

Research paper

Diversity and species-specificity of brood pollination of leafflower trees (Phyllanthaceae: *Glochidion*) by leafflower moths (Lepidoptera: *Epicephala*) in tropical Southeast Asia (Cambodia)

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ARTICLE INFO

Article history:

Received 26 January 2021

Received in revised form

18 June 2021

Accepted 1 July 2021

Available online 16 July 2021

Keywords:

*Glochidion**Epicephala*

Obligate pollination mutualism

Leafflower–leafflower moth

Phyllanthaceae

Southeast Asia

ABSTRACT

Glochidion (Phyllanthaceae; leafflower trees) is a genus of trees which is widely reported to be pollinated by leafflower moths (Gracillariidae: *Epicephala*) in temperate and subtropical Asia, Australia, and the Pacific islands. However, the pollination ecology of *Glochidion* is not well described from tropical Asia, the region where it is most species-rich at both local (≤ 9 spp.) and regional (~ 200 spp.) scales. Here we report investigations of pollination biology and species-specificity of five *Glochidion* species in tropical Southeast Asia (Cambodia). Through nocturnal observations and fruit dissections, we find that at least three and likely five *Glochidion* species in Cambodia are pollinated by seed-parasitic leafflower moths. We find no evidence that any of these leafflower moths are non-mutualistic parasites, despite known examples of such parasites of this mutualism elsewhere in Asia. While the presence of a single larva in a fruit results in only a fraction of seeds being consumed, the presence of more than one larva per fruit—a frequent occurrence in some species—can result in almost all seeds within the fruit being infested. Multilocus phylogenetic analysis indicates that there are five different minimally monophyletic leafflower moth clades, each of which pollinates a unique *Glochidion* host species. Our results indicate that in its center of diversity in tropical Asia this system is an obligate pollination mutualism as previously described at the global margins of its distribution. These findings provide insights into the processes that generate and maintain biodiversity and maintain mutualism stability in plant–insect interactions in this biodiversity hotspot.

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1. Introduction

Symbiotic relationships between plants and animals have always captivated evolutionary biologists (Darwin, 1862; Thompson, 1994). To understand the evolution of specialization, many studies have focused on brood pollination mutualisms between plants and pollinators as model study systems. Such mutualisms include the

associations between figs and fig wasps (Moraceae: *Ficus* L., Hymenoptera: Agaonidae; Janzen, 1979; Herre et al., 2008), yuccas and yucca moths (Asperagaceae: *Yucca* L., *Hesperoyucca* (Engelmann) Baker; Lepidoptera: Prodoxidae: *Tegeticula* Zeller, *Parategeticula* Davis; Riley, 1892; Pellmyr, 2003; Rentsch and Leebens-Mack, 2014) and leafflower plants and leafflower moths (Phyllanthaceae: *Phyllanthus* L. s.l., Lepidoptera: Gracillariidae: *Epicephala* Meyrick; Kato et al., 2003; Kawakita and Kato, 2006; Hembry et al., 2013a, 2018; Luo et al., 2017).

Brood pollination mutualisms are mutualisms in which the plant provides a portion of its developing seeds as nourishment for the pollinator's offspring (larvae) as a reward for pollination

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Peer review under responsibility of Editorial Office of Plant Diversity.

services (Hembry and Althoff, 2016; Kawakita and Kato, 2017). It has been reported that leafflower moths (Lepidoptera: Gracillariidae: *Epicephala* Meyrick) and several host plant species in the family Phyllanthaceae are engaged in obligate pollination mutualisms (OPMs) (Kato et al., 2003; Kawakita and Kato, 2004a; Okamoto et al., 2013). To date, these OPMs have been observed in species from the genera *Glochidion* J.R. Forst. & G. Forst., *Breynia* J.R. Forst. & G. Forst., and *Phyllanthus* (Kato et al., 2003; Kawakita and Kato, 2004a,b; Hembry et al., 2012; Zhang et al., 2012; Okamoto et al., 2013; Kawakita et al., 2015).

Glochidion is a monoecious tree genus in Phyllanthaceae which has tiny apetalous female flowers with extremely specialized styles (Airy Shaw, 1978; Chakrabarty and Gangopadhyay, 1995). This genus comprises over 300 described species that are distributed in the subtropical and tropical Asia–Pacific region, with a few species in temperate Asia (Govaerts et al., 2000; Hoffmann and McPherson, 2003; Webster, 2014; Angiosperm Phylogeny Group, 2016). Species in this genus engage in obligate pollination mutualisms with species-specific, seed-parasitic leafflower moths (Kato et al., 2003; Kawakita et al., 2010). At night, female leafflower moths actively pollinate the flowers, collecting pollen from male flowers using their specialized proboscises and depositing the pollen on the stigmas of female flowers. After pollination, female leafflower moths oviposit in pollinated flowers so that when the flowers develop into fruits, hatched larvae can eat a portion of the developing seeds and leave a subset of seeds intact for plant reproduction (Kato et al., 2003). The pollination behavior of leafflower moths associated with *Glochidion* shares similarities to the obligate associations between other species of leafflower moth and close relatives of *Glochidion* in the family Phyllanthaceae (Kawakita and Kato, 2004a,b).

