

Multiple Factors Maintaining High Species-Specificity in *Macaranga-Crematogaster* Myrmecophytism: Higher Mortality in Mismatched Ant-Seedling Pairs

by

Kaori Murase^{1,2*}, Seiki Yamane³, Takao Itino⁴ & Takao Itioka^{2,5}

ABSTRACT

Myrmecophytism, a type of mutualistic symbiosis, occurs between certain species of *Macaranga* trees and *Crematogaster* ants in the tropics of Southeast Asia. Several recent studies have revealed that myrmecophytism in *Macaranga-Crematogaster* involves a highly species-specific partnership, but there is less specificity at the beginning of the symbiosis when ant foundress queens settle into host seedlings. However, how ‘mis-matched’ species combinations of ants and plants are selected against in the field has not yet been investigated. We therefore experimentally swapped foundress queens among three sympatric species of myrmecophytic *Macaranga* and subsequently examined growth and survival of the ants and recipient plants. Our results suggest that severe mortality occurs among ants and seedlings of unmatched pairs throughout multiple processes, such as initial worker production, early colony growth after initial worker appearance, and defense by ants against herbivores. This contributes to the maintenance of high species-specificity in *Macaranga-Crematogaster* myrmecophytism.

Keywords: myrmecophytes, plant-ants, ant-plant interactions, symbiosis, mutualism, coevolution, Borneo

INTRODUCTION

Myrmecophytism is a type of mutualistic symbiosis between plants and ants (Janzen 1966; Buckley 1982; Beattie 1985; Huxley & Cutler 1991;

¹ Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo 113-8657, Japan

² Graduate School of Bioagricultural Sciences, Nagoya University 464-8601, Japan

³ Faculty of Science, Kagoshima University, Kagoshima 890-0065, Japan

⁴ Faculty of Science, Shinshu University, Nagano 390-8621, Japan

⁵ Graduate School of Human and Environmental Studies, Kyoto University, Kyoto 606-8501, Japan

*Corresponding author. Lab. of Biometry and Bioinformatics, Graduate School of Agriculture and Life Science, University of Tokyo, Yayoi1-1-1, Bunkyo-ku, Tokyo, 113-8657, JAPAN. Email: murase@lbm.ab.a.u-tokyo.ac.jp

Davidson & Mckey 1993). In this symbiosis, plants (myrmecophytes or ant-plants) provide nest sites and food for their symbiont ants, while the ants (plant-ants) protect the plants from herbivores and plant competitors such as clinging vines.

Myrmecophytism between *Macaranga* plants and *Crematogaster* ants in the tropics of Southeast Asia is richly diversified in terms of the species involved, and the modes and tightness of the relationships (Whitmore 1969, 1975; Fiala *et al.* 1989, 1994, 1999; Fiala & Maschwitz 1990, 1991, 1992; Itioka *et al.* 2000; Blattner *et al.* 2001; Davies *et al.* 2001; Itino & Itioka 2001; Itino *et al.* 2001a; Quek *et al.* 2004). Myrmecophytic *Macaranga* species harbor their partner *Crematogaster* ant species inside hollow stems and provide them with food resources. *Macaranga-Crematogaster* myrmecophytism is often obligate; host plants and ants in the symbioses fully depend on each other for survival and growth, the symbioses are maintained throughout most of their life span, and the characteristics of both symbionts are thought to be adapted and highly specialized to each other (Fiala *et al.* 1989, 1994; Fiala & Maschwitz 1990; Itino *et al.* 2001a). These obligate systems show high species-specificity in the partnerships between host ant-plants and symbiotic plant-ants (Fiala *et al.* 1999; Itino *et al.* 2001b). This high species-specificity is often seen even in a single locality where several species of obligate myrmecophytic *Macaranga* coexist, i.e., one *Macaranga* species maintains mutualistic relations with only one or a few *Crematogaster* species, and one plant-ant species colonizes only one or a few *Macaranga* species (Fiala *et al.* 1999; Itino *et al.* 2001b). To better understand the coevolutionary process (Thompson 1994) of the myrmecophytic mutualism, it is interesting and important to elucidate which factors maintain this high species specificity in *Macaranga-Crematogaster* myrmecophytism.

