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Host-plant dissections reveal contrasting distributions of *Crematogaster* ants and their symbionts in two myrmecophytic *Macaranga* species

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Abstract. 1. Ant–plant mutualisms are among the most widespread and ecologically important insect–plant interactions in the tropics. The multitrophic mutualism involving *Macaranga* plants (Euphorbiaceae) and *Crematogaster* ants (Formicidae) is the most diverse in Southeast Asia. This interaction also includes trophobiotic scale insects (Coccidae) and nematodes inhabiting ant refuse piles.

2. Here two myrmecophytic systems were compared, *Macaranga trachyphylla* with *Crematogaster captiosa* (*Mt* + *Cc*) and *Macaranga beccariana* with *Crematogaster decamera* (*Mb* + *Cd*), using a fine-scale dissection of the stems. For the two plant species, for each internode, both contents (ants, coccids, refuse piles) and structure (internode height, numbers of open and occluded ant holes) were recorded.

3. There were significant patterns in the vertical distribution of ant colonies and their symbionts in the plant stems. Most coccids were kept in the highest sections of both systems, although *Mb* + *Cd* hosted a broader range of coccid species than *Mt* + *Cc*. Three nematode species were recorded, but with a rather low specificity to plant or ant species. Furthermore, the fine-scale distribution showed aggregation of closed holes with ant brood and separation of nematode-infested refuse piles from eggs.

4. The results of this study indicate that ants manipulate spatial colony structure via distribution of brood, holes and the symbionts. It is suggested that ants optimise the location of refuse piles and occluded holes via spatial heterogeneity in their distribution among internodes. This paper discusses the protective role of occluded holes and demonstrates some general interactions with other symbiotic fauna.

Key words. Co-occurrence, mutualism, myrmecophytes, nematode, scale insect, symbiosis.

Introduction

Plant–insect mutualisms are widespread and ecologically important (Bronstein *et al.*, 2006). In particular, interactions of ants with plants are extremely common in tropical forests, where they involve a large proportion of woody species (Schupp & Feener, 1991), including canopy trees (Klimes, 2017). Plant species that have the strongest mutualistic relationships with ants (myrmecophytes) span a high diversity of tropical lineages (Davidson & McKey, 1993). One of those, *Macaranga* (Euphorbiaceae), is a species-rich genus of tropical Old World pioneer trees that play an important role in forest regeneration due to their fast growth. They are found in riparian areas and gaps in primary forest, but even more abundantly in logged forest and oil palm plantations (Slik *et al.*, 2003; Whitmore, 2008; Fiala *et al.*, 2016). Approximately 30 species of *Macaranga* (Davies *et al.*, 2001; Bänfer *et al.*, 2004) are obligately associated with a group of ant species from the *Crematogaster borneensis* group (Formicidae). Each of these species of *Crematogaster* associates with either one or several different *Macaranga* species, and

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almost all myrmecophytic species of *Macaranga* are associated with up to three species of *Crematogaster* (Feldhaar *et al.*, 2016).

These specialist *Crematogaster* ants defend *Macaranga* trees against herbivores and from encroachment by vines by intensively biting them (Fiala *et al.*, 1994; Federle *et al.*, 2002). In exchange, plants provide a variety of benefits to the ant: nesting places (domatia) inside hollow stems (or stems that can be hollowed out by the ants), extrafloral nectar, and lipid-rich food bodies produced in recurved stipules or on leaf surfaces (Fiala *et al.*, 1994; Davies *et al.*, 2001). Other symbionts are an integral part of the system. Inside the stem, the ants cultivate scale insects, in particular *Coccus* species (Hemiptera: Coccidae), which are largely restricted to this myrmecophytic system and provide honeydew to the ants (Heckroth *et al.*, 1998, 2001). Recently, bacteria-eating nematodes have been shown as additional frequent associates that live inside ant nests in *Macaranga* (Maschwitz *et al.*, 2016). However, the function, diversity, distribution, and specificity of these various symbiont assemblages within these ant-plants are still largely unknown. Here we explore differences and similarities in the symbiont communities and the structural functioning of the ant colonies in two highly species-specific *Macaranga*–*Crematogaster* systems in Southeast Asia.

At our study site, the two plant species studied here, *Macaranga trachyphylla* Airy Shaw and *Macaranga beccariana* Merr. each have single ant partners: *Crematogaster captiosa* Forel and *Crematogaster decamera* Forel, respectively. However, the ants are not restricted to these *Macaranga* species. *Crematogaster captiosa* has also been found in four other species of *Macaranga: M. angulata* S.J. Davies, *M. glandibracteolata* S.J. Davies, *M. bancana* (Miq.) Müll. Arg., and *M. hullettii* King ex Hook. f., whereas *C. decamera* has been recorded from four additional species (which do not overlap with those inhabited by *C. captiosa*): *M. constricta* Whitmore & Airy Shaw, *M. havilandii* Airy Shaw, *M. hypoleuca* (Reichb. f. & Zoll.) Müll. Arg., and *M. motleyana* (Müll. Arg.) Müll. Arg. (Feldhaar *et al.*, 2016). Although both plant species studied here belong to the section *Pachystemon* s.s. of *Macaranga*, they are not closely related (Davies *et al.*, 2001; Bänfer *et al.*, 2004) and differ substantially in leaf and stipule appearance. *Macaranga beccariana* produces food bodies on the underside of its young leaves, whereas *M. trachyphylla* produces them on the abaxial surface of stipules. However, both are true myrmecophytic species (Fiala & Maschwitz, 1992a,b) with production of food bodies, extrafloral nectar and self-hollowing domatia. Both ant species provide similar defensive services to the two *Macaranga* species, and have comparable nesting behaviour in which they dynamically open and plug holes in the stem and build up refuse piles in which nematodes are found (Maschwitz *et al.*, 2016). However, due to the high level of specificity of the ant species to different plant species, there might be differences in the symbionts present, and hence also in the spatial use of the plant internodes (i.e. of the hollow sections formed between the externally visible stem nodes) by both ants and these symbionts. Previous surveys of ants and coccids in the *Macaranga* system have only been conducted at the level of whole plants and did not compare the individual internodes (see Heckroth *et al.*, 1998; Itino *et al.*, 2001). Hence, the within-plant distribution of ants, coccids, and other aspects of the system remain unknown.

