

# A Novel, Enigmatic Basal Leafroller Moth Lineage Pollinating a Derived Leafroller Host Illustrates the Dynamics of Host Shifts, Partner Replacement, and Apparent Coadaptation in Intimate Mutualisms

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**ABSTRACT:** Leafroller plant/leafroller moth brood pollination mutualisms are widespread in the Palearctic. Leafroller moths pollinate leafroller plants, but their larvae consume a subset of the hosts' seeds. These interactions are highly phylogenetically constrained: six clades of leafroller plants are each associated with a unique clade of leafroller moths (*Epicephala*). Here, we report a previously unrecognized basal seventh pollinating *Epicephala* lineage—associated with the highly derived leafroller clade *Glochidion*—in Asia. *Epicephala lanceolaria* is a pollinator and seed predator of *Glochidion lanceolarium*. Phylogenetic inference indicates that the ancestor of *E. lanceolaria* most likely shifted onto the ancestor of *G. lanceolarium* and displaced the ancestral allospecific *Epicephala* pollinator in at least some host populations. The unusual and apparently coadapted aspects of the *G. lanceolarium*/*E. lanceolaria* reproductive cycles suggest that plant-pollinator coevolution may have played a role in this displacement and provide insights into the dynamics of host shifts and trait coevolution in this specialized mutualism.

**Keywords:** *Epicephala*, *Glochidion*, host shift, intimate mutualism, specialized pollination, coadaptation.

## Introduction

Intimate mutualisms (those with a high degree of biological intimacy between partners, including symbiotic mutualisms; Ollerton 2006) are often observed to be extremely specialized in ecological time (Thompson 1994; Guimarães et al. 2007; Thompson et al. 2013). Consequently, the extent to which these interactions are phylogenetically constrained

over evolutionary timescales has been of interest since the days of the earliest cophylogenetic analyses (Chenuil and McKey 1996; Herre et al. 1996; Itino et al. 2001; Lopez-Vaamonde et al. 2001; Piercey-Normore and DePriest 2001; Weiblen 2001; Kawakita et al. 2004; Quek et al. 2004; Kawakita and Kato 2009; Althoff et al. 2012; Cruaud et al. 2012a; Hembry et al. 2013; Chomicki et al. 2015; Yang et al. 2015). It is clear that in many cases, mutualisms between eukaryotic hosts and microbial endosymbionts are vertically transmitted and consequently show significant phylogenetic congruence (e.g., Moran et al. 1993; Baumann and Baumann 2005; Urban and Cryan 2012). Interestingly, cophylogenetic patterns and evidence for clade-level phylogenetic conservatism in interactions have also been found in a range of intimate yet not fully symbiotic eukaryotic mutualisms. Examples include pollinating seed-predation, ant-myrmecophyte, and leafcutter ant-fungus interactions (Currie et al. 2003; Kawakita and Kato 2009; Althoff et al. 2012; Cruaud et al. 2012b; Chomicki et al. 2015). In these eukaryotic mutualisms, evidence for strict cospeciation is limited (Cruaud et al. 2012a; Hembry and Althoff 2016; but see Yang et al. 2015), and at some scales in some interactions, phylogenetic congruence is even lacking (e.g., Quek et al. 2004; Hembry et al. 2013). However, the evidence for both cophylogenetic structure and phylogenetic conservatism across different intimate mutualisms is striking, given the obvious potential for (and clear evidence of) horizontal transfer. This would suggest that patterns of partner use in intimate yet nonsymbiotic mutualisms frequently remain constrained over macroevolutionary timescales. This pattern is interesting and could potentially be due to opportunities within intimate associations for mutualistic partners to exert stabilizing selection, as suggested by theory (Kopp and Gavrillets 2006; Yoder and Nuismer 2010; Rai-

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munro et al. 2014), or due to antagonistic components of these mutualistic interactions (Thompson 1994; Althoff 2016). Such phylogenetic conservatism would also be in contrast to less intimate mutualisms, such as generalized pollination or seed dispersal, in which theory has suggested that unrelated lineages are readily pulled into interaction networks of unrelated species (Thompson 2009; Guimarães et al. 2011).

Brood pollination mutualisms between leafflowers (Phyllanthaceae: *Phyllanthus* s.l., including *Glochidion* and *Breynia*) and leafflower moths (Lepidoptera: Gracillariidae: *Epicephala*) exemplify this combination of constraint and dynamics in the evolution of intimate mutualisms. Six distantly related clades of leafflower plants in the Old World tropics and subtropics are each, in turn, pollinated by a reciprocally associated clade of leafflower moths that is exclusive to it (Kawakita and Kato 2009). These moths gather pollen from male flowers, actively transfer pollen to a recessed stigmatic surface in female flowers, and then oviposit into the flowers, where moth larvae subsequently hatch and consume (in most species known) a subset of the developing seeds (Kato et al. 2003; Kawakita and Kato 2004a, 2004b). In the best-studied pair of reciprocally associated leafflower and moth clades—*Glochidion* trees and their monophyletic group of *Epicephala*—phylogenies are more similar than would be expected by chance, although inconsistent with strict cospeciation (Hembry et al. 2013). Thus, while patterns of interaction among species in this mutualism may be labile over evolutionary timescales, they are still strongly phylogenetically constrained.

Such phylogenetic conservatism in this intimate mutualism may in part be due to the evolution of functional traits that mediate the interaction across the phylogenies of *Phyllanthus* s.l. and *Epicephala*. For instance, floral odor blends emitted by leafflowers are known to vary both among species within a clade (Okamoto et al. 2007) as well as among clades (Okamoto et al. 2013), and these blends are responsible for mediating host recognition by female moths. More derived *Epicephala* females possess a sclerotized ovipositor, which is used in most cases for laying eggs inside ovary tissue of leafflower flowers rather than on the external surface of the carpels (Kawakita and Kato 2009, 2016; but see Zhang et al. 2012b). Finally, processes such as biogeographic isolation of some pairs of subclades (such as Madagascar *Phyllanthus* and their *Epicephala*) may reduce the likelihood of host shifts among subclades. It is highly likely that additional factors not previously examined—these may include morphological traits of flowers and developmental traits of *Epicephala* larvae—may also mediate and constrain host shifts by *Epicephala* among very distantly related leafflower species.

Of all reciprocally associated subclades of leafflowers and leafflower moths, the clade *Glochidion* and its clade

of *Epicephala* are the best studied. The 300 species of *Glochidion* and their presumably similar number of *Epicephala* species are widely distributed in east, south, and southeast Asia, Australia, New Guinea, and the Pacific Islands and have received concerted attention from multiple groups of investigators, particularly in East Asia and Polynesia (Kato et al. 2003; Kawakita and Kato 2006, 2016; Okamoto et al. 2007, 2013; Goto et al. 2010; Hembry et al. 2012, 2013; Zhang et al. 2012a; Mochizuki et al. 2014; Okamoto 2014; Li et al. 2015). In East Asia, *Glochidion* and their *Epicephala* show very high species specificity (Kawakita and Kato 2006, 2016; Zhang et al. 2012a; Li et al. 2015), which is mediated by species-specific floral odor blends (Okamoto et al. 2007, 2013). *Epicephala* females actively pollinate *Glochidion* flowers, inserting pollen into the recessed stigmatic surface and ovipositing inside the locules of the flowers' ovaries. Morphologically, these *Glochidion*-associated *Epicephala* species are part of a larger derived clade within the genus in which adult females have sclerotized ovipositors. Larvae consume a subset of developing seeds before exiting the fruit to pupate in the leaf litter (Kato et al. 2003). Cophylogenetic analyses between *Glochidion* and their *Epicephala* show greater congruence than would be expected by chance between the two clades, although evidence for host shifts is also seen (Hembry et al. 2013).