In most obligate *Macaranga-Crematogaster* associations, the symbiosis begins with foundress queens settling on the host plants at the early seedling stage. Inui *et al.* (2001) reported that foundress queens of a few ant species were able to discriminate partner plants from other species by recognizing contact-chemicals on the stem surface of the seedlings. However, Murase *et al.* (2002) revealed that the highly species-specific partnership, which was recognized in host plants on which ant colonies had produced workers that defended the hosts (Fiala *et al.* 1999; Itino *et al.* 2001b), was not complete during host-plant selection by foundress queens in the field; some *Macar-*

anga seedlings were colonized by queens that did not belong to the specific-partner ant species. (Here, we use ‘specific-partner’ to indicate that the highly species-specific symbiont [ant-plant or plant-ant] that is observed on mature trees and healthy seedlings, as described by Fiala *et al.* [1999] and Itino *et al.* [2001b].) Seedlings colonized by foundress queens of non-specific-partner ants suffer much higher mortality than those colonized by foundress queens of their specific-partner species after the queens have settled onto the host plants. Consequently, the symbiotic system collapses before mutualistic symbiosis, or anti-herbivore defenses by ants can begin to work effectively. We hypothesized that these relationships facilitate the highly species-specific partnerships observed in sympatric myrmecophytes.

To test the hypothesis, we experimentally swapped foundress queens among three sympatric species of obligate myrmecophytic *Macaranga*. We then examined the growth and survival of the swapped ants and recipient plants from the time of queen settlement (introduction of queens into seedlings) through the establishment of a colony, i.e., the production of adequate numbers of adult post-initial workers (workers reared by older workers rather than by queens) that could protect new leaves from herbivores. Among the three *Macaranga* species we used, the species-specificity in the partnership is known to be very high at the time when ant colonies produce workers that defend the host plants (Fiala *et al.* 1999; Itino *et al.* 2001b), but it is somewhat more relaxed just after the foundress queens have settled (Murase *et al.* 2002).

MATERIALS AND METHODS

Study site

This study was conducted in a primary lowland mixed dipterocarp forest in Lambir Hills National Park (4°2'N, 113°50'E, 60 m a.s.l.), c. 20 km south of Miri, Sarawak, Malaysia. This region lies in the humid tropics and experiences no pronounced dry season and little seasonal variation in temperature (Kato *et al.* 1995). The study site was described in detail by Inoue and Hamid (1994) and Kato *et al.* (1995).

Life history

The genus *Macaranga* comprises c. 280 tree species, distributed from west Africa to the South Pacific, with a center of diversity in Borneo and New Guinea; this genus includes many obligate myrmecophytes (Whitmore 1969,

1975; Fiala *et al.* 1989, 1994, 1999; Blattner *et al.* 2001; Davies *et al.* 2001). At least 20 *Macaranga* species of which at least 11 are myrmecophytic, have been recorded from this study site (Nagamasu & Momose 1997). The target species of this study were three obligate myrmecophytes, *Macaranga winkleri* Pax. and Hoffm., *M. trachyphylla* Airy Shaw, and *M. beccariana* Merr. In the study site, although the preferred light and soil conditions differed somewhat among the three species (Davies *et al.* 1998), seedlings and mature trees were usually found sharing the same microhabitats, including forest gaps.

In the study site, each *Macaranga* species has a specific partner ant species; investigations of mature trees and healthy seedlings have shown that *M. winkleri*, *M. trachyphylla*, and *M. beccariana* harbor *Crematogaster* sp. A, *C. borneensis*, and *C. decamera* respectively (Fiala *et al.* 1999; Itino *et al.* 2001b). These three *Crematogaster* species can be clearly separated morphologically (Sk. Yamane, *personal observation*) and are distinguishable by mtDNA sequences (Itino *et al.* 2001b). Murase *et al.* (2002) provided details of the taxonomic status of these ants.

From the time of seed dispersal until foundress queens have settled, the three *Macaranga* species grow in the absence of a symbiosis with ants. When seedlings reach a height of about 10-30 cm, the stems begin to swell and the piths degrade. Alate foundress queens then arrive at the seedlings and settle inside the hollow stem internodes (*i.e.*, domatia) (Fiala *et al.* 1989; Murase *et al.* 2002). Upon settling into seedlings, a foundress chews a hole in the stem surface, enters the domatia through the hole, and plugs the hole with parenchyma scraped from the inside walls of the domatia. The foundress then begins to feed her initial workers with regurgitated food. About one to two months after settlement, the initial ant workers reach adulthood, emerge from the domatia by reopening the holes, and begin collecting extrafloral nectar (EFN) on the leaf surface and food bodies (FBs) inside the domatia-shaped stipules (*M. winkleri* and *M. trachyphylla*) or on the leaf surface (*M. beccariana*). Thereafter, workers in these ant colonies harvest FBs and EFN as their main food sources and always tend to plant parts that appear vulnerable to herbivores. Several queens are often observed attempting to found their nests on a single seedling; however only one ant colony eventually occupies the entire seedling, by killing the other queens and their workers. Settlements by foundress queens were commonly observed on all *Macaranga* species year

round (Murase *et al.* 2002). Murase *et al.* (2002) reported that 1-30 % of queens that settled into seedlings of the three *Macaranga* myrmecophytes belonged to non-specific-partner ant species.