Here, for the first time, we assess symbiont distribution at the internode scale. For practicality, we focus specifically on the internal structure of young trees. For each internode, we measure the height, record the presence of the different developmental stages of ants (eggs, larvae, pupae, workers and dead/live queens) and coccids (nymphs and adults), and the presence of refuse piles and open or occluded holes. Our novel approach is intended to provide more information on the structural specificity of each partner in the mutualism, and to reveal how ant colonies and their symbiotic communities self-organise inside the plant stem. We expect that the distribution of ant workers, brood, coccids, refuse piles, and open and occluded holes (hereafter referred to collectively as 'elements' of the symbiosis) will differ vertically between the two ant–plant systems, because the two plant species are not the most closely related and are colonised by different ant species. Furthermore, we assess distributions of symbiotic coccid and nematode assemblages found in the two systems to estimate their degree of host specificity. We also expect that the co-occurrence of the different elements will be non-random at the internode scale; they should reveal structuring patterns that relate to functional aspects of these elements.

Materials and Methods

Study site

Field sampling was conducted in undisturbed rainforest in the Batu Apoi Forest Reserve near to Kuala Belalong Field Studies Centre (KBFSC) in the Temburong district of Brunei, Borneo (N 04°32′50″, E 115°09′30″, ~30 m above sea level). The region has a perhumid equatorial climate with average annual rainfall > 4000 mm and monthly mean air temperatures of approximately 26 °C in all months (Cranbrook & Edwards, 1994). KBFSC is situated in lowland dipterocarp rainforest with emergent trees of up to 62 m (Ashton, 1964; Poulsen *et al.*, 1996). The surrounding area consists of deeply dissected gullies and ridges with steep slopes and silty clay soils. *Macaranga*, locally referred to as mahang, are common in disturbed habitats. Eight species of *Macaranga* commonly occur near KBFSC, including our two study species, *M. beccariana* and *M. trachyphylla* (Cranbrook & Edwards, 1994), and all are myrmecophytic (Bänfer *et al.*, 2004).

Sampling methods

Plants of the studied *Macaranga* species can reach 10–18 m in height. In order to access and study whole stems, we sampled only young understorey plants ranging from 50 to 335 cm. A complete census of the contents of ant nests in 23 entire plants was made in August 1995 by PJG (11 of *M. beccariana* and 12 of *M. trachyphylla*), which were chosen randomly in a 1-km^2 area, with 10 m minimal distance between plants. For each plant, the following parameters were measured: (i) total

trunk height along the main stem; (ii) number of side branches and their lengths; (iii) number of hollow internodes, including those in branches; and (iv) length of every internode. Each harvested plant was held horizontally and taken immediately to the KBFSC laboratory, where internodes were cut open progressively from the base of the plant, and the various elements relating to the mutualism in every internode were recorded. The internode chambers narrow at the nodes and are usually separated from each other, minimising movement of ants and other colony contents between sections after collection and dissection. The occurrences of coccids, live and dead queen ants (dealate and presumably fertile in both cases), ant brood (eggs, larvae and pupae), ant workers, refuse piles and open or occluded exit holes were recorded for each internode chamber. In addition, individual counts were made for each internode of the numbers of exit holes (open and occluded), adult and nymphal coccids, and refuse piles (Appendix 2 and 3). The occasional cases of alates were excluded from analyses (Appendix 2). All ant nests contained refuse piles, most of which contained nematodes. These piles were scraped from the inner surfaces of the chambers of ant nests inside the 23 host plants and preserved in separate, labelled vials of formalin, 70% ethanol or a mixture of formalin and ethanol (one sample per plant individual). These samples were later slide-mounted and identified by specialists (see Acknowledgements). Quantitative analysis of the occurrence of nematode species in each plant stem could not be made because of the poor preservation of some samples and the immaturity of most nematodes. Therefore, species identification of nematodes is provided hereafter only at plant species level (Appendix 1), not for plant individuals as done for coccids and ants (Table 1). The scale insects (all coccids) present in each plant stem were preserved in lactic-alcohol (Upton, 1991) for mounting on microscope slides to allow identification to species (i.e. for each plant individual). The slide-mounting was based on the protocol of Williams and Granara de Willink (1992), except that xylene was used instead of clove oil. Identifications were made using keys in Morrison (1921) and Hodgson (1994), and PJG's voucher collection from the only known former study (Heckroth *et al.*, 1998). Ant brood, workers, and queens from each individual plant were preserved in separate vials of 90% ethanol for identification to species, which was done using keys in Feldhaar *et al.* (2016). Voucher specimens of coccids and ants are held by PJG and will be deposited in the Australian National Insect Collection, CSIRO, Canberra, Australia. We refer to the plant–ant partnerships of *M. beccariana* + *C. decamera* and *M. trachyphylla* + *C. captiosa* as *Mb* + *Cd* and *Mt* + *Cc*, respectively.

Data analysis

For vertical distribution and multivariate analyses we consider only the occurrence of each element of the symbiosis, and we tested their responses as the presence/absence in each internode (see later). However, for analyses of mean differences per plant, we also report means of counts per internode, wherever possible (see Table 1). Average occupancy rates (per internode) for all elements between the two systems were compared using *t*-tests. Trees were similar in their architecture in both species and usually lacked side branches, except for two trees of *M. trachyphylla* and three of *M. beccariana* (Fig. 1). These branches were not considered in the analysis of vertical sections and co-occurrences, where our focus was on the main stems for comparability, but they were included in all other univariate and multivariate analyses which look at total insect abundances and total plant lengths, including branches.

Table 1. Species composition of symbiotic associates in the two *Macaranga* species; values are the number of plants of each species that contained each associate (23 individuals were dissected in total).

		Macaranga beccariana ($n = 11$)	*Macaranga trachyphylla* ($n = 12$)
Ants	*Crematogaster decamera*	11	0
	Crematogaster captiosa	0	12
Nematodes	*Diploscapter lycostoma*	p	p
	Rhabditonematinae sp.	p	p
	Dolichorhabditis dolichura	a	p
Coccids	*Coccus macarangae*	8	5
	Coccus penangensis	5	5
	Coccus secretus	2	2
	Coccus sp. 'pseudo'	1	6
	Myzolecanium sp.	3	0
	Coccus sp. 42	1	0
	Coccus sp. Q	1	0
	Coccus nr *formicarii*	1	0

p, present; a, absent.

