Mutualism favours higher host specificity than does antagonism in plant–herbivore interaction

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Coevolved mutualisms often exhibit high levels of partner specificity. Obligate pollination mutualisms, such as the fig–fig wasp and yucca–yucca moth systems, represent remarkable examples of such highly species-specific associations; however, the evolutionary processes underlying these patterns are poorly understood. The prevailing hypothesis suggests that the high degree of specificity in pollinating seed parasites is the fortuitous result of specialization in their ancestors because these insects are derived from endophytic herbivores that are themselves highly host-specific. Conversely, we show that in the *Glochidion–Epicephala* obligate pollination mutualism, pollinators are more host-specific than are closely related endophytic leaf-feeding taxa, which co-occur with *Epicephala* on the same *Glochidion* hosts. This difference is probably not because of shifts in larval diet (i.e. from leaf- to seed-feeding), because seed-eating lepidopterans other than *Epicephala* do not show the same degree of host specificity as *Epicephala*. Species of a tentative sister group of *Epicephala* each attack several distantly related plants, suggesting that the evolution of strict host specificity is tied to the evolution of pollinator habit. These results suggest that mutualists can attain higher host specificity than that of their parasitic ancestors and that coevolutionary selection can be a strong promoter of extreme reciprocal specialization in mutualisms.

Keywords: *Caloptilia*; *Cuphodes*; *Diphtheroptila*; Gracillariidae; Phyllanthaceae

1. INTRODUCTION

Parasitic lifestyles usually favour extreme specialization to one or few host species because they require complex adaptations to circumvent host defences and sustain life on a single host (Ehrlich & Raven 1964; Price 1980; Thompson 1994; Strauss & Zangerl 2002). Although specific mechanisms underlying host specialization may vary among taxa, the broad general understanding is that host–parasite coevolution promotes specialization in parasitic organisms (Thompson 1994, 2005). Strict host specificity of parasites is often linked to high species diversity because specialization to different hosts can result in host-associated speciation (Mitter *et al.* 1988; Farrell 1998; Schluter 2000; Coyne & Orr 2004).

In contrast, the evolutionary processes that determine the level of specialization in mutualisms are far less understood. Although many mutualisms do not evolve to exhibit high degrees of specificity (e.g. most plant–pollinator and plant–seed disperser interactions), reciprocal partner specialization is often found in intimate mutualisms, such as those between myrmecophytic plants and their resident ants (Davidson & McKey 1993; Heil & McKey 2003; Guimarães *et al.* 2007), ants/termites and their cultivated fungi (Mueller *et al.* 1998; Aanen *et al.*

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2002; Currie *et al.* 2003) or various invertebrates and their endosymbiotic micro-organisms (Moran & Telang 1998; Hosokawa *et al.* 2006). Both ultimate and proximate causes of specialization have been proposed, including selection for elimination of less-cooperative partners (Heil *et al.* 2005; Poulsen & Boomsma 2005) and chemical or physical mechanisms of partner discrimination (Federle *et al.* 1997; Brouat *et al.* 2001; Edwards *et al.* 2006; Grangier *et al.* 2009). However, the general understanding of the evolutionary conditions favouring specialization in mutualisms is still very limited (Thompson 1994, 2005), and modern molecular approaches continue to refine our view of how mutualists are associated with one another on both local and broad geographical scales (Molbo *et al.* 2003; Mikheyev *et al.* 2006; Quek *et al.* 2007; Visser *et al.* 2009).

Perhaps, the most remarkable cases of reciprocal specialization between mutualists are found in obligate pollination mutualisms (Janzen 1979; Pellmyr 2003; Herre *et al.* 2008). The fig–fig wasp and yucca–yucca moth mutualisms are well-known examples of such highly species-specific associations, in which the plants are pollinated by one or, rarely, two insect species, which in turn are highly host-specific seed parasites of the plants they pollinate. Figs and yuccas have diversified into more than 700 and 40 species, respectively, and a corresponding high diversity of pollinator species have evolved, each of which is obligately mutualistic with one or few fig/yucca hosts (Weiblen 2002; Pellmyr 2003; Herre *et al.* 2008). This level of specificity is unusual

among pollination mutualisms because, although selection may favour plants depending on specialized visitors for effective conspecific pollination, pollinators are generally expected to maximize the range of plants they visit to optimize resource use (Pellmyr 2002; Gómez & Zamora 2006). The high specificity of pollinating seed parasites is therefore considered to be the result of their inherently parasitic lifestyle (Thompson 1994, 2005) because seed-feeding insects commonly specialize to a narrow range of host plants. Indeed, detailed ecological and phylogenetic studies of the yucca moth family Prodoxidae have found that close relatives of the pollinators are also highly host-specific herbivores (Pellmyr & Thompson 1992; Pellmyr 1999; Pellmyr *et al.* 2006), suggesting that the high degree of pollinator-specificity is driven by the parasitic part of the interaction and cannot be attributed to mutualistic selection (Thompson 1994, 2005).

