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Difference in leaf herbivory between two plant-ant taxa associating with a myrmecophytic species, *Macaranga lamellata*

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ABSTRACT. *Macaranga lamellata* (Euphorbiaceae) is a myrmecophytic species that is protected against herbivorous insects by two plant-ant taxa, *Colobopsis macarangae* (Formicinae) and *Crematogaster* spp. (Myrmicinae). Although a single *M. lamellata* tree houses one plant-ant colony of either of the two taxa, both coexist in a population of *M. lamellata* in a Bornean rainforest. To elucidate the extent of herbivory damage upon *M. lamellata* trees associated with *Colobopsis* relative to trees associated with *Crematogaster*, we counted the number of leaf galls and measured the leaf loss area chewed by leaf-chewing insects on *M. lamellata* in the forest. The occurrence of gall midges was not significantly different between the trees associated with the two plant-ants, while the degree of leaf-chewing herbivory was significantly higher on *Crematogaster*-associated trees than *Colobopsis*-associated trees. The data gathered on chewing traces observed on *Crematogaster*-associated trees indicated that most herbivory damage was caused by a phasmid species. These results suggest that the herbivory pressures and occurrences of different herbivore species differ between *Crematogaster*-associated and *Colobopsis*-associated trees within a population of *M. lamellata*.

Keywords	ant defense, ant-plant interactions, Bornean tropical rainforests, *Colobopsis macarangae*, *Crematogaster*, *Camponotus*, Cecidomiidae, mutualistic relationships, *Orthomeria alexis*
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INTRODUCTION

Myrmecophytic plants have hollow spaces (domatia) that are utilized by their partner ants (plant-ants) as their nest sites (Beattie 1985, Davidson & McKey 1993). Plant-ants nesting in myrmecophytes can reduce herbivory of their host plants by excluding herbivores; the anti-herbivore defense mechanism via ants is called "ant-defense" (Heil & McKey 2003). This type of defensive mutualism has evolved in various taxa of both plants and ants, mainly in tropical regions (Davidson & McKey 1993, Chomicki & Renner 2015). Although meta-analyses have revealed that ant-defense of myrmecophytic species is generally highly effective (Rosumek *et al.* 2009, Zhang *et al.* 2015), there remains an insufficient number of empirical studies investigating variations of behavioral and ecological traits in interactions between plant-ants and herbivores on myrmecophyte populations in which there may be genetical, environmental and ontogenetic variation in eco-physiological traits.

In Southeast Asia, there are approximately 25 myrmecophytic species of *Macaranga* (Euphorbiaceae) (Davies *et al.* 2001). They have hollow stems used by their plant-ants as nest spaces and are usually colonized by highly species-specific ants, most of which belong to the myrmicinae genus *Crematogaster* (Fiala *et al.* 1999, Feldhaar *et al.* 2016). Plant-ants of *Macaranga* myrmecophytes feed on extra-floral nectar and food bodies produced by their host plants and usually cannot survive away from their hosts (Fiala & Maschwitz 1990, 1992). Similarly, when their plant-ants are absent, *Macaranga* myrmecophytes suffer heavy herbivory that is likely to increase their mortality (Fiala *et al.* 1989, Itioka *et al.* 2000). This evidence indicates that ant-defense is important for the survival of *Macaranga* myrmecophytes.

It is known that ant-defense varies in intensity among myrmecophytic *Macaranga* species associated with different plant-ant species (Itioka *et al.* 2000, Nomura *et al.* 2011). The intensity of non-ant defenses, which consist of chemical and physical mechanisms, also varies depending on the *Macaranga* species, and is negatively correlated among species with the intensity of ant-defense (Nomura *et al.* 2000, 2011). Thus, the intensity of anti-herbivore defenses of *Macaranga* myrmecophytes are regarded as highly species-specific to the plant-ant species (Itioka *et al.* 2000, Itino & Itioka 2001, Itioka 2005), which themselves are rather species-specific to the host *Macaranga* species (Fiala *et al.* 1999, Feldhaar *et al.* 2016).