Comparing within-internode spatial distribution patterns across different plant height sections

From preliminary inspection of the data, we decided that five height sections would adequately capture the variation in response variables related to the section of the tree analysed, while retaining sufficient sample size within each section. The five sections per tree were based on total number of internodes rather than relative stem heights (as internodes differed in length; see Fig. 1), with each section representing 20% of the total number of internodes for that plant. Numbering started from the base of the tree, i.e. from the first (bottom) section, up to the tip of the tree as the fifth (last) section. For each section, we calculated the overall proportion of occupancy (of ants, coccids, brood, etc.) based on the total number of internodes occupied divided by total number of internodes. This enabled us to compare all response variables both among sections within one plant species, and between the two species. One-way ANOVA with *post hoc* Tukey honest significant difference tests were performed to compare occupancy of different sections. For each response variable, *t*-tests were used to compare sections of the same level between different plant species.

Co-occurrence between elements of symbiont community structure

Analyses using vertical distributions across plant sections may not reveal patterns at the level of individual internodes.

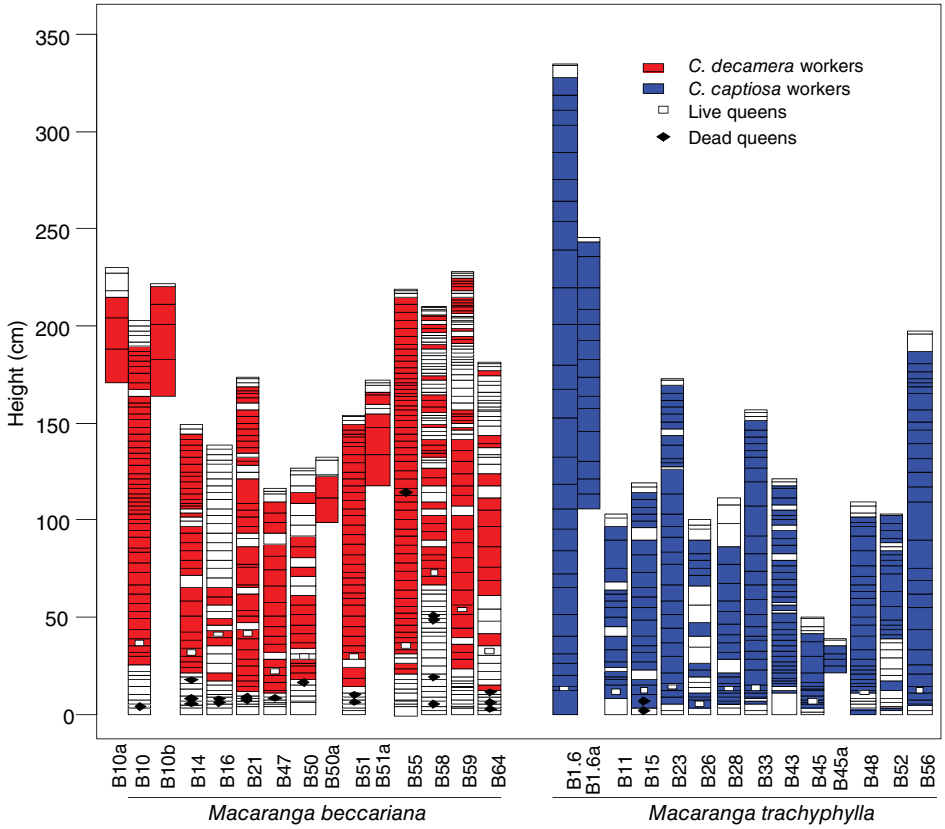

Fig. 1. Diagram visualising the sampled plants and their individual internodes and branches, with plant and internode heights on the *x*-axis. Lateral branches are represented vertically for clarity and are denoted with the main tree number and "a" or "b". Ant workers are in red (*Macaranga beccariana* + *Crematogaster decamera* mutualistic system) or blue (*Macaranga trachyphylla* + *Crematogaster captiosa* system) and living and dead dealate queens (gynes) are marked with squares and black diamonds, respectively. The *y*-axis shows each plant's unique code, referring to the collector's (PJG) voucher numbers. [Colour figure can be viewed at wileyonlinelibrary.com].

Hence we also used null model species co-occurrence analyses on all pairs of elements, using each internode as a unit. We tested whether pairs of elements co-occurred more or less frequently than expected at random (Ellwood *et al.*, 2016). The different elements of ant–symbiont community structure were considered to co-occur when found in the same internodes. We analysed co-occurrence separately for each *Macaranga* species. Each analysis was based on a presence/absence matrix where each internode is a row and each column is one of the factors. For each element, we calculated the number of realised co-occurrences as the number of times it co-occurred with any other element in the same internode. To test whether this observed pattern differed from what would be expected if the two elements were independently distributed in the plant, a null model was run. The occurrences of each element were randomly assigned to the different internodes, such that the total number of occurrences per element equalled those in the original matrix, but each internode had the same probability of being assigned an element occurrence (i.e. the fixed-equiprobable algorithm sensu Gotelli, 2000). We then performed 1000 randomisations per analysis, and compared the real number of co-occurrences with this distribution of randomised values. The aggregation and segregation quantiles reflect the number of randomised values falling in either the upper or lower tail (both tails: $\alpha < 0.025$) of the number of randomized values. Segregation indicates that there were fewer associations between a pair of elements than expected, and aggregation that there were more associations. This analysis, based on the checker-board concept (Gotelli 2000), only focuses on the location of each element and does not account for lack of independence of adjacent internodes, and so we interpret our results with caution. The analyses should be considered as supplementary to our vertical section analysis, which tests for vertical patterns at larger scales.

Variation in coccid assemblages and their drivers

To test for differences between the coccid assemblages of the two *Macaranga* species and also their response to the other environmental predictors (host-plant and ant-colony traits) we used multivariate analyses in CANOCO ver. 5 (Šmilauer & Lepš, 2014). We compiled a matrix of occurrence of eight coccid species across 23 plant individuals as samples (Appendix 3). As coccid assemblage turnover was relatively high (detrended correspondence analysis gradient length = 3.47) and data were presence/absence, a unimodal constrained ordination method

was used (Šmilauer & Lepš, 2014). We conducted two analyses: (i) the effects of multiple predictors measured for each plant (numbers of occluded and open holes per internode, proportions of internodes with ants and brood, total plant length, i.e. height including length of branches, and plant species identity) were analysed simultaneously, and forward selection with permutation of all rows and columns in the matrix was performed to test which predictors were significant (999 permutations, $P < 0.05$ at first axis); and (ii) the effect of plant species alone was analysed (with the same permutation procedure). Analyses were conducted both with and without singletons (three of eight species were singletons) to test for effects of rare species.