However, a growing body of evidence suggests that the current view of host specificity in pollinating seed parasites may require revision. Within the yucca moth lineage, two cheater species have independently lost their pollinating behaviour and oviposit in young fruits to exploit the seeds that other yucca moth species have pollinated (Pellmyr *et al.* 1996; Pellmyr 1999). In contrast to their pollinating relatives, each of these cheater species has evolved to use four to six yucca hosts (Pellmyr 1999; Segraves & Pellmyr 2004), suggesting that host specificity in the pollinators may not be determined solely by the herbivorous habit of the moths (Pellmyr 2003). In the fig system, non-pollinating agaonid wasps that are closely related to and co-occur with pollinating fig wasps tend to be less host specific than are the pollinators (Weiblen & Bush 2002; Marussich & Machado 2007; but see Lopez-Vaamonde *et al.* 2001; Jousselin *et al.* 2006, 2008). In addition, fig herbivores in general are dominated by insects that feed on several locally available fig hosts (Novotny *et al.* 2002, 2006). Given that shared pollinators can result in hybridization among closely related, co-occurring figs (Machado *et al.* 2005), selection may favour figs relying on specialist pollinators to achieve effective conspecific pollination. Thus, these observations indicate that pollinating seed parasites may in fact attain a higher degree of host specificity than that of their parasitic ancestors owing to coevolutionary selection arising after the evolution of pollination mutualism.

We tested whether host specificity is greater in pollinating seed parasites than in their herbivorous ancestors in a recently discovered mutualism between Phyllantheae plants (Phyllanthaceae) and *Epicephala* moths (Gracillariidae; Kato *et al.* 2003; Kawakita & Kato 2004*a*,*b*, 2009). Currently, an estimated 500 species of Phyllantheae plants exist that are pollinated by the ovipositing females of *Epicephala* moths (Kawakita & Kato 2009; Kawakita 2010). Among them are plants of the genus *Glochidion*, which is the largest radiation and comprises more than 300 species distributed throughout the Asian–Australian tropics (Govaerts *et al.* 2000). Previous detailed assessment of pollinator specificity in the Japanese species of *Glochidion* has found that, although some *Epicephala* species are associated with two closely related *Glochidion* hosts in different parts of their ranges, each *Epicephala* species is specialized to only one of the several co-occurring hosts in all populations studied, thus showing very strict local host specificity (Kawakita & Kato 2006).

The *Glochidion–Epicephala* system is ideal for studying the evolution of high host-specificity in pollinating seed parasites because *Glochidion* plants are host to two other genera of herbivores (*Diphtheroptila* and *Caloptilia*) that belong to the subfamily Gracillariinae together with *Epicephala* (Kumata 1982; Kuroko 1982); this situation allows for comparison of host specificity among phylogenetically related genera that share the same host plants. *Diphtheroptila* are leaf miners that use young *Glochidion* leaves, whereas *Caloptilia* are leaf miners as early instar larvae and, as they develop into late instars, construct leaf rolls or induce leaf galls, depending on species (Kumata 1982). Furthermore, *Glochidion* plants are attacked by lepidopteran seed parasites that belong to the families Carposinidae, Tortricidae and Pyralidae, whose host specificity may be determined by a common mechanism with that of *Epicephala* owing to their shared larval diet.

In this study, we first conducted a molecular phylogenetic analysis of Gracillariinae to determine the relative phylogenetic positions of *Epicephala*, *Diphtheroptila* and *Caloptilia* within the subfamily. We then analysed host specificity of the abovementioned *Glochidion*-associated herbivores (*Diphtheroptila*, *Caloptilia* and non-gracillariid seed-feeding lepidopterans) to test whether the level of host specialization is indeed higher in *Epicephala*. We also investigated the degree of host specificity in a candidate sister genus of *Epicephala* to determine whether the high pollinator specificity is an ancestral condition predating the evolution of the pollination mutualism in *Epicephala*.

2. MATERIAL AND METHODS

(a) *Sampling*

To determine the phylogenetic positions of *Epicephala*, *Diphtheroptila* and *Caloptilia* within the subfamily Gracillariinae, we first conducted a molecular phylogenetic analysis of the subfamily based on the mitochondrial cytochrome oxidase subunit I (*COI*) and the nuclear elongation factor-1 alpha (*EF-1α*), arginine kinase (*ArgK*) and *18S rRNA* genes. Within Gracillariinae, *Epicephala* and *Diphtheroptila* belong to the *Parectopa* group as proposed by Kumata (1988), which is characterized by a highly distinct morphological synapomorphy (i.e. the female ostium bursae opens on the sternite of the seventh abdominal segment). *Caloptilia* belongs to the *Gracillaria* group (Kumata 1982) and thus is probably distantly related to *Epicephala*. We sampled a total of 45 non-*Epicephala* gracillariine species for phylogenetic analysis with a particular emphasis on the *Parectopa* group, including putative new taxa that have morphological affinities to *Epicephala* (full list of species are provided in the electronic supplementary material, table S1). An effort was made to sample moths from a broad range of angiosperm hosts to avoid sampling bias in our inference of generic relationships. Representatives of the subfamilies Oecophyllembiinae (*Eumetriochroa hederae*) and Lithocolletinae (*Cameraria niphonica*) were also sampled, and species of Bucculatricidae (*Bucculatrix* spp.) were used to root the entire gracillariid tree. Because we were unable to include many gracillariine genera in this analysis, our phylogenetic results remain inconclusive with regard to the sister group of *Epicephala*. However, firmly establishing the closest extant relative of *Epicephala* is not straightforward because a large number of

unnamed lineages continue to be discovered (Vargas & Landry 2005; A. Kawakita & M. Kato 2009, personal observation) and phylogenetic relationships among gracillariiine genera are difficult to resolve even by analyses of large molecular data sets (A. Kawahara, University of Maryland 2009, personal communication), probably owing to rapid radiation of extant genera. Rather, our purpose was to determine how closely *Diphtheroptila* and *Caloptilia* are related to *Epicephala* within the subfamily and to identify genera, if any, that are more closely related to *Epicephala*. Such genera share similar evolutionary background with *Epicephala* and thus provide an opportunity to test whether the level of host specificity in *Epicephala* is in fact exceptional in light of the overall evolutionary trend among its closest relatives.