There is the possibility that many more species in Phyllanthaceae engage with OPMs that remain to be described (Kawakita and Kato, 2004a; Cooper and Cooper, 2013). So far, the mutualistic relationship between *Glochidion* and leafflower moths has been documented from Japan (Kato et al., 2003; Kawakita and Kato, 2006), China (Luo et al., 2017), Australia (Henderson et al., 2020) and French Polynesia (Hembry et al., 2012, 2013a, 2018). All of these locations are around the margins of the global distribution of *Glochidion* in the Asia–Pacific region. The center of diversity of *Glochidion* (~200 species; Govaerts et al., 2000) is tropical Southeast Asia and New Guinea, where up to nine species can co-occur at one locality (Hembry et al., 2013a). Although leafflower moths have been reported reared from *Glochidion* fruits in several Southeast Asian countries (Laos, Vietnam, Myanmar, and Malaysia; Kawakita et al., 2004; Hembry et al., 2013a), basic biological observations of its behavior and host-specificity are lacking from this region where its hosts, and presumably it as well, are most diverse. Consequently, it is not clear if the biology of this interaction as described from temperate and subtropical Asia and Australia, and the Pacific islands, are representative of this association in its center of diversity. Furthermore, even if mutualistic, we have little data on interspecific differences in the ecology of the interactions between different *Glochidion* and different leafflower moths, particularly with regards to the number of larvae per fruit and the extent of their seed damage, or the presence of parasitic (non-pollinating) species (Kawakita et al., 2015; Li et al., 2015), which has great implications for the stability and diversity of this mutualism. Here, we report the first detailed descriptions of the ecology and species-specificity of the *Glochidion*–leafflower moth association in a tropical Asian country, Cambodia. Cambodia has thirteen reported species of *Glochidion* (Cho et al., 2016). Here we report investigations of the pollination biology and interactions with leafflower moths of five Cambodian *Glochidion* species. Specifically, we aimed to: (a) observe pollination behavior, (b) investigate the cost

of mutualism (the fraction of seeds consumed by leafflower moth larvae and number of larvae per fruit) to the host plant, and (c) assess phylogenetic relationships among the moths associated with different *Glochidion* hosts. Taken together, these three aims allow us to obtain fundamental information about the biology of this interaction: the diversity of *Epicephala* moths associated with five species of Cambodian *Glochidion*, whether or not they are mutualistic pollinators of their hosts, and the extent to which the ecology of this interaction varies among different species pairs.

2. Methods

2.1. Research sites and organisms

We studied the pollination biology of five *Glochidion* species, *Glochidion coccineum* (Buch.-Ham.) Müll.Arg., *Glochidion littorale* Blume, *Glochidion* sp. 1, *Glochidion glomerulatum* (Miq.) Boerl., and *Glochidion rubrum* Blume, at six sites across Cambodia (Preah Vihear Heritage Site [PVHS], Phnom Kulen National Park [PKNP], Kirirrom National Park [KRNP], Veal Renh [VR], Ream National Park [RNP], and Bokor National Park [BKNP]; Fig. 1; Table 1) during the periods 12th October to 13th November 2019 and 7th January to 28th March 2020.

Glochidion coccineum is an evergreen shrub or treelet, usually about 4–10 m high. This species usually grows in disturbed forests, along roadsides or near streams (Fig. 2a). Male flowers are yellow with 6 sepals and 6 stamens (Fig. 2b). The male flowers are aggregated at the proximal ends of branches, whereas female flowers typically are more common along the distal parts of branches and have very short pedicels and 6 sepals (Fig. 2c). The fruit is lobed, and the ovary has 7 to 12 locules (Fig. S1c) (van Welzen, 2007). The flowering period is from February to October, and the fruiting period is from August to January (at our study site). This species was found and collected from Preah Vihear Heritage Site [PVHS] (104°42'54.70" E, 14°16'44.85" N) (Fig. 1) where the forest holds a diversity of deciduous plant species representative of tropical deciduous forest ecosystems in the northern and northeastern regions of Cambodia. The fruits were collected to rear *Epicephala* moths in October 2019. The total of 370 sampled fruits from 15 individual trees were dissected and the pollination behavior observation was made on 13th to 18th January 2020 and on 1st to 5th March 2020.