Data availability

Data for main variables per plant individual are provided in Appendices 2 and 3. Original full data per internode will be available from the Dryad Digital Repository (doi:10.5061/dryad.8qr6qf4).

Results

Species composition of the mutualistic associations of the two Macaranga *species*

As expected, the ant species *C. decamera* and *C. captiosa* were exclusive to *M. beccariana* and *M. trachyphylla*, respectively. This was not the case for nematodes and coccids, where species compositions (at least partly) differed (Table 1, Appendix 1).

Plant stem architecture. The average height of trees did not differ between plant species (*t*-test, $P = 0.13$, d.f. $= 16.39$, $t = 1.598$; *M. beccariana*: $n = 11$, mean $= 177.2$ cm, SE $= 12.2$; *M. trachyphylla:* $n = 12$, mean $= 139.8$ cm, SE $= 20.9$) but the internode length was greater in *M. trachyphylla* (*t*-test, $P = 0.042$, d.f. $= 12.049$, $t = -2.274$).

Ants. Even though worker occupancy of internodes was higher in *Mt* + *Cc*, proportions of internodes occupied by ant eggs, larvae, and pupae did not differ between species (Table 2). However, when these developmental stages were combined under one variable 'ant brood', occupancy was also greater in *Mt* + *Cc* (Table 2).

Scale insects (Coccidae). The species richness of the coccids ranged from one to four species per plant, with a total of eight species recorded (Table 1, Fig. 2). Although the ratio of adult coccids to nymphs showed high variability between trees (min $= 0.06$, max $= 22$), there were, on average, more nymphs than adults, but the abundances did not differ between the two systems (Table 2, *t*-test, $P = 0.33$; d.f. $= 11.07$; t $= -1.0153$). Half of the coccid species were found in both plant species, whereas the remainder were exclusive to *Mb* + *Cd*. However, three of the four coccid species unique to *Mb* + *Cd* were singletons. The fourth species was from a different coccid genus, *Myzolecanium*, an interaction not previously observed in *Macaranga* (cf. *Coccus* associated typically with *Macaranga*).

Table 2. Mean values of each element of the symbiosis for the two mutualistic systems.

Element of symbiosis	*Macaranga beccariana*	*Macaranga trachyphylla*	*t*-test
Proportions of internodes occupied			
Ant workers	0.53	0.75	**
All ant brood	0.27	0.37	*
Ant eggs	0.09	0.09	n.s.
Ant larvae	0.24	0.26	n.s.
Ant pupae	0.16	0.24	∼
Average number per internode			
Coccid adults	0.79	0.7	n.s.
Coccid nymphs	2.73	3.8	n.s.
Refuse piles	0.24	0.64	**
Occluded holes	0.79	0.98	n.s.
Open holes	0.51	0.7	n.s.

Significant differences (according to *t*-tests) are indicated as follows: ∼$P < 0.1$; *$P < 0.05$; **$P < 0.01$. n.s., not significant.

Refuse piles and nematodes. In freshly opened chambers, the refuse piles were dark brown in colour and each pile was usually 5–10 mm in diameter. Chambers with multiple refuse piles were rare in both species. The percentage of internodes with at least one pile was significantly greater in *Mt* + *Cc* (20–69%) than in *Mb* + *Cd* (11–33%, Table 2). These piles appeared to be composed of fine particulate matter and plant fibres, with occasional fungal hyphae and remains of ants. Cuticular remains of coccids were not observed. Almost all refuse piles swarmed with nematodes. Only three of the trees had no nematodes in the preserved samples examined and some trees had up to three species, among which *Dolichorhabditis dolichura* (Schneider) (Rhabditidae) was found only in *Mt* + *Cc*. Unfortunately, as some samples were not well preserved, we could not test statistically for nematode assemblage differences between the two plant species as we did for the coccids (data in Appendix 1). Nevertheless, the same two species, *Diploscapter lycostoma* Völk (Rhabditidae s.l. or Diploscapteridae) and an undetermined species of subfamily Rhabditonematinae (Rhabditidae), were the most common in both symbioses (Table 1).

Comparing within-internode spatial distribution patterns across height sections

Except for overall occupation (Table 2) and the first section (the first section being closest to the ground, the fifth section being at the top of the plant), *Mb* + *Cd* and *Mt* + *Cc* showed similar distributional patterns for ant workers (Fig. 3), with a gradual increase in internode occupancy that reached a maximum halfway up the stem, i.e. around the third or fourth height section out of five. A single dealate (wingless) queen was found in all trees, except for two *M. trachyphylla*, in which no queens were found (Fig. 1). Queen location (height) differed