In this article, we examine the widespread tree *Glochidion lanceolarium*, native to mainland tropical and subtropical Asia. The moth *Epicephala lanceolaria* has been reported from *G. lanceolarium* in Guangdong and Hainan Provinces and Hong Kong, China (Zhang et al. 2012a). *Glochidion lanceolarium* is unusual (although not unique) within *Glochidion* with regard to the seasonality of its reproductive phenology (see “Results”) and the presence of empty sinuses/locules (carpel chambers) within its mature fruit (S.-X. Luo, unpublished data). Together these observations suggest that the interaction between *G. lanceolarium* and *E. lanceolaria* may have a different origin from that of other *Glochidion* with their *Epicephala*. Here, we report observations of the biology and life cycle of *E. lanceolaria* and the phenology of *G. lanceolarium* to determine whether the former is a pollinator of the latter. Additionally, we examine the phylogenetic relationships of both species within their respective genera to determine whether this species pair shares a common evolutionary history with other *Glochidion* and their previously known, reciprocally associated clade of pollinating *Epicephala*. We find that *G. lanceolarium* is indeed part of the clade *Glochidion* and is exclusively pollinated by *E. lanceolaria* at multiple sites in south China. However, we also find that *E. lanceolaria* is distantly related to the clade containing all other *Epicephala* known to pollinate *Glochidion*. Instead, *E. lanceolaria* represents a previously unknown, enigmatic basal lineage within *Epicephala* with no known close rel-

atives. We infer that the ancestor of *E. lanceolaria* most likely shifted from an unknown host onto *G. lanceolarium* or its ancestor and, in doing so, displaced any previous *Epicephala* pollinator(s) that may have been present, at least in part of the range of *G. lanceolarium*. The many apparently coadapted aspects of the *G. lanceolarium* phenology and *E. lanceolaria* life cycle that we report here may indeed have rapidly evolved following this host shift or may have previously existed in either or both of the ancestors of these taxa before the host shift.

## Material and Methods

### Study Sites and Plant Species

The study was carried out at South China Botanical Garden (SCBG), Guangzhou; Wutongshan, Shenzhen; Xiangtoushan, Huizhou; and Gaobangshan, Huizhou, all in Guangdong Province, China, from 2007 to 2013. *Glochidion lanceolarium* (vouchers Yao 073, 134, and 144, deposited in the herbarium IBSC) is a monoecious tree (fig. 1A) that occurs in China, Vietnam, India, Laos, Cambodia, and Thailand (Nguyen 2007; Li and Gilbert 2008; van Welzen and Chayamarit 2016). In China, it usually grows in low vegetation or along forest edges at 100–800-m elevation. The plants attain a height of 2–5 m and produce tens to hundreds of thousands of male and female flowers from early April to early May (S.-X. Luo, unpublished data; see “Results”).

### Flower Observation

We made diurnal and nocturnal observations of flower visitors for a total of more than 300 h during the study periods. Particular effort was made to conduct nocturnal observation to study flower visitation by *Epicephala* moths. Moths that visited the plants' flowers were collected for identification and examination of their pollen loads under a stereoscope (Stemi Dv4; Zeiss, Germany) or under SEM (JSM-6360LV, KEOL, Japan). Visiting and oviposition behavior by moths was carefully observed, recorded, and photographed. Adults and larvae were identified using morphology and molecular methods; vouchers have been deposited at SCBG, Guangzhou, Guangdong, China, and the Center for Ecological Research, Kyoto University, Otsu, Shiga, Japan. In flower seasons, female flowers were collected randomly to check for the pollen grains on the stigmas and eggs in the flowers, using a microscope. To determine the extent of seed infestation by seed-parasitic moths, we also collected mature fruits in three populations. For each fruit, we counted the number of destroyed seeds, intact seeds, and unfertilized ovules. Sample sizes are given in the results.

### Flowering-Fruiting Phenology and Moth Life Cycle

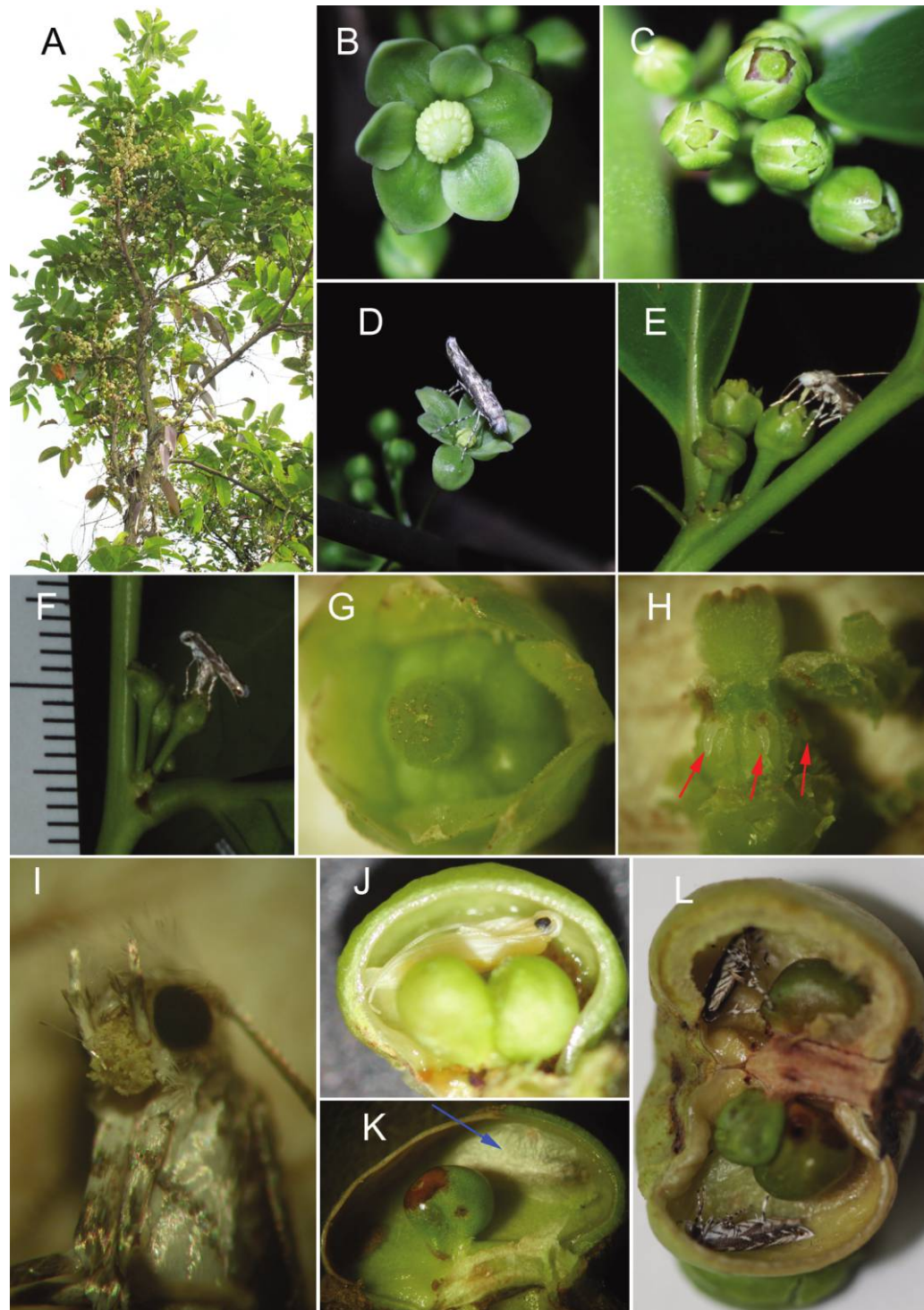
Since *G. lanceolarium* has only one flowering-fruiting cycle per year, we monitored the flowering and fruiting phenology by checking the flowers and fruits developing each week in April and May and each month from June to the following March at SCBG. In order to understand the life cycle of the *Epicephala* moths, we randomly collected female flowers, immature fruits, and mature fruits from the trees in three of the four study populations. We used these samples to determine the egg hatching time and the number of larvae, pupae, and adult moths across the annual phenological cycle of the plants. Since there are up to seven eggs or adults in one female flower or in one fruit, we also counted and analyzed the frequency distribution of the number of eggs, larvae, and adult moths per flower and fruit at four populations. Since preliminary observations showed that some of the larvae did not spin a protective cocoon around their pupae, we collected data on the distribution and frequency of pupae with or without cocoons. Preliminary observations also found that the adult moths fly out from the mature fruits concurrently with the dehiscing of the mature fruits. Consequently, we marked different fruits on different tree individuals and monitored them each night in April.