Previous studies on variations in anti-herbivore defenses and herbivory in *Macaranga* myrmecophytes have focused on interspecific differences among plants, whilst intraspecific differences (e.g., among individual plants occupied by different ants) have been poorly investigated. Although studies have explored intraspecific differences in anti-herbivore defenses of myrmecophytic *Macaranga* species (Murase *et al.* 2003, Houadria *et al.* 2020), these studies compared differences between trees in two different habitats where herbivore pressure and abiotic conditions differed from each other, such as primary forest and degraded forest. Few studies have demonstrated how anti-herbivore defenses differ among conspecific myrmecophyte individuals occupied by different plant-ant species in the same habitat. The study of the intraspecific differences in anti-herbivore defenses on myrmecophytes would contribute to our understanding of ant-plant-herbivore interactions in tropical rainforests and evolutionary ecology of associations between ants and *Macaranga* myrmecophytes.

Macaranga lamellata has symbiotic relationships with a few *Crematogaster* species, as well as with the formicine ant *Colobopsis macarangae* (Maschwitz *et al.* 1996, Fiala *et al.* 1999). In Bornean rainforests, although a single *M. lamellata* tree is occupied by a single plant-ant colony of either of the two ant taxa, and both plant-ant taxa can coexist in a population of *M. lamellata*. Maschwitz *et al.* (1996) reported that both *Crematogaster* and *Colobopsis* plant-ant species patrol the surfaces of their host, and attack intruders by biting. However, their means of chemical attack and exclusion of intruders differ (Maschwitz *et al.* 1996), although how those differences affect the degree of herbivory on the host *M. lamellata* has not been examined. In the present study, we investigated herbivory damage and occurrence of herbivorous insects on *M. lamellata* trees associated with *Crematogaster* and on those associated with the *Colobopsis* in a Bornean rainforest.

MATERIAL AND METHODS

Study site

This study was conducted in Lambir Hills National Park, Sarawak, Malaysia (4°13’N, 113°59’E) from 2012 to 2014. The park is mainly covered by mixed lowland dipterocarp forest (Hazebroek & bin Abang Morshidi 2006). Mean annual temperature in the site was approximately 26°C and mean annual rainfall was approximately 2,600 mm (Kume *et al*. 2011). There are no clear dry seasons in a year (Kume *et al.* 2011).

Plants and ants

Macaranga lamellata, which grows in the understory of intact primary forests, is a small tree up to 15 m tall with large entire peltate leaves (see Electronic Supplementary Material: Fig. S1; Davies 2001). It is an obligate myrmecophytic species that has mutualistic associations with specific plant-ant colonies from the early seedling stage (i.e., from approximately 10 cm in height; Maschwitz *et al.* 1996, Fiala *et al.* 1999). *Macaranga lamellata* secretes extra-floral nectar on the leaf margins and produces food bodies on the abaxial surfaces of stipules and young leaves. Plant-ants feed on both extra-floral nectar and food bodies as their main food source (Fiala & Maschwitz 1992). The plant-ant workers open small entrance holes on the stems, from which they emerge to forage on the surfaces of the host plants, which they never leave (Maschwitz *et al.* 1996).

Previous work conducted in the study site showed that *M. lamellata* was associated with *Colobopsis macarangae*, which is known only from this host plant (Maschwitz *et al.* 1996), and with several undescribed *Crematogaster* (*Decacrema*) species (Maschwitz *et al.* 1996, Fiala *et al.* 1999). *Colobopsis macarangae* was originally placed under the genus *Camponotus* (Dumpert 1996). Ward *et al.* (2016) combined the species in the genus *Colobopsis* following the elevation of *Colobopsis* to the rank of genus from subgenus under *Camponotus*. Thus, the species described as *Camponotus macarangae* by Maschwitz *et al.* (1996) is called *Colobopsis macarangae* in this study. In the study site, approximately 80% of *M. lamellata* trees are occupied by *Co. macarangae* and the rest are occupied by *Crematogaster* (*Decacrema*) species (Maschwitz *et al.* 1996). Considering the taxonomic difficulty of identifying these *Crematogaster* species, along with the possibility that they hybridize (Feldhaar *et al.* 2016), we treated all *Crematogaster* plant-ants, which might belong to more than one species, as one group in the present study. Hereafter, we refer to *M. lamellata* trees housing *Co. macarangae* as “*Colobopsis*-associated trees”, and those housing *Crematogaster* species as “*Crematogaster*-associated trees”.

