

An ancient tripartite symbiosis of plants, ants and scale insects

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In the Asian tropics, a conspicuous radiation of *Macaranga* plants is inhabited by obligately associated *Crematogaster* ants tending *Coccus* (Coccidae) scale insects, forming a tripartite symbiosis. Recent phylogenetic studies have shown that the plants and the ants have been codiversifying over the past 16–20 million years (Myr). The prevalence of coccoids in ant–plant mutualisms suggest that they play an important role in the evolution of ant–plant symbioses. To determine whether the scale insects were involved in the evolutionary origin of the mutualism between *Macaranga* and *Crematogaster*, we constructed a cytochrome oxidase I (COI) gene phylogeny of the scale insects collected from myrmecophytic *Macaranga* and estimated their time of origin based on a COI molecular clock. The minimum age of the associated *Coccus* was estimated to be half that of the ants, at 7–9 Myr, suggesting that they were latecomers in the evolutionary history of the symbiosis. *Crematogaster* mitochondrial DNA (mtDNA) lineages did not exhibit specificity towards *Coccus* mtDNA lineages, and the latter was not found to be specific towards *Macaranga* taxa, suggesting that patterns of associations in the scale insects are dictated by opportunity rather than by specialized adaptations to host plant traits.

Keywords: *Coccus*; coevolutionary radiation; mitochondrial DNA cytochrome oxidase I gene phylogeny; myrmecophytic *Macaranga*; southeast Asia

1. INTRODUCTION

Symbiosis has been a pervasive theme in the evolution of living systems. Indeed, much of life comprises composite organisms cobbled together from ancient interactions that evolved into obligate partnerships, exemplified at the cellular level by the mitochondria of eukaryotic cells. At higher levels of biological organization, organisms enlist the help of other species in extracting nutrients from the environment, such as the intestinal flora of animals and the fungi cultivated by leaf-cutter ants (Mueller *et al.* 2001). The need to defend against exploitation has likewise elicited creative solutions involving members of other species, as seen in ant-defended plants (Davidson & Mckey 1993) and fungus-defended termite eggs (Matsuura *et al.* 2000). So successful are some of these associations that they have persisted over geological time (e.g. Pellmyr & Leebens-Mack 1999; Machado *et al.* 2001; Mueller *et al.* 2001). The need to simultaneously exploit and protect oneself against exploitation has led to the evolution of intricate webs of associations involving multiple species. It is becoming evident that the long-term persistence of such associations is prevalent not only

in two-party interactions, but also in more complex systems comprising three or more associates, such as the *Attine* ant–fungus–parasite (and microbe) system (Currie *et al.* 2003).

A frequently encountered phenomenon in tropical forests is the association between ants, plants and homopterans (sap-sucking insects). One such tripartite system comprises the obligate association between *Macaranga* trees, *Crematogaster* (subgenus *Decacrema*) ants and *Coccus* scale insects in southeast Asia (figure 1). The plants are protected against vines and herbivores by the ants that, in turn, gain residence in domatia created by hollow stems and food bodies secreted by the plants (Fiala *et al.* 1989). The plants do not survive to maturity without their *Decacrema* ants, and the ants are never found without their *Macaranga* hosts. In addition, the ants are able to further extract nutrients from the plants indirectly via the exudates of *Coccus* scale insects feeding on *Macaranga* sap and cohabiting with the ants in the confines of the *Macaranga* stem domatia (Heckroth *et al.* 1998).

Recent phylogenetic studies have suggested that *Macaranga* plants and *Decacrema* ants have been codiversifying over the past 16–20 million years (Myr; Davies *et al.* 2001; Itino *et al.* 2001; Quek *et al.* 2004, 2007). The host plant assemblage comprises 26 *Macaranga* species in two closely related clades (Blattner *et al.* 2001; Davies *et al.* 2001) and the ants fall into a single mitochondrial DNA

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Figure 1. A *Crematogaster* (*Decacrema*) ant colony nesting inside a stem of *Macaranga beccariana* with *Coccus* symbiotic scale insects on the inner surface.