To compare the degree of host specialization in *Epicephala* with those of *Diphtheroptila*, *Caloptilia* and the non-gracillariid seed feeders, we analysed the mitochondrial *COI* and nuclear *EF-1α* genes for these moths. Sampling was conducted within precisely the same geographical range as that used to assess pollinator specificity in our previous study (Kawakita & Kato 2006); this enabled a direct comparison of host specialization among herbivores using the same sets of *Glochidion* species. *Diphtheroptila* and *Caloptilia* were collected by sampling the leaves containing the larvae and were reared to adults in the laboratory. Seed-parasitic lepidopterans belonging to Carposinidae, Tortricidae and Pyralidae were sampled by collecting the fruits and were also reared to adults. To minimize the possibility of analysing multiple siblings, only one moth per tree per sampling event was used for the molecular analysis. *Diphtheroptila* sp. attacking *Bridelia balansae* and *Caloptilia recitata* infesting *Rhus succendanea* were used to root the *Diphtheroptila* and *Caloptilia* trees, respectively, based on the results of the phylogenetic analysis of Gracillariiinae.

We also sampled moths of the leaf-miner genus *Cuphodes* because this genus was identified as the putative sister taxon of *Epicephala* in the Gracillariiinae phylogeny (see §3). *Cuphodes* moths are known to occur on species of *Diospyros* (Ebenaceae) (Issiki 1957), Fabaceae (Kuroko 1982; Robinson *et al.* 2001) and Rhamnaceae (M. Kato 1991, personal observation), but their host ranges have not been investigated in detail. We therefore sampled *Cuphodes* from various species of *Diospyros*, Fabaceae and Rhamnaceae that occur in approximately the same geographical region as the above-sampled *Glochidion* herbivores to determine the degree of host specificity in a close relative of *Epicephala*. Analysis was done using *COI*, *EF-1α* and additionally, *ArgK* because we initially found one of the exemplars to fall into largely different clades in the *COI* and *EF-1α* phylogenies, which we suspected as the result of genetic introgression.

Full details of host association, sample size and locality information for the sampled gracillariid moths are provided in figure S1 and electronic supplementary material, tables S1 and S2.

(b) Molecular phylogenetic analysis

We extracted moth genomic DNA from thoracic muscle using a NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). PCR amplification and direct sequencing of the *COI*, *EF-1α*, *ArgK* and *18S rRNA* genes were conducted using the primers and protocols detailed in Kawakita *et al.* (2004) and Kawakita & Kato (2006, 2009). Heterozygous sites in *EF-1α* and *ArgK* were identified as double peaks of similar height in the chromatograms of both forward and reverse strands and accordingly coded using degenerate bases. Obtained

sequences have been deposited in the GenBank database under accession numbers GU816251–GU816796.

Sequences of the protein-coding genes (*COI*, *EF-1α* and *ArgK*) contained no introns, and thus the alignment was straightforward. The alignment of *18S rRNA* sequences was performed using CLUSTALX 2.0 (Larkin *et al.* 2007) software with default settings. Phylogenetic trees were constructed for each of the following four datasets: the four-gene dataset of Gracillariiinae, the *COI + EF-1α* datasets of *Diphtheroptila* and *Caloptilia*, and the *COI + EF-1α + ArgK* dataset of *Cuphodes*. We focus on the analyses of combined datasets because initial analyses of individual genes suggested no strongly conflicting phylogenetic relationships among genes. However, there was a major incongruence in the placement of one *Cuphodes* specimen between the mitochondrial (*COI*) and nuclear gene datasets (*EF-1α* and *ArgK*; separate phylogenies are provided in the electronic supplementary material, figure S2), suggestive of mitochondrial introgression. We therefore performed the combined analysis excluding this anomalous *Cuphodes* individual. We did not construct phylogenetic trees for non-gracillariid seed parasites because each of the three taxonomic groups (Carposinidae, Tortricidae and Pyralidae) was represented by a single species having minimal sequence variation (see §3).

Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian methods. We performed ML analyses using the program TREEFINDER (Jobb 2008) with substitution model chosen and fitted separately for each gene. Nodal support was assessed using bootstrap analyses with 1000 replications. Bayesian analyses were performed using MRBAYES 3.1.2 (Ronquist & Huelsenbeck 2003) with substitution parameters unlinked among gene partitions. Appropriate models of base substitution were selected for individual genes using MRMODELTEST 2.3 (Nylander 2004).

Because neither *Diphtheroptila* nor *Caloptilia* was recovered as sister to *Epicephala* on the Gracillariiinae phylogeny, we tested the robustness of this reconstruction using the likelihood-based approximately unbiased (AU) test (Shimodaira 2002) as implemented in TREEFINDER. We also determined whether *Epicephala*, *Diphtheroptila* and *Caloptilia* each colonized Phyllanthaceae plants independently by reconstructing the ancestral host association using BAYESTRAITS (Pagel *et al.* 2004). Each terminal taxon was coded as either Phyllanthaceae or non-Phyllanthaceae feeder, and ancestral states were reconstructed on the above-obtained ML phylogeny using the ML criterion. To account for phylogenetic uncertainty, we also used a Bayesian framework by integrating post-burn-in trees resulting from the Bayesian phylogenetic analysis. Likelihood ratio or Bayes factor of greater than 5 was considered significant evidence for the occurrence of either state at ancestral nodes (Pagel 1999; Pagel *et al.* 2004).