Glochidion littorale is a shrub up to 6 m in height, which grows at low elevations near the coast (Fig. 2h). Male flowers have 6 sepals and 5 or 6 stamens and a pedicel 5.5–6 mm long (Fig. 2i). The fruit is round, apically flattened, and depressed in the center, sometimes also basally flattened (Fig. S1b). The ovary has 10 to 14 locules (van Welzen, 2007). This species was found and collected from three locations: Veal Renh (103°48'59.98" E, 10°42'34.28" N), Bokor National Park (104° 1'39.51" E, 10°38'22.93" N), and Ream National Park (103°41'40.07" E, 10°31'57.05" N) (Fig. 1). These three locations are representative of evergreen forest in southern Cambodia near the ocean with both low- and medium-elevation vegetation (Emerton et al., 2002). The fruits were collected to rear *Epicephala* moths in November 2019 from Veal Renh, Bokor National Park and Ream National Park. The total of 212 dissected fruits from 9 individuals were sampled from Veal Renh and Ream National Park on 17th to 28th February 2020. Pollination observations were made on 23rd to 28th February 2020 and on 11th to 13th March 2020 at Veal Renh and Ream National Park.

Glochidion sp. 1 is a shrub which is determined to be a distinct species by us and by personal communication with Dr. Peter van Welzen (Naturalis Biodiversity Center, Netherlands). It grows along roadsides and in open forest (Fig. S1a). The male flowers are yellow with 6 sepals and 3 stamens (Fig. 3a). The ovary has 3 locules. The

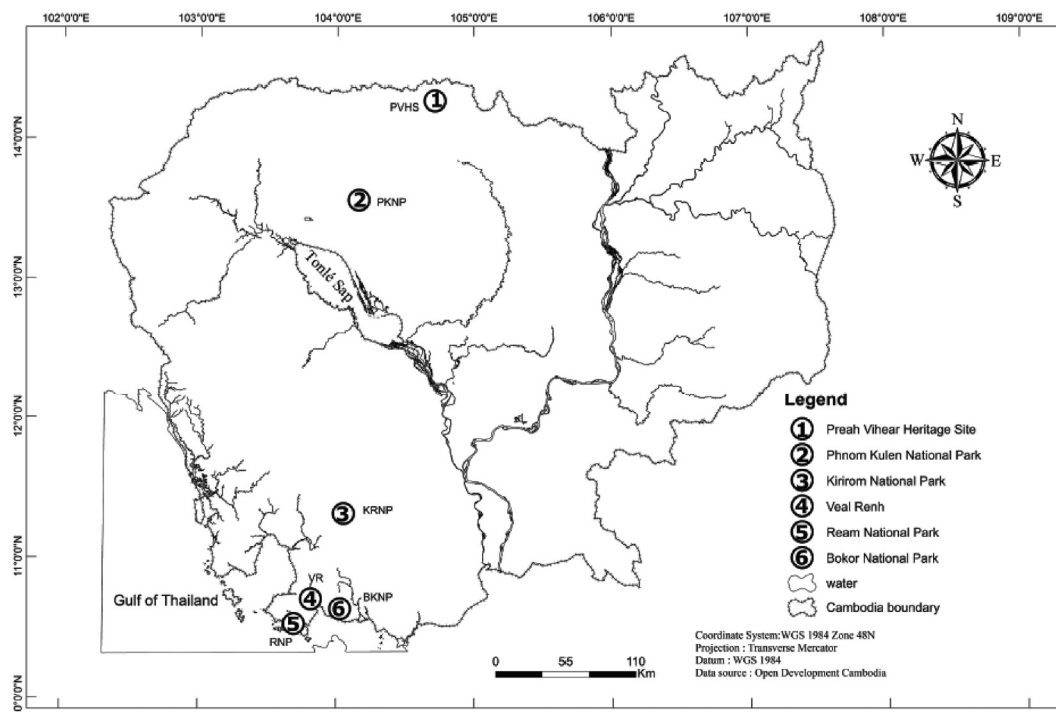


Fig. 1. Map of study sites in Cambodia. The six sites, Preah Vihear Heritage Site (PVHS), Phnom Kulen National Park (PKNP), Kirirom National Park (KRNP), Veal Renh (VR), Ream National Park (RNP), and Bokor National Park (BKNP), are indicated.

Table 1
Distribution of the five species of *Glochidion* among the six sites in Cambodia where the study was conducted.

<i>Glochidion</i> species	Preah Vihear Heritage Site (PVHS)	Phnom Kulen National Park (PKNP)	Kirirom National Park (KRNP)	Veal Renh (VR)	Ream National Park (RNP)	Bokor National Park (BKNP)
<i>G. coccineum</i>	✓					
<i>G. littorale</i>				✓	✓	✓
<i>G. sp. 1</i>	✓	✓		✓	✓	✓
<i>G. glomerulatum</i>		✓				
<i>G. rubrum</i>			✓			

A check or tick mark (✓) indicates the presence of a species at a site.

fruit is hairy and slightly lobed (Fig. 3e). This species was found at almost all of our study areas except for Kirirom National Park. The fruits were collected to rear *Epicephala* moths in October and November 2019. The total of 320 dissected fruits from 10 individual trees were collected from Preah Vihear Heritage Site, Veal Renh and Ream National Park. Pollination observations were made on 17th to 22nd February 2020 and on 8th to 10th March 2020.