(mtDNA) clade consisting of 17 distinct mtDNA lineages (Quek *et al.* 2007). Although the ants and plants disperse independently of each other, most of the *Decacrema* mitochondrial lineages are specific to *Macaranga* species or clades (Itino *et al.* 2001; Quek *et al.* 2004, 2007). Coadaptations facilitating species specificity between the ants and plants include (i) species-specific defence systems in the ants (Itino & Itioka 2001; Itino *et al.* 2003), (ii) host selection using chemical cues by *Decacrema* founding queens (Inui *et al.* 2001), (iii) associated life-history traits of ants and plants (Feldhaar *et al.* 2003), and (iv) specialized stem textures in the plants and ant adaptations to host stem texture (Federle *et al.* 1997, 2000, 2002).

Scale insects appear to be a common associate of myrmecophytes, such as those in the Neotropical genera *Cecropia* and *Cordia* (Gullan 1997). Ward (1991) proposed that one route to the evolutionary origin of ant–plant mutualisms may have involved the tending of scale insects by ants in cavities bored by beetle or lepidopteran larvae. Davidson (1997) additionally proposed that the carbohydrate-rich exudates of homopterans fuel the high-tempo activities of their tending ants. Collectively, these propositions imply an important role for scale insects in the origin and evolution of ant–plant mutualisms.

Extensive sampling of *Coccus* from *Macaranga* and other plant genera in southeast Asia has revealed that they are highly specialized on myrmecophytic *Macaranga* (Heckroth *et al.* 1998), but have occasionally been found with phytoecious (i.e. dwelling in live plant cavities) *Cladomyrma* ants (Moog *et al.* 2005). The *Coccus* from *Macaranga* are believed to constitute a monophyletic group (P. J. Gullan 2008, personal communication), but this has yet to be ascertained. An intriguing but thus-far unanswered question is: how do the scale insects colonize the plant? Are they brought in by a founding ant queen, as observed in several phytoecious ant taxa (Moog *et al.* 2005), or do they arrive of their own accord? Moog *et al.* (2005) suggest that the former is unlikely in ant–plant–homopteran systems, such as the *Decacrema*–*Macaranga*–*Coccus* alliance, in which a founding queen must first chew a hole into the stem (since the mandibles are needed to secure the homopteran until entry into the domatium). Even more intriguing is to contemplate the question in evolutionary time: how did the tripartite system originate—did *Coccus* become part of the

association only after *Decacrema* and *Macaranga* formed the mutualism or was *Coccus* already in association with *Macaranga* trees when *Decacrema* ants entered the scene? Alternatively, were the ants and scales already in association before colonizing *Macaranga* plants? Is it indeed the case that coccoids are an integral component in the origin of ant–plant mutualisms, as proposed by Ward (1991)? To address these questions, we reconstructed a gene phylogeny of the *Macaranga*-inhabiting scale insects and estimated a time frame for their diversification using nucleotide sequences from the mitochondrial gene cytochrome oxidase I (COI), the same gene used in dating the ant phylogeny (Quek *et al.* 2004, 2007). If the scales are brought in by founding queens (i.e. vertically transmitted), then we might expect to observe some influence of the *Decacrema* ant phylogeny on the *Coccus* scale phylogeny. And if the scales colonize independently of the ants, then the null expectation is a pattern of association that is independent of the ant phylogeny. Thus, we also characterize the specificity of the *Coccus* mtDNA lineages reported here to *Macaranga* clades, and the specificity of *Decacrema* mtDNA lineages (from Quek *et al.* 2007) towards the *Coccus* mtDNA lineages.

2. MATERIAL AND METHODS

(a) Sampling

Scale insects were collected from 204 trees representing 22 *Macaranga* species in 13 locations throughout Borneo and Malaya (listed in the electronic supplementary material). We focused on myrmecophytic *Macaranga* trees because the *Macaranga*-inhabiting *Coccus* are reported to be specific to these (Heckroth *et al.* 1998). Most of the scale insects were collected from the ant colonies reported in Quek *et al.* (2007). For out-groups we sequenced (i) three free-living *Coccus* species (*Coccus celatus*, *Coccus hesperidum* and *Coccus pseudomagnoliarum*) believed to be close relatives of the *Coccus* on *Macaranga* (P. J. Gullan & T. Kondo 2008, personal communication), (ii) a member of the genus *Pukwinaria* within the same subfamily, Coccinae (Hodgson 1994), and (iii) two genera, *Eulecanium* and *Parthenolecanium*, in the subfamily Eulecaninae within Coccidae (Hodgson 1994).

