

## CHEMICAL RECOGNITION OF PARTNER PLANT SPECIES BY FOUNDRESS ANT QUEENS IN *Macaranga*–*Crematogaster* MYRMECOPHYTISM

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**Abstract**—The partnership in the *Crematogaster*–*Macaranga* ant–plant interaction is highly species-specific. Because a mutualistic relationship on a *Macaranga* plant starts with colonization by a foundress queen of a partner *Crematogaster* species, we hypothesized that the foundress queens select their partner plant species by chemical recognition. We tested this hypothesis with four sympatric *Macaranga* species and their *Crematogaster* plant-ant species. We demonstrated that foundress *Crematogaster* queens can recognize their partner *Macaranga* species by contact with the surface of the seedlings, that they can recognize compounds from the stem surface of seedlings of their partner plant species, and that the gas chromatographic profiles are characteristic of the plant species. These findings support the hypothesis that foundress queens of the *Crematogaster* plant-ant species select their partner *Macaranga* species by recognizing nonvolatile chemical characteristics of the stem surfaces of seedlings.

**Key Words**—partner-plant selection, partner-plant recognition, ant–plant interactions, mutualism, chemical recognition, myrmecophyte, ant dispersal, insect–plant coevolution.

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## INTRODUCTION

Myrmecophytism, a type of ant–plant interaction, is one of the most conspicuous examples of the coevolution of mutualism between an animal and a plant (Huxley and Cutler, 1991; Davidson and McKey, 1993). In the mutualistic interactions, the ants (plant-ants) protect their host plants from herbivores and clinging vines, while the host plants (myrmecophytes) provide nest sites and, in some cases, food for the ants (Janzen, 1966; Buckley, 1982; Davidson and McKey, 1993). Myrmecophytism is often obligate: plant-ants and myrmecophytes cannot survive or have difficulty in surviving without each other. Many characteristics of both plant-ants and myrmecophytes are highly specialized and adapted for the mutualistic interaction (Davidson and McKey, 1993).

*Macaranga* (Euphorbiaceae) is a tree genus including more than 25 species of myrmecophytes, found predominantly in the tropical areas of South East Asia (Whitmore, 1969; Fiala and Maschwitz, 1991, 1992a). Most myrmecophytes have obligate mutualistic relationships with the species-specific specialist ant (plant-ant) species of the genera *Crematogaster* and *Camponotus*. The plants harbor partner plant-ants in their hollow stems and produce food bodies that the plant-ants harvest as their main food (Fiala et al., 1989; Fiala and Maschwitz, 1991, 1992b). In many obligate myrmecophytic species, the mutualistic and symbiotic interactions begin with the foundress ant queens colonizing the plant partners at a very early stage of plant growth. The queens produce ant workers who patrol the host trees and exclude herbivores (Fiala et al., 1989). Both host plants and plant-ants are dependent on each other for survival (Fiala and Maschwitz, 1990; Fiala et al., 1999).

In some cases, many myrmecophytic species have evolved within a particular genus, such as *Acacia* (Leguminosae), *Cecropia* (Cecropiaceae), and *Macaranga* (McKey and Davidson, 1993). The *Macaranga*–*Crematogaster* system, however, is the only case where the partnership between plant and ant species is highly species-specific in a wide variety of myrmecophytic species within a single genus (Fiala et al., 1999). One species of *Macaranga* has mutualistic relationships mainly with only one or two species of *Crematogaster* plant-ants, and one ant species interacts with a narrow range of *Macaranga* species, in some cases just one or two species (Fiala et al., 1999). Although several species of obligate myrmecophytic *Macaranga* species often coexist in similar habitats in a locality (Davies et al., 1998; Itioka et al., 2000), the species-specificity is well maintained (Itino et al., unpublished data). What factors affect the species-specific partnership in *Macaranga*–*Crematogaster* myrmecophytism? The first objective of this study was to test whether foundress queens of *Crematogaster* plant-ants can distinguish between *Macaranga* species.