### Molecular Phylogeny

We extracted total DNA from adults and larvae following previously described methods (Kawakita et al. 2004; Kawakita and Kato 2006). Five loci were amplified and Sanger sequenced (for *Epicephala*, mitochondrial cytochrome oxidase I [COI] and nuclear EF1- $\alpha$  and ArgK; for *Glochidion*, nuclear ribosomal internal transcribed spacer [ITS] and external transcribed spacer [ETS]) using primers from previous phylogenetic work on these genera (Kato et al. 2003; Kawakita et al. 2004). Sequences are GenBank numbers KY078233–KY078291. Initial sequencing and phylogenetic analysis using a single individual of *Epicephala lanceolaria* suggested that it occupied an unexpected position within the *Epicephala* phylogeny (A. Kawakita, personal communication, 2009), so we sampled 20 additional moths from four sites in Guangdong Province, China, to confirm this result. We made sure to sample moths that had emerged from pupae with and without cocoons, in case these moths represented different taxa. For specimen information, see table 1.

We combined these sequences with 100 additional *Glochidion* operational taxonomic unit (OTU) sequences and 71 *Epicephala* OTU sequences from earlier studies (Kawakita et al. 2004; Kawakita and Kato 2009; Hembry et al. 2013). We added three species of non-*Glochidion* *Phyllanthus* s.l. and three non-*Epicephala* Gracillariidae in these analyses, setting one taxon in each as an outgroup on the basis of previous phylogenetic analyses. Our data set





**Figure 1:** Flowers, fruit, and pollinator of *Glochidion lanceolarium*. A, *Glochidion lanceolarium* individual bearing many fruits and flower buds. B, Male flower. C, Female flowers. D, Female *Epicephala lanceolaria* moth visiting a male flower. E, *Epicephala lanceolaria* actively pollinating a female flower. F, Ovipositing *E. lanceolaria*. G, Pollinated stigma, showing the pollen grains on the stigma. H, Cross section of a female flower with three eggs (arrows). I, Proboscis of a female *E. lanceolaria* moth bearing pollen. J, *Epicephala lanceolaria* pupa in a carpel chamber without cocoon. K, *Epicephala lanceolaria* cocoon in a carpel chamber with a pupa inside. L, Cross section of a mature fruit with two enclosed *E. lanceolaria* in the carpel chamber.

**Table 1:** List of *Epicephala lanceolaria* samples sequenced in this study

Sampling locality and sample no.	Cocoon presence/absence	Life stage	GenBank no.		
			COI	ArgK	EF-1 $\alpha$
Longdong (SCBG; LD):					
7	...	Larva	KY078239	KY078271	KY078279
15	No	Adult	KY078246	KY078256	KY078286
16	No	Adult	KY078247	KY078266	KY078287
17	No	Adult	KY078248	KY078259	KY078288
18	Yes	Adult	KY078249	KY078260	KY078289
19	Yes	Adult	KY078250	KY078268	KY078290
20	Yes	Adult	KY078251	KY078255	KY078291
Gaobangshan (GB):					
5	No	Pupa	KY078237	KY078262	KY078277
6	Yes	Adult	KY078238	KY078270	KY078278
13	No	Pupa	KY078244	KY078258	KY078284
14	Yes	Adult	KY078245	KY078267	KY078285
Qiao Island (QA):					
3	...	Larva	KY078235	KY078257	KY078275
4	...	Larva	KY078236	KY078261	KY078276
9	No	Pupa	KY078240	KY078264	KY078280
10	Yes	Adult	KY078241	KY078269	KY078281
Wutongshan (WT):					
1	No	Pupa	KY078233	KY078254	KY078273
2	...	Larva	KY078234	KY078272	KY078274
11	Yes	Adult	KY078242	KY078263	KY078282
12	Yes	Adult	KY078243	KY078265	KY078283

Note: COI, cytochrome oxidase I; SCBG, South China Botanical Garden.

included all available *Glochidion* and other Phyllanthaceae ITS and ETS sequences, as well as sequences from all available *Epicephala* associated with *Glochidion* and other Phyllanthaceae. We detected a possible labeling error for two insular East Asian *Epicephala* taxa (three OTUs from *Glochidion obovatum* and *Glochidion rubrum* in Japan and Taiwan) in an early study (Kawakita et al. 2004) that was unintentionally propagated to a later study (Hembry et al. 2013). Sequences from those OTUs in the study by Kawakita et al. (2004) were replaced with other sequences from the same taxa from a different study (Kawakita and Kato 2006). We updated the nomenclature of some East Asian *Epicephala* OTUs in the phylogeny following recent species descriptions (Kawakita and Kato 2016). Sequences were either aligned by eye (lacking gaps) or using MUSCLE (Edgar 2004). We inferred phylogenetic relationships using Bayesian inference in MrBayes (ver. 3.2; Ronquist and Huelsenbeck 2003) and maximum likelihood implemented in RAxML-HPC2 (Stamatakis 2014). For *Glochidion*, we ran the analyses with two partitions, one for each gene region (with the models HKY + G for both partitions in MrBayes and GTR + G for both partitions in RAxML). For *Epicephala*, we ran the analyses with seven partitions (identified using Bayesian informa-

tion criterion implemented in PartitionFinder; Lanfear et al. 2012). This partition combined first-codon positions for both nuclear genes and second-codon positions for both nuclear genes, leaving all remaining codon positions (third positions and mitochondrial COI) as independent partitions. In MrBayes, each partition received its own model based on the PartitionFinder results (ArgK position 1 + EF1 -  $\alpha$  position 1: GTR + I + G; ArgK position 2 + EF1 -  $\alpha$  position 2: F81 + I + G; ArgK position 3: GTR + G; EF1- $\alpha$  position 3: SYM + G; COI position 1: SYM + I + G; COI position 2: HKY + I + G; COI position 3: GTR + I + G), and in RAxML, each was set to the model GTR + G. Most of these phylogenetic inferences used computational infrastructure from the Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway on the Extreme Science and Engineering Discovery Environment (XSEDE; Miller et al. 2010).

