

Recognition of Host Plant Volatiles by *Pheidole minutula* Mayr (Myrmicinae), an Amazonian Ant-Plant Specialist

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ABSTRACT

In the tropics, several ant species are obligate inhabitants of leaf pouches and other specialized structures in plants known as myrmecophytes. However, the cues used by ant queens to locate suitable host-plants following dispersal remain poorly understood. Here we tested the hypothesis that *Pheidole minutula* queens use volatiles to distinguish their host *Maieta guianensis* (Melastomataceae) from other sympatric myrmecophytes. To do so, we used a Y-tube olfactometer to quantify the preference for volatiles of different plant species. Our results indicate that *P. minutula* queens discriminate the chemical volatiles produced by its host-plant from those of other sympatric ant-plant species. However, queens failed to distinguish the volatiles of *Maieta* from those of the ant-plant *Tococa bullifera* (Melastomataceae), with which *P. minutula* is not mutualistically associated. Nevertheless, a strong preference for *Maieta* over *Tococa* was observed during a subsequent bioassay, where the ants had physical contact with a domatium of each plant species. These results suggest that additional, short distance mechanisms are also necessary for host discrimination. Overall, our findings suggest that the high degree of compartmentalization observed in symbiotic ant–plant relationships is achieved, at least in part, by the relatively high degree of specificity in host selection displayed by foundress queens.

Abstract in Portuguese is available at <http://www.blackwell-synergy.com/loi/btp>.

Key words: ant–plant interactions; chemical cues; Formicidae; host-plant location; *Maieta guianensis*; mutualisms; myrmecophytes.

ANT–PLANT MUTUALISMS, in which ants defend plants from herbivores in exchange for rewards such as food or shelter, are typical of tropical forests and have become model systems with which to investigate the evolutionary ecology of mutualisms. In nonsymbiotic associations between ants and plants, as those mediated by plants with extrafloral nectaries, many species can interact with each other (Bluthgen *et al.* 2007, Guimarães *et al.* 2007). In contrast, ants of symbiotic systems are obligately associated with their specific host plants – they establish colonies only in swollen thorns, leaf pouches, hollow stems, or other specialized structures collectively referred as domatia. Thus, symbiotic ant–plant associations are much more compartmentalized and the presence of a partner can determine the presence of the other partner (Bluthgen *et al.* 2007, Guimarães *et al.* 2007). Since the pioneering work of Janzen (1966, 1967), considerable attention has focused on understanding the dynamics of these interactions and their consequences for host plants (Beattie 1985, Bronstein 1998, Heil & McKey 2003). However, our understanding of the means by which these horizontally transmitted mutualisms are reassembled each generation, and in parti-

cular the cues by which ants locate suitable host-plants following dispersal, remain poorly understood (but see Edwards *et al.* 2006, Grangier *et al.* 2009).

Volatile chemicals emitted by plants are used as cues by insects ranging from herbivores to parasitoids (Strong *et al.* 1984, Bernays & Chapman 1994, Combes 2005, Heil 2008). It has been hypothesized that these volatiles are also used by mutualistic ants to identify potential host plants (Longino 1989, Yu & Davidson 1997, Christianini & Machado 2004). In the first test of this hypothesis, Edwards *et al.* (2006) found that *Allomerus* and *Azteca* queens were significantly more attracted to the volatiles of their host-plant species (*Cordia nodosa* Lam.) than to understory plant species that do not form associations with ants. Similarly, experimental trials in which foundress queens were exposed to seedlings of myrmecophytic and nonmyrmecophytic *Macaranga* suggest volatile cues are important for host-plant recognition by dispersing queens (Jürgens *et al.* 2006).

The results of Edwards *et al.* (2006) and Jürgens *et al.* (2006) provide strong support for the hypothesis that ant queens use volatile cues to discriminate their host-plants from nonmyrmecophytic species. However, an ant's host-plant species is rarely the only myrmecophyte to which a dispersing queen is exposed

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(Murase *et al.* 2002, Grangier *et al.* 2009). For instance, up to 16 ant-plants can co-occur in Central Amazonian forest stands (Fonseca & Ganade 1996, Bruna *et al.* 2005), of which only a limited subset are suitable host-plants for each plant-ant species (Fonseca & Ganade 1996). Queens must therefore have mechanisms—visual, tactile or olfactory—to distinguish their particular host plant species from among the suite of sympatric myrmecophytes to which they are exposed (Inui *et al.* 2001, Murase *et al.* 2002, Edwards *et al.* 2006, Jürgens *et al.* 2006). While previous work has documented the ability of ant workers to distinguish among the volatiles of different myrmecophytes (Bruna *et al.* 2008), to date little work has explored the mechanism that queens use to distinguish among sympatric ant-plants (Jürgens *et al.* 2006).