Analyses of *Diphtheroptila* and *Caloptilia* datasets recovered several well-defined clades that are each associated with two or more plant species (see §3). To detect any host-associated divergence within each of these putative species, we performed an analysis of molecular variance (AMOVA) on each of *COI* and *EF-1α* datasets using ARLEQUIN 2.0 software (Schneider *et al.* 2000). *EF-1α* sequences with multiple heterozygous sites were analysed as genotypes with unknown gametic phase. Analyses were not performed for *Cuphodes* and non-gracillariid seed feeders owing to small sample sizes per clade/species.
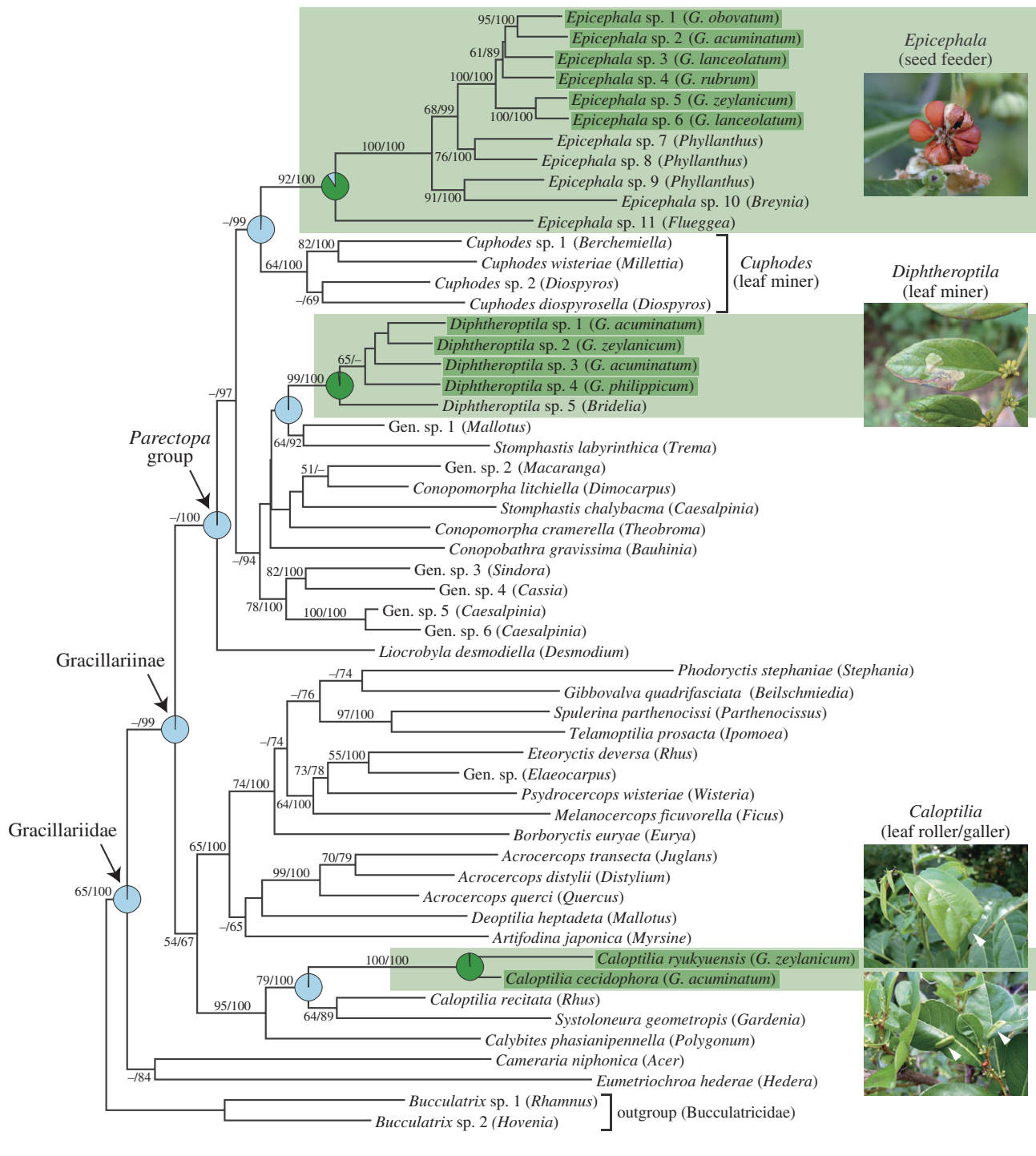
Figure 1. Maximum-likelihood (ML) phylogeny of Gracillariinae based on 2548 bp of the combined mitochondrial *COI* and nuclear *EF-1α*, *ArgK* and *18S rRNA* genes. Nodal numbers indicate ML bootstrap values followed by Bayesian posterior probabilities. Clades boxed in light green are those feeding on Phyllanthaceae plants, and species highlighted individually in dark green are those associated with *Glochidion* plants. Pie graphs show the relative likelihoods of alternative host associations at selected ancestral nodes: green, Phyllanthaceae host; blue, non-Phyllanthaceae host. Asterisks indicate significant differences in likelihoods (i.e. likelihood ratio or Bayes factor of greater than 5). Taxon names in parentheses indicate host plants (species name for *Glochidion* hosts and genus name for non-*Glochidion* hosts). Photographs show *Glochidion* fruit/leaves infested by *Epicephala*, *Diphtheroptila* and *Caloptilia* larvae; arrows indicate a leaf roll and leaf galls. Scale bar, 0.005 substitutions per site.

