigma*	*Cyphomyrmex*	*Trachymyrmex*	*Acromyrmex*	*Atta*
Licking before contact with garden?	No	No	No	Yes	Yes
Licking within garden?	Yes	Yes	Yes	Yes	Yes
Cutting?	No	No	Yes	Yes	Yes
Chewing?	No	No	Yes	Yes	Yes
Inoculation with fungus?	Yes	Yes	Yes	Yes	Yes

Note: The species observed were *Apt. dentigerum*, *Apt. cf. pilosum*, *C. longiscapus*, *C. costatus*, *T. zeteki*, *T. bugnioni*, *T. cornetzi*, *Ac. octospinosus*, *Ac. laticeps*, *Ac. hispidus*, *Ac. echinatior*, *At. sexdens*, *At. colombica*, and *At. cephalotes*.

relocated during the preparation process, even after being set on the garden or inoculated with fungus. We did not detect any differences in the types of substrate-preparation behaviours engaged in by *Apterostigma* and *Cyphomyrmex* workers. Workers in these genera initially investigated substrate by moving their antennae back and forth across the surface. They then picked up pieces in their mandibles and either carried them directly onto the fungus garden or set them on the plaster of Paris near the garden. Although the glossa was often extended by an individual ant during the investigation phase, substrate was not licked and the glossa was retracted once the substrate was picked up. Thus, pieces of substrate receive no form of preparation by *Apterostigma* or *Cyphomyrmex* before being relocated to the fungus garden. Once substrate was moved to the garden, the first stage of preparation involved workers licking it. Although licking of substrate occurred in both genera, it was significantly more common in *Cyphomyrmex* (observed 16 times in 25 h) than in *Apterostigma* (observed 1 time in 25 h). No further preparation was carried out before the substrate was inoculated with cultivar. Ant workers inoculated new substrate with fungal cultivar by ripping off hyphae from within an established garden, carrying it in their mandibles, and either pushing and rocking the cultivar onto the new substrate or patting it repeatedly with both forelegs. This behaviour was observed 3 and 4 times in *Apterostigma* and *Cyphomyrmex* colonies, respectively. The same preparation behaviours were observed for oak catkin, polenta, and oat substrates. One observation of an *Apterostigma* worker cutting substrate was made. The worker cut an oat crumb by pulling it with its mandibles while pushing it with its forelegs. This behaviour may be more common than observed here, as most pieces of substrate material were small enough that cutting was not necessary.

Trachymyrmex workers employed a similar substrate-preparation procedure until just prior to the incorporation of substrate into the fungus garden, when they cut, chewed (by opening and closing their mandibles in a clamping fashion), and moulded substrate into balls. Cutting was observed 11 times during at least 20 h of observation, while chewing was observed 15 times. Chewing and moulding behaviours often occurred simultaneously. The workers then placed fecal droplets on the surface of the balls. This was observed 7 times during these 20 h of observation. Fecal-droplet deposition was achieved by pausing from chewing and then moving the gaster cranioventrally towards the substrate. *Trachymyrmex* workers cut large pieces of substrate by holding individual pieces against a stationary mandible and sliding the other mandible repeatedly and continuously in a sawing motion across the substrate surface.

Time elapsed during and between stages in the substrate-preparation process varied. In general, *Apterostigma*, *Cyphomyrmex*, and *Trachymyrmex* workers began foraging within a half hour of substrate presentation, and typically foraged for at least 4 h. At that point, most of the substrate had been moved into the nest chamber and was at various stages of treatment, with some pieces having already been inoculated. Twenty-four hours after initial presentation, with few exceptions substrate had been incorporated into the fungus garden.

Atta and *Acromyrmex* exhibited a third form of substrate preparation. Workers investigated leaves with their antennae, then licked and cut them into smaller pieces before the leaves ever came into contact with fungus gardens or garden chambers. After a varying length of time (2–120 min), the licked and cut leaf fragments were carried to the garden, where they sat for a second variable time interval (2–180 min). Pieces were then licked again, cut into smaller pieces, chewed, and sometimes wadded into a

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Table 3. Isolations of mutualistic actinomycetous bacteria from leaf material that had undergone different degrees of substrate preparation by workers of leaf-cutting ant species.