(b) DNA extraction, polymerase chain reaction and sequencing

DNA was extracted from single ethanol-preserved scale insects using a ‘salting-out’ protocol (Sunnucks & Hales 1996). A partial COI gene (521 bp) corresponding to positions 1718–2239 in the *Drosophila yakuba* mtDNA genome was amplified by polymerase chain reaction (PCR) with the primers mtD-6 (5′-GGA TCA CCT GAT ATA GCA TTC CC-3′; Simon *et al.* 1994) and CI-N4 (5′-CCT GGT AGG ATT AAA ATA TAT AC-3′), and using the following PCR temperature profile: 35 cycles of 95°C for 30 s, 42°C for 30 s and 72°C for 40 s. After amplification, PCR products were purified using Qiagen PCR purification kits. Cycle sequencing reactions for both strands were performed with the BIGDYE TERMINATOR cycle sequencing ready reaction kit on an ABI 377 automated sequencer. Upon the inspection of the COI sequences, it was discovered that a pseudogene had been amplified in the out-group *C. celatus* (as indicated by a frame-shift mutation with an ensuing stop codon near the 5′ end) and thus it was removed from analyses.

(c) Phylogenetic analyses

Maximum-likelihood (ML) analyses were performed with PHYML (Guindon & Gascuel 2003), using the general time-reversible substitution model with a proportion of invariant sites and a gamma-distributed rate heterogeneity (GTR+I+G), as selected by hierarchical likelihood ratio tests (LRTs; Huelsenbeck & Rannala 1997) implemented in MODELTEST v. 3.06 (Posada & Crandall 1998). Clade support was assessed with 1000 bootstrapped datasets in PHYML. The GTR+I+G model was also used in Bayesian analysis, conducted in MRBAYES v. 3.1.2 (Huelsenbeck & Ronquist 2001), using the default run settings that perform two independent analyses with four Markov chain Monte Carlo chains each (one cold and three heated). The Bayesian analysis was run for five million generations, with a burn-in of four million generations, well after stationarity was reached. Parsimony analyses were performed with PAUP* v. 4.0.b10 (Swofford 2002), using the heuristic search option with 1000 random addition replicates and TBR branch swapping. Clade support was assessed from 1000 bootstrap replicates using 10 random addition replicates.

(d) Partner preference

Specificity of ant lineages to *Coccus* lineages (using ant lineage designations from Quek *et al.* 2007) was determined by chi-squared tests of the extent of departure of the observed *Coccus* lineage's proportion from its expected proportion as indicated by its frequency in all the locations where the ant lineage was sampled. Specificity of *Coccus* lineages to host plant clades (sections *Pachystemon*, *Pruinosae* and *Winklerianae*) was also determined in a similar manner. *Macaranga* section *Pachystemon* contains the majority of the myrmecophytes (21 species) and the closely related section *Pruinosae* contains five myrmecophytic species; section *Winklerianae*, more distantly related, has two myrmecophytes (see Davies *et al.* 2001). Five thousand Monte Carlo simulations of the sampling distribution were also performed to assess the observed sample against the null distribution. Ant clades C and E ($n=1$ in both clades) were removed from the analyses.