Glochidion glomerulatum is a treelet up to 8 m in height. It grows along streams at medium elevation (up to 300 m) at our study site (Fig. S1f). Flowers are pale green with 6 sepals and 3 stamens (Figs. S1g and h). The ovary has 3 locules. Fruits are hairy, slightly lobed, circular, and flattened (Fig. S1i) (van Welzen, 2007). This species was found at Phnom Kulen National Park (104° 9'37.35" E, 13°34'10.53" N) (Fig. 1) where there is a combination of evergreen, semi-evergreen, and deciduous dipterocarp forest (Hayes et al., 2013). The fruits were collected to rear *Epicephala* moths in November 2019 and the total of 25 sampled fruits from 2 individual trees were collected for dissection in January 2020.

Glochidion rubrum is a shrub or treelet up to 5 m in height. It grows along roadsides and forest edges at medium to high elevations (about 500–700 m) at our study site (Fig. S1k). Its male flowers have 6 sepals and 3 stamens (Fig. S1l). Its sepals are strongly ovate to elliptic to obovate. The ovary has 3 locules, and the fruit is glabrous (Fig. S1o). This species was found and collected from

Kirirom National Park (104°3'10.56" E, 11°19'12.87" N) (Fig. 1). The park comprises lowland evergreen and deciduous forest and some medium-altitude evergreen forest (Emerton et al., 2002). The fruits were collected to rear *Epicephala* moths in October 2019 and the total of 50 sampled fruits from 2 individual trees were collected on 8th January 2020 for dissection.

2.2. Pollinator observations

Pollination behavior was observed, focusing on three species: *Glochidion coccineum*, *G. littorale*, and *G. sp. 1*. We conducted observations of flower visitors. Particular effort was made to focus on any flower visitation by *Epicephala* moths at night. After they had been observed pollinating female flowers, *Epicephala* moths were caught to check if pollen grains were attached to their proboscises.

Due to the lack of prior detailed studies of *Glochidion* species at our study sites, we used photos of *Glochidion* plants to interview local people in order to locate trees growing in the wild. Then plant phenology was reported to us by local people; we aimed to keep track of flowering time in order to be able to make flower observations at suitable times. Nocturnal flower observations were made during the period 7:00 pm to 11:00 pm using a yellow flashlight to see flower-visiting insects and any pollination behavior. We spent