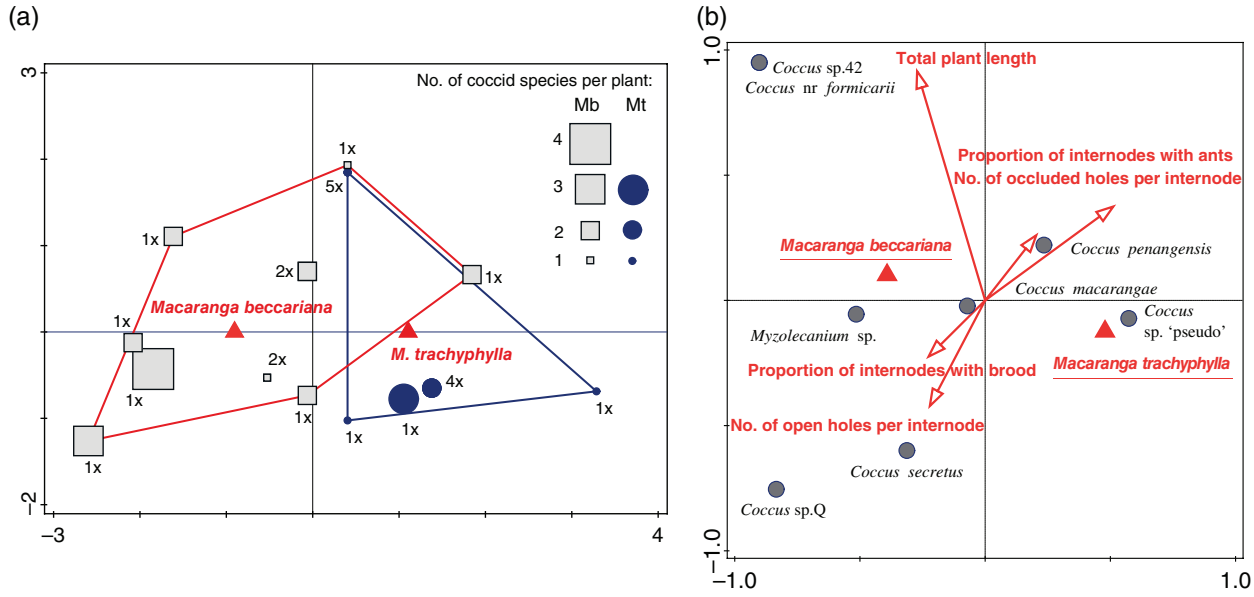

Fig. 2. Constrained ordination method diagrams demonstrating the effects of the explanatory variables on distribution of coccid assemblages in plant individuals of two *Macaranga* species: (a) sole effect of plant species; (b) effects of all variables (see Methods for more details). Variation in coccid species is related to the first two ordination axes, with environmental predictors in red, as triangles, and with arrows representing continuous predictors. In (a) the symbols show the individual plants [*Macaranga beccariana* (Mb) + *Crematogaster decamera* system, squares; *Macaranga trachyphylla* (Mt) + *Crematogaster captiosa* system, circles] and are sized according to increasing coccid species richness (1–4); the polygons visualise differences between the coccid assemblages of the two plant species (9.1% variability explained by the first two axes, pseudo-*F* first axis = 2.1, $P = 0.02$). The number by each symbol refers to the number of plants with the same coccid species richness. In (b) individual coccid species are represented by circles, and predictors that were significant in forward selection are underlined (31.6% variability explained by the first two axes in total; pseudo-*F* first axis = 2.9, $P = 0.17$). Note that only the plant species effect was significant ($P = 0.04$) in (b). [Colour figure can be viewed at wileyonlinelibrary.com].

between the systems (*t*-test, $P < 0.001$; d.f. = 11.07; $t = -6.33$) with *C. decamera* queens occupying higher internodes (Fig. 1). The most pronounced difference between species and height sections was for refuse piles (Fig. 3d), with *Mb* + *Cd* always having lower occupancy, in agreement with the plant-level analysis (Table 2). For most elements of the symbiosis, either the first and/or fourth sections contrasted the most between the two plant species (Fig. 3a–d). Notably, the fourth section of *Mt* + *Cc* had higher internode occupancy for ant workers, ant brood, and refuse piles compared with *Mb* + *Cd*. In the first section, *Mb* + *Cd* also displayed lower occupancy for ant workers, fewer refuse piles and fewer open holes compared with *Mt* + *Cc*. *Crematogaster captiosa* kept brood in the first four stem sections, whereas *C. decamera* concentrated its brood in the second and third sections (Fig. 3b). Ant larval occupation was significantly higher in the second section of *M. beccariana* than in *M. trachyphylla*. *Crematogaster captiosa* maintained its eggs and queen mainly in the first section, whereas *C. decamera* used both the first and second sections (Fig. 1 and Appendix 4). Interestingly, *Mb* + *Cd* regularly had dead queens in at least one internode of each tree, typically in the lowest part of the plant, but this occurred only in a single tree for *Mt* + *Cc* (Fig. 1).

For coccids, the same trends were observed for both mutualist systems regardless of the section and plant species observed (Fig. 3c). In both systems there was a general increase in coccid internode occupancy that levelled out at the third section and remained similar above that.

Occluded and open holes showed very similar patterns in the two plant–ant systems (Figs. 3e,f). Nevertheless there was a more distinct increase in the number of occluded holes between the first and second sections in *Mb* + *Cd* (not significant in *Mt* + *Cc*). The difference for open holes was significant between systems, although only in the first stem section (Fig. 3f), with *C. decamera* maintaining fewer open holes.

Comparing co-occurrence patterns at the scale of individual internodes

There were similar within-internode co-occurrence patterns in the two plant species. Ant workers were aggregated with all other elements (Table 3). Other aggregations were observed between developmental stages, such as between ant larvae and eggs and between coccid adults and nymphs. However, ant pupae and eggs were not aggregated in *Mb* + *Cd*. The main aggregation difference between the mutualist interactions was found for refuse piles, which in *Mt* + *Cc* were aggregated with coccid nymphs and adults, open and occluded holes and ant workers, but in *Mb* + *Cd* this was only the case for open holes and ant workers. Ant eggs were segregated from refuse piles in both systems, but ant larvae were segregated from refuse piles only for *Mb* + *Cd*, this being mainly driven by differences in vertical stratification (Appendix 4). In the two systems, open and occluded holes showed opposite patterns in relation to ant brood: occluded holes were aggregated with ant brood (eggs, larvae,