There is no clear difference in habitat between *Colobopsis*- and *Crematogaster*-associated trees (Maschwitz *et al.* 1996). Both *Co. macarangae* and *Crematogaster* plant-ants attack intruders similarly by biting, while only *Co. macarangae* release citral-like terpenes from their mandibular glands towards intruders (Maschwitz *et al.* 1996). *Colobopsis macarangae* lack a major worker subcaste (Dumpert 1996) and are larger than those of *Crematogaster* plant-ant workers are (see Electronic Supplementary Material: Table S1).

Field observation

In the field, we surveyed all the 39 undisturbed and unbranched *M. lamellata* trees of 0.7–4.0 m in height that we found in the study site. Each tree was observed once. Of the 39 trees, 26 were occupied by *Co. macarangae* and 13 by *Crematogaster* ants. The average number of leaves per tree were 9.5 ± 0.5 and 8.8 ± 0.4 for *Colobopsis*- and *Crematogaster*-associated trees, respectively. We refer to newly developing leaves as “young leaves” and to fully developed leaves as “mature leaves”.

On every leaf of the 39 target trees, we checked in daylight whether herbivorous insects were present and whether leaves showed damage inflicted by herbivores, and then we visually measured the percentage of lost leaf area, just after we selected the trees. According to the percentage of lost leaf area, we categorized all of the surveyed leaves into three groups of different herbivory levels: 1) the group of heavily chewed leaves, on which the percentage of lost leaf area was obviously more than 20%; 2) the group of less chewed leaves, on which the percentage of lost leaf area was approximately 20% or less than 20%, but not intact; and 3) the group of intact leaves, on which no lost leaf area was observed.

In addition, based on the characteristics of some types of feeding marks, for which we identified during preliminary surveys in the study site the responsible herbivorous insects that caused the damage (Shimizu-kaya *et al.* 2015), we categorized all of the observed herbivory damage on the leaves into five types: 1) lepidopteran: rounded margin and regularly placed along leaf vein; 2) phasmid: angular along leaf vein; 3) orthopteran: raggedly margin or net-like; 4) unknown leaf-chewer: leaf loss with uncertain and unclear characteristics; and 5) gall type. The first four types were caused by chewing herbivores (see Electronic Supplementary Material: Fig. S2).

Galls on *M. lamellata* leaves are known to be induced by two undescribed species of Cecidomyiidae (Cecidomyiidae sp. 4 and sp. 10 in Shimizu-kaya *et al.* 2015). The forms of galls differ between the two species. For each of the two gall-making species, we recorded the number of galls with emergence holes and that of galls without emergence holes.

When we found herbivorous insects on the target trees, we collected and reared them in the laboratory at the study site to check whether these insects fed on *M. lamellata* or were just transitory visitors on the trees. They were provided with leaves of *M. lamellata* in a plastic case for at least one week to check their feeding behaviors. Once determined, we recorded the number of herbivores on each leaf. For two species of gall midges, we considered the number of galls to be the number of gall midges, because in both of the gall-making midge species, one gall contains one individual midge larva.

Statistical analysis

In order to examine the effect of plan-ant taxa on occurrence of herbivorous insects, we employed generalized linear mixed models (GLMM) with number of herbivorous insects per leaf as the response variable, leaf age and plant-ant taxa as fixed effects, and tree as a random effect, using a negative binomial distribution with a log link function. GLMM was implemented using the statistical software R ver. 4.0.2 (R Core Team 2020) with package lme4 (Bates *et al.* 2015). We selected the best model based on Akaike's Information Criterion scores that were obtained by comparison of the models containing all possible subsets of fixed effects and random effect.

We used Fisher's exact-tests to examine the effect of plant-ant taxa on the frequency of the occurrence of herbivorous insects, number of three leaf types of herbivory, and number of observation times for chewing marks caused by four types of chewing herbivores, respectively. Fisher's exact tests were also implemented using with R ver. 4.0.2.

RESULTS

Galls

In total, 527 gall midges inside galls were observed on 23.1% of the *Colobopsis*-associated trees and 256 gall midges inside galls on 53.8% of all *Crematogaster*-associated trees (Table 1). Of all the gall midges found on *Colobopsis*-associated trees, 97.5% were Cecidomyiidae sp. 10 and the rest were Cecidomyiidae sp. 4. Of all the galls found on *Crematogaster*-associated trees, 96.5% were Cecidomyiidae sp. 10 and the rest were Cecidomyiidae sp. 4.