Full details of the molecular analyses are provided in the electronic supplementary material.

3. RESULTS AND DISCUSSION

Phylogenetic analysis of Gracillariinae using the four-gene dataset suggested that *Epicephala*, *Diphtheroptila* and *Caloptilia* are not monophyletic and that they occupy separate positions in the phylogeny (figure 1). These relationships were recovered by both ML and Bayesian analyses, and an AU test rejected the hypothesis of either *Diphtheroptila* or *Caloptilia* forming a monophyletic group with *Epicephala* ($p < 0.01$ for both tests). Furthermore, Bayesian reconstruction of ancestral character

states provided strong support for non-Phyllanthaceae plants as the ancestral host for Gracillariinae (figure 1). These results indicate that pollinating seed parasites (*Epicephala*) are not derived from leaf herbivores with which they share host plants, and that leaf-feeding *Diphtheroptila* and *Caloptilia* each colonized *Glochidion* plants independently. This contrasts with the situation in yucca moths, for which direct sisters of the pollinators are non-pollinating herbivores that feed on yuccas (Pellmyr & Leebens-Mack 1999; Pellmyr 2003). Nevertheless, the shared use of *Glochidion* by the three genera allows for a rigorous test of how different life histories affect patterns of host specificity in different herbivore clades by controlling for the effect of host plant species.

The analysis *Diphtheroptila* and *Caloptilia COI + EF-1α* datasets indicated that they each comprise four distinct clades throughout our sampling range (figure 2). Both *COI* and *EF-1α* recovered clades consisting of the same sets of individuals, suggesting that there is no gene flow among clades and thus they represent distinct species. Individuals belonging to different clades can also be distinguished by wing pattern, male genitalia morphology and larval feeding habit (A. Kawakita & M. Kato 2009, personal observation). The phylogenies of *Diphtheroptila* and *Caloptilia* further indicated that these moths commonly use more than one coexisting *Glochidion* species. We found no evidence for host race formation in these moths because the AMOVA analysis failed to detect host-associated genetic differentiation in either the *COI* or *EF-1α* gene ($p > 0.1$ for all tests). Although we cannot completely rule out the possibility of hidden divergence associated with *Glochidion* species, we consider it very unlikely that all the *Diphtheroptila* and *Caloptilia* species under consideration are at incipient stages of such host-associated divergence. Moreover, the level of host-associated differentiation, if any, is overwhelmingly lower than that found in *Epicephala*, in which individuals attacking different *Glochidion* hosts in any population are morphologically distinct and divergent by at least 4 per cent uncorrected pairwise sequence difference in the *COI* gene (Kawakita & Kato 2006). Therefore, these results provide strong evidence that *Epicephala* are more highly host specific than are their leaf-feeding relatives that use the same sets of *Glochidion* hosts.

The observed increase in the level of host specialization in *Epicephala*, however, may simply be the result of a shift to seed feeding, rather than coevolutionary selection resulting from being a pollinator. We therefore determined the level of host specificity in seed-infesting lepidopterans that share the same larval food with *Epicephala* moths. Non-gracillariid moths that emerged from *Glochidion* fruits were morphologically identified as either *Peragrarchis syncolleta* (Carposinidae) or as undescribed species of *Tritopterna* (Tortricidae) or *Cryptoblabes* (Pyralidae). Analysis of the *COI* and *EF-1α* sequences in each of these moth taxa suggested that there is very little sequence variation among individuals sampled from four to five different *Glochidion* hosts and that none of the base substitutions found were diagnostic to host species (table 1). Although it is not straightforward to directly compare host specificity between *Epicephala* and non-gracillariid moths, the level of host specialization found in these groups is at the opposite extreme from the pattern expected if seed feeding is to promote higher host-specificity. Therefore, these results do not provide positive evidence that seed feeding favours a higher degree of host specialization and are consistent with the view that *Epicephala* host specificity is determined by factors other than larval diet.

Another explanation for strict host specialization in *Epicephala* is that such high host specificity is a common feature among all the closest relatives of *Epicephala*, and that pollinator habit evolved against a background of high host specificity. To test this possibility, we examined host specificity in species of *Cuphodes*, which was tentatively suggested as the putative sister group of *Epicephala* in the Gracillariinae phylogeny (figure 1). Although an AU test did not reject the non-monophyly of the *Epicephala + Cuphodes* clade ($p > 0.1$), *Epicephala* and *Cuphodes* share distinct apomorphies not otherwise found in any genera of the *Parectopa* group (i.e. two pairs of bristles on the seventh and eighth abdominal segments in the males and anteriorly tilted posture of resting adults; A. Kawakita & M. Kato 2009, personal observation), suggesting that *Cuphodes* is probably one of the closest relatives of *Epicephala*.