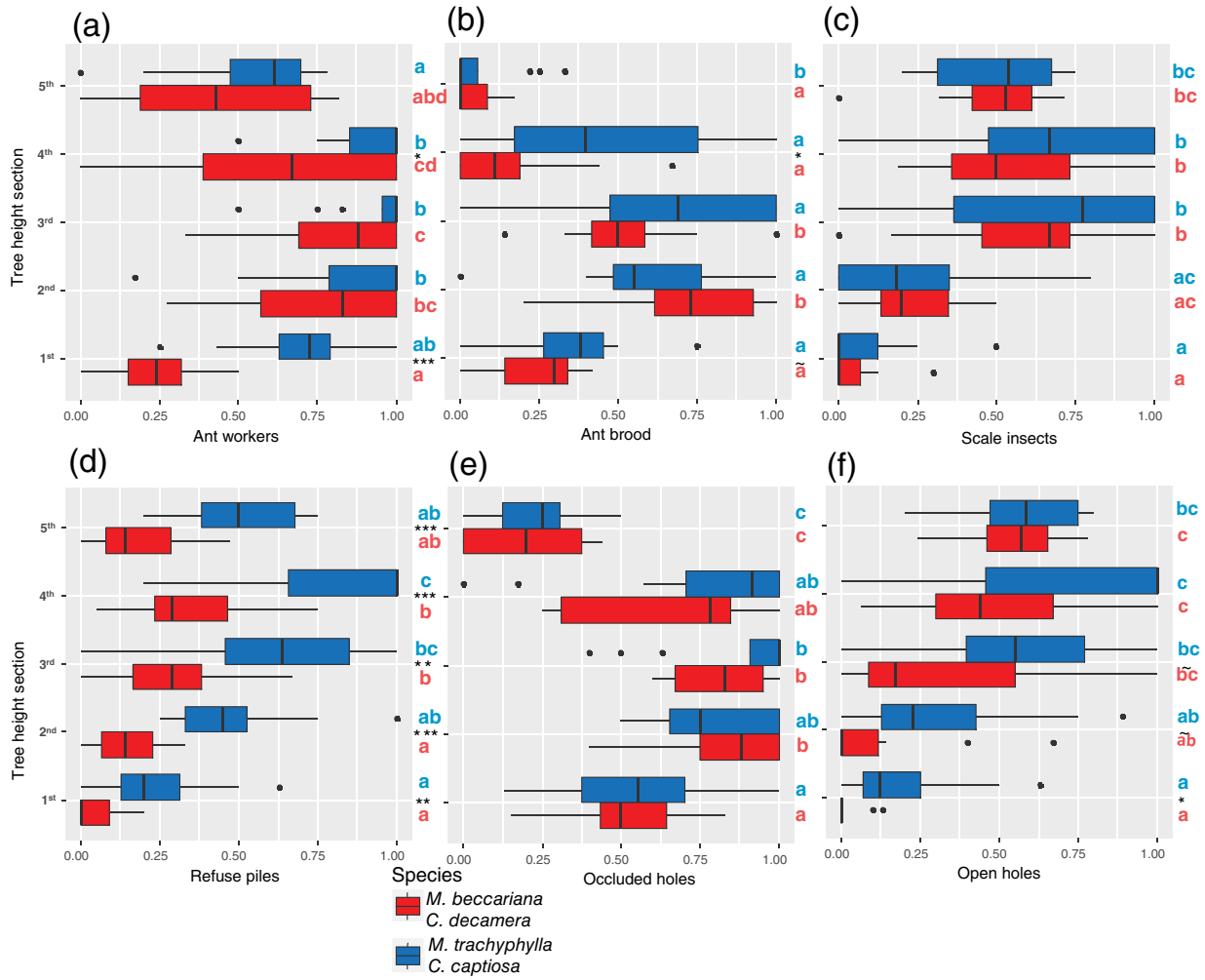

Fig. 3. Proportion of internodes occupied (on *x*-axis) for each height section (*y*-axis, from first at the bottom to fifth at the top) of the two *Macaranga* species for: (a) ant workers; (b) ant brood (eggs, larvae and pupae); (c) coccids (nymphs and adults); (d) refuse piles; (e) occluded holes; (f) open holes. Significant differences (*t*-tests) between plant species are indicated as follows: $\sim P < 0.1$; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Plots with the same letters within each plant–ant system are not significantly different according to Tukey honest significant difference. [Colour figure can be viewed at wileyonlinelibrary.com].

pupae), and open holes were segregated from most of the brood, except for pupae in *Mb* + *Cd* where they co-occurred randomly (Table 3). Surprisingly, in spite of segregation between open and occluded holes in *Mb* + *Cd*, both types of holes were found aggregated to coccid adults. However, open and occluded holes were not segregated in *Mt* + *Cc* and closed holes were not found to be aggregated with coccid adults (Table 3). The only completely opposite trend between mutualist interactions was for coccid nymphs, which were segregated from occluded holes in *Mb* + *Cd* but aggregated in *Mt* + *Cc*.

What are the drivers of coccid community assemblages, and are coccids specific to the mutualistic interactions?

The most common scale insect species across all plant individuals was *C.macarangae* (in 13 plants). The effect of plant species on the composition of the scale insects was significant, if assessed as the sole effect (Fig. 2a), and was also marginally significant when other predictors were included (Fig. 2b). None of the additional predictors tested was significant in forward selection, although there was a trend for some coccid species to occur in the larger plants, while others occurred in plants with relatively more occluded or open holes (Fig. 2b). The statistical power of the plant species was driven mainly by the singletons, however, as after their exclusion the effect was not significant (first axis pseudo-$F = 2.4$, $P = 0.052$).

Discussion

This study provides the first fine-scale dissection of *Macaranga* plants to document the distribution of the ants, refuse piles and coccids inside each internode, as well as the location of open and occluded holes made by the ants.

Table 3. Null model co-occurrence analyses of different elements involved in the mutualistic relationships.

	Brood: eggs	Brood: larvae	Brood: pupae	Ant workers	Refuse piles	Coccid adults	Coccid nymphs	Open holes	Occluded holes
Brood: eggs		A	**A**	A	S	**S**	S	S	A
Brood: larvae	A		A	A				S	A
Brood: pupae		A		A			**A**	**S**	A
Ant workers	A	A	A		A	A	A	A	A
Refuse piles	S	**S**		A		**A**	**A**	A	**A**
Coccid adults			**A**	A			A	A	
Coccid nymphs	S			A		A		A	**A**
Open holes	S	S		A	A	A	A		
Occluded holes	A	A	A	A		**A**	**S**	**S**	

Macaranga trachyphylla–Crematogaster captiosa (Mt + Cc)

M. beccariana–C. decamera (Mb + Cd)

A, pairs of elements that were significantly aggregated; S, pairs of elements that were segregated. An empty cell indicates that the pattern of co-occurrence did not differ from random. Green bold letters represent differences between the two systems and bold underlined purple letters represent opposing results. For details on the model see Methods. [Colour table can be viewed at wileyonlinelibrary.com].