(e) Age estimation

A general conservatism in COI substitution rates in arthropods (Gaunt & Miles 2002) makes this gene useful for dating divergences within arthropod groups (e.g. Degnan *et al.* 2004). In addition, the pairwise divergence rate of COI was found to cluster approximately $1.5\% \text{ Myr}^{-1}$ (uncorrected pairwise distance) in several arthropod groups (Quek *et al.* 2004); thus we used this rate to estimate divergences within the COI phylogeny. Homogeneity of substitution rate on the ML phylogeny of the scales was tested using the LRT (Huelsenbeck & Rannala 1997) with the GTR+I+G model, implemented in PAUP* v. 4.0b10. We removed all duplicate haplotypes from the LRT. Because the LRT showed significant deviations from rate constancy ($p < 0.05$), branch lengths were subjected to non-parametric rate smoothing (Sanderson 1997), implemented in TREEEDIT v. 1.0 (Rambaut & Charleston 2002). Three well-supported nodes of varying genetic divergences (5.8–14.2%) were chosen as fixation points to obtain the relative ages of the other nodes. This range of divergences was chosen to approximate that used for dating divergences in various arthropod groups (table 4 in Quek *et al.* 2004). This approach produces a range of ages rather than a point

estimate, and is thus more conservative. Mean uncorrected pairwise distances between sister taxa were calculated using MEGA v. 2.1 (Kumar *et al.* 2001).

3. RESULTS AND DISCUSSION

Phylogenetic analyses reveal six well-supported primary mtDNA lineages, L1–L6 (figure 2). The monophyly of the in-group (L1–L6) was recovered in the ML topology, but was poorly supported by ML bootstrapping and not supported by parsimony bootstrapping and Bayesian posterior probability; in parsimony and Bayesian analyses, L6 was placed sister to the two *Coccus* out-groups, albeit with poor support. The branch uniting L3 and L4 in the ML topology was similarly poorly supported—in Bayesian and parsimony analyses, L3 was placed sister to the node uniting L1 with L2, but again with low support. The membership among lineages varied considerably (from $n=5$ in L6 to $n=87$ in L5), probably reflecting the natural abundance of the scale lineages, because our sampling was carried out with no prior knowledge of the scales' distribution and without regard to their morphology.

The chi-squared test for the ant lineages' preference for the scale lineages indicated that none of the ant lineages showed significant preferences for particular scale lineages (table 1), and specificity between the scale lineages and *Macaranga* taxa was also not observed (table 2). By contrast, Quek *et al.* (2007) revealed that most ant mtDNA lineages showed specificity towards *Macaranga* taxa. The non-specific association of scales with ants and plants has similarly been documented based on morpho-species of the ants and scales (Heckroth *et al.* 1998), and is consistent with independent colonization of *Macaranga* plants by scales and ants. Thus, in contrast to the ants, the scales' association within the system, while obligate, is dictated by opportunity rather than by physiological, behavioural or ecological adaptations to specific ant or host plant taxa.

The minimum estimated age of the *Coccus* inhabiting *Macaranga* is 7.3–8.8 Myr (table 3; figure 3, node a), while that of the ants is 15.9–22.6 Myr (Quek *et al.* 2007). The latter estimate is consistent with the origin of ever wet forests in western Malesia at approximately 20 Myr ago (Morley 2000) and also with the origin of myrmecophytic *Macaranga*, since myrmecophytic *Macaranga* are confined to the aseasonal ever wet climatic zone there (Davies *et al.* 2001). Thus, taken at face value, the time-scaled phylogeny suggests that the scales are only approximately half as old as the ants. Certainly, we expect that the true pairwise divergence rate of COI in the scales would vary somewhat around the assumed $1.5\% \text{ Myr}^{-1}$. Allowing a similar age for the ants and scales would require halving the scales' rate to approximately $0.6\% \text{ Myr}^{-1}$. Such an extensive departure seems unlikely, given that a survey of geologically calibrated COI rates encompassing beetles, cicadas and crustaceans yielded a range between 1.3 and $1.9\% \text{ Myr}^{-1}$ (see table 4 in Quek *et al.* 2004). Furthermore, if we take into account the possibility that the scales collected from *Macaranga* are not monophyletic (figure 2) and that L6 represents an independent colonization of the *Decacrema*–*Macaranga* system by *Coccus*, then, based on the estimate at node b (for the clade comprising L1–L5 in figure 3), rather than node a, the minimum age estimate is even younger at 5.3–6.4 Myr. However, studies by