It is well known that ants use various semiochemicals, such as pheromones and cuticular hydrocarbons, for communication in their socialized colonies and

for recognition of nestmates, trophobionts, and prey organisms (Traniello, 1980; Jessen and Maschwitz, 1986; Bonavita et al., 1987; Hölldobler and Carlin, 1987). We also know that closely related but different plant species have different nonvolatile chemicals on their surfaces. Thus, it is likely, in the *Macaranga*–*Crematogaster* system, that foundress queens may recognize their host plant species chemically. The second objective of this study was to examine whether chemical cues, characteristic of each *Macaranga* species, are used for host plant recognition by *Crematogaster* foundress queens.

To examine these two objectives, we conducted three experiments on four obligate myrmecophytic species of *Macaranga* and their specific *Crematogaster* plant-ants: (1) preference experiments to observe how foundress queens of each ant species respond to seedlings presented experimentally; (2) chemical analyses of extracted from the stem surfaces of *Macaranga* seedlings; and (3) preference experiments using esters extracted from the stem surfaces of *Macaranga* seedlings to test whether foundress queens use these as chemical cues for host plant recognition.

#### METHODS AND MATERIALS

*Study Site.* All experimental plants and ants were collected in lowland mixed dipterocarp forest in Lambir Hills National Park, Sarawak, Malaysia (4°20'N, 113°50'E, altitude 55–70 m). The details of the study site were described by Watson (1985), Inoue and Hamid (1994) and Kato et al. (1995). At least 20 species, including at least 11 myrmecophytic species, of *Macaranga* occur at the study site (Nagamasu and Momose, 1997).

*Life History.* We investigated four *Macaranga* species: *M. winkleri*, *M. trachyphylla*, *M. beccariana*, and *M. hypoleuca*, which are all abundant at the study site. Although the preferred light and soil conditions differ widely among the four species (Davies et al., 1998), it is not uncommon to find seedlings and mature trees of multiple species sharing a single microhabitat, such as a tree gap or a riverbank (Itioka, personal observation). All the experiments, except for chemical analysis, were conducted during September and October 1998 and March 1999.

The four species are all obligate myrmecophytes that harbor specific plant-ant species of the *Crematogaster* genus. The details of the basic biology of the mutualistic interactions between typical obligate myrmecophytes of *Macaranga* and their *Crematogaster* plant-ants have been described by Fiala et al. (1989) and Fiala and Maschwitz (1990, 1991, 1992a,b). The *Macaranga* plants produce pearly secreted, called food bodies, on the backs of young leaves or stipules. These food bodies, which the symbiont ants utilize as their main food resource, are rich in carbohydrates, sugars, and lipids (Rickson, 1980; Fiala and Maschwitz, 1992b; Heil et al., 1997). In return, the symbiont ants protect their host plants from

phytophagous insects and clinging vines (Fiala et al., 1989). When they reach 10–30 cm in height, the hollow stems of *Macaranga* seedlings swell and then form domatia between the nodes. At this stage, foundress ant queens settle on the seedlings, although the average heights of seedlings that ant queens begin to colonize depends on species. A foundress makes a hole in the wall of a domatium, enters the domatium, closes the hole, and starts to make her new colony. Thereafter, the mutualism begins.

Each of the four *Macaranga* species associates with a unique plant-ant species at the study site. The *Crematogaster* ant species associated with the four *Macaranga* species have not yet been described, although they have been distinguished by analysis of mtDNA (Itino et al., unpublished data). At the study site, the ant species inhabiting *M. beccariana* and *M. hypoleuca* are almost identical to *Crematogaster decamera*. Those inhabiting *M. trachyphylla* and *M. winkleri* are a species closely related to *C. borneensis* and an undescribed non-*decamera* *Crematogaster* species, respectively (Seiki Yamane, personal communication; see Itioka et al., 2000 for further details). Although the two species resembling *C. decamera* are morphologically indistinguishable, they can be clearly distinguished from the species inhabiting *M. trachyphylla* and *M. winkleri* by the morphology of queens and workers.