We tested the hypothesis of biphyly of *Glochidion*-associated *Epicephala* using a Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999) implemented in the package phangorn (Schliep 2011) in the R programming language (ver. 3.0.2; R Development Core Team 2013). These tests used consensus trees of *Epicephala* obtained in MrBayes

(ver. 3.1; Ronquist and Huelsenbeck 2003), constrained to force *Glochidion*-associated *Epicephala* to be either monophyletic or not monophyletic, respectively. These consensus trees were generated using a different partitioning (GTR + G for each codon position for each of three genes, for a total of nine partitions) on the same *Epicephala* data set. The resulting trees were topologically nearly identical to those generated using the seven-partition scheme above and shared the same placement of *E. lanceolaria* relative to all other major lineages of *Epicephala* in the negative constraint analysis (that in which all *Glochidion*-associated *Epicephala* were constrained to be not monophyletic). These analyses were performed for an earlier version of this manuscript, and we report them here because we were unable to repeat the analysis using the newly generated trees in the new release of phangorn (ver. 2.0.4; June 2016) because of an apparent bug in the function phyDat. Data underlying phylogenetic and Shimodaira-Hasegawa analyses—and the resulting trees—are deposited in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.t9140> (Luo et al. 2016).

## Results

### *Floral Morphology and Pollination*

Trees of *Glochidion lanceolarium* (fig. 1A) have male and female flowers in separate axillary clusters on different branches. Male flowers usually occur in the axils, which have mature fruits; each such cluster on average has  $9.7 \pm 2.7$  (mean  $\pm$  SD; range 5–13,  $N = 11$ ) male flowers. Female flowers are also produced in axillary clusters, and each cluster typically consists of  $3.47 \pm 1.7$  (range 1–7,  $N = 18$ ) female flowers. Male flowers have long (8–10 mm; Li and Gilbert 2008) pedicels, unfolded yellow perianths, and connate ellipsoid stamens (fig. 1B), whereas female flowers are inconspicuously green, have short (2–4 mm; Li and Gilbert 2008) pedicels, and are composed of reduced perianths and fused styles; the styles form a narrow pit at the tip of the fused column (fig. 1C). In SCBG, on average, female flowers of *G. lanceolarium* have 6–8 carpels ( $6.78 \pm 0.55$ ,  $N = 60$ ), and each has two ovules. The average ovule number per flower is  $13.5 \pm 1.11$  ( $N = 60$ ). Flowering is synchronous both between sexes and among individuals, and no nectar is produced by male or female flowers.

During our 380 h of field observations over 4 years, *Epicephala lanceolaria* moths (Zhang et al. 2012a) were the only visitors and pollinators of *G. lanceolarium*. From the beginning of the evening and continuing until around midnight, the flowers were visited frequently by female *E. lanceolaria*. Female moths visited male flowers to collect pollen by inserting their proboscis into the anthers (fig. 1D). On the female flowers, female moths actively deposited pollen on the cryptic stigmas by using their proboscises

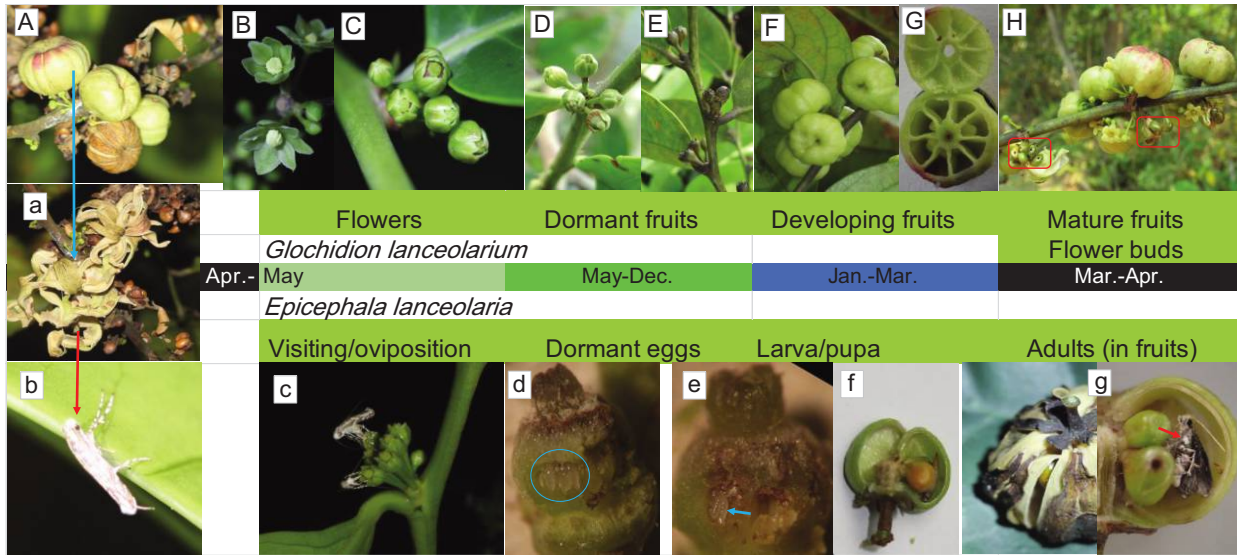
(fig. 1E) and then inserted their abdomens alongside the styles and laid eggs into the ovary walls, while not directly damaging the ovules (fig. 1F, 1H). A video of pollination and oviposition behavior is provided (video A1, available online) and is also available in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.t9140>. Females walked along branches, visiting each female flower sequentially and repeating the pollination and oviposition behavior. A female moth often repeated her pollinating and ovipositing behavior more than once (up to four times) in the same flower. Three (out of 150) and four (out of 150) monitored female flowers were pollinated and oviposited in by a second moth at SCBG in 2010 and 2013, respectively. Oviposited flowers were consistently pollinated (fig. 1G), whereas unoviposited flowers were very rarely pollinated, and unpollinated female flowers abscised shortly thereafter. All female *E. lanceolaria* moths collected from male and female flowers and leaves had *G. lanceolarium* pollen grains attached to their proboscides (fig. 1I), whereas proboscides of newly emerged *E. lanceolaria* from dehisced fruits had no pollen grains (fig. 2B;  $N = 30$ ).

### *Moth Eggs in Flowers and Adult Moths in Fruit*

We examined moth eggs in female flowers and larvae in young fruit by dissecting ovary or fruit walls under a microscope. Normally, a female moth laid more than one egg in a flower, and on average female flowers received around three eggs (table 2). The mean number of pollen grains deposited on an oviposited flower was  $27.29 \pm 10.53$ , significantly greater than that of an unoviposited flower ( $1.7 \pm 3.6$ ).

The mean number of eggs per female flower, larvae per unmaturing fruit, and adults per matured fruit are  $2.55 \pm 1.65$ ,  $2.53 \pm 1.85$ , and  $2.78 \pm 1.64$  at SCBG, respectively. No significant difference was found among the average number of eggs, larvae, and adults per flower or per fruit at different developmental stages of flowers or fruits or among different populations (S.-X. Luo and D. H. Hembry, unpublished data). One female flower received up to seven eggs, whereas 14.75% of pollinated flowers at SCBG, 12.9% at Gaobangshan, and 18.2% at Wutongshan were not infested by larvae (fig. 3). On average, one fruit had seven carpels (each containing two ovules), of which a mean number of 2.2–3.6 ovules were infested by moth larvae in different populations, 3.2–9.9 ovules were intact and viable, and 1.1–7.2 ovules were sterile (did not develop). Larvae hatched in the developing flowers, each consuming only one seed ( $N = 300$ ) within a carpel to complete larval development, and then escaped from the seed to pupate in the carpel chamber. Roughly equal numbers of moths formed and did not form cocoons in two populations (fig. 4).