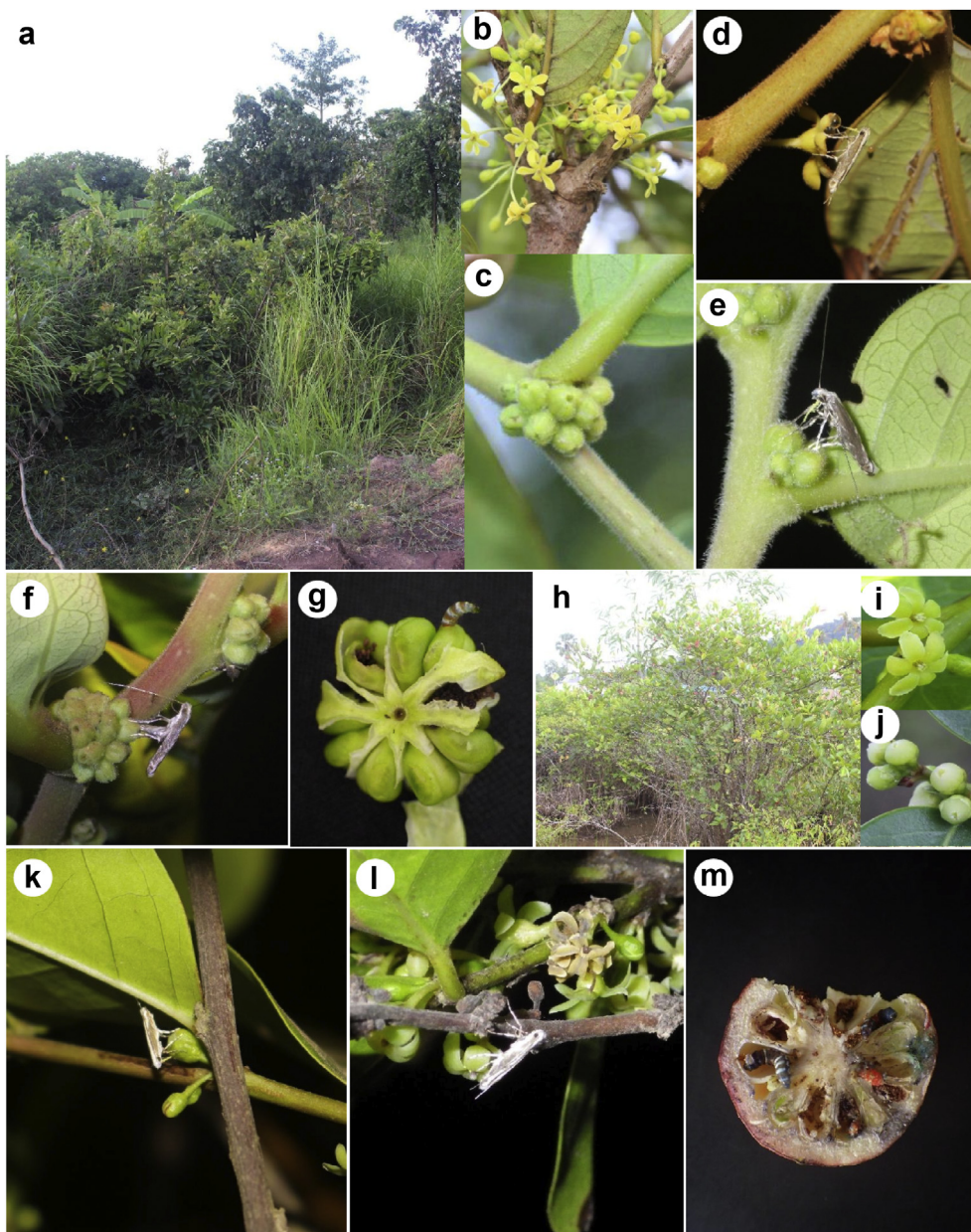


Fig. 2. Flowers and associated insects of *Glochidion coccineum* and *G. littorale*. (a) habitat of *G. coccineum*; (b) male flower of *G. coccineum*; (c) female flower of *G. coccineum*; (d) female *Epicephala* moth collecting pollen at a male flower of *G. coccineum*; (e) female *Epicephala* moth pollinating a female flower of *G. coccineum*; (f) female *Epicephala* moth ovipositing into a female flower of *G. coccineum*; (g) *Epicephala* larva in a dissected fruit of *G. coccineum*; (h) Habitat of *G. littorale*; (i) male flower of *G. littorale*; (j) female flower of *G. littorale*; (k) *Epicephala* moth pollinating female flower of *G. littorale*; (l) *Epicephala* moth visiting *G. littorale*; (m) three *Epicephala* larvae in a dissected fruit of *G. littorale*.

60 h for nocturnal observations and 20 h for diurnal observations. To detect pollen grains on *Epicephala* proboscises and record their pollination behavior, we used a PENTAX WG-3 model camera to record both photos and videos.

2.3. Fruit dissections

To assess the extent of seed destruction by *Epicephala* larvae, mature fruits of these five *Glochidion* species were collected from various individuals and locations for dissection (Table 2). When dissecting, we recorded the number of infested seeds within each mature fruit and noted the cause of seed destruction and the number of *Epicephala* larvae per each fruit. Seeds which were infested by other insect larvae were discarded and not included in the datasets presented in this study. To avoid a situation in which

larvae emerge from the fruits before dissection, we tried to dissect the fruits immediately after they were collected from the field, although we recognize that this may undercount total seed damage by larvae that had not completed development. Our data were then compared with equivalent data from other studies on Phyllanthaceae-*Epicephala* interactions (Kato et al., 2003; Kawakita and Kato, 2004a,b; Goto et al., 2010; Furukawa et al., 2017; Luo et al., 2017; Finch et al., 2019; Henderson et al., 2020).

2.4. Molecular phylogenetic analysis

To determine the phylogenetic relationship of the moths associated with different *Glochidion* hosts, we initially conducted fine-scale sampling of 1–2 larvae or moth individuals per tree, for 4–13 trees per species per site (similar to methods of Hembry et al.,



Fig. 3. Flowers, fruits and associated insects of *Glochidion* sp. 1. (a) male flower; (b) female flower; (c) female *Epicephala* moth pollinating a female flower; (d) female *Epicephala* moth ovipositing into female flower; (e) fruits; (f) two *Epicephala* larvae in a dissected fruit.

Table 2
Comparison of seed infestation by *Epicephala* among five *Glochidion* species.