Experimental swapping of foundress queens

To conduct the queen-swapping experiment, 60 seedlings of each *Macaranga* species were haphazardly collected in May 1999 from several forest gaps and riverbanks where saplings and seedlings of each *Macaranga* species were observed. Only seedlings that were <10 cm tall and not yet colonized by ants were targeted. The collected seedlings were planted into 1 L plastic pots with local soil and then placed in a shade house near the study site. We used black cloth to match the light levels in the shade house to those of a typical forest gap. In the shade house, herbivorous insects, if any, were manually removed from plants every other day. Based on the finding of Murase *et al.* (2002) that the number of domatia on seedlings with at least one “foundress queen-confined domatia” ranged from one to eight, we used the seedlings when they contained two to five domatia.

Nulliparous foundress queens were collected in June and July 1999 from other intact *Macaranga* seedlings 10-40 cm tall. We considered a queen with no offspring in the domatia to be a nulliparous foundress, and we identified the ant species based on the morphology of the queen. When we found non-specific-partner ant species in the collections, we abandoned them. In total, 60 nulliparous foundress queens were collected for each ant species from its specific-partner host *Macaranga*. The 60 queens of each species were used for the experimental swap among the three *Macaranga* species; they were assigned to 20 seedlings of their specific-partner plant species (as controls), and 20 seedlings of each of the two non specific-partner plant species. The former type of species combination was considered a ‘matched’ pairs while the latter type was an ‘unmatched’ pairs.

The day after queen collecting, each nulliparous queen was introduced into one of the potted *Macaranga* seedlings in the shade house. We made a hole of c. 7 mm in diameter on the stem surface of each seedling and inserted a queen through each hole. Upon inserting the queens, we anaesthetized them with CO₂ and filled the holes with stem tissues. We also dug a hole of c. 1 mm in diameter in the domatia in which the queens were confined, to con-

firm their arousal from anaesthesia 24 h later. When the confined foundress queens were observed being active in the domatia, they also filled these holes with plant tissues.

We then checked whether initial ant workers appeared on the plant surface by carefully observing each seedling every other day. In particular, we looked for new holes that had to be made by initial ant workers when they emerged on the surface. When we found ant workers on the surface, we checked whether the ants collected FBs. In addition, we confirmed the growth of the ant colonies and seedlings; we checked whether plant condition is healthy or not every week. We supplied sufficient water and soil nutrients (N, P and K fertilizer) for the ant-seedling pairs in the shade house and removed almost all herbivores by hand.

For each ant-seedling pair, six months after we confirmed that initial workers emerged from the domatia for the first time, we checked whether workers were still alive and working on the plant surface. If we were unable to find any ant workers on the surface, we checked for the presence of live workers by shaking the seedlings. If we then still could not confirm the presence of ants, we harvested and dissected the seedlings to ascertain the death of the ant colonies. In this way, we confirmed that in seedlings on which we could not find any adult workers, all ant colonies failed to survive for six months after producing initial workers.

Of all experimental seedlings ($N = 180$), 37 contained ant colonies that had survived and grown for more than six months in the shade house (see Table 3, 4). On these seedlings, at least 20 workers were observed tending to the apical parts of the seedlings. We transplanted these seedlings to riparian forest gaps to expose them to herbivory in the field. We observed growth, survival, and herbivory of the seedlings and activities of the ant colonies at intervals of about two months until one year after transplanting. If we could not find any workers on the plant surface, we shook the seedlings and checked whether any workers emerged from the domatia. At the end of the experiment, all seedlings were harvested and dissected to measure the sizes of the ant colonies. This series of swapping experiments was conducted from 1999 to 2001.

Statistics

We used a chi-square test for independence to compare among three species, and Fischer's exact tests to compare between matched pairs and unmatched pairs. For some comparisons, only Fischer's exact test was used, since we were unable to obtain a sufficient number of replications to conduct a chi-square test.

RESULTS

Success in producing adult initial workers

For colonies derived from queens of *M. winkleri* specific-partner ants, the number of introduced queens that succeeded in raising adult initial workers that appeared on the surface of seedlings was significantly higher when the queens were introduced onto matched plants (*M. winkleri*) than when the queens were introduced onto the other two non-specific-partner *Macaranga* species (chi-square test of independence, $df = 2, \chi^2 = 10.83, P < 0.005$; Fisher's exact test, $P < 0.05$, Table 1). Similarly, for *M. beccariana* specific-partner ants, the number of introduced queens that succeeded was significantly higher when the queens were introduced onto matched plants (*M. beccariana*) than when the queens were introduced onto the two other non-specific-partner *Macaranga* species (Fisher's exact test, $P < 0.05$), although there was not significant difference among three plant species (chi-square test of independence, $df = 2, \chi^2 = 5.84$). Although *M. trachyphylla* specific-partner ants also performed better on matched pairs, the difference was not significant (chi-square test of independence, $df = 2, \chi^2 = 1.76$; Fisher's exact test, $P > 0.10$).