**Figure 2:** Plant phenology and flower functional phases in *Glochidion lanceolarium* (A–H) and life cycle of *Epicephala lanceolaria* (a–g) over 1 year. A, Branch with mature fruits. B, Male flowers. C, Female flowers. D, E, Dormant developing fruits. F, Fruits just before the phase of rapid growth. G, Carpel chambers and dormant seeds of the developing fruit. H, Mature fruit and adjacent flower buds, and seeds on the carpopodium (squares). a, Dehiscence of mature fruits. b, Adult moth that has just exited a fruit. c, Pollinating moth (bottom left) and ovipositing moth (top). d, Dormant eggs (circle) in a dormant fruit. e, Larva (arrow) in an immature fruit. f, Pupa in a carpel chamber. g, Exterior view of mature fruit (left) and adult moth (arrow) in an opened carpel chamber (right).

*Plant Phenology, Fruit Morphology, and Moth Life Cycle*

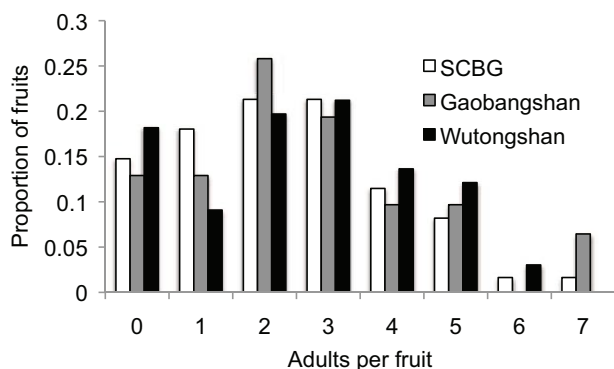
The main flowering time for *G. lanceolarium* is from early April to early May in south China. Each year, *G. lanceolarium* has only one flowering and fruiting season, and the pollinator *E. lanceolaria* has only one generation (fig. 2). Both the fruiting phenology and moth life cycle have distinct stages. From April to May (the flowering stage), *E. lanceolaria* actively collect and transport pollen with specialized proboscises and oviposit eggs into the pistils after pollination (fig. 2c). From May (in some cases, from late April) to December is an extended stage during which developing fruit and eggs inside them are dormant (fig. 2D, 2E, 2d). Fruits develop rapidly in January and February, although seed development is still dormant at this time (fig. 2F, 2G). As seeds remain dor-

mant inside rapidly expanding fruit, an empty carpel chamber (sinus) forms between the seeds and the carpel wall (fig. 2G); *Epicephala* eggs hatch, and larvae enter this carpel chamber. From mid-late January to early February, seeds develop rapidly. Larvae enter and consume one of two seeds in the carpel (there is often asynchrony in ovule development, and the larva will consume the more rapidly developing of the two ovules; fig. 2e, 2f). From mid-February to early March, most of the larvae in the fruit crawl out of the seeds and pupate inside the carpel chamber. Some larvae spin cocoons (fig. 1k), whereas others do not (fig. 1j). Adult moths eclose in the fruit beginning in March (fig. 2g) and persist for roughly 20 days inside the fruit (S.-X. Luo, unpublished data); subsequently from mid-March to early April, mature fruits dehiscence and adult moths fly out (fig. 2A, 2a, 2b). Most of

**Table 2:** Comparison of *Epicephala lanceolaria* pollination, oviposition, and seed infestation among four populations of *Glochidion lanceolarium*

Population	Laid eggs	Carpels	Intact seeds	Infested seeds	Inviabile seeds
Gaobangshan	2.4 ± 1.8	7.2 ± .7	8.4 ± 2.8	2.4 ± 1.9	3.6 ± 3.2
Qiao Island	2.6 ± 1.2	6.5 ± .6	3.2 ± 1.3	2.6 ± 1.2	7.2 ± 2.1
SCBG	2.2 ± 1.8	6.7 ± .6	9.9 ± 3.1	2.2 ± 1.8	1.1 ± 2.0
Wutongshan	3.5 ± 1.8	6.8 ± .7	5.2 ± 2.6	3.6 ± 1.9	4.8 ± 2.1

Note: Numbers indicate average values per flower (mean ± SE). SCBG, South China Botanical Garden.



**Figure 3:** Frequency distribution of the number of adult *Epicephala lanceolaria* per fruit of *Glochidion lanceolarium*. The number of fruits sampled from South China Botanical Garden (SCBG), Gaobangshan, and Wutongshan are 61, 31, and 21, respectively.

the mature fruits in one branchlet often dehisce in the same time at night. Wild-collected fruits at this time of year often contain enclosed adult moths that have not yet departed the fruits (fig. 1L). The carpel chambers present in the fruit of *G. lanceolarium* are unusual (although not unique) within *Glochidion*; the moths' use of these chambers for pupation and posteclosion behavior is also known in *Epicephala colymbetella* in Australia (Turner 1913, 1940), but detailed documentation of that species' life history has never been made. Since the pupae and adults are protected by the fruit, the life cycles of both the plant and its pollinator moth are linked to a degree not previously known in leafflowers and leafflower moths (fig. 1J–1L).

#### Molecular Phylogenetic Analysis

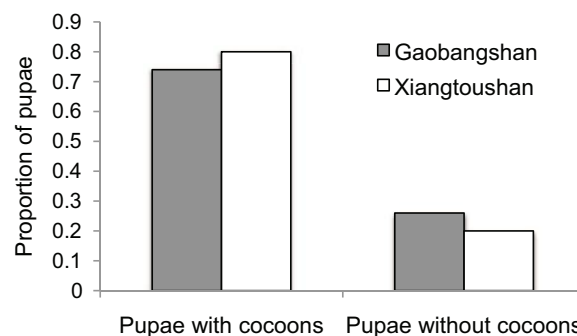
Both maximum likelihood and Bayesian phylogenetic analyses indicate that *G. lanceolarium* is nested within the clade *Glochidion*, as predicted by morphological taxonomy (Li and Gilbert 2008; van Welzen and Chayamarit 2016; figs. 5, A1; figs. A1, A2 are available online). All moths sampled from *G. lanceolarium* belong to *E. lanceolaria*; however, in both the maximum likelihood and the Bayesian analyses, this moth is found to not be part of the clade to which all other previously reported *Glochidion*-associated *Epicephala* belong (figs. 6, 7, A2; Kawakita et al. 2004; Kawakita and Kato 2006; Hembry et al. 2013). Rather, *E. lanceolaria* is a previously unknown seventh major lineage of pollinating *Epicephala* (alongside the *Epicephala* clades associated with all other *Glochidion*, *Breynia*, Madagascar *Phyllanthus*, the two subclades of *Gomphidium*, and the *Phyllanthus reticulatus* complex; Kawakita and Kato 2009). Our phylogeny suggests that *E. lanceolaria* occupies a relatively basal position within *Epicephala*, and it is consistent with previous morphological findings (Zhang et al. 2012a) that

suggest that the sacculi of the valvae in *E. lanceolaria* male genitalia may be less well developed and lack the conspicuous ornamentation that are characteristic of many other *Glochidion*-associated *Epicephala* (Zhang et al. 2012a; Li et al. 2015; T. Kumata, personal communication, 2010; D. H. Hembry, unpublished data). Female *E. lanceolaria* do, however, possess sclerotized ovipositors like all other derived *Epicephala* (Zhang et al. 2012a). Shimodaira-Hasegawa tests, three nodes with posterior probabilities >0.90 (in the Bayesian analyses), and three nodes of which two have bootstrap values >80 (in the maximum likelihood analyses) separating the two clades indicate that the biphyly of *Epicephala* associated with *Glochidion* is more strongly supported than monophyly of all *Glochidion*-associated *Epicephala* (Shimodaira-Hasegawa tests:  $P = .0080$ ).