<i>Glochidion</i> species	Number of sampled fruits	Number of infested fruits	Mean number of intact seeds per fruit (SD)	Mean percentage of intact seeds per fruit (SD)	Mean number of infested seeds per fruit (SD)	Mean percentage of infested seeds per fruit (SD)	Mean number of ovules per fruit (SD)	Mean number of sterile/aborted seeds per fruit (SD)
<i>G. coccineum</i>	370 (N = 15)	328	7 ± 3.52	58 ± 22.81	5 ± 2.6	*42 ± 22.81	16 ± 2.45	5 ± 2.51
<i>G. littorale</i>	212 (N = 9)	192	13 ± 5.55	55 ± 23.07	11 ± 5.75	*45 ± 23.07	24 ± 1.79	2 ± 1.23
<i>G. sp. 1</i>	320 (N = 10)	244	3 ± 1.19	52 ± 20.39	3 ± 1.23	*48 ± 20.39	6 ± 0.0	1 ± 0.42
<i>G. glomerulatum</i>	25 (N = 2)	18	3 ± 0.43	54 ± 7.13	3 ± 0.43	46 ± 7.13	6 ± 0.0	1 ± 0.0
<i>G. rubrum</i>	50 (N = 4)	31	3 ± 0.6	54 ± 10.3	3 ± 0.6	46 ± 10.3	6 ± 0.0	2 ± 0.5

Asterisk (*) indicates that totals include data from fruits with one larva and fruits with more than one larva. N indicates the number of tree individuals.

2018). Sites are defined as a circle of area 1000 km² (radius 18 km), to accommodate dispersal by *Epicephala* adults or *Glochidion* seeds, following a previous study (Hembry et al., 2018).

We analyzed nucleotide sequence variation among the larvae and moths reared from fruits of five different *Glochidion* species from various locations: *G. coccineum* ($N = 13$), *G. littorale* ($N = 28$), *G. sp. 1* ($N = 30$), *G. glomerulatum* ($N = 5$), and *G. rubrum* ($N = 7$). Total DNA was extracted from adults and larvae using a standard DNeasy Tissue Kit (Qiagen, Hilden, Germany) protocol. For each larva and adult, we amplified and Sanger-sequenced three loci, mitochondrial cytochrome oxidase I (COI), nuclear elongation factor 1- α (EF1- α) and arginine kinase (ArgK), following protocols from past studies on *Epicephala* (Hembry et al., 2013a, 2018; Kato et al., 2003; Kawakita et al., 2004; Luo et al., 2017). A fragment of COI, ArgK, and EF1- α were PCR-amplified by using the following primers: for COI, 5'-ATAATTTTTT-TATAGTTATAC-3' and 3'-GATGGGCTCATACAATAAATCCTA-5' (Kato et al., 2003); for ArgK, 5'-ATTTAGACTCTGGTGTGG -3' and 3'-ATGCCGTCGTACATCTCCTT-5' (Kawakita et al., 2004); for EF1- α , 5'-CCCATTTCGGCTGGCAYGGAGA-3' and 3'-GATTACCRGWACGACGR TC-5' (Kawakita et al., 2004). To this novel dataset (GenBank accession numbers MZ393203–MZ393363, MZ393691–MZ393770), we added a sequence dataset of the same loci for other *Epicephala* taxa globally from previous studies (Hembry et al., 2013a; Kawakita et al., 2004; Kawakita and Kato, 2009; Luo et al., 2017), ensuring one representative for each *Epicephala* taxon reared from *Glochidion* for which published sequence data is available. We used GenBank sequences of the moth *Calybites phasianipennella* (Hübner) (Gracillariidae: Gracillariinae; a different subfamily than *Epicephala*) as the outgroup (Kawakita and Kato, 2009). We aligned sequences using MUSCLE (Edgar, 2010) and inferred phylogenetic relationships using maximum likelihood implemented in RAxML-HPC2 (Stamatakis et al., 2006) on the CIPRES server (Miller et al., 2010) setting the model of evolution to GTR + G.

3. Results

3.1. Observations of pollination behavior

Flowers were seldom visited by insects by day, whereas at night a few insects were observed walking past inflorescences. At the beginning of the evening at 7:00 pm, flowers started to release their

odor, which attracts *Epicephala* moths; the odor was noticeable to human observers as a fragrant scent. Although we observed some insects (true bugs, Hemiptera) visiting male flowers and appeared to be eating pollen and tepals, *Epicephala* moths were the only flower visitors observed pollinating *Glochidion* flowers. *Epicephala* moths were observed being active from 8:00 pm to 10:00 pm (although we observed flowers from 7:00 pm to 11:00 pm). Female *Epicephala* moths first visited male flowers to collect numerous pollen grains (Fig. 2d) using their proboscises. After collecting pollen, *Epicephala* moths remained stationary on male flowers for a few minutes before flying to a female flower. After detecting a female flower, an *Epicephala* moth walked around the flower, then started to pollinate it using its specialized proboscis to deposit pollen into the recessed surface of its fused styles (Fig. 2e,k, and 3c). We observed *Epicephala* pollination behavior on three species of *Glochidion*: *G. littorale* ($N = 2$), *G. coccineum* ($N = 7$), and *G. sp. 1* ($N = 5$). For *G. coccineum* and *G. sp. 1*, the process of pollination by a female moth took about 2–4 s, whereas one *Epicephala* moth observed pollinating *G. littorale* took about 10 min. Subsequently, after pollination behavior was finished, the female *Epicephala* moth bent its abdomen and inserted its long ovipositor into the narrow stigmatic pit of the female flower and laid eggs (Figs. 2f and 3d). During the day, we observed braconid wasps probing *Glochidion* fruit with their ovipositors, probably to oviposit in early instar *Epicephala* larvae (Fig. S1e).