Ant spatial occupation of the stem

Ant workers were found to aggregate with all other elements of the symbiosis that we quantified in the trees (Table 3). This was to be expected, as the workers actively interact with their brood, the coccids, the refuse piles and the holes of their nest environment. A previous study (which included both of our *Macaranga* study species) found that in trees taller than 2 m, the most ant-occupied part of *Macaranga* is in the upper section of the stem (Itino *et al.*, 2001). From our detailed dissections we found that in both *Macaranga* species most ants are found in the mid-section and the neighbouring sections (second and fourth sections) of the stems; however, our analyses covered trees shorter than 2 m as well. Internode occupation by ant workers and brood was much higher in *Mt + Cc* system than in *Mb + Cd* in general (Table 2), which agrees with the higher biomass of ants previously found in *M. trachyphylla* (Itino *et al.*, 2001) and could explain the higher number of internodes occupied by refuse piles. In *Mt + Cc*, the occupation of ant workers was higher in the first and fourth sections than was the case in *Mb + Cd*. This is partly paralleled by the distribution of the brood, which is also higher for *Mt + Cc* in the fourth section, although differences for brood are only marginal in the first section. It therefore seems that there are differences between species in the way workers manage their brood in this vertically structured habitat. Broadly we concur with the Itino *et al.* (2001), who found that *C. decamera* maintain their queens in the lowest part of the tree. Similar findings on queen position have been reported in other *Macaranga* and *Crematogaster* species (Feldhaar *et al.*, 2003). Yet on a finer scale we demonstrated differences: while *C. captiosa* maintains most of its eggs and queens in the first stem section, more larvae and queens of *C. decamera* are found in the second section. When considering size of these young trees, this strategy of *C. captiosa*, which is maintaining the most important part of the colony (queens and eggs) in the lowest section, may increase survival of the ant colony, as the thickest part of the tree is the least likely to break or be attacked. Another possibility is that queens are more successful in colonising trees in *C. captiosa* and establish colonies earlier, and therefore are found at lower levels of the tree. But because of the snapshot way in which we sampled these trees, we are not able to control for late colonisation or recolonisation after potential first colonisation failures. Hence we are unable to conclude that colonisation attempts were more successful in *Mt + Cc*, even though more dead queens were observed in *Mb + Cd* (Fig. 1). This could also be the result of higher worker occupancy in the first section of *Mt + Cc* (where most dead queens were found), as these workers could have disposed of any dead queens that were previously present. Note that most of the trees we sampled housed monogynous (single queen) ant colonies (in two trees queens were not found), confirming previous observations that *C. decamera* and *C. captiosa* colonies only have one fertile queen, at least when trees are small. However, when trees are larger (> 10 cm diameter at breast height), colonies have been shown to become secondarily polygynous (Feldhaar *et al.*, 2000).

Coccid distribution and specificity

Coccid abundance and distribution both per internode and per plant section did not differ between the two *Macaranga* species (Table 1, Fig. 3c), even when adult and nymphal coccids were considered separately (Appendix 5). The coccid species found in *Mt + Cc* formed a subset of those found in *Mb + Cd*. Our results thus suggest that *C. captiosa* is stricter (more specialised) in its mutualistic coccid partners, or that *M. beccariana* is a more suitable host for some coccids than *M. trachyphylla*. However, sampling across more than one locality is needed to test such hypotheses. The fact that the coccid assemblage of *Mb + Cd* had more unique species supports the possibility of differences in the functional use of coccid nymphs depending on the plant–ant system (Itino *et al.*, 2001). Only one other study to date has thoroughly examined the coccid species assemblages

of *Macaranga* (Heckroth *et al.*, 1998). This showed similar species assemblages to the ones reported from our studied plants with *C. macarangae* Morrison, *C. penangensis* Morrison and *C. secretus* Morrison being host-plant generalists, present in both *M. beccariana* and *M. trachyphylla*, as well as other *Macaranga* species. We also found one non-*Coccus* species, an undescribed species of *Myzolecanium*, in three *Mb* + *Cd* individuals. To our knowledge this is the first record of this genus occupying *Macaranga. Myzolecanium* is widespread though in Australia and New Guinea, where its species typically occur inside hollow branches or trunks of a wide taxonomic range of other plants and appear to be associated facultatively with a high diversity of ant genera (Gullan *et al.*, 1993; Klimes & McArthur, 2014).

It is well known that coccids in the *Macaranga* provide carbohydrates to ants in the form of honeydew (Heckroth *et al.*, 2001; Handa & Itioka, 2011). In addition, Itino *et al.* (2001) suggested that a population distribution skewed to adult coccids is a sign of predation by the ants on coccid nymphs. However, these authors only mentioned this distribution, but did not provide the data on different coccid life stages (or any direct evidence for predation). Based on the study (Itino *et al.*, 2001), we were expecting the abundance of coccids to differ between systems as ants in *M. beccariana* were reported to rely more on coccids as a food resource (rather than food bodies). However, we found rather similar coccid ratios in both systems, and only a few adults, compared with many nymphs in general (i.e. the opposite pattern; Table 2). The ratio of coccid nymphs to adults also varied between tree individuals, indicating other possible influences, such as varying reproduction rates of different coccid species. A study by Heckroth *et al.* (2001) found that the ants living in *M. hypoleuca* with *C. tumuliferus* Morrison, even when starved, never ate the coccids in their nests. We found ant remains but no coccid remains in refuse piles, suggesting no apparent consumption of the coccids in these two systems. Our study therefore supports the idea that no coccid predation occurs in *Mb* + *Cd* or *Mt* + *Cc*. Further evidence is needed to broaden this aspect to all *Macaranga*–*Crematogaster* systems, as ant diets may differ depending on the system involved (M.Y.I. Houadria, unpublished).

Nematode specificity in Macaranga

A recent study has shown that each refuse pile found inside the stems of certain *Macaranga* species, including *M. trachyphylla*, can contain over 3000 myrmecophilous nematodes (Maschwitz *et al.*, 2016). These nematodes could be seeded by the ant queens, as they are already present in the first refuse pile produced in claustral nests. The nematodes in the study of Maschwitz *et al.* (2016) were identified as belonging to two new (undescribed) species of the rhabditid genera *Diploscapter* and *Sclerorhabditis* which are believed to feed mainly on bacteria and fungi. Thanks to the support of specialists (see Acknowledgements), we were able to identify two new nematode species for the *Macaranga* system, *Diploscapter lycostoma* and *Dolichorhabditis dolichura*, both of which are known from ant postpharyngeal glands (Markin & McCoy, 1968). This supports the study by Maschwitz *et al.* (2016), which found that 30% of queen alates from *C. captiosa* nests had nematodes in their postpharyngeal glands. However, more direct evidence is still required, in particular from the dissection of foundress queen ants prior to them entering *Macaranga* plants, in order to confirm this mechanism for colonisation of nematodes.