Table 1. Percentage of foundress queens that succeeded in producing initial workers on seedlings of three different myrmecophytic *Macaranga* species. The number of successful queens (numerator) and the number of queens used for the swapping experiment (denominator) are indicated in parentheses. Foundress queens belonged to three *Crematogaster* ant species specific to one of the three *Macaranga* species. All foundress queens were collected from seedlings of the three *Macaranga* species when they were confined by themselves in the stem domatia.

Plant species of recipient seedlings	Ant species of introduced foundress queens (specific-partner <i>Macaranga</i> species)		
	<i>Crematogaster</i> sp. A (<i>Macaranga winkleri</i>)	<i>C. borneensis</i> (<i>M. trachyphylla</i>)	<i>C. decamera</i> (<i>M. beccariana</i>)
<i>Macaranga winkleri</i>	80.0 (16/20)	40.0 (8/20)	30.0 (6/20)
<i>M. trachyphylla</i>	30.0 (6/20)	55.0 (11/20)	35.0 (7/20)
<i>M. beccariana</i>	65.0 (13/20)	35.0 (7/20)	65.0 (13/20)

For *M. winkleri*, the percentage of introduced queens that succeeded was significantly higher when *M. winkleri* specific-partner ants were introduced than when the two other non-specific-partner ant species were introduced (chi-square test of independence, $df = 2$, $\chi^2 = 11.20$, $P < 0.005$; Fisher's exact test, $P < 0.005$). However, for *M. trachyphylla* and *M. beccariana*, there was no significant difference between matched and unmatched pairs (chi-square test of independence, $df = 2$, $\chi^2 = 2.92$; Fisher's exact test, $P > 0.05$ for *M. trachyphylla*; chi-square test of independence, $df = 2$, $\chi^2 = 4.85$; Fisher's exact test, $P > 0.10$ for *M. beccariana*).

On all seedlings where adult initial workers were found on the plant surface following the introduction of queens, they were observed walking actively on stems and leaves, bringing FBs into the domatia, and tending to the apical parts of the seedlings.

Survival rate of ant colonies after initial worker production

For each ant species, the number of colonies that survived for six months after the appearance of initial workers was significantly higher on the specific-partner *Macaranga* species than on non-specific-partner *Macaranga* species (Fisher's exact test, $P < 0.0001$ for queens of *M. winkleri* specific-partner ants; $P < 0.005$ for *M. trachyphylla*; and $P < 0.01$ for *M. beccariana*, Table 2).

For each plant species, the number of colonies that survived for six months after the appearance of initial workers was significantly higher when specific-partner ants were introduced than when the two other non-specific-partner ant species were introduced (Fisher's exact test, $P < 0.005$ for *M. winkleri*; $P < 0.05$ for *M. trachyphylla*; and $P < 0.0001$ for *M. beccariana*).

Total ant mortality on seedlings under herbivore-free conditions

Combining the first two time periods, the number of introduced queens that succeeded in growing post-initial workers and survived for six months after being introduced onto experimental seedlings under herbivore-free conditions (in the shade house) was significantly higher for specific-partner *Macaranga* species than for non-specific-partner *Macaranga* species (Fisher's exact test, $P < 0.0001$ for queens of *M. winkleri* specific-partner ants; $P < 0.005$ for queens of *M. trachyphylla* specific-partner ants; $P < 0.0005$ for queens of *M. beccariana* specific-partner ants, Table 3). About half of the queens introduced into their specific-partner *Macaranga* species survived for

Table 2. Percentage of ant colonies that succeeded in producing sufficient post-initial workers to tend to young leaves and that survived for six months after the appearance of initial workers on seedlings of three different myrmecophytic *Macaranga* species. The number of successful colonies (numerator) and the number of colonies used for the analysis (denominator) are indicated in parentheses. The number of colonies used corresponds to the number of successful queens shown in Table 1. See Table 1 for details of the ant and plant species.