3.2. Seed loss and the cost of mutualism

Out of the sampled mature fruits of *Glochidion coccineum* ($N = 370$), *G. littorale* ($N = 212$), *G. sp. 1* ($N = 320$), *G. glomerulatum* ($N = 25$), and *G. rubrum* ($N = 50$), 88.6%, 90.56%, 76.25%, 72%, and 62%, respectively, were infested by *Epicephala* larvae. *Epicephala* larvae were easily distinguished by their morphology (Figs. 2g, 2m, 3f, and S1p). Interestingly, we often found multiple larvae per fruit in *G. coccineum*, *G. littorale*, and *G. sp. 1*, while *G. glomerulatum* and *G. rubrum* had no more than one larva per fruit. 24%, 27%, and 3% of *G. coccineum*, *G. littorale*, and *G. sp. 1* fruit, respectively, contained two *Epicephala* larvae, while 7% of *G. coccineum* fruit and 23% of *G. littorale* fruit contained three *Epicephala* larvae (Fig. 4a). The number of seeds consumed per fruit by *Epicephala* larvae varied across the five *Glochidion* species, while the number of seeds

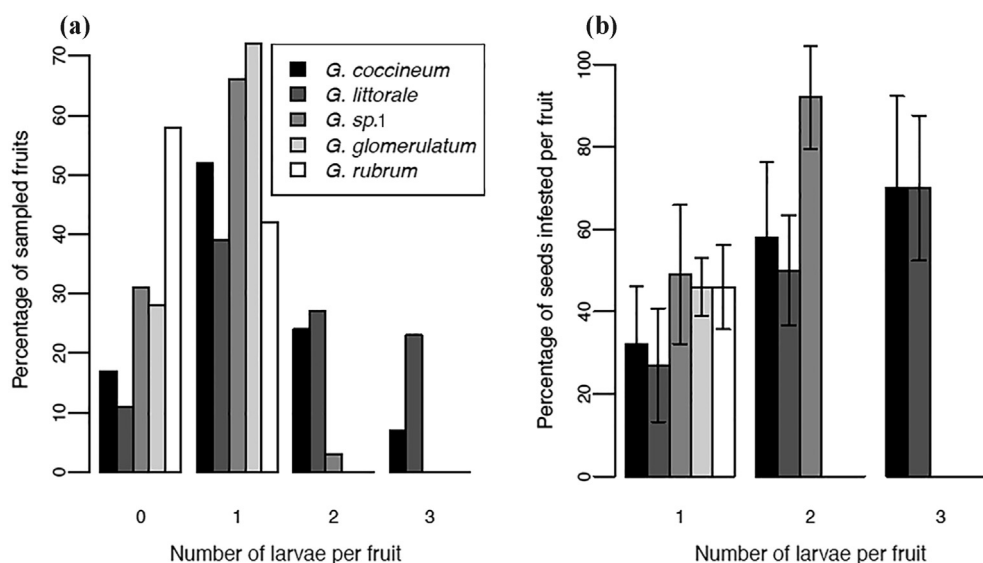


Fig. 4. (a) Percentage of sampled fruits with different numbers of *Epicephala* larvae in five *Glochidion* species. (b) Mean percentages of seeds infested by different numbers of *Epicephala* larvae per fruit, in five *Glochidion* species. Error bars indicate standard deviations.

(Table 2) and the number of larvae per fruit also varied among species (Fig. 4a). Typically, a single larva did not consume all seeds within the fruit, while more than one *Epicephala* larvae often but not always destroyed all seeds within a fruit (Fig. 4b). The mean percentage of infested seeds consumed by a single larva were 32%, 27%, 49%, 46%, and 46% for *G. coccineum*, *G. littorale*, *G. sp. 1*, *G. glomerulatum*, and *G. rubrum*, respectively, whereas the mean percentage of infested seeds consumed by two larvae were 58%, 50%, and 92% for *G. coccineum*, *G. littorale*, and *G. sp. 1*, respectively. The mean percentage of infested seeds consumed by three larvae was 70% for both *G. coccineum* and *G. littorale* (Fig. 4b). Furthermore, the mean percentage of seeds consumed by all larvae within a fruit were 43%, 45%, and 48% for *G. coccineum*, *G. littorale*, and *G. sp. 1*, respectively (Table 2).