*Host Plant Recognition by Ant Queen (Preference Experiments).* To examine whether foundress ant queens recognize and differentiate among different congeneric plant species, we offered seedlings of each of the four *Macaranga* species to foundress ants of each *Crematogaster* plant-ant species. Seedlings that were less than 30 cm in height and not yet colonized by ants were collected at random from several tree gaps and riverbank areas within the study site. The seedlings were potted in a shade house at a distance from the forest ridge. We used cloth shades to match the light levels in the shade house to those of a typical forest gap. We allowed the seedlings to grow until they bore one to three domatia and used them for the preference experiments described below. Nulliparous ant queens were collected from domatia of intact and 10- to 20-cm-high seedlings of each *Macaranga* species in the field, and kept in a plastic bag for one day before they were used. We considered a queen with no offspring in the domatia to be nulliparous. We omitted queens that were in domatia with lignified entrance holes, which indicated that a long time had elapsed after the queens' settlement. We checked the species of the ant queens morphologically. When we found nonspecific plant-ant queens in the collection, which was rare, we omitted them. Single ant queens were placed on the youngest well-developed leaf of a potted seedling and observed to see whether they entered the stem of the offered seedling within an hour or within a day. Each individual queen was used in the experiments only once or twice; each queen was placed once on a seedling of the same species from which she was collected and once on a seedling of one of the other three species. The order of the two trials was randomized and the interval between the trials was an hour. The experimental seedlings were used only once for the experiments.

*Chemical Analyses of Surface Extracts of Macaranga Seedlings.* To investigate the differences in chemical characteristics that might be used as cues for host recognition by the foundress ant queens, we obtained extracts from the stem surfaces of seedlings of each species. Seedlings were collected and cultivated in the shade house as above, then the hollow stems were cut out. The stem part that covered two or three consecutive nodes (domatia) was sampled from each seedling. Each stem sample was soaked in ethyl acetate for 30 min. The extracts from each stem sample were analyzed separately by gas chromatography (GC) and used as samples in the bioassays (see below). We sampled stem pieces from at least seven seedlings of each species.

Each ethyl acetate extract was evaporated completely and then dissolved in 40  $\mu$ l hexane. We added 100 ng docosane as an internal standard and then analyzed 2  $\mu$ l of each sample by GC in a GC-14A gas chromatograph (Shimadzu Inc.) equipped with a flame ionization detector and a DB-1 glass capillary column (30 m  $\times$  0.25 mm ID; 0.25  $\mu$ m film thickness; J&W Scientific Inc.). Helium was used as the carrier gas. The oven temperature was programmed as follows: 80°C for 1 min, increasing by 20°C/min to 320°C and held there for 10 min.

*Bioassays Offering Extracts of Macaranga Seedling to Ant Queens.* To examine whether the chemicals extracted from *Macaranga* seedlings can be used as chemical cues for host plant recognition by ant queens, we offered the extracts from the stem surfaces to foundress queens. Ethyl acetate extracts of each *Macaranga* species were applied to tube-shaped filter papers (60 mm  $\times$  5 mm ID) and allowed to dry completely. The quantity of extract was equivalent to the extract from the stem part of a node. About 1 hr after the application of the extract, single ant queens were placed on the center of a glass tray (90 mm ID  $\times$  20 mm) with the extract-impregnated paper tube, and their behavior was observed for 30 min. Talc was applied to the wall of the glass tray so that the ant queen inside could not climb up the wall. When the ant queen climbed the paper tube and touched it with her antennae, we regarded the behavior as showing interest and recorded the time during which she showed such behavior and stayed on the paper tube. A paper tube treated with pure ethyl acetate was used as a control treatment. Experimental ants were prepared and collected in the same way as in the preference experiments, and the trial schedule was also the same. Each ant queen was used twice: she was offered a paper tube containing the extract of her host species once and one containing the extract of one other species or a control once, in random order.

## RESULTS

*Host Recognition by Ant Queen.* We defined a partner *Macaranga* species for a particular experimental ant queen as the *Macaranga* species of the seedling from which the queen was derived. The frequencies with which ant queens of the

TABLE 1. COMPARISON OF ANT QUEEN PREFERENCES TO SEEDLINGS OF DIFFERENT *Macaranga* SPECIES<sup>a</sup>

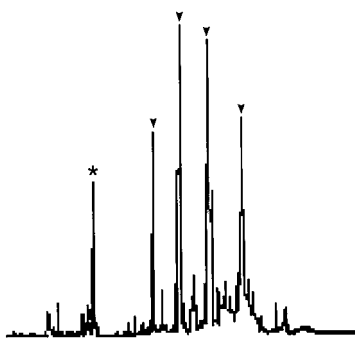
Plant species of offered seedlings	Ant species (plant species that ant queens associate with)			
	<i>M. winkleri</i>	<i>M. trachyphylla</i>	<i>M. beccariana</i>	<i>M. hypoleuca</i>
<i>M. winkleri</i>	77.8 (18)	15.3 (13)	42.9 (7)	40.0 (5)
<i>M. trachyphylla</i>	0 (8)	71.0 (31)	50.0 (4)	60.0 (5)
<i>M. beccariana</i>	0 (7)	13.3 (15)	91.3 (23)	81.3 (25)
<i>M. hypoleuca</i>	0 (7)	0 (4)	77.8 (18)	84.0 (25)

<sup>a</sup>Percentage of ant queens that entered the offered seedlings of each species; the number of experimental ant queens is given in parenthesis.