The frequency with which any gall midges were found to occur in trees was not significantly different between *Colobopsis*- and *Crematogaster*-associated trees (Fisher's exact-test: $P = 0.208$). For Cecidomyiidae sp. 10, the average number of galls without emergence holes in a leaf was significantly higher on young leaves than on mature leaves irrespective of plant-ant taxa (GLMM: $P < 0.001$; Table 2, Fig. 1). Plant-ant taxa did not significantly affect their occurrence (GLMM: $P = 0.208$), but the difference between young and mature leaves was significantly larger on *Colobopsis*-associated trees (GLMM: $P = 0.009$). The average number of both perforated and unperforated Cecidomyiidae sp.10 galls per leaf was also significantly higher on young leaves than on mature leaves irrespective of plant-ant taxa (GLMM: $P < 0.001$). Plant-ant taxa did not significantly affect their occurrence (GLMM: $P = 0.854$).

For Cecidomyiidae sp.4, we omitted any further analyses because of their small sample size on both *Colobopsis*- and *Crematogaster*-associated trees.

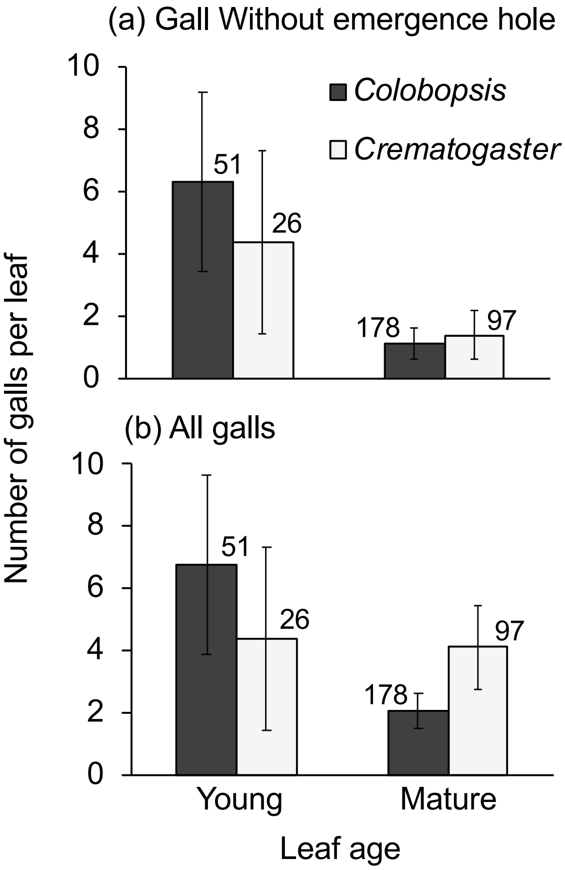

Fig. 1. Density (numbers per leaf) of Cecidomyiidae sp. 10 galls without emergence holes (a) and all of Cecidomyiidae sp. 10 galls (b) on young (left) and mature (right) leaves of *Macaranga lamellata* saplings associated with *Colobopsis macarangae* plant-ants and those associated with *Crematogaster* spp. plant-ants. Bars indicate mean values and vertical lines indicate the standard errors. A numeral above each bar indicates the number of surveyed leaves.

Table 1. Numbers of trees or leaves on which at least one individual of each of the three herbivorous insect species were observed for *Macaranga lamellata* associated with two plant-ant taxa. A numeral in parentheses beside each number of trees or leaves indicates the total number of individuals of the herbivorous insect species on the whole leaves.

Species of insect herbivore	*Colobopsis macarangae*			*Crematogaster* spp.		
	Tree (*n*=26)	YL (*n*=51)	ML (*n*=178)	Tree (*n*=13)	YL (*n*=26)	ML (*n*=97)
Cecidomiidae sp. 4						
Gall without emergence hole	3	2 (3)	1 (10)	3	2 (6)	1 (3)
Gall with emergence hole	1	0 (0)	1 (2)	3	3 (3)	0 (0)
Cecidomiidae sp. 10						
Gall without emergence hole	5	7 (322)	6 (192)	5	3 (114)	4 (133)
Gall with emergence hole	7	2 (23)	14 (177)	3	0 (0)	7 (267)
Orthomeria alexis	0	0 (0)	0 (0)	1	1 (1)	0 (0)

YL: young leaf, ML: mature leaf

Table 2. Results of analyses on factors affecting the occurrence of Cecidomyiidae sp. 10 galls, using generalized linear mixed models. Significant effects are shown in italics.