State of preparation[a]	Total no. of plates	No. with actinomycetous bacterium present
Fresh initial substrate	50	0
Licked	50	0
Atta cephalotes	9	0
At. colombica	8	0
At. sexdens	7	0
Acromyrmex echinatior	11	0
Ac. hispidus	4	0
Ac. laticeps	1	0
Acromyrmex sp.	10	0
Chewed	50	5
At. colombica	5	0
At. sexdens	7	0
Ac. echinatior	11	0
Ac. octospinosus	10	2
Acromyrmex sp.	17	3
Inoculated with fungus	50	13
At. cephalotes	14	4
At. colombica	9	2
At. sexdens	8	1
Ac. hispidus	1	
Ac. laticeps	7	2
Ac. octospinosus	4	0
Acromyrmex sp.	7	4

[a]The stages sampled included (*i*) fresh substrate prior to any treatment by ants, (*ii*) leaf material cut and licked by ants, without any substrate-garden contact, (*iii*) leaves chewed, with substrate garden contact, and (*iv*) substrate incorporated into the fungus garden and freshly inoculated with fungal mutualist.

ball. After this preparation, leaf fragments were inoculated with fungal cultivar.

Inoculation of substrate with actinomycetous bacteria

Isolations of actinomycetous bacteria from leaf material sampled from *Acromyrmex* and *Atta* gardens revealed a significant increase in the prevalence of actinomycetes as the substrate-preparation process progressed ($\chi^2_3 = 27.6$, $P < 0.001$; Table 3). No actinomycetous bacteria were present on fresh leaf pieces or those that were only licked in the outer chamber; bacteria were isolated only from pieces of prepared leaf material that were in direct contact with the gardens. More specifically, 5 and 13 CFUs of the mutualistic bacterium were obtained in the chewed and inoculated stages of substrate preparation, respectively.

Discussion

Preparation of the substrate used to cultivate the fungal mutualist is apparently an important component of the success of fungiculture by attine ants. Previously, garden substrate preparation studies have focused on the two most phylogenetically derived attine genera, *Acromyrmex* and *Atta* (Quinlan and Cherrett 1977; Andrade *et al.* 2002). Similar to what was recorded for those genera, in this study we found that workers in the more phylogenetically basal genus *Apterostigma* and the phylogenetically "intermediate" genera *Cyphomyrmex* and *Trachymyrmex* also engage in specific behaviours to prepare substrate before incorporating it into the fungus garden. This indicates that these behaviours span the phylogenetic range of fungus-growing ants. Additionally, the finding of garden substrate preparation behaviours in species of *Apterostigma* that cultivate fungi of the family Pterulaceae, rather than Lepiotaceae like all other attine ants (Chapela *et al.* 1994; Villesen *et al.* 2004), indicates that the occurrence of the behaviour is independent of fungal-cultivar lineage. Our results indicate that substrate preparation is essential for successful fungiculture, and is likely a key component of ant–fungus agricultural mutualism.

Although preparation of garden substrate occurred in all five ant genera studied, there were differences between genera. First, the specific procedures that are employed are different. *Apterostigma* and *Cyphomyrmex* workers employed the least elaborate preparation process, the main observed behaviour being licking. In *Trachymyrmex*, *Acromyrmex* and *Atta*, procedures became more complex, and included behaviours such as cutting and chewing. Placing fecal droplets on substrate was observed only in *Trachymyrmex*, but was previously observed in *Apterostigma*, *Cyphomyrmex*, *Acromyrmex*, and *Atta* (Martin 1970; Rønhede *et al.* 2004; Poulsen and Boomsma 2005). A second major distinction was the licking behaviour. *Apterostigma*, *Cyphomyrmex*, and *Trachymyrmex* began licking their substrate only after it was carried into the garden chamber. This was not the case for *Atta* and *Acromyrmex*, which regularly prepared their substrate prior to garden contact. Licking was