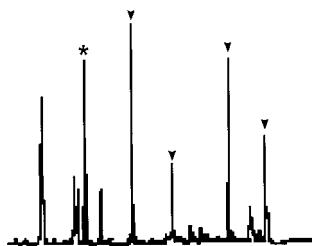
three species that associate with *M. winkleri*, *M. trachyphylla*, and *M. beccariana* entered the stem domatia of the partner *Macaranga* species were higher than those at which queens entered the stem domatia of the other two nonpartner *Macaranga* species (Table 1; Fischer's exact test,  $\chi^2 = 20.263$ ,  $P < 0.0001$  for queens from *M. winkleri* and  $\chi^2 = 19.177$ ,  $P < 0.0001$  for queens from *M. trachyphylla*, and  $\chi^2 = 8.694$ ,  $P = 0.0069$  for queens from *M. beccariana*). The queens derived from *M. winkleri* rejected all seedlings of the three nonpartner *Macaranga* species, including *M. hypoleuca*. Some individual ant queens derived from *M. trachyphylla* and *M. beccariana*, however, entered the seedling domatia of nonpartner *Macaranga* species, although they showed a significant preference for their original partner species as shown above. Ant queens from *M. trachyphylla* did not enter the seedlings of *M. hypoleuca* at all, whereas queens from *M. beccariana* entered them frequently (77.8%, 14/18 queens). For queens derived from *M. beccariana*, there was no difference between the frequency of entrance into *M. beccariana* seedlings and into *M. hypoleuca* seedlings (Fischer's exact test,  $\chi^2 = 1.479$ ,  $P = 0.377$ ). The frequency with which ant queens derived from *M. hypoleuca* entered the seedling domatia of *M. hypoleuca* was not higher than the frequency with which they entered the domatia of the three nonpartner species (Fischer's exact test,  $\chi^2 = 1.545$ ,  $P = 0.324$ ). The difference was smallest in the case of *M. beccariana*; ant queens derived from *M. hypoleuca* entered the seedling domatia of *M. hypoleuca* and *M. beccariana* with almost equal frequency.

*Chemical Analyses of Extracts of Surfaces of Macaranga Seedling.* Figure 1 shows gas chromatograms of the extracts from the seedling stem surfaces of the

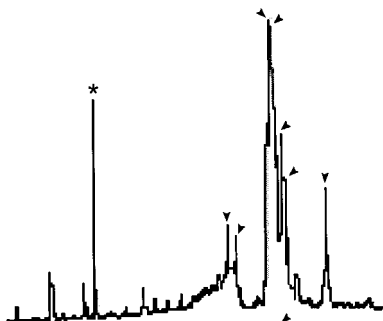
FIG. 1. Gas chromatograms of ethyl acetate extracts from the surface of the four *Macaranga* species. The chromatograms presented are those that contain the least "noisy" peaks of all the individual seedlings of each species. Arrows mark the main peaks that are found in the samples from each seedling of that species in notable amounts. Asterisks indicate the peaks of the internal standard, docosane C<sub>22</sub> (100 ng).