Maieta guianensis Aubl. (Melastomataceae) is an Amazonian understory myrmecophyte that grows to 1 m in height. Leaves of *M. guianensis* have two pouches at their base in which ant queens establish colonies (Michelangeli 2000, Vasconcelos & Davidson 2000). Although *M. guianensis* can be colonized by multiple ant species, over 90 percent of the plants are associated with only a single ant species: *Pheidole minutula* Mayr. These ants forage for insects on the host plant's leaves and tend scale insects for honeydew (Vasconcelos & Davidson 2000); plants without ants are quickly attacked by herbivores and have dramatically reduced fruit production (Vasconcelos 1991). The density of *M. guianensis* in our field sites ranges from *ca* 25 plants/ha in plateaus to > 300 plants/ha in streamside valleys, but vacant seedlings in which queens can establish colonies are rare (T. J. Izzo, unpubl. data). Furthermore, *M. guianensis* occurs in sympatry with several other ant-plant species (Fonseca & Ganade 1996, Bruna *et al.* 2005). It is therefore an excellent system to investigate the potential for queens to use volatile cues to both distinguish their host-plant from nonmyrmecophytes and discriminate their host-plant from other sympatric myrmecophytes.

Here we present the results of bioassays used to test the following hypotheses: (1) *P. minutula* queens use volatiles to distinguish *M. guianensis* from the con-familial nonmyrmecophytic species *Miconia nervosa* Triana (Melastomataceae) and (2) *P. minutula* queens use volatiles to distinguish *M. guianensis* from three sympatric myrmecophytes with which they do not form mutualistic associations: *Tococa bullifera* Mart. ex Schrank (Melastomataceae), *Cordia nodosa* Lam. (Boraginaceae), and *Hirtella myrmecophila* Pilg. (Chrysobalanaceae).

METHODS

The study was conducted at Reserve No. 1501 of the Biological Dynamics of Forest Fragments Project, an 800-ha terra firme preserve located approximately 70 km north of Manaus, Brazil (2°25' S, 59°48' W). While tree diversity in the site is extremely high (> 1600 species), the understory is relatively species-poor and dominated by stemless palms. For a description of the field site and its ant-plant communities (see Bierregaard *et al.* 2002, Bruna *et al.* 2005, Fonseca & Ganade 1996).

The *P. minutula* queens used in our bioassays were collected by opening the domatia of wild *M. guianensis* seedlings. We collected only those mated queens that had not yet produced

workers; prior to being used in assays the queens were kept for 48–72 h in petri dishes and supplied with water *ad libitum*. *Maieta guianensis* and *T. bullifera* seedlings used in the experiments were grown from seed in a shadehouse located at the field site and remained uncolonized, *M. nervosa*, *H. myrmecophila* and *C. nodosa* seedlings were collected in the field; we opened the domatia of all *Hirtella* and *Cordia* and removed any ants or brood present. Hereafter all species will be referred to by their generic names only.

All bioassays were conducted using a glass Y-olfactometer using a modified version of the protocols described by Edwards *et al.* (2006). The arms of the olfactometer were 13 cm long (2.6 cm internal diameter) and were offset by 130°, while the shaft had the same internal diameter but was 16 cm long. The entrances to the arms were covered with mesh and connected to plastic tubes where the leaves that were the source of volatiles were located. An air current of 800 mL/min was produced by an air pump, regulated by a flowmeter (Vigoar[®] 110 V, Vigo-Flex Ltda., São Paulo, Brazil), with 400 mL passing through each of the arms. All olfactometer-tube connections were sealed with Magipack[®] plastic PVC film (Inproco Ltda., Campinas, Brazil), and the Y-tube olfactometer and tubes were covered with red acetate to prevent light contamination.

Bioassays consisted of a queen being simultaneously exposed to volatiles of *Maieta* in one arm and an alternative plant species in the other. In all cases, the source of the volatiles was three fully expanded leaves free of herbivory that had been washed under running water with a brush for 10 min immediately before the start of the trial. In the case of *Cordia* and *Hirtella* leaves, this procedure was used to reduce the potential influence of a scent other than that of leaves on queen's choice. We did the same with *Miconia*, *Maieta* and *Tococa*, which never harbored colonies, to control for any potential effects of washing leaves on queen choice. After running the air pump for 5 min, the trial started by placing the leaves in the tubes and a queen in the shaft of the olfactometer. We registered a choice for a particular species when a queen had passed more than halfway down that arm of the olfactometer and spent > 2 min in the arm. In all experiments, we considered only the first choice for each queen. If after 20 min the queen had failed to make a choice, the trial was recorded as 'no choice' and subsequently excluded from the statistical analysis (following Edwards *et al.* 2006). After each trial, the three leaves of each species used in the trial were replaced to avoid pseudoreplication. We used *G*-tests to compare the frequency with which *P. minutula* chose *Maieta* over the plant species with which it was being compared (Zar 1996).