Plant species of recipient seedlings	Ant species of introduced foundress queens (specific-partner <i>Macaranga</i> species)					
	<i>Crematogaster</i> sp. A (<i>Macaranga winkleri</i>)	<i>C. borneensis</i> (<i>M. trachyphylla</i>)		<i>C. decamera</i> (<i>M. beccariana</i>)		
<i>Macaranga winkleri</i>	75.0	(12/16)	12.5	(1/ 8)	16.7	(1/ 6)
<i>M. trachyphylla</i>	0	(0/ 6)	72.7	(8/11)	42.6	(3/ 7)
<i>M. beccariana</i>	0	(0/13)	14.3	(1/ 7)	84.6	(11/13)

Table 3. Percentage of foundress queens that succeeded in producing initial workers, producing sufficient post-initial workers, and surviving for six months after the appearance of initial workers on seedlings of three different myrmecophytic *Macaranga* species. The number of successful colonies (numerator) and the number of colonies used for the analysis (denominator) are indicated in parentheses. The data was based on those in Table 1 and 2. See Table 1 for details of the ant and plant species.

Plant species of recipient seedlings	Ant species of introduced foundress queens (specific-partner <i>Macaranga</i> species)					
	<i>Crematogaster</i> sp. A (<i>Macaranga winkleri</i>)	<i>C. borneensis</i> (<i>M. trachyphylla</i>)		<i>C. decamera</i> (<i>M. beccariana</i>)		
<i>Macaranga winkleri</i>	60.0	(12/20)	5.0	(1/20)	5.0	(1/20)
<i>M. trachyphylla</i>	0	(0/20)	40.0	(8/20)	15.0	(3/20)
<i>M. beccariana</i>	0	(0/20)	5.0	(1/20)	55.0	(11/20)

more than six months after introduction and succeeded in raising post-initial workers that tended to the apical parts of the seedling under herbivore-free conditions. However, on seedlings of non specific-partner *Macaranga* species, few queens survived to the time of post-initial worker production.

For each plant species, combining the first two time periods, the number of introduced queens that succeeded in growing post-initial workers and survived for six months (in the shade house) was significantly higher when specific-partner ants were introduced than when the two other non-specific-partner ant species were introduced (Fisher's exact test, $P < 0.0001$ for *M. winkleri*; $P < 0.005$ for *M. trachyphylla*; and $P < 0.0001$ for *M. beccariana*).

Survival rate of ant colonies after being transplanted to the field

Of the 37 seedlings on which ant colonies survived for more than six months

Table 4. Percentage of foundress queens that succeeded in producing initial workers, producing sufficient post-initial workers, surviving for six months after the appearance of initial workers, and surviving for one year after transplanting in the field. The number of surviving colonies (numerator) and the number of transplanted colonies (denominator) are indicated in parentheses. The number of transplanted colonies corresponds to the number of successful colonies shown in Table 2. See Table 1 for details of the ant and plant species.

Plant species of recipient seedlings	Ant species of introduced foundress queens (specific-partner <i>Macaranga</i> species)					
	<i>Crematogaster</i> sp. A (<i>Macaranga winkleri</i>)		<i>C. borneensis</i> (<i>M. trachyphylla</i>)		<i>C. decamera</i> (<i>M. beccariana</i>)	
<i>Macaranga winkleri</i>	100.0	(12/12)	0	(0/1)	0	(0/1)
<i>M. trachyphylla</i>	-		100.0	(8/8)	0	(0/3)
<i>M. beccariana</i>	-		0	(0/1)	100.0	(11/11)

in the shade house, all six unmatched pairs of ants and plants disappeared in one year, whereas all matched pairs survived (Fisher's exact test, $P < 0.0001$, Table 4). The former suffered from severe herbivory, while the latter were subject to very few herbivore attacks and survived for at least one year in the field. One month after transplantation, 5.57% of the leaf area was lost on average in unmatched pairs, whereas $< 1.9\%$ of the leaf area was lost in the three types of matched pairs. The leaf damages after one month in fields, however, were not significant difference between matched pair and unmatched pairs (Mann-Whitney test, $P > 0.05$ for *M. trachyphylla* specific-partner ants; $P > 0.05$ for *M. beccariana* specific-partner ants). Six month after transplantation, we confirmed that the apical meristem in unmatched pairs had been broken several times as a result of herbivory on each of the seedlings, and $> 70\%$ of the remaining leaves were found to be necrotic.