	Estimate	SE	*z* value	*P*
Gall without emergence hole				
(Intercept)	-12.426	1.917	-6.481	*< 0.001*
Leaf age	-4.315	0.652	6.613	*< 0.001*
Ant species	-2.885	2.291	1.259	0.208
Leaf age×Ant species	-2.303	0.881	-2.615	*0.009*
All galls				
(Intercept)	-7.117	2.123	-3.353	*< 0.001*
Leaf age	1.324	0.343	3.857	*< 0.001*
Ant species	-0.363	1.979	-0.184	0.854
Leaf age×Ant species	-0.265	0.635	-0.417	0.677

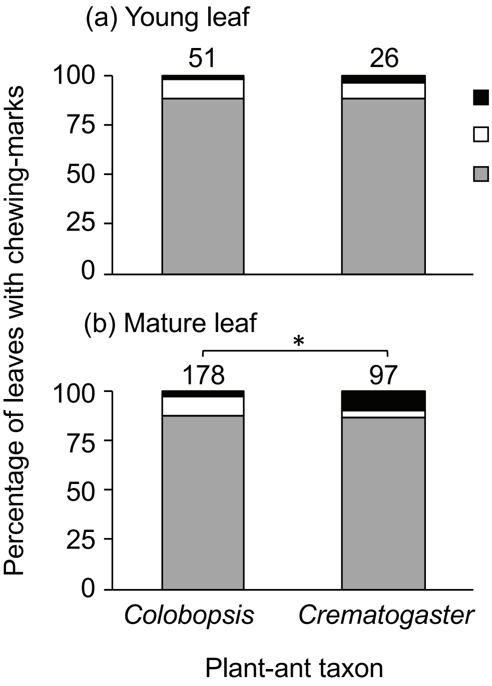

Fig. 2. Proportions of three categories of leaf loss caused by chewing herbivory on young (a) and mature (b) leaves of *Macaranga lamellata* saplings associated with *Colobopsis macarangae* plant-ants (left) and *Crematogaster* spp. plant-ants (right). A numeral above each column indicates the number of observed leaves. An asterisk (*) indicates significant difference at $P < 0.01$ (Fisher's exact test).

Chewing herbivory

One nymph of the phasmid *Orthomeria alexis* was the only herbivore that was observed on the target trees, besides gall midges inside their galls, throughout this study (Table 1).

On *Colobopsis*-associated trees, chewing marks were found on 11.8% of the observed young leaves and on 12.9% of mature leaves. On *Crematogaster*-associated trees, chewing marks were found on 11.5% of the observed