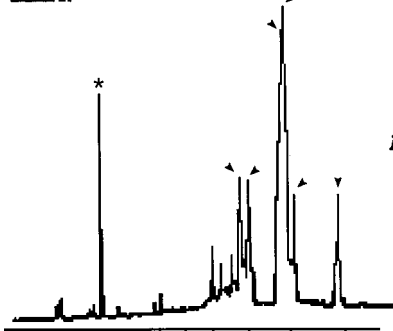
*M. winkleri*



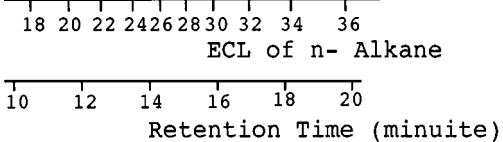
*M. trachyphylla*



*M. beccariana*



*M. hypoleuca*



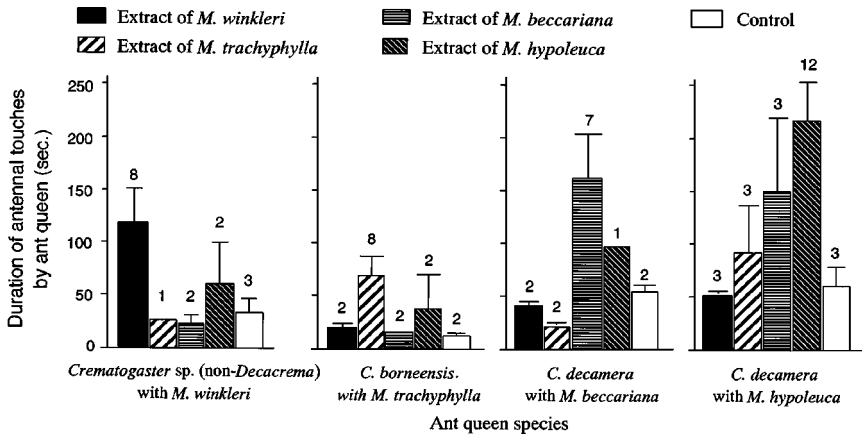


FIG. 2. The ant queen preferences among the extracts of the four *Macaranga* species. The preference is expressed by the mean duration of antennal touches by the ant queen of the given paper tube. Vertical lines on the bars indicate standard errors. Sample sizes are indicated above the bars.

four *Macaranga* species. All seedlings of a species had the same main GC peaks without exception. The four main GC peaks of *M. winkleri* were not found in any gas chromatograms of the other three species, and similarly the four main peaks in the gas chromatograms of *M. trachyphylla* were not found in the other three species. *M. hypoleuca* and *M. beccariana* showed seven and six main GC peaks, respectively; six were the same for these two species and were not found in the other two species. Moreover, the relative proportions of the six peaks (peak heights) were similar in *M. hypoleuca* and *M. beccariana*.

*Bioassays Offering Extracts of Macaranga Seedlings to Ant Queens.* More than 90% of bioassayed ant queens stayed longer on the paper tubes treated with the extracts of their partner species than on those treated with the nonvolatile extracts of their nonpartner species or controls (Figure 2; *t* test: *df* = 14, *t* = 5.791, *P* < 0.0001 for ants from *M. winkleri*; *df* = 14, *t* = 2.162, *P* = 0.0484 for ants from *M. trachyphylla*; *df* = 12, *t* = 6.315, *P* < 0.0001 for ants from *M. beccariana*; *df* = 22, *t* = 6.963, *P* < 0.0001 for ants from *M. hypoleuca*).

## DISCUSSION

Recent work on the specificity in the partnership between *Macaranga* myrmecophytes and *Crematogaster* plant-ants (Fiala et al., 1999; Itino et al., unpublished data) has verified that the *Macaranga*–*Crematogaster* myrmecophytism is highly species-specific. To clarify the mechanisms of this species specificity, it



is necessary to investigate the colonizing process of the partner myrmecophytes by foundress queens, since a mutualistic relationship (symbiosis) on a particular *Macaranga* plant starts with colonizing or settling of a seedling by a foundress queen. The recognition of the partner plants by foundress queens is a crucial phase in the process.

The results of the preference experiments, in which foundress queens were artificially placed on seedlings, suggest that foundress queens of some *Crematogaster* species can recognize their partner *Macaranga* species by contact with the plant surface at the colonizing phase. Foundress queens derived from *M. winkleri* and *M. trachyphylla* seedlings showed clear acceptance of their original partner *Macaranga* species but rejected nonpartner species. Simultaneously, however, the results suggest that the recognition of or preference for the original partner myrmecophyte is not complete in some cases, especially with foundress queens derived from *M. beccariana* and *M. hypoleuca* seedlings. These foundress queens more frequently accepted the nonoriginating *Macaranga* myrmecophytes as nest sites than did the ant queens derived from *M. winkleri* and *M. trachyphylla*. Moreover, foundress queens from *M. beccariana* and *M. hypoleuca* seemed almost equally to accept *M. beccariana* and *M. hypoleuca*. The ability to distinguish between *M. beccariana* and *M. hypoleuca* by contact with seedlings seems to be weaker in the foundress queens derived from either of these two species than in the other two ant species. Thus, the ants vary in the intensity of their preferences for or in their ability to recognize particular partner species of *Macaranga* by contact with the plant surface.