DISCUSSION

The swapping experiment demonstrated that more than 70% of foundress queens succeeded in establishing colonies when they colonized seedlings of their specific-partner *Macaranga* species, whereas most (c. 95-100 %) foundress queens failed to establish colonies with sufficient post-initial workers when they colonized seedlings of non-specific-partner *Macaranga* species. Moreover, even when unmatched pairs of ants and seedlings survived under herbivore-free conditions through the colony-establishment phase, or when ant colonies appeared to produce sufficient numbers of post-initial workers

to effectively defend plants against herbivores, all of these pairs died within a year after being transplanted to the field, probably due to heavy damage from herbivory. These results supported our hypothesis that high species-specificity in the partnership between the time of settlement through the establishment of a colony is maintained by higher mortality imposed on ‘unmatched’ *Macaranga-Crematogaster* pairs, which could result from foundress queens ‘mis-settling’ onto seedlings of non-specific-partner species in the field.

The remarkably higher mortality evident in unmatched pairs could be separated into the following three processes: 1) failure of foundress queens to breed the initial workers, 2) failure to maintain the colonies after raising initial workers, and 3) insufficient anti-herbivore defenses after establishment of ant colonies. Our results suggested that all three processes contributed to the maintenance of high specific partnerships.

Of these three processes, the first is likely to be independent of food services from the host plant, because a confined foundress queen probably feeds her initial workers with regurgitated food using her flight muscle. This suggests that there is an incompatibility between unmatched pairs of *Crematogaster* plants and *Macaranga* ant-plant species during the period from queen settling until the beginning of FB harvesting, likely due to physical and physiological conditions that are unsuitable for the foundress queens inside the domatia. How the physical and physiological conditions within the domatia – such as temperature, humidity, and presence of volatile chemicals – vary among *Macaranga* species and how each plant-ant species is specialized for these possible variations remain to be determined quantitatively or experimentally.

Even if foundress queens on seedlings of unmatched *Macaranga* species succeed in producing initial workers that can make access FBs, colony growth is notably restricted compared with queens on matched *Macaranga* species, which may be attributable to the second process. In the second process, the asymmetry in mortality among unmatched species combinations suggests that a shortage of nutrients is not the only causal factor involved. Of the three *Macaranga* species, *M. winkleri* provides the most abundant and most nutritious FBs, while *M. beccariana* provides the least abundant FBs (Itioka *et al.* 2000; Hatada *et al.* 2002). If the amount of FBs produced was the only limiting factor, the asymmetric pattern should have been the opposite to the one that was observed. Irrespective of mechanisms underlying the second

process, our results suggest that the incompatibility of unmatched pairs, probably in terms of utilizing FBs, seems to be more intensive in the second than in the first process.

The herbivory observed on seedlings colonized by unmatched ant species after transplantation to the field suggests that herbivory pressure could be an additional important factor that maintains species-specificity in the *Macaranga-Crematogaster* partnership through the third process. In unmatched pairs, it is likely that anti-herbivore defenses are not sufficient to allow the host plants to continue to grow and survive; plants are heavily damaged by herbivores and wither as a result, and the symbiont ants die in response to the death of the host plants. Insufficient anti-herbivore defenses may be due to deficient colony growth as a result of the same mechanisms involved in the first or second processes. Although the leaf damages after one month in fields were not significant difference between matched pair and unmatched pairs, the leaf damage of *M. winkleri* with unmatched ant species is highest among all seedlings (the leaf damage of *M. winkleri* within *C. borneensis* is 8.0%, the leaf damage of *M. winkleri* within *C. decamera* is 5.2%). This remains to be tested, since we did not quantitatively compare colony size and ant activity in unmatched pairs to those in matched pairs at the onset of the third process.

Itioka *et al.* (2000) and Nomura *et al.* (2000) demonstrated that the three *Macaranga* species have different strategies for anti-herbivore defenses in terms of the balance between ant- and non-ant defense mechanisms. *Macaranga winkleri* is defended from herbivores by the most effective ant defenses and the weakest non-ant (chemical and physical) defenses; *M. beccariana* is defended by the least effective ant defenses and the strongest non-ant defenses; and *M. trachyphylla* is defended by intermediate ant and non-ant defenses. Corresponding to this relationship, patterns of ant defense appear to vary among species combinations. For example, in matched pairs, most ant workers preferentially tend to and defend young leaves around apical parts on *M. beccariana*, whereas young leaves are given less priority on *M. winkleri* (*unpublished data*). The complementarity of the interspecific variation in the combination of ant- and non-ant defenses may be disrupted in unmatched pairs.

Although we assessed colony mortality through the second process under experimentally herbivore-free conditions, herbivory pressure would be im-

posed on *Macaranga* seedlings even during the second process in the field. While queens keep themselves confined in the domatia for breeding initial workers, the three *Macaranga* species defend themselves from herbivores through their own chemical and physical defenses without the help from the ants (Nomura *et al.* 2001). Hence, the relative impact from herbivory pressure is likely not very high on seedlings during the self-confinement in queens.