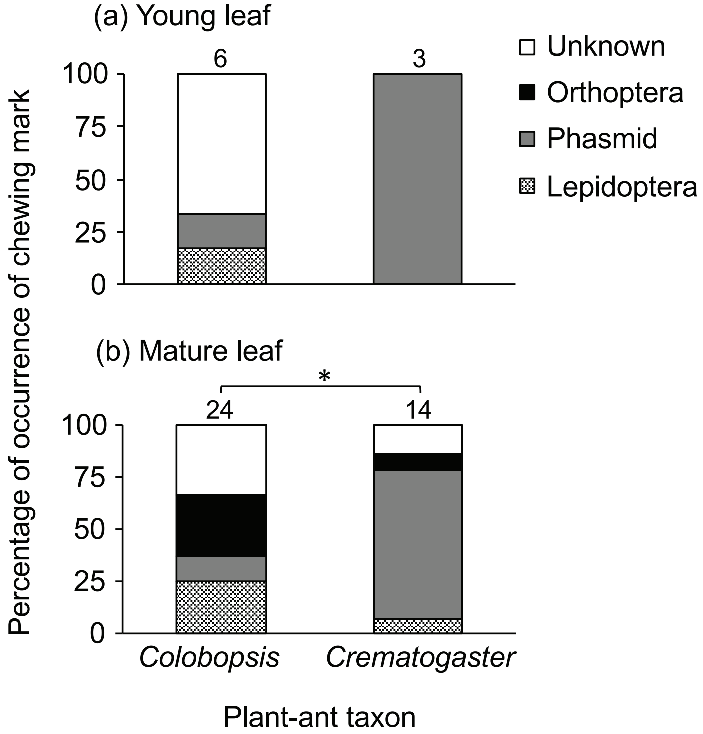

Fig. 3. Frequency of occurrence of leaves with any damage inflicted by chewing herbivores of four types on young leaves (a) and mature leaves (b) on *Macaranga lamellata* saplings associated with *Colobopsis macarangae* plant-ants (left) and *Crematogaster* spp. plant-ants (right). A numeral above each column indicates the number of leaves that were damaged by leaf-chewers. An asterisk (*) indicates significant difference at $P < 0.01$ (Fisher's exact test).

young leaves and on 13.4% of mature leaves. For young leaves, the proportions of "intact", "less chewed" and "heavily chewed" leaves were not significantly different between *Colobopsis*- and *Crematogaster*-associated trees (Fisher's exact-test: $P = 1.000$, Fig. 2a). For mature leaves, the ratio of "heavily chewed" to the other leaves on *Crematogaster*-associated trees was significantly higher than that on *Colobopsis*-associated trees (Fisher's exact-test: $P = 0.006$, Fig. 2b).

The percentages of the types of chewing marks were significantly different between *Colobopsis*- and *Crematogaster*-associated trees for mature leaves, with the percentage of chewing marks of phasmids being remarkably higher on *Crematogaster*-associated trees than on *Colobopsis*-associated trees (Fisher's exact-test: $P = 0.004$, Fig. 3b). Although they were not significantly different between *Colobopsis*- and *Crematogaster*-associated trees for young leaves (Fisher's exact-test: $P = 0.095$, Fig. 3a), the number of chewed leaves was likely to be insufficient on both trees for getting any statistically robust results. However, the major type of chewing marks on young leaves was considerably different; all the chewing marks were categorized into phasmid type on *Crematogaster*-associated trees while the percentage of phasmid-type chewing marks was less than 20% on *Colobopsis*-associated trees. In contrast, the percentage of chewing marks of lepidopterans was considerably higher on *Colobopsis*-associated trees than on *Crematogaster*-associated trees, both upon young leaves and mature leaves. The percentage of chewing marks of orthopterans was considerably higher on *Colobopsis*-associated trees than on *Crematogaster*-associated trees for mature leaves (no chewing mark of orthopterans was observed on young leaves).

DISCUSSION

The results of this study suggest that the difference of plant-ant taxa affects both the degree of leaf chewing herbivory and the species composition of leaf-chewing insects on *M. lamellata*. *Crematogaster*-associated trees were likely to suffer more chewing herbivory damage than *Colobopsis*-associated trees (Fig. 2), which were mainly inflicted by the phasmid *O. alexis*. *Colobopsis*-associated trees seemed to escape herbivory damage by phasmid more effectively than *Crematogaster*-associated trees, although the former received herbivory damages more frequently from lepidopteran and orthopteran leaf-chewers (Fig. 3).

As for most plant-ants on other *Macaranga* myrmecophytes, stimulated by physical damages and presence of intruders on leaves, ant workers of both plant-ant taxa on *M. lamellata* become active and show aggressive behaviors against intruders (confirmed by *our personal observation*). Therefore, the observed difference in leaf-chewing herbivory is presumed to reflect differences in the two plant-ant taxa in how their ant workers attack, or in how they interact with each species of leaf-chewing insects. Anti-herbivore defense through aggressive behaviors by *Co. macarangae* is presumably more effective in excluding the phasmid *O. alexis* from their host trees than the method utilized by *Crematogaster* plant-ants, but less effective in excluding lepidopteran and orthopteran leaf-chewers (Fig. 3). Nymphs and adults of *O. alexis* are known to be able to circumvent *Crematogaster* plant-ants' attacks by a series of behaviors consisting of a quick raise of their legs and rapid walking on other myrmecophytic species of *Macaranga* (Shimizu-kaya & Itioka 2015, Shimizu-kaya *et al.* 2015). Although such kinds of behaviors are useful to evade *Crematogaster* plant-ants, they may be insufficient to circumvent anti-herbivore defenses by *Co. macarangae.* On the other hand, some lepidopteran and orthopteran leaf-chewers may have different types of mechanisms that are effective to circumvent anti-herbivore defenses by *Co. macarangae* but not effective in circumventing the defenses by *Crematogaster* plant-ants. The details of counter-adaptation by each leaf-chewing herbivore to each of the plant-ant taxa and the consequences of those interactions requires further study.