We conducted five comparisons: (1) *Maieta* in one arm of the olfactometer versus no plant in the other ($N = 50$ trials), (2) *Maieta* versus *Miconia* ($N = 30$ trials), (3) *Maieta* versus *Tococa* ($N = 50$ trials), (4) *Maieta* versus *Hirtella* ($N = 30$ trials), and (5) *Maieta* versus *Cordia* ($N = 30$ trials). All queens and leaves were used in only one trial, and for each trial we alternate the arm in which the *Maieta* leaves were located. We also cleaned the inner surface of the olfactometer and the plastic tubes following each trial using 90 percent ethanol and replaced the PVC film.

Because we found no preference for *Maieta* over the sympatric myrmecophyte *Tococa* (see "Results" section), we conducted an additional bioassay in which we assessed the ability of *P. minutula*

queens to use short-distance visual, tactile, or volatile cues to identify their host plants. To do so, we used 44 *P. minutula* queens collected and maintained in the same conditions as for other trials. Each queen was placed in the center of a 10-cm-diam. petri dish containing a domatium (never inhabited) of each plant species on opposite sides of the dish. After 5 h, the domatia were opened to determine which one had been colonized by the queen. We then used *G* tests to compare the frequency with which each plant species was selected by queens.

RESULTS

Pheidole minutula queens strongly preferred their host-plant species over the alternative plant species in all bioassays except those conducted with *Tococa* (Table 1). When given the choice between *Maieta* odor and an odor-free treatment, *Maieta* was selected by the ants in 80 percent of the trials (Table 1). Similarly, *Maieta* was chosen by *P. minutula* queens over *Miconia* in 78 percent of the trials, and over the myrmecophytic *Cordia* and *Hirtella* in 78 and 77 percent of trials, respectively (Table 1). When faced with a choice between *Maieta* and *Tococa*, *P. minutula* queens did not show a significant preference, although here again, the arm emanating the volatiles of *Maieta* was chosen in most (63%) trials. However, when queens were allowed to choose between the domatia of *Tococa* or *Maieta*, then a highly significant difference was detected. *Maieta* was chosen in 36 of the 44 trials ($G = 15.2$, $P < 0.001$; Fig. 1). *Tococa* was selected in only four trials, whereas in the remaining four trials, no choice was made (*i.e.*, the queen did not enter any of the domatia).

DISCUSSION

Our olfactometer bioassays indicate that *P. minutula* queens clearly discriminate the chemical volatile cues produced by its host-plant

TABLE 1. Results of bioassays comparing the preference of *Pheidole minutula* queens for volatiles of different sympatric plant species. Using a Y-olfactometer, queens were simultaneously presented with either *P. minutula*'s host plant (*Maieta guianensis*) or one of the following four species: the nonmyrmecophytic *Miconia nervosa* or the myrmecophytes *Cordia nodosa*, *Hirtella myrmecophila*, and *Tococa bullifera*. Although we present the number of times no choice was made by queens, statistical analyses were conducted using only those trials in which a choice for one of the volatiles was made (*sensu* Edwards *et al.* 2006).

Bioassay	<i>Maieta</i> chosen	Alternative chosen	No choice	<i>G</i>	<i>P</i>
<i>Maieta</i> vs. empty chamber	36	9	5	9.57	0.002
<i>Maieta</i> vs. <i>Miconia</i>	21	6	3	4.83	0.028
<i>Maieta</i> vs. <i>Cordia</i>	21	6	3	4.83	0.028
<i>Maieta</i> vs. <i>Hirtella</i>	20	6	4	4.41	0.036
<i>Maieta</i> vs. <i>Tococa</i>	26	15	9	1.65	0.198

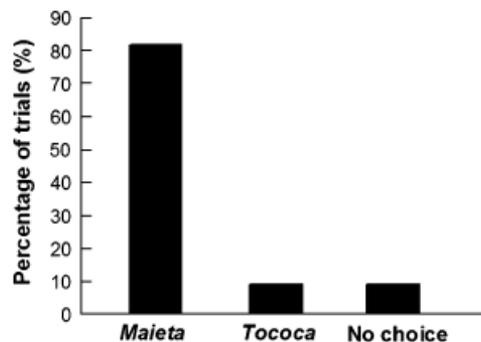


FIGURE 1. Percentage of trials in which *Pheidole minutula* queens choose to colonize the domatium of their host plant *Maieta guianensis* or the domatium of the sympatric myrmecophyte *Tococa bullifera*. The trials were conducted by simultaneously presenting the domatia of both species. Cases where queens remained immobile throughout the 5-h trial, and therefore failed to colonize either domatium, were recorded as 'no choice' ($N = 44$ trials).