The results of this study indicate that multiple mechanisms and processes select against symbiosis pairs of 'unmatched' *Macaranga* myrmecophytes and *Crematogaster* plant-ants after 'erroneous' host-plant selection by foundress queens. These mechanisms and processes play important roles in maintaining highly species-specific *Macaranga-Crematogaster* myrmecophytism in an area where multiple *Macaranga* species share similar microhabitats.

Do the mechanisms maintaining the high species-specificity have some adaptive value for either symbiont? It is possible that the incompatibility in the use of FBs between unmatched pairs may be beneficial to *Macaranga* myrmecophytes. In the field, multiple foundress queens are frequently observed sharing a seedling as a settling-site, and then compete for occupation of the seedling (K. Murase, *unpublished data*). If *Macaranga* seedlings can select against competing foundress queens of non-specific-partner species based on incompatibility and can subsequently select for competing foundress queens of their specific-partner species, the seedlings would have a higher probability of survival, because ant colonies derived from foundress queens of non-specific-partner species cannot effectively defend the seedlings against herbivores even if they succeed in producing some workers. For the ants, the ability to correctly recognize seedlings of their specific-partner species, e.g., through chemical cues (Inui *et al.* 2001), may be selected for, preventing the fitness loss due to 'erroneous' selection of host plants. It remains to be examined whether settling into non-specific-partner *Macaranga* species is truly erroneous or does in fact confer some fitness benefits.

Even though flower-bee associations that have been receiving increasing attention, prior attempts to test the mechanisms to maintain the species-specificity have been inconclusive. What mechanisms operate to maintain the species-specificity in other symbiosis? We want this interesting question widely known and solved in other species-specificity associations.

ACKNOWLEDGMENTS

We thank Hua-Seng Lee, Tohru Nakashizuka and Takayuki Ohgushi for providing opportunities for this study. Tomoaki Ichie provided a few ideas for maintenance of the seedlings in a shade house. Thanks are also due to Masahiro Nomura, Yoko Inui and the staff of the Entomological Section, Forest Research Centre, Sarawak, for helpful assistance at the fieldwork. This work was supported partly by Grants-in-Aids for Scientific Research (No. 14405004 and No. 09NP1501) from the Ministry of Education, Science, and Culture, Japan, by CREST of JST (Japan Science and Technology Corporation) and by JSPS Research Fellowships for young scientists.

REFERENCES

- Beattie, A. J. 1985. *The Evolutionary Ecology of Ant-plant Mutualisms*. Cambridge University Press, Cambridge.
- Blattner, F. R., K. Weising, G. B. Baenfer, U. Maschwitz & B. Fiala. 2001. Molecular analysis of phylogenetic relationships among myrmecophytic *Macaranga* species (Euphorbiaceae). *Molecular Phylogenetics and Evolution* 19: 331-344.
- Buckley, R. C. (ed) 1982. *Ant-plant Interactions in Australia*. Dr. W. Junk, The Hague.
- Davidson, D. W. & D. McKey. 1993. The evolutionary ecology of symbiotic ant-plant relationship. *Journal of Hymenoptera Research* 2: 13-83.
- Davies, S. J., P. A. Palmiotto, P. S. Ashton, H. S. Lee & J. V. LaFrankie. 1998. Comparative ecology of 11 sympatric species of *Macaranga* in Borneo: tree distribution in relation to horizontal and vertical resource heterogeneity. *Journal of Ecology* 86: 662-673.
- Davies, S. J., S. K. Y. Lum, R. Chan & L. K. Wang. 2001. Evolution of myrmecophytism in western Malesian *Macaranga* (Euphorbiaceae). *Evolution* 55: 1542-1559.
- Fiala, B. & U. Maschwitz. 1990. Studies on the South East Asian ant-plant association *Crematogaster borneensis* / *Macaranga*: adaptations of the ant partner. *Insectes Sociaux* 37: 212-231.
- Fiala, B. & U. Maschwitz. 1991. Extrafloral nectaries in the genus *Macaranga* (Euphorbiaceae) in Malaysia: comparative studies of their possible significance as predispositions for myrmecophytism. *Biological Journal of the Linnean Society* 44: 287-306.
- Fiala, B. & U. Maschwitz. 1992. Food bodies and their significance for obligate ant-association in the tree genus *Macaranga* (Euphorbiaceae). *Botanical Journal of the Linnean Society* 110: 61-75.
- Fiala, B., U. Maschwitz, T. Y. Pong & A. J. Helbig. 1989. Studies of a South East Asian ant-plant association: protection of *Macaranga* trees by *Crematogaster borneensis*. *Oecologia* 79: 463-470.