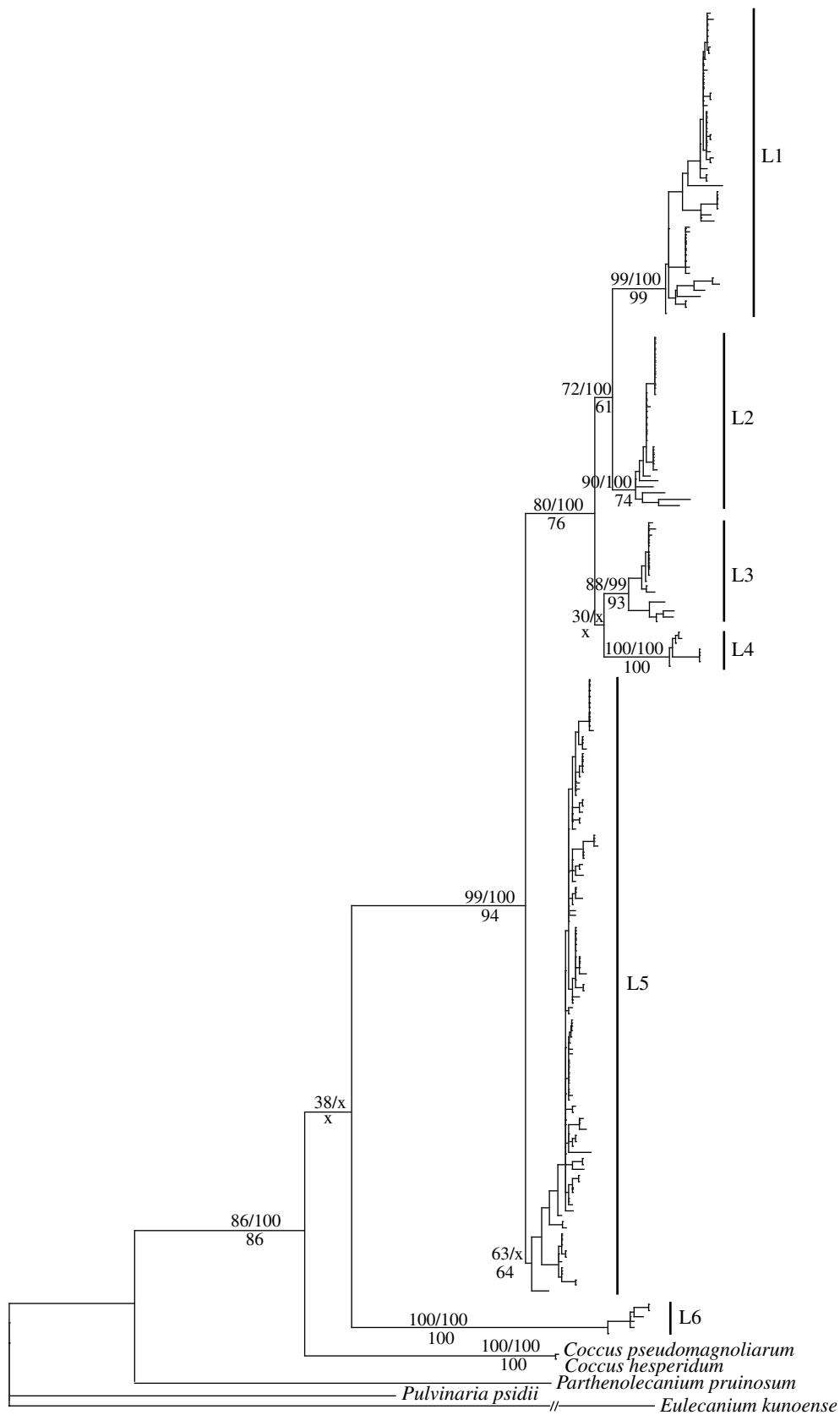


Figure 2. Phylogeny of 220 *Macaranga*-inhabiting *Coccus* scale insects estimated from 521 bp mtDNA sequences (COI) using ML. The six primary mtDNA lineages of *Coccus* scales are indicated (L1–L6). The numbers above branches and left of slash indicate ML bootstrap support (1000 replicates), numbers right of slash indicate Bayesian posterior probabilities, and numbers below branches indicate parsimony bootstrap support (1000 replicates). An ‘x’ in the node support values indicates the absence of the node in the parsimony bootstrap or Bayesian consensus topology.