In our study, one species, *D. dolichura*, was occasionally present in refuse piles of *M. trachyphylla* but absent from the refuse piles of *M. beccariana*, a *Macaranga* species not sampled by Maschwitz *et al.* (2016). Future studies should focus on the variability of nematode species among individual plants and internodes, which we could not explore due to the poor preservation of some samples and the immaturity of most of the nematodes. However, our data and those of Maschwitz *et al.* (2016) suggest that some of the nematodes at least are unlikely to be species-specific at the level of *Macaranga*–*Crematogaster* systems. For both coccids and nematodes, future studies could also attempt to identify them to species for each internode to allow more interpretation regarding their distribution inside stems. Molecular techniques (i.e. metabarcoding) might be useful in these respects (Pimm *et al.*, 2014).

Refuse piles as the most contrasting element between the two myrmecophytic systems

The proportion of internodes occupied by refuse piles was the greatest difference between the two mutualistic systems, as, regardless of the stem height section, occupancy was lower in *Mb* + *Cd* than in *Mt* + *Cc*. This might be caused by the higher observed worker and brood occupancy in *Mt* + *Cc*. However, refuse piles presumably also play an important role in the breeding of the nematodes and it has been suggested that the nematodes might be an alternative source of nitrogen for *Crematogaster* ants (Maschwitz *et al.*, 2016). However, whether ants really can feed on nematodes remains unknown. If this were the case, it would be likely that the nematodes would be fed to the ant larvae, as nitrogen intake is most important for them (for development). Nevertheless, the presence of refuse piles might be detrimental to egg development, due to increased risk of pathogen infections. Indeed, in both *Macaranga*–*Crematogaster* systems, eggs were always kept separately from the refuse piles (Table 3), and in *Mb* + *Cd*, larvae were also kept in separate internodes from refuse piles. Overall, it seems that location of refuse piles are more structured *Mt* + *Cc* than is the case with *Mb* + *Cd*. In *Mb* + *Cd* the location of the piles was more random, while in *Mt* + *Cc* they were found to co-occur with open holes and coccid adults and nymphs. Further observation is needed to assess if ants actively transport nematodes to feed larvae, or if nematodes are simply a way of controllinmg the densities of bacteria and fungi.

The functional role of open and occluded holes

Open holes are likely to facilitate ant worker access to internodes, consistent with our finding that open holes co-occurred with ant workers. Also, there was lower worker occupancy and fewer open holes in the lowest parts of *Mb* + *Cd* plants (Fig. 3a,f). Hence there seems to be some detrimental effect of

open holes, as ants actively occlude them and workers were also found in aggregation with occluded holes. However, for both brood and eggs of the two ant species there was clear aggregation with occluded holes, but segregation from open holes. We suggest that occluding holes afford brood some protection from exterior threats such as desiccation, parasitism or even predation. However, this would need to be confirmed by additional experiments that compare survival rates of brood maintained in stem internodes with those found in open and occluded holes.

For the coccids, the relationship with stem holes seems to be more complex. In both mutualistic systems, there was aggregation of coccids with open and occluded holes. Coccids may be brought in by the ants as they land on leaves, or actively enter open holes in the stem (Handa *et al.*, 2012). Subsequently, ants may close those holes to provide protection to the coccids from desiccation or attack from natural enemies. This might also be a way of managing the coccid population per internode. Interestingly, occluded holes were aggregated with nymphs in *Mt* + *Cc*, whereas they were segregated from them in *Mb* + *Cd*, the only analysis where we found opposite tendencies of segregation for the same element between the two systems (Table 3). We tentatively suggest that this could be due to differences in the management of the coccid populations, with *C. captiosa*, but not *C. decamera*, sealing holes to control movement of nymphs. Furthermore, the ants might adapt their reaction depending on the specific coccid species requirements and ecology.

Conclusion

Taken together, our results demonstrate the presence of fine-scale vertical organisation inside host plants for a widespread and ecologically important ant–plant system. Ant worker occupation of internodes differed between the two systems, and coccid assemblages occupied specific sections of the plant regardless of its overall height or ant-plant species. Our study provides evidence of structural organisation in both systems: occluded holes are found more frequently in internodes containing brood, and nematode-infested refuse piles are separated from eggs. This indicates that ants manipulate colony structure via distribution of brood, holes, refuse piles, and the coccids. We confirm that most common coccids in these two systems are generalists, although the ant *C. decamera* has a broader range of mutualistic partners than does *C. captiosa*, suggesting potential differences in the functional use of these symbionts. To further understand the ecology of each specific system, in future studies, symbionts, including ants, should be identified and quantified for each internode, because 'the devil is in the detail'.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Nematodes were not found in three instances; not all piles from a plant were preserved if there were many piles present. For plants for which no nematodes were recorded (B14, B16, and B50), the lack of nematodes may reflect failure to sample all piles

Appendix S2. Overall information on plant structure as well as abundance for male alates and queens. P was noted for presence of queen pupae and larvae. PP was when many individuals were observed without a precise count.

Appendix S3. Presence (P) of different coccid species, number of coccid species, and overall abundance per plant of coccid adults and nymphs

Appendix S4. Proportion of internodes occupied (on *y*-axis) for each height section (*x*- axis: from first at the plant bottom to fifth at the top) of the two *Macaranga* species for: (a) ant eggs; (b) ant larvae ; (c) ant pupae. Significant differences (*t*-tests) between plant species are indicated as follows: $\sim P < 0.1$; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Plots with the same letters within the graphs are not significantly different according to Tukey HSD.

Appendix S5. Proportion of internodes occupied (on *y*-axis) for each height section (*x*-axis: from first at the plant bottom to fifth at the top) of the two *Macaranga* species for: (a) coccid nymphs; (b) coccid adults. Significant differences (*t*-tests) between plant species are indicated as follows: $\sim P < 0.1$; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Plots with the same letters within the graphs are not significantly different according to Tukey HSD.

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