- Fiala, B., H. Grunsky, U. Maschwitz & K. E. Linsenmair. 1994. Diversity of ant-plant interactions: protective efficacy in *Macaranga* species with different degrees of ant association. *Oecologia* 97: 186-192.
- Fiala, B., A. Jakob, U. Maschwitz & K. E. Linsenmair. 1999. Diversity, evolutionary specialization and geographic distribution of a mutualistic ant-plant complex: *Macaranga* and *Crematogaster* in South East Asia. *Biological Journal of the Linnean Society* 66: 305-331.
- Hatada, A., T. Itioka, R. Yamaoka & T. Itino. 2002. Carbon and nitrogen contents of food bodies in three myrmecophytic species of *Macaranga*: implications for antiherbivore defense mechanisms. *Journal of Plant Research* 115: 179-184.
- Huxley, C.R. & D. F. Cutler (eds) 1991. *Ant-plant Interactions*. Oxford University Press, Oxford.
- Inoue, T. & A. A. Hamid (eds) 1994. *Plant reproductive systems and animal seasonal dynamics: long-term study of dipterocarp forests in Sarawak*. Center for Ecological Research, Kyoto University, Otsu.
- Inui, Y., T. Itioka, K. Murase, R. Yamaoka & T. Itino. 2001. Chemical recognition of partner plant species by foundress ant queens in *Macaranga-Crematogaster* myrmecophytism. *Journal of Chemical Ecology* 27: 2029-2040.
- Itino, T. & T. Itioka 2001. Interspecific variation and ontogenetic change in anti-herbivore defense in myrmecophytic *Macaranga* species. *Ecological Research* 16: 765-774.
- Itino, T., T. Itioka, A. Hatada & A. A. Hamid. 2001a. Effects of food rewards offered by ant-plant *Macaranga* on the colony size of ants. *Ecological Research* 16: 775-786.
- Itino, T., S. J. Davies, H. Tada, Y. Hieda, M. Inoguchi, T. Itioka, Sk. Yamane & T. Inoue. 2001b. Cospeciation of ants and plants. *Ecological Research* 16: 787-793.
- Itioka, T., M. Nomura, Y. Inui, T. Itino & T. Inoue. 2000. Difference in intensity of ant defense among three species of *Macaranga* myrmecophytes in a Southeast Asian dipterocarp forest. *Biotropica* 32: 318-326.
- Janzen, D. H. 1966. Coevolution of mutualism between ants and acacias in Central America. *Evolution* 20: 249-275.
- Kato, M., T. Inoue, A. A. Hamid, T. Nagamitsu, M. B. Merdek, A. R. Nona, T. Itino, Sk. Yamane & T. Yumoto. 1995. Seasonality and vertical structure of light-attracted insect communities in a dipterocarp forest in Sarawak. *Researches on Population Ecology* 37: 59-79.
- Murase, K., T. Itioka, Y. Inui & T. Itino. 2002. Species specificity in settling-plant selection by foundress ant queens in *Macaranga-Crematogaster* myrmecophytism in a Bornean dipterocarp forest. *Journal of Ethology* 20: 19-24.
- Nagasasu, H. & K. Momose. 1997. Flora of Lambir Hills National Park, Sarawak, with special reference to the Canopy Biology Plot. pp. 20-67 In: Inoue T, Hamid AA (eds) *General Flowering of Tropical Rainforests in Sarawak*. Center for Ecological Research, Kyoto University, Otsu.

- Nomura, M., T. Itioka & T. Itino. 2000. Variations in abiotic defense among myrmecophytic and non-myrmecophytic species of *Macaranga* in a Bornean dipterocarp forest. *Ecological Research* 15: 1-11.
- Nomura, M., T. Itioka & K. Murase. 2001. Non-ant anti-herbivore defenses before plant-ant colonization in *Macaranga* myrmecophytes. *Population Ecology* 43: 207-212.
- Tompson, J. N. 1994. *The Coevolutionary Process*. The University of Chicago Press, Chicago.
- Quek, S. P., S. J. Davies, T. Itino & N. E. Pierce. 2004. Codiversification in an ant-plant mutualism: stem texture and the evolution of host use in *Crematogaster* (Formicidae: Myrmicinae) inhabitants of *Macaranga* (Euphorbiaceae). *Evolution* 58: 554-570.
- Whitmore, T. C. 1969. First thoughts on species evolution in Malayan *Macaranga* (Studies in *Macaranga* III). *Biological Journal of the Linnean Society* 1: 223-231.
- Whitmore, T. C. 1975. *Macaranga*. pp 140-159 In: Shaw HKA (ed) *The Euphorbiaceae of Borneo* (Kew Bulletin Additional Series 4). The Royal Botanic Garden, London.