T. Kondo (2003–2007, unpublished data) indicate that the *Macaranga* coccids form a morphologically coherent group; thus the hypothesis of independent colonization

events seems unlikely. It is also possible that there are older *Coccus* lineages not detected by our sampling, which might bring our age estimate closer to that of the ants but this

Table 1. Chi-squared tests for biased association between ant lineages and scale insect lineages. (The *p*-values are from 5000 Monte Carlo simulations; n.s., not significant.)

ant lineage (sample size)	scale insect lineage	expected proportion	observed proportion	<i>p</i>
A (3)	L2	28/160 (0.18)	0/3 (0.00)	n.s.
	L3	14/160 (0.09)	0/3 (0.00)	n.s.
	L4	7/160 (0.04)	0/3 (0.00)	n.s.
	L5	107/160 (0.67)	2/3 (0.67)	n.s.
	L6	4/160 (0.03)	1/3 (0.33)	n.s.
D (12)	L2	28/160 (0.18)	4/12 (0.33)	n.s.
	L3	14/160 (0.09)	2/12 (0.17)	n.s.
	L4	7/160 (0.04)	0/12 (0.00)	n.s.
	L5	107/160 (0.67)	6/12 (0.50)	n.s.
	L6	4/160(0.03)	0/12 (0.00)	n.s.
F (10)	L2	28/160 (0.18)	0/10 (0.00)	n.s.
	L3	14/160 (0.09)	4/10 (0.40)	n.s.
	L4	7/160 (0.04)	0/10 (0.00)	n.s.
	L5	107/160(0.67)	6/10 (0.60)	n.s.
	L6	4/160 (0.03)	0/10 (0.00)	n.s.
G (47)	L2	28/160 (0.18)	6/47 (0.13)	n.s.
	L3	14/160 (0.09)	1/47 (0.02)	n.s.
	L4	7/160 (0.04)	2/47 (0.04)	n.s.
	L5	107/160 (0.67)	37/47 (0.79)	n.s.
	L6	4/160 (0.03)	1/47(0.02)	n.s.
H (61)	L2	28/160 (0.18)	10/61(0.16)	n.s.
	L3	14/160 (0.09)	2/61 (0.03)	n.s.
	L4	7/160 (0.04)	4/61 (0.07)	n.s.
	L5	107/160 (0.67)	44/61 (0.72)	n.s.
	L6	4/160 (0.03)	1/61 (0.02)	n.s.
K (37)	L1	53/60 (0.88)	35/37 (0.95)	n.s.
	L2	2/60 (0.03)	1/37 (0.03)	n.s.
	L3	3/60 (0.05)	0/37 (0.00)	n.s.
	L6	2/60 (0.03)	1/37 (0.03)	n.s.

Table 2. Chi-squared tests for biased association between scale insect lineages and host plant section. (The *p*-values are from 5000 Monte Carlo simulations; n.s., not significant.)

<i>Coccus</i> lineage (sample size)	<i>Macaranga</i> section tested	expected proportion	observed proportion	<i>p</i>
L1 (49)	<i>Pachystemon</i>	51/60 (0.85)	45/49 (0.92)	n.s.
	<i>Pruinosae</i>	5/60 (0.08)	4/49 (0.08)	n.s.
L2 (30)	<i>Pachystemon</i>	174/216 (0.81)	21/30 (0.70)	n.s.
	<i>Pruinosae</i>	38/216 (0.18)	7/30 (0.23)	n.s.
	<i>Winklerianae</i>	4/216 (0.02)	2/30 (0.07)	n.s.
L3 (17)	<i>Pachystemon</i>	174/216 (0.81)	14/17 (0.82)	n.s.
	<i>Pruinosae</i>	38/216 (0.18)	1/17 (0.06)	n.s.
	<i>Winklerianae</i>	4/216 (0.02)	2/17 (0.12)	n.s.
L4 (7)	<i>Pachystemon</i>	123/160 (0.77)	6/7 (0.86)	n.s.
	<i>Pruinosae</i>	33/160 (0.21)	1/7 (0.14)	n.s.
	<i>Winklerianae</i>	4/160 (0.03)	0/7 (0.00)	n.s.
L5 (107)	<i>Pachystemon</i>	123/160 (0.77)	83/107 (0.78)	n.s.
	<i>Pruinosae</i>	33/160 (0.21)	24/107 (0.22)	n.s.
	<i>Winklerianae</i>	4/160 (0.03)	0/107 (0.00)	n.s.
L6 (6)	<i>Pachystemon</i>	174/216 (0.81)	5/6 (0.83)	n.s.
	<i>Pruinosae</i>	38/216 (0.18)	1/6 (0.17)	n.s.
	<i>Winklerianae</i>	4/216 (0.02)	0/6 (0.00)	n.s.