#### Discussion

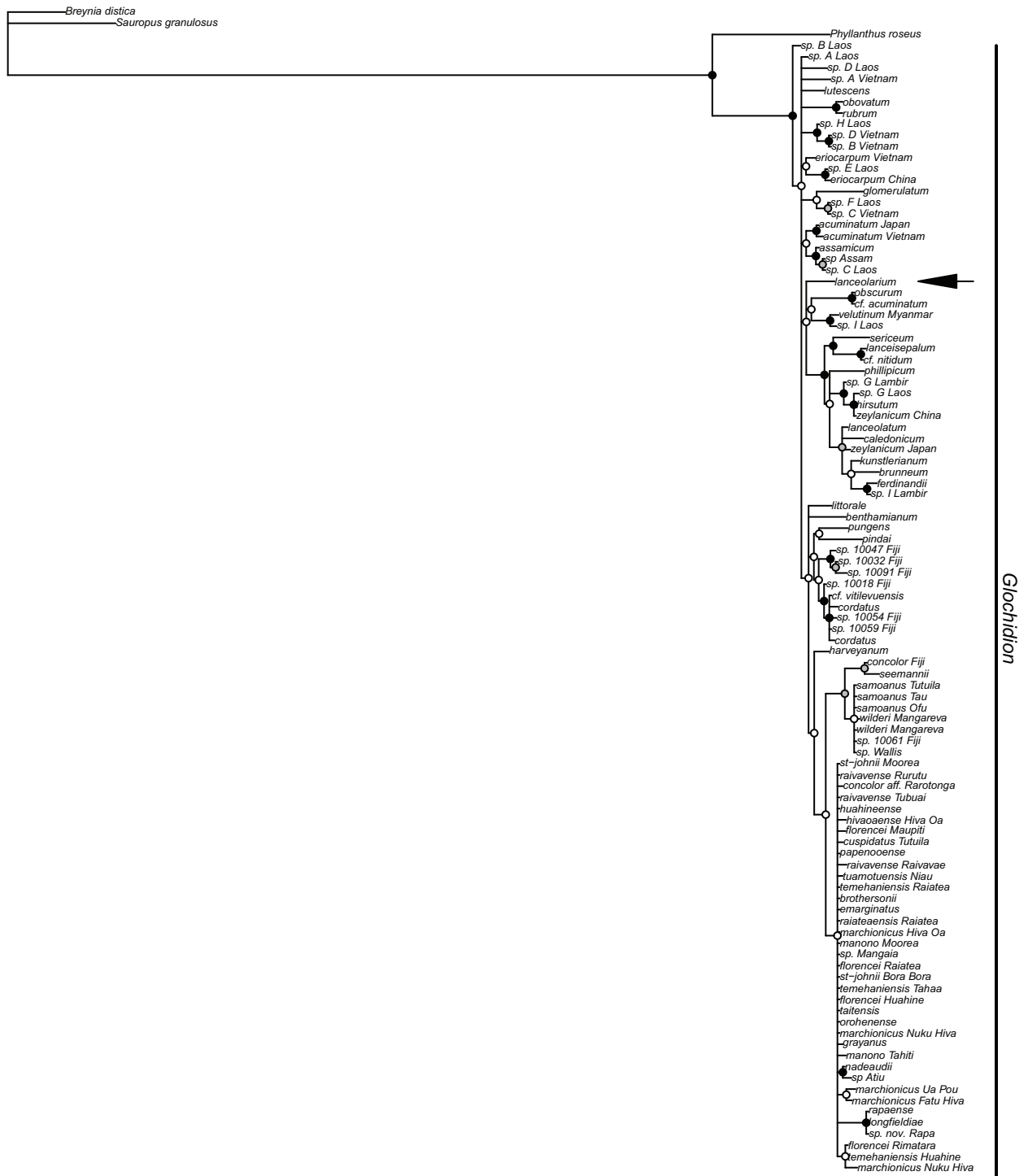
We find that the leafflower tree *Glochidion lanceolarium* is pollinated by a mutualistic leafflower moth (*Epicephala lanceolaria*) in south China. Although *G. lanceolarium* falls within the monophyletic group *Glochidion*, its pollinator *E. lanceolaria* does not form a monophyletic group with all other *Epicephala* species that have been previously reported in association with *Glochidion* (Kawakita et al. 2004; Kawakita and Kato 2006; Hembry et al. 2013). Thus, we find that *Epicephala* associated with *Glochidion* are a biphyletic group (fig. 7). This is the first reported exception to the pattern of each subclade of pollinating leafflower moths being associated with a unique subclade of leafflower hosts (Kawakita and Kato 2009).

The close relatives of *E. lanceolaria* are unknown. Phylogenetically, *E. lanceolaria* is sister to a clade containing nearly all other pollinating *Epicephala* associated with other leafflower plants. Because of the lack of known close relatives