Analysis of the *COI + EF-1α + ArgK* dataset in *Cuphodes* suggested that the sampled moths consist of eight putative species (figure 3; also see electronic supplementary material, figure S2), which can be distinguished by wing pattern, male genitalia morphology and larval mining pattern (A. Kawakita & M. Kato 2009, personal observation). The obtained phylogeny demonstrated that these species regularly use two to four closely related plants (figure 3), suggesting that the closest relatives of *Epicephala* do not show the same degree of host specificity as *Epicephala*. Although the use of different host plant families in *Epicephala* and *Cuphodes* may make direct comparison difficult, available evidence suggests that *Cuphodes* species exhibit much broader host ranges than do the species of *Epicephala*. For example, *C. wisteriae* uses *Wisteria* and *Millettia*, which are distantly related genera within Fabaceae, having diverged at least 50 Ma (Lavin *et al.* 2005), whereas the age of the *Glochidion* crown group is estimated to be less than 10 Ma (Kawakita & Kato 2009). Similarly, *Cuphodes* sp. 4 feeds on two genera (*Berchemia* and *Berchemiella*) of the Rhamnaceae family, although the antiquity of their divergence is unknown. Thus, these results indicate that the high degree of host specialization found in *Epicephala* is probably not an ancestral condition predating the evolution of pollinator habit.