seems unlikely, given the broad geographical area sampled. It is also plausible that such older lineages may have become extinct. Alternatively, the mutualism between *Macaranga* and *Decacrema* may have originated with a taxon of coccoids that have since become replaced by those in this study. Unfortunately, hypotheses involving the possibility of extinct or replaced taxa are untestable.

If we accept the data at face value, then we infer that the scale insects were indeed latecomers and, therefore, that the origin of the mutualism between *Decacrema* and *Macaranga* did not involve scale insects, in contrast to Ward's (1991) model for the origin of ant-plant mutualisms. If so, the ants may have relied on extrafloral nectaries (EFNs) in place of coccoids or they may have relied only

Table 3. Mean uncorrected pairwise distances and inferred ages of nodes on the *Coccus* chronogram (figure 3) obtained by non-parametric rate smoothing, based on a pairwise divergence rate of 1.5% Myr⁻¹. (Fixed ages are indicated in italics.)

node	mean pairwise distance (%)	age (node o fixed)	age (node b fixed)	age (node e fixed)
o (out-group/L1–L6)	14.2	9.47	7.86	7.84
a (L6/L1–L5)	15.4	8.78	7.28	7.27
b (L5/L1–L4)	7.9	6.35	5.27	5.26
c (L3–L4/L1–L2)	6.7	5.17	4.29	4.28
d (L3/L4)	5.6	4.91	4.07	4.06
e (L1/L2)	5.9	4.75	3.94	3.93