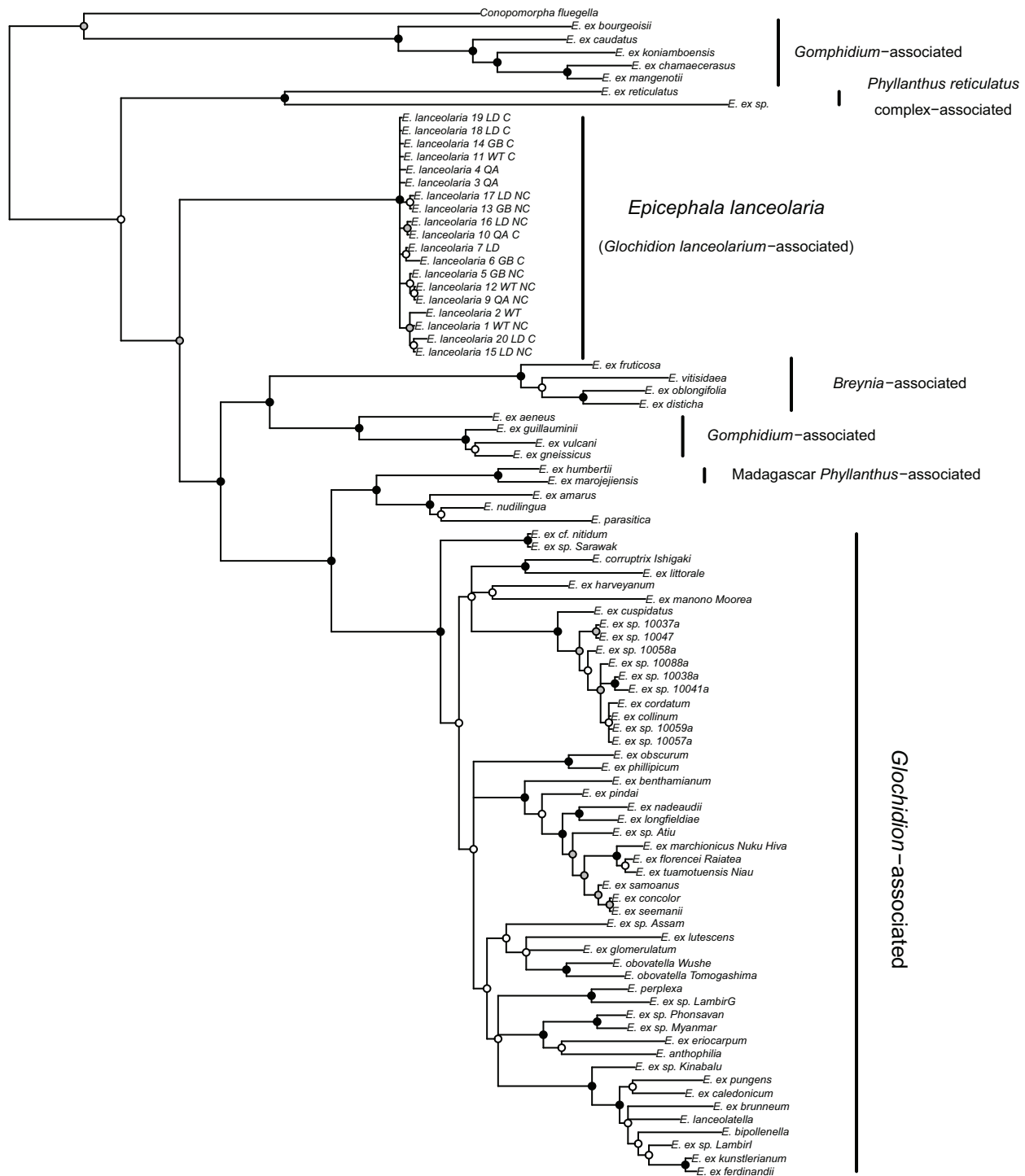


**Figure 4:** Frequency distribution of *Epicephala lanceolaria* pupae with or without cocoons. Number of pupae with cocoons: Gaobangshan, 58; Xiangtoushan, 175. Number of pupae without cocoons: Gaobangshan, 20; Xiangtoushan, 45.

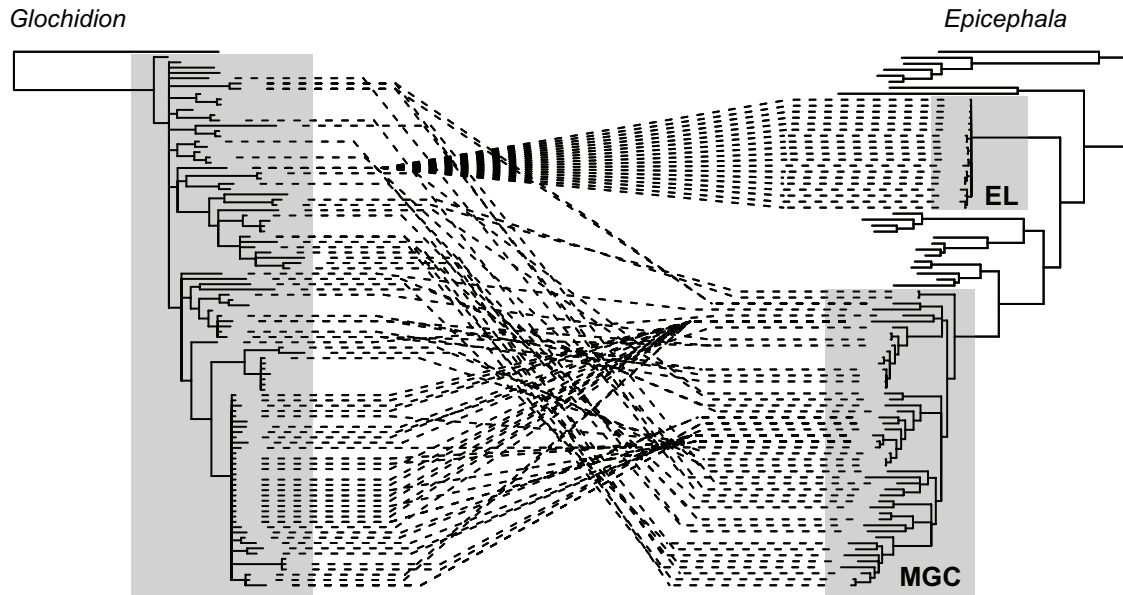




**Figure 5:** Phylogenetic hypothesis recovered using Bayesian inference for *Glochidion*. Tip labels represent specific epithets for *Glochidion* species. *Phyllanthus roseus* is a very close relative to *Glochidion* but is not pollinated by *Epicephala* (Kawakita and Kato 2009). *Glochidion lanceolarium* is nested within the clade *Glochidion* (arrow). Circles indicate posterior probabilities: white  $< 0.90$ ;  $0.90 \leq$  gray  $< 0.99$ ; black  $\geq 0.99$ .



**Figure 6:** Phylogenetic hypothesis for *Epicephala*, recovered using Bayesian inference. Tip labels indicate either described species (where names exist) or the species epithet of the host plant from which the specimen was reared. Outgroups (three non-*Epicephala* Gracillariidae) pruned. The seven major clades of pollinating *Epicephala* associated with different clades of *Phyllanthus* s.l. (in the genera *Phyllanthus*, *Breyenia*, and *Glochidion*) are indicated. The unlabeled clade containing *Epicephala parasitica*, *Epicephala nudilingua*, and *Epicephala* sp. ex *Phyllanthus amarus* is a group of nonpollinating seed-predatory *Epicephala* associated with species of *Phyllanthus* that lack pollinating *Epicephala* (Kawakita and Kato 2009). *Epicephala lanceolaria*, despite being associated with a species of *Glochidion*, does not form part of the same clade as all other known *Glochidion*-associated *Epicephala*. Circles indicate posterior probabilities: white < 0.90; 0.90 ≤ gray < 0.99; black ≥ 0.99. For *E. lanceolaria* operational taxonomic units, numbers refer to particular specimens (table 1); two-letter codes indicate collection localities (GB, Gaobangshan; LD, South China Botanical Garden; QA, Qiao Island; WT, Wutongshan); C, pupa with cocoon; NC, pupa with no cocoon.



**Figure 7:** Phylogenies of *Glochidion* (and its sister taxon *Phyllanthus roseus*) and *Epicephala*. Lines between tips indicate associations between taxa. EL, *Epicephala lanceolaria*; MGC, main *Glochidion*-associated clade. Other *Epicephala*-pollinated *Phyllanthus* s.l. (which are outgroups to *Glochidion* + *Phyllanthus roseus*) omitted for clarity. Unmarked *Epicephala* operational taxonomic units indicate taxa associated with *Phyllanthus* s.l. other than *Glochidion*.

to *E. lanceolaria*, inferences about the history of association between *E. lanceolaria* and *G. lanceolarium* are difficult. The lineage containing *E. lanceolaria* may represent a relatively recent host shift onto *Glochidion*. Alternately, the lineage containing *E. lanceolaria* may have been associated with *Glochidion* for a long time. The age of crown *Glochidion* is not particularly clear; middle Miocene fossils of *Glochidion* are known from India (Prasad 1994; Antal and Prasad 1996); molecular dating using these or *Phyllanthus* fossils has found similarly old dates (Kawakita and Kato 2009; Hembry et al. 2013; van Welzen et al. 2015). Fossil calibrations for *Epicephala* are not available, but molecular dating done using the arthropod COI molecular clock suggests that the main *Glochidion*-associated clade of *Epicephala* is no older than about 10 Ma, roughly the same age as the oldest known *Glochidion* fossils (Kawakita and Kato 2009). Since *Glochidion* is considered a relatively derived clade within Phyllanthaceae (Kathriarachchi et al. 2006; Kawakita and Kato 2009), even if the ancestor of *E. lanceolaria* has been associated with *Glochidion* for a long time, it may still have been associated with some other plants in Phyllanthaceae before its association with *Glochidion*.