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observed to be much less frequent in *Apterostigma*. Third, *Trachymyrmex* colonies were generally faster at substrate integration than *Apterostigma* or *Cyphomyrmex* colonies, but slower than *Atta* and *Acromyrmex* colonies. This can be attributed to the greater colony size (number of workers) of *Trachymyrmex* as well as the general trend within the fungus-growing ants for activity levels to be higher in the more phylogenetically derived genera (Weber 1972; Hölldobler and Wilson 1990). *Apterostigma* and *Cyphomyrmex* colonies were the least active, requiring starvation and provision of oak catkins directly into the nest chamber to encourage faster, and more evident, foraging and substrate preparation. Although it was not examined in this study, a final distinction is that substrate-preparation tasks are partitioned in the leaf-cutters, *i.e*, different worker morphs are involved in different tasks (Quinlan and Cherrett 1977; Andrade *et al.* 2002). All species of *Apterostigma*, *Cyphomyrmex*, and *Trachymyrmex* used in this study have monomorphic workers, thus no size-based task partitioning is possible, although it is possible that an age-based division of tasks does occur.

Substrate-preparation behaviours are not dependent upon substrate type: all substrate types (oak catkins, polenta, ground orange peels, tea leaves, oats, and fresh leaf material) underwent some form of preparation by at least some colonies, although not all individual substrate samples were treated in the same fashion. In addition, some were not treated at all, especially by *Apterostigma* colonies. Each attine ant genus employed a general procedure, but there were discrepancies between individual foraging and substrate-preparation events. The difference between the ways in which individual pieces of a substrate were treated may indicate that attine ants tailor their response to the individual conditions of specific substrate sources. Some support for this is seen in the findings of Quinlan and Cherrett (1977): they found that the extent of licking and the duration of substrate preparation in *Acromyrmex* depends upon the substrate type and the amount of cuticular wax on leaves. In addition, we have found that leaf-cutter ants avoid microbe-contaminated substrate and do not incorporate it into their gardens (D.M. Mangone and C.R. Currie, unpublished data).

Isolations of actinomycetous bacteria from leaf-cutter ant substrate revealed an increase in the presence of actinomycetes during the process of substrate preparation by *Atta* and *Acromyrmex* (Table 3). Substrate inoculated with the fungal mutualist had the highest prevalence of actinomycetous bacteria, with 13 out of 50 samples yielding at least 1 CFU. This is a relatively high rate of isolation: the bacterium involved grows slowly in culture and is difficult to isolate (Cafaro and Currie 2005). It is unlikely that the isolated bacteria had acidentally contaminated the substrate via contact with the ants; *Atta* spp. workers are not known to carry the bacterium on their exoskeleton (Currie *et al.* 2006). It is not completely clear how or at what stage in substrate preparation actinomycete inoculation occurs. Little *et al.* (2006) showed that the mutualistic actinomycete is present in the infrabuccal pocket in the heads of *Trachymyrmex cf. zeteki*. If the actinomycete is also present in the infrabuccal pocket in the heads of *Atta* spp. workers, it would be relatively easy for the substrate to be inoculated with it during licking and chewing. Alternatively, it is possible that the cultivar is the immediate source of the mutualist actinomycetous bacterium, and the increase in actinomycetes between the chewing and inoculating stages may be a function of the length of time the substrate had been in contact with the cultivar. Although the exact source of the mutualist bacterium on the leaf substrate is still unclear, the bacterium was absent from initial substrate that had not had any contact with ants or cultivar, and became significantly more prevalent as preparation progressed. In addition, our finding, in combination with those of Poulsen *et al.* (2003), suggests that in addition to occurring on the cuticle of the ants (Currie *et al.* 2006), the mutualistic actinomycetous bacterium is also present within the ants' fungus garden.

Our study demonstrates that substrate preparation occurs throughout most of the phylogenetic range of fungus-growing ants, and is present regardless of the type of substrate utilized. This indicates that preparation is a key component of fungus growing by attine ants, and was likely present in the earliest stages of attine fungiculture. Although this illustrates the importance of substrate preparation for the cultivation of fungus by attine ants, the functional significance is still not completely clear. As suggested by Quinlan and Cherrett (1977), there appear to be two primary functions: (1) to promote the ability of the fungus to break down the garden substrate and (2) to remove microbes associated with the substrate used to culture the fungal mutualist. Our finding that leaf-cutting ants inoculate the substrate with mutualistic actinomycetous bacteria further indicates the

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potential role this behaviour plays in nest hygiene. Future work focusing on the importance of substrate preparation in nest hygiene will be valuable.

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