The frequencies of leaves with heavy area loss due to leaf-chewing (Fig. 2) suggest that the total amount of leaf loss inflicted by leaf-chewing insects is higher on *Crematogaster*-associated trees than on *Colobopsis*-associated trees. We can propose two alternative, but not mutually exclusive, hypotheses on causal factors influencing this difference. The first is that *Co. macarangae* provides more intensive anti-herbivore defenses for the host plants than *Crematogaster* plant-ants. The second hypothesis is that the higher amount of leaf-chewing herbivory was caused by higher herbivory pressure imposed by the phasmid *O. alexis,* which was the most dominant leaf-chewing insect in this study, on *Crematogaster*-associated trees. Even if the intensity of anti-herbivore defenses by plant-ants is equal among individual trees, the amount of herbivory on a tree might be affected by the abundances of particular leaf-chewing insects. The relatively higher abundance of phasmid *O. alexis*, compared to the abundance of other leaf-chewing herbivores that are able to more effectively circumvent plant-ants' attack on *Colobopsis*-associated trees than on *Crematogaster*-associated trees, might cause the higher frequency of heavy leaf area loss on *Crematogaster*-associated trees. These hypotheses remain to be tested by some in-depth analyses of the population dynamics of major leaf-chewing insects in the field, as well as those on the behavioral interactions between each ant-taxon and each leaf-chewing insect under various ecological and environmental conditions.

The observations of gall occurrence demonstrated that most of the galls were formed by Cecidomyiidae sp. 10 and that the occurrence of Cecidomyiidae sp. 4 was too low to statistically analyze. Therefore, by focusing on galls of Cecidomyiidae sp. 10, we can discuss the effect of plant-ant taxa on gall occurrence. In contrast to leaf-chewing insects, the frequency of occurrence of Cecidomyiidae sp. 10 galls was not affected by plant-ant taxa (Table 2). It is likely that the intensity of anti-herbivory defense against the gall midge is not considerably different between the two plant-ant taxa. Although we have not observed ovipositing female adults of the midge, the observed disproportionate occurrence of unperforated galls on young leaves (Table 1) and our observation of oviposition by closely-related gall midge species on a few other species of *Macaranga* myrmecophytes (*personal observation* by US and TI) suggests that female adults of Cecidomyiidae sp. 10 oviposit mainly on young leaves. Thus, around young (developing) leaves, workers of both of the ant taxa are presumed to equally interfere with the oviposition by Cecidomyiidae sp. 10 on *M. lamellata*.

Davidson & McKey (1993) hypothesized that *Camponotus* ants, and specifically those now classified as *Colobopsis*, were the first associates of *Macaranga* myrmecophytes and have been progressively displaced by more aggressive *Crematogaster* ants in south-east Asia. On the basis of this hypothesis, Maschwitz *et al.* (1996) speculated that, on *M. lamellata, Camponotus* plant-ants are being gradually replaced by *Crematogaster* plant-ants. As mentioned above, our results suggest that *Co. macarangae* exclude *O. alexis* from, and reduce leaf loss due to leaf-chewing herbivores on, leaves of their host plant more effectively than do *Crematogaster* plant-ants. This means that *M. lamellata* trees occupied by *Co. macarangae* would have an advantage over those occupied by *Crematogaster* plant-ants in survival rate in sites where *O. alexis* is abundant. Considering that plant-ants and myrmecophytic *Macaranga* depend on each other to survive, this advantage could be a factor counteracting the replacement of *Camponotus* or *Colobopsis* by *Crematogaster*, and could subsequently allow the two plant-ant taxa to coexist in a population of *M. lamellata.* However, because the present study focused only on young *M. lamellata* trees, the growth rate, mortality, and reproductive success of *M. lamellata* should be measured throughout the lifetime of the trees associated with both plant-ant taxa, in order to discover the environmental and ecological mechanisms that affect the coexistence of the two plant-ant taxa in a local population of their host plant, and in order to elucidate the method behind partners choice between species.

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