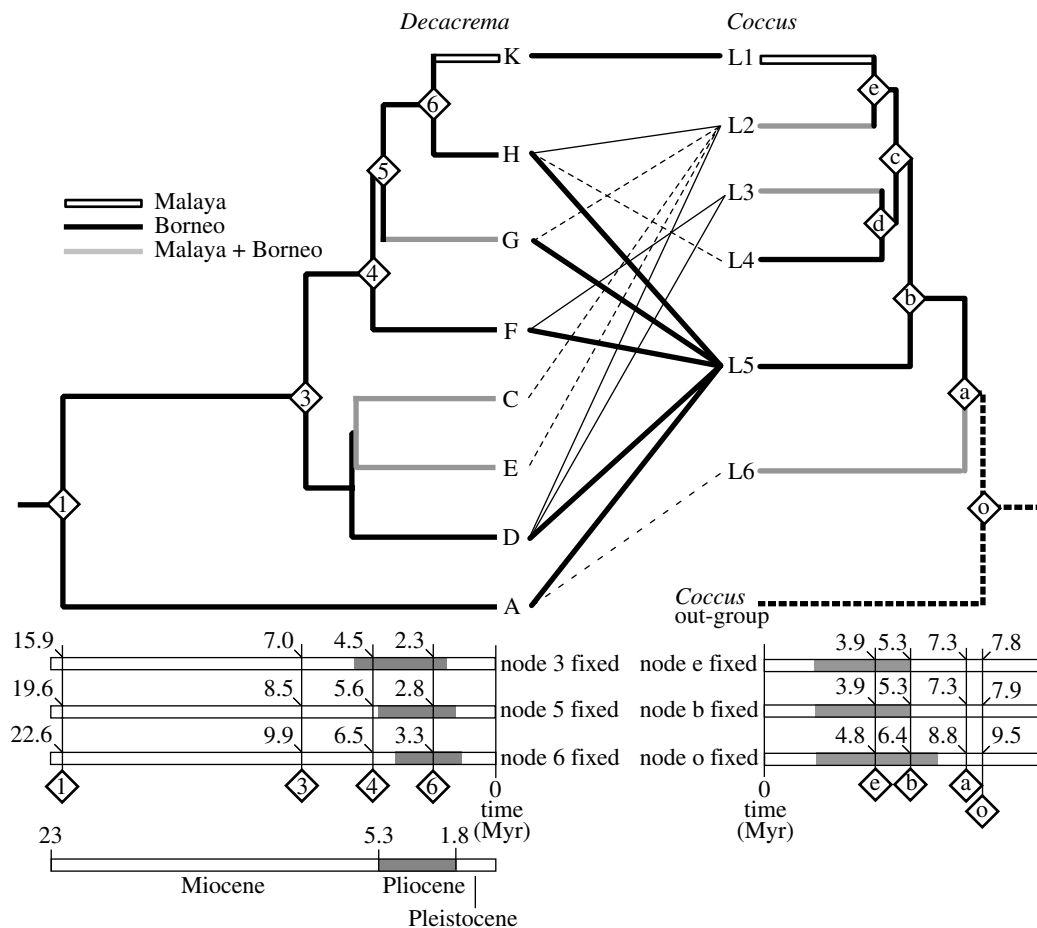


Figure 3. Comparison of the *Coccus* scale insect chronogram with that of *Decacrema* ants (modified from Quek *et al.* 2007). A thick black line connecting an ant lineage to a scale lineage indicates that the scale lineage constitutes 50% or more of the ant's total scale associates; a narrow line indicates 16–50% and dashed line 7–13% (or single sample); scales constituting less than 5% are not shown.

on food bodies provisioned by the plants (as seen in *Acacia*–ant mutualisms, Janzen 1966). Interestingly, Davies *et al.* (2001) showed that myrmecophytism evolved only in *Macaranga* lineages in which EFNs on mature leaf surfaces had been lost and in which leaf margin (as opposed to leaf surface) EFNs became enlarged, suggesting a role for carbohydrate-rich plant exudates, whether in the form of EFNs or homopteran honeydew, in the evolution of myrmecophytism.

The single-locus dataset used here does not lend itself to inferring species boundaries, which can conflict with mitochondrial lineages due to introgression or incomplete lineage sorting (Avice 1994; Sota & Vogler 2001; Shaw 2002; Linnen & Farrell 2007). Indeed, preliminary sequence data from nuclear loci are in disagreement with the mtDNA phylogeny presented here (S. Ueda 2008,

unpublished data). However, our main objective in this paper is to infer the antiquity of the *Macaranga*-associated scale insects. Certainly, introgression and incomplete lineage sorting can introduce errors into our age estimation, but the relative contribution of this error decreases with evolutionary time, especially when measured using fast-evolving genes such as metazoan COI. At the time frame of 7–9 Myr (the older end of the time scale on our phylogeny), the effect of introgression or incomplete lineage sorting should not contribute to any worrying deviation that is not already contained by our bracketing method (where we obtain three time frames based on three fixed nodes).

The obligate and ancient association of *Macaranga* plants, *Decacrema* ants and *Coccus* scale insects shown here appears to be unique in contrast to the tropical American

ant-plant systems in which numerous plant and ant genera are involved, and intergeneric host shifts are not uncommon (Davidson & Mckey 1993). Furthermore, other taxa appear to have evolved obligate associations with the *Macaranga* symbiotic system, such as phytophagous *Arhopala* butterflies (Maschwitz et al. 1984). The *Macaranga*-based symbioses thus offer a promising system for the study of coevolutionary radiations of multipartite and obligate associations. Further empirical and theoretical treatment of this complex association may provide a reflection of the evolutionary dynamics of coevolving communities in the rainforests of southeast Asia.

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