There are two possible reasons for the differences among the ant species and among the plant species in the results of the preference experiments: the phylogenetic relationships between the *Macaranga* species and the strength of the mutualistic relationships. Because *M. beccariana* and *M. hypoleuca* are considered to be phylogenetically closely related species (S. J. Davies, personal communication), the queens derived from these two species tend to have a weaker preference for a particular one. The characteristics that ant queens use to discriminate among partner plants may be similar in the two plant species. In fact, as mentioned below, the characteristics of nonvolatile chemicals on the stem surfaces of these two plant species are almost identical. On the other hand, *M. winkleri* is phylogenetically more remote from the other three species, which may be why ant queens derived from *M. winkleri* are the most persistent in their preference for their original partner species.

The strength of the mutualistic relationship or interdependency may be an important factor in the persistent preference by foundress queens for a particular partner species. It is reasonable to infer that ant species that are most dependent on partner myrmecophytes have been selected for their superior ability to distinguish their own partner plant species because they have a particularly strong preference for the partner species. Among *M. winkleri*, *M. trachyphylla*, and *M. beccariana*,

the myrmecophytism—the intensity of the mutual dependency between plant and ant—is highest on *M. winkleri* and next highest on *M. trachyphylla* (Itioka et al., 2000; Nomura et al., 2000). Although the intensity of the interdependency between *M. hypoleuca* and its plant-ant has not yet been investigated, it seems to be the weakest in these four *Macaranga* species. Because the microhabitat of *M. hypoleuca* is the most shady and, therefore, its growth is slowest, the food supply provided for its plant-ants appears to be lower than that of the others (Murase, personal observation), and the plant-ant is observed to be the least aggressiveness (Itioka, unpublished data).

Together with the clear results of the preference experiments, the results of the bioassay offering extracts of *Macaranga* seedlings to ant queens suggest that foundress queens recognize their own specific partner-plant species from the chemical characteristics of the plant surface and that they use compounds of low volatility on the stem surfaces of *Macaranga* seedlings in the recognition. These findings are supported by the differences among the gas chromatographs of the surface extracts. The fact that the GC profiles of *M. winkleri* and *M. trachyphylla* are distinct from those of the other species is consistent with the finding that foundress queens derived from these two species much less frequently failed to recognize their own original partner species. Further, the similarity of the chemical patterns of *M. beccariana* and *M. hypoleuca* is consistent with the finding that these two species are equally accepted as partner plants by foundress queens derived from either of them. It may be difficult for these two ant species to distinguish their own partner species from a closely related species. Thus, the extent to which the chemical patterns differ correlates with the ability of foundress queens to distinguish the partner plant in the preference experiment.

Our results demonstrate that high-boiling compounds on the stem surface play an important role as semiochemicals in the recognition of the partner-plant species by plant-ant foundress queens; however, this does not exclude other mechanisms from being involved in the process of selection and recognition of the partner plant species. Differences in microhabitat; in the ant's reproductive, dispersal, and colonization seasons (or the plant's receptive season for ant colonization); characteristics of volatile chemicals of seedlings; morphology, coloration, and physical features of seedling surfaces, etc., might all be used by foundress queens. These factors should be investigated in the future. However, differences in microhabitat are unlikely to be involved in the selection of the partner species by foundress queens in our system, because, except for *M. hypoleuca*, the spatial distribution of seedlings of the *Macaranga* species studied overlapped markedly at the microhabitat level (unpublished data). This is different from the habitat segregation in the myrmecophytism between *Cecropia* plants and their plant-ants reported by Yu and Davidson (1997). In the case of *M. hypoleuca*, differences in microhabitat, coloration of foliage, and leaf surface texture might be

cues in selection and recognition by plant-ants, because only *M. hypoleuca* favors a shady habitat, such as the forest floor, and has whitish, lustrous and waxy leaves.

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