3.3. Molecular phylogenetics

Sanger-sequencing yielded 1775 bp of three combined genes (ArgK, COI, and EF1- α) per larva. Molecular phylogenetic analysis using ML revealed that *Epicephala* sampled in this study fall into five minimally monophyletic clades with high bootstrap support values (>99), likely indicating five different *Epicephala* species (here, “taxa”) associated with five different *Glochidion* species (Fig. 5). Each of these Cambodian *Epicephala* taxa is uniquely associated with a single species of host *Glochidion*; many of these *Epicephala* samples come from localities (PVHS, PKNP, VR, RNP, BKNP) where two *Glochidion* species co-occur (Table 1). The *Epicephala* specimens collected from *G. littorale* group in the phylogeny with a single specimen collected from the same host in Malaysia (Sarawak) in a previous study (Hembry et al., 2013a). Aside from the *Epicephala* associated with *G. littorale*, no Cambodian *Epicephala* specimens form minimally monophyletic clades with specimens collected in previous studies elsewhere in Asia. The *Epicephala* associated with *G. glomerulatum* in Cambodia are separated by several strongly supported nodes from an *Epicephala* specimen collected from the same host in Malaysia (Sarawak). All *Epicephala* collected in this study fall within the main *Glochidion*-associated clade of *Epicephala*; none are sister to the enigmatic and more distantly related *E. lanceolaria* (the pollinator of *Glochidion lanceolarium* in southern China; Luo et al., 2017).

4. Discussion

4.1. Pollination behavior

Our study revealed that at least three and likely five Cambodian *Glochidion* species are pollinated by *Epicephala* moths. At night, *Glochidion* flowers attract *Epicephala* moths by releasing their odor (see Okamoto et al., 2007, 2013). After detecting the male flowers, the *Epicephala* moths actively transport the pollen between the flowers. Female *Epicephala* used their specialized proboscises to collect pollen and deposit it into the styles of the female flowers, and then laid eggs in the carpels of the female flower. This behavior is similar to that observed in *Glochidion* in temperate and subtropical Asia (Kato et al., 2003; Kawakita and Kato, 2006; Luo et al., 2017) and the Pacific islands (Hembry et al., 2012), as well as in *Phyllanthus* (Kawakita and Kato, 2004a) and *Breynia* (Kawakita and Kato, 2004b; Zhang et al., 2012; Finch et al., 2018). In most cases, we noticed that both pollination and oviposition behaviors were repeated at the same female flower. This constancy might ensure successful pollination and oviposition. Due to the difficulty of pollination behavior observation at night, we still do not know exactly whether moths collect pollen from male flowers and pollinate female flowers on the same tree, or move to another new tree after collecting pollen. Notably, our observation is consistent

with those of Kawakita (2010) that a female *Epicephala* moth visits male flowers only once and successively visits several female flowers to pollinate and oviposit without revisiting male flowers. These five *Glochidion* species share similar basic pollination ecology and behavior with *Glochidion acuminatum* Müll.Arg. as described in Kato et al. (2003) and Kawakita (2010). Interestingly, despite the recent discovery of non-pollinating or galling non-mutualistic *Epicephala* in Asia (Kawakita et al., 2015; Li et al., 2015), we find no evidence that any of the moths discovered here are not mutualistic pollinators. These findings indicate that in the center of diversity of *Glochidion* in tropical Asia, the pollination ecology of this plant clade is the same in its broad aspects to that reported at the margins of its distribution in temperate and subtropical Asia, Australia, and the Pacific islands. Alongside the specialized, fused stylar morphology seen in of all *Glochidion*, it constitutes further evidence to suggest that all the ~300 species of *Glochidion* are pollinated by *Epicephala* moths (Kato et al., 2003).

4.2. Seed loss and the cost of mutualism

A number of studies have measured the extent of seed damage by *Epicephala* larvae. It is difficult to compare these values directly across studies as they vary in the developmental stage of fruit, time after collection at which seed damage was assessed, the presence or absence of other Lepidoptera or braconid wasps (Hembry et al., 2013b), number of larvae per fruit, whether data are zero-inflated, and whether means or ranges, or standard deviations or standard errors, are reported for the data. Furthermore, some studies do not specify all of these aspects of data collection and analysis. Nevertheless, the ranges of seed infestation per fruit reported here for single *Epicephala* larvae (~20–70%) (Fig. 4b and Table S1) roughly overlap with those previously reported for other *Epicephala* associated with *Glochidion* (~15–80%) (Kato et al., 2003; Goto et al., 2010; Luo et al., 2017; Henderson et al., 2020) and other leafrollers (Tables S1 and S2) (Kawakita and Kato, 2004a,b; Furukawa et al., 2017; Finch et al., 2019). Intriguingly, the mean percentage of intact seeds per fruit is roughly similar across all five species (52–58%), despite variation in the number of total seeds and larvae per fruit (Table 2). Some fruits, however, in this study were found to lack *Epicephala* larvae (Fig. 4a). This is may be due to egg mortality (perhaps a result of egg predation). Another possibility is that *Epicephala* moths do not always oviposit in pollinated flowers, as reported in *Phyllanthus* (Kawakita and Kato, 2004a) and *Breynia* (Kawakita and Kato, 