Taken together, the present results on host specificity of *Diphtheroptila*, *Caloptilia*, *Cuphodes* and *Glochidion*-feeding, non-gracillariid lepidopterans all indicate that the level of host specialization in *Epicephala* is higher than would be expected if host specificity were determined solely by the herbivorous habit of the moths. Thus, our data are more consistent with the view that pollinator habit favours higher host specificity than the ancestral parasitic lifestyle. What, then, is the ultimate cause driving the strict host specificity of *Epicephala*? A previous analysis of floral scent in *Glochidion* (Okamoto *et al.* 2007) found clear differences in the chemical composition of floral volatiles among co-flowering *Glochidion* species. These differences are perceived by host-seeking *Epicephala* females (Okamoto *et al.* 2007) and probably facilitate the attraction of species-specific pollinators.
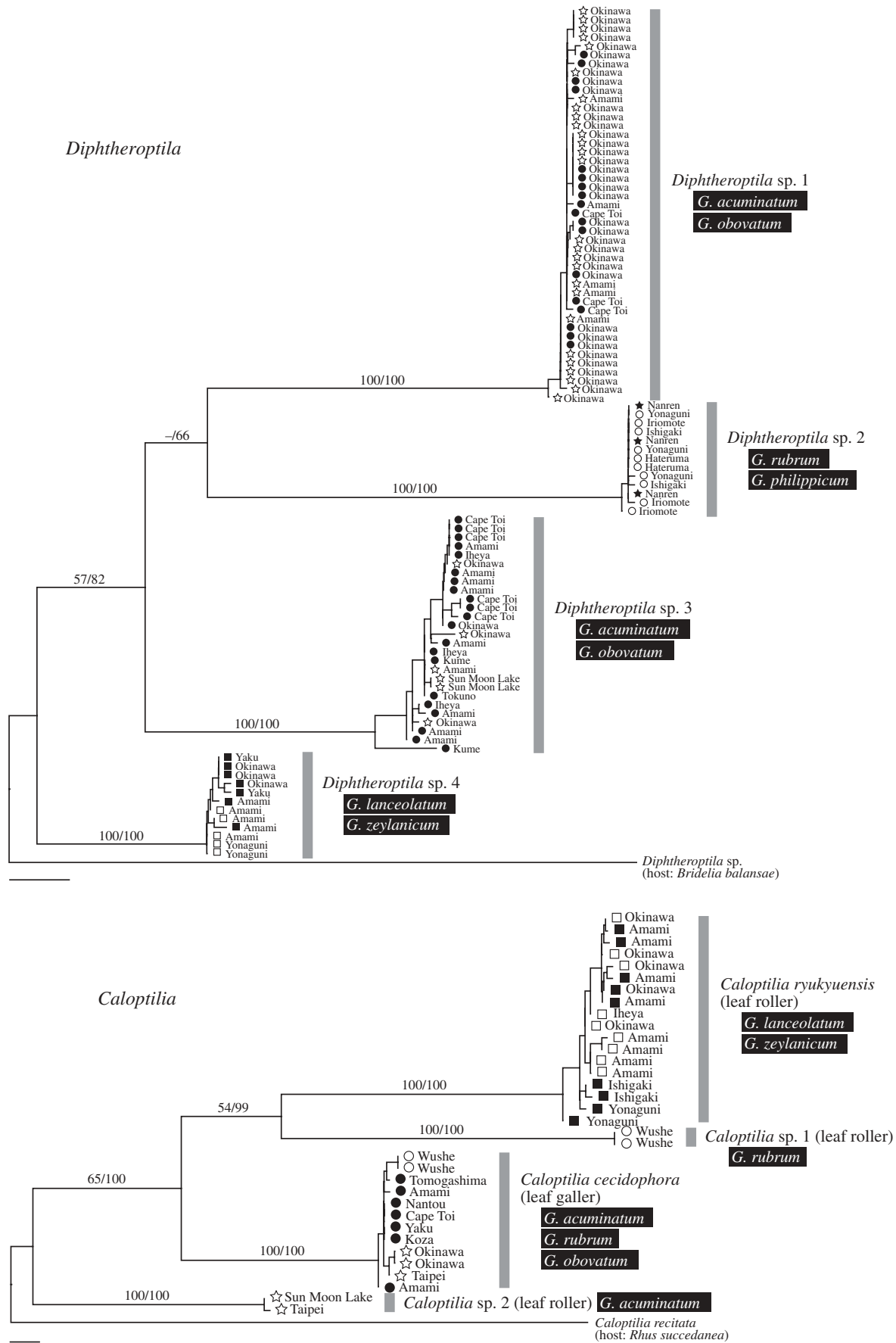
Figure 2. Maximum-likelihood (ML) phylogeny of *Diphtheroptila* and *Caloptilia* moths based on 1058 bp of the combined mitochondrial *COI* and *EF-1α* genes. Terminal symbols represent host *Glochidion* species followed by locality names. Numbers above branches indicate ML bootstrap values followed by Bayesian posterior probabilities. Individuals belonging to two of the *Caloptilia* clades were each identified morphologically as *Caloptilia ryukyuensis* and *Caloptilia cecidophora*. Symbols: filled circle, *G. obovatum*; open circle, *G. rubrum*; filled square, *G. lanceolatum*; open square, *G. zeylanicum*; filled star, *G. philippicum*; open star, *G. acuminatum*. Scale bars, 0.005 substitutions per site.
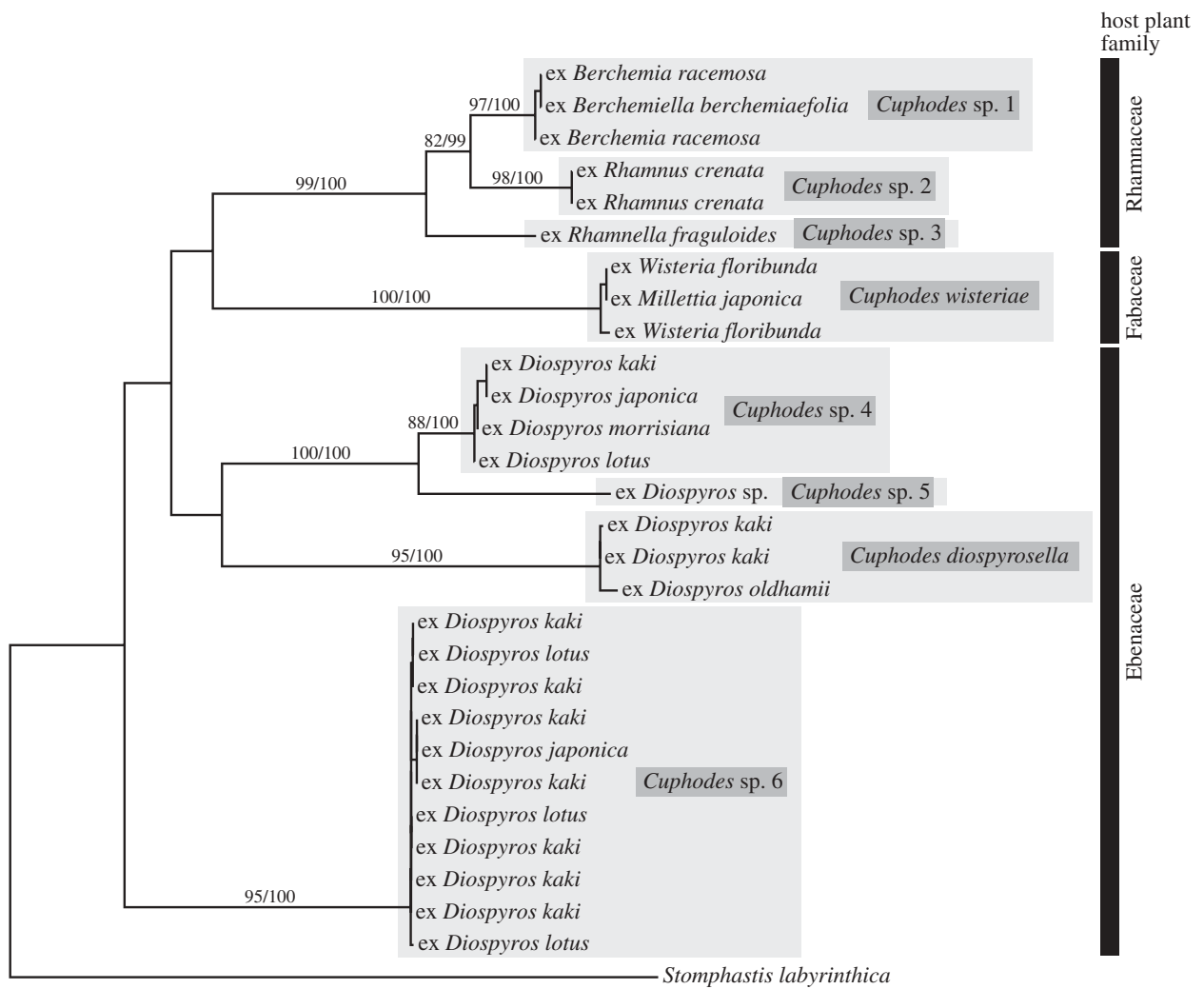
Figure 3. Maximum-likelihood (ML) phylogeny of *Cuphodes* based on 1601 bp of the combined mitochondrial *COI* and nuclear *EF-1α* and *ArgK* genes. Terminal taxon labels indicate host plant names. Host plant families are indicated using bars on the right. Numbers above branches indicate ML bootstrap values followed by Bayesian posterior probabilities. Scale bar, 0.01 substitutions per site.