One might hypothesize that, rather than representing a long-distance host shift by the ancestor of *E. lanceolaria* onto *G. lanceolarium*, the most recent common ancestor of *E. lanceolaria* and the main *Glochidion*-associated clade of *Epicephala* was actually associated with *Glochidion*. This would imply that the clade descended from this most recent

common ancestor underwent at least three host shifts off *Glochidion* onto other groups of Phyllanthaceae (onto *Breynia*, onto at least one lineage of herbaceous *Phyllanthus*, and onto Madagascar *Phyllanthus*; fig. 6) and that although *Glochidion* appears to be relatively derived within Phyllanthaceae, it is actually much older than its phylogenetic affinities and fossil record would suggest. We note that although such a scenario would require at least three shifts off *Glochidion*, the actual number of shifts required could be higher. First, in the maximum likelihood analyses, the bootstrap values for the clade uniting *E. lanceolaria* with its sister clade is relatively low (56; fig. A2), indicating that a fourth shift—onto the *Phyllanthus reticulatus* complex—might also need to be invoked. Second, we note that it is likely that some Afrotropical and Neotropical *Phyllanthus* have *Epicephala* associations, although sequence data are not available. Multiple *Epicephala* have been described from Africa (Vári 1961), and Kawakita and Kato (2009, 2016) allude to the possibility of Neotropical *Epicephala*; if any of these lineages share a most recent common ancestor with all *Glochidion*-associated *Epicephala*, the number of shifts off *Glochidion* required under this scenario might additionally increase. On the basis of available evidence, we consider the hypothesis—that *Glochidion* and its main clade of pollinating *Epicephala* diversified contemporaneously and that the ancestor of *E. lanceolaria* is a relic lineal lineage that independently shifted onto *Glochidion*—to be a simpler and more likely explanation for the observed patterns of host association.



Understanding better the evolution of host associations in the lineage represented by *E. lanceolaria* would require additional data from the field on *Glochidion-Epicephala* associations, particularly in southeast Asia, and on Phyllanthaceae-*Epicephala* associations elsewhere in the world. Approximately one-fourth of described *Glochidion* species—primarily in insular East Asia, mainland Southeast Asia, and Oceania—have been sampled for *Epicephala* moths in a phylogenetic context (Kawakita et al. 2004; Kawakita and Kato 2006; Hembry et al. 2013). It is therefore quite possible that additional species of *Glochidion* in the Asia-Pacific region are pollinated (exclusively or partially) by moths from the *E. lanceolaria* clade. Because *G. lanceolarium* is widely distributed in southern China, mainland southeast Asia, and south Asia, it may be that other populations of this species have associations with *Epicephala* more closely related to those that pollinate other *Glochidion*. It is, however, interesting that work by multiple investigators in regions where *G. lanceolarium* is present have yet failed to find any close relatives of *E. lanceolaria*. Additional fieldwork elsewhere in the Old World tropics may find close relatives of *E. lanceolaria* associated with other leafy plants in the tribe Phyllanthaceae.

The striking aspects of the *G. lanceolarium-E. lanceolaria* interaction are the morphological and behavioral traits that are not known from other *Glochidion-Epicephala* interactions and the synchronized nature of the tree's reproductive phenology and moth's life cycle. Since no close relatives of *E. lanceolaria* are previously known, it is tempting to conclude that the unusual aspects of its interaction with *G. lanceolarium* mutualism represent rapid coadaptations following a long-distance host shift. However, we caution against such an interpretation in the absence of additional comparative data. *Glochidion lanceolarium* flowering and fruiting phenology—in which trees flower synchronously only once per year and fruits dehisce immediately before flower opening—may appear to be unusual within the genus. For instance, flowering and fruiting periods are longer and do not appear to have such narrow synchrony in most Polynesian and Japanese *Glochidion* (Florence 1997; D. H. Hembry, unpublished data). However, a similar specialized phenology—in which flowers persist on trees for several months before developing into fruits simultaneously—is known from *G. acuminatum* in Japan, which is pollinated by an *Epicephala* (*Epicephala anthophilia*) belonging to the main *Glochidion*-associated clade (Goto et al. 2010; Hembry et al. 2013; Kawakita and Kato 2016; fig. 6). *Glochidion acuminatum*, is, however, not particularly closely related to *G. lanceolarium* (fig. 5). Likewise, the carpel chambers of *G. lanceolarium* fruit are unusual within the genus but not completely unknown; for instance, *Glochidion ferdinandii* in Australia (distantly related to *G. lanceolarium*; fig. 5) and a distantly related and unidentified *Glochidion* from Fiji (10047

in fig. 5) also possess such carpel chambers. The former is associated with *Epicephala colymbetella* that also ecloses within the carpel chamber (Turner 1913, 1940), although it is not clear whether the accession of *Epicephala* from *G. ferdinandii* in our analysis (from Kawakita et al. 2004)—which is part of the main *Glochidion*-associated clade (fig. 6)—is indeed *E. colymbetella* or another species. The latter, unidentified Fijian *Glochidion* has a moth associated with the main *Glochidion*-associated clade, of which no pupae were found in the dissected fruit (*E. ex. sp.* 10047 in fig. 6; Hembry et al. 2013; D. H. Hembry, unpublished data). Carpel chambers may thus evolve for reasons unrelated to *Epicephala*. Presence or absence of cocoons is likely a behavioral polymorphism in *E. lanceolaria* (fig. 4), and it may be that cocoon formation is in the process of being lost.

More importantly, we point out that cophylogenetically enigmatic patterns of association have been reported elsewhere. Comparative divergence time estimations in other insect-plant mutualisms have revealed likely examples in which insect clades may have replaced each other sequentially in association with the same host plant lineages over macroevolutionary timescales (Althoff et al. 2012; Chomicki et al. 2015). In a similar and striking example to that reported here, a basal leafcutter ant lineage, *Apterostigma megacephala*—known from only a few localities in South America—cultivates a highly derived fungus, *Leucoagaricus gongylophorus* (Schultz et al. 2015). Similar to leafy-plant-leafcutter moth interactions, leafcutter ants show strong fidelity and constraint to particular fungal lineages, with more derived ants being associated with derived, domesticated fungi. The authors hypothesize that either ancestral ants were more plastic in their fungal associations—but subsequently evolved in parallel to become more specialized—or the ancestor of the basal ant species was somehow preadapted to acquiring a highly derived fungal lineage distantly related to its previous cultivars. Such phylogenetically unusual patterns of host association may seem to be a challenge to our understanding of how organisms engaged in intimate mutualism select and coevolve with their symbiotic partners, but they may be more common within specialized mutualisms than generally recognized. Mutualistic interactions between *G. lanceolarium* and *E. lanceolaria*, both of which are common and widely distributed in south China, provide a promising study system for understanding the currently unknown biological constraints on host-pollinator association, the process by which hosts change partners, and the dynamics of these associations over evolutionary time.

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