M. guianensis from those produced by several other plants found in our study site, including both myrmecophytic and nonmyrmecophytic species. These results support the hypothesis that volatile chemicals are used as cues by obligate plant-ants to find their hosts (Longino 1989, Yu & Davidson 1997, Edwards *et al.* 2006). The existence of a chemical mechanism that helps obligate ants to find their host plants indicates that mated queens do not fly at random, but rather perform a directional dispersal to their specific hosts. This is an obvious advantage to the ant queens, as the time and energy to find the host is minimized. The use of chemical cues for host localization have been reported in wide range of insects, including especially herbivores (Strong *et al.* 1984, Bernays & Chapman 1994, Heil 2008) and parasitoids (Combes 2005, Heil 2008), and such cues are likely to be one of the determinants for the maintenance of species-specific relationships (Grangier *et al.* 2009).

When *P. minutula* queens were challenged with volatiles of *Tococa*, they chose their normal host-plant, *Maieta*, comparatively less often than when the nonmyrmecophytic *Miconia* was the alternative. Similarly, *P. minutula* workers were attracted to volatiles produced by experimentally damaged leaves of *M. guianensis* and *T. bullifera*, but not those of the nonmyrmecophytic Melastomataceae *Miconia albicans* and *Clidemia japurensis* (Bruna *et al.* 2008). Collectively, these results suggest that there is a greater similarity in the volatiles produced by related myrmecophytic species than those produced by myrmecophytes and closely related nonmyrmecophytes. Previous work has found no difference in the profile of cyanogenic glycosides found in myrmecophyte and nonmyrmecophyte Melastomataceae (Michelangeli & Rodrigues 2005), and there are no obvious differences between the chemical composition of *Macaranga* that do and do not have mutualistic interactions with ants (Jürgens *et al.* 2006). Nevertheless, we suggest that more comprehensive chemical analyses evaluating both the type and quantity of volatile compounds, coupled with a robust phylogeny of the Melastomataceae (Michelangeli *et al.* 2004, Stone 2006), are needed to test the hypotheses that myrmecophytic species have similar volatile profiles.

In our collective 20 yr of research with this system, including the inspection of > 1000 plants, only one colony of *P. minutula* has been found in *T. bullifera* (H. L. Vasconcelos, E. M. Bruna, and T. J. Izzo, pers. obs.). Given the inability of *P. minutula* queens to distinguish among its host *Maieta* and the nonhost *Tococa* in olfactometer trials, what mechanisms help promote the specificity of this association? Our experiment in which queens were allowed to choose among domatia of the two species suggests that short-distance cues also play an important role in host-plant identification. The use of contact, nonvolatile chemicals has been demonstrated in some ant-plant systems. For instance, in *Crematogaster-Macaranga* associations, the recognition of specific hosts by queens requires contact with the seedling surface, where characteristic nonvolatile chemicals unique to each *Macaranga* species are produced (Inui *et al.* 2001). We therefore propose that the identification of putative host-plants by *P. minutula* is initially based on volatile cues, while the final selection relies on nonvolatile, surface chemicals or other tactile/visual cues.

In a recent analysis of ant-plant mutualist networks, Guimarães *et al.* (2007) suggested that filtering mechanisms eliminate opportunist ants and are responsible for the high levels of compartmentalization observed in ant-plant communities (Fonseca & Ganade 1996). A diversity of plant structures that act as exclusion filters in ant-plant symbioses have been reported (Davidson *et al.* 1989, Federle *et al.* 1997, Yu & Davidson 1997, Quek *et al.* 2004). In African *Leonardoxa* (Brouat *et al.* 2001) and some Asian *Macaranga* (Quek *et al.* 2004), variations in size and shape of the entrance of domatia act as a filter that limits the access of some ant species. Nevertheless, such physical structures are not ubiquitous and, in at least one case (Grangier *et al.* 2009) do not prevent plant colonization by generalist ants. Furthermore, no such mechanism exists in *T. bullifera*—the entrances to domatia are large enough for *P. minutula* queens, and they are located on a similar position at the base of the leaf as those of *M. guianensis*. In fact, four *P. minutula* queens entered the *Tococa* domatia in our experimental trials. However, even if queens did colonize plants, previous experimental work has shown *P. minutula* colonies have very poor rates of growth and survivorship when queens are experimentally forced to establish colonies in *T. bullifera* (Nery & Vasconcelos 2003).

Our results support the hypothesis that chemical volatiles—and the recognition of these volatiles by ant queens—is an important filter that serves to promote compartmentalization in ant-plant communities (Jürgens *et al.* 2006, Grangier *et al.* 2009). Studies testing for specificity in host-plant localization and selection in other symbiotic ant-plant systems are necessary to test the generality of our results and evaluate this hypothesis, as are studies describing the composition and functional importance of plant chemical profiles (Jürgens *et al.* 2006).

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