Table 1. Summary of genetic variations in non-gracillariid *Glochidion* seed parasites. Each moth species was sampled from four to five *Glochidion* hosts, and the number of base substitutions that were unique to individuals associated with a particular host (diagnostic sites) are given for each gene.

family/species	host species(locality number)	moths sampled	bases sequenced		diagnostic sites (variable sites)	
			COI	*EF-1α*	*COI*	*EF-1α*
Carposinidae						
Peragrarchis syncolleta	*G. acuminatum*	2	580	498	0 (1)	0 (3)
	G. lanceolatum	1				
	G. obovatum	3				
	G. rubrum	1				
Tortricidae						
Tritopterna sp.	*G. lanceolatum*	4	580	498	0 (2)	0 (14)
	G. obovatum	1				
	G. philippicum	1				
	G. rubrum	3				
	G. zeylanicum	4				
Pyralidae						
Cryptoblabes sp.	*G. lanceolatum*	1	580	498	0 (0)	0 (3)
	G. obovatum	2				
	G. rubrum	2				
	G. zeylanicum	2				

Thus, selection may operate on *Glochidion* plants to produce distinct floral scents and attract specific pollinators and thereby to avoid incompatible hybridization. Although further experimentation is needed to determine whether interspecific crosses result in fruit production, any decrease in quantity and/or quality of hybrid fruits is likely to facilitate reciprocal specialization by *Epicephala* to species-specific floral volatiles. Thus, it is possible that the high plant–pollinator-specificity in obligate pollination mutualisms is driven by a plant's interest to avoid less advantageous hybridization.

Overall, our finding of strict host specificity in *Epicephala* is in marked contrast with previous findings in the yucca moth lineage (Pellmyr & Thompson 1992; Thompson 1994, 2005). The closest relatives of the pollinating yucca moths, *Prodoxus*, feeds on inflorescence stalks, fruit or, rarely, leaves of yucca plants and have very similar degrees of host specificity with the pollinating yucca moths (Pellmyr *et al.* 2006). We suggest that this difference is because of contrasting patterns of flowering phenology between yuccas and *Glochidion*. Because both pollinating (*Tegeticula* and *Parategeticula*) and non-pollinating (*Prodoxus*) yucca moths are short-lived (Powell 1984) and their life histories are strongly associated with yucca flowers (Pellmyr 1999, 2003; Pellmyr *et al.* 2006), the adult moths must emerge during a short period when host flowers are available. However, coexisting yucca species typically exhibit largely non-overlapping flowering periods (Pellmyr 2003); thus, there is little opportunity for both pollinating and non-pollinating yucca moths to select among multiple hosts within a single population. In contrast, most *Glochidion* species produce flowers and leaves continuously from spring to autumn, and as many as four species flower at the same time within our study area. Under such circumstances, both leaf-feeding and flower-infesting moths are provided with multiple available hosts, but the latter are more selective in their host choice owing to a broader range of coevolutionary traits with which they are constrained. The occurrence of multiple co-flowering host species is also the case in figs, for which preliminary analysis of host specificity in pollinating and non-pollinating fig wasps suggested that the former tends to be more host-specific (Weiblen & Bush 2002; Marussich & Machado 2007; but see Lopez-Vaamonde *et al.* 2001; Jousselin *et al.* 2006, 2008).

Although our data clearly indicate that pollinating seed parasites exhibit higher degrees of host specialization than those of their parasitic ancestors, a more direct test of host specificity would be to include non-pollinating *Epicephala* seed parasites in the analysis. The most basal lineage of *Epicephala* is a non-pollinator that attacks the seeds of *Flueggea*, a close relative of *Glochidion* within the tribe Phyllantheae. However, this species is currently known only from *Flueggea suffruticosa* in southwestern Japan (Kawakita & Kato 2009), where there are no other co-occurring *Flueggea* species. Also, a derived clade of *Epicephala* has secondarily lost the pollinating habit, and currently there are three species that are each specific to a single *Phyllanthus* host (Kawakita & Kato 2009). However, closely related *Phyllanthus* hosts are rarely available within the same population, which precludes a direct comparison of host specificity with pollinating *Epicephala* in this case as well. Within the yucca moth lineage, two derived species have independently lost their pollinating

behaviour and oviposit in young fruits to exploit the seeds that other yucca moth species have pollinated (Pellmyr *et al.* 1996; Pellmyr 1999). These cheater species evolved to use four to six yucca hosts (Pellmyr 1999, 2003), which is consistent with our hypothesis that pollinator habit promotes host specificity in pollinating seed parasites. The cheater yucca moths are likely to have a broader phenological window for successful oviposition (Pellmyr 2003); thus, selection for host specialization may have been relaxed in these derived non-pollinators.

Although further research is required to identify coevolutionary forces driving pollinator specificity, our findings indicate that mutualistic selection probably favours strict host specificity of pollinating seed parasites in obligate pollination mutualisms. As shown in recent population-level analyses of gene flow in figs and yuccas (Machado *et al.* 2005; Smith *et al.* 2008, 2009), pollinator specificity is likely to strongly impact patterns of gene flow between coexisting plant species and play an important role in facilitating reproductive isolation between diverged populations. Thus, future studies of coevolution and codivergence in the *Glochidion–Epicephala* mutualism, as well as comparative analysis across systems, are likely to substantially improve our understanding of the role of coevolution in promoting speciation and diversification in obligate pollination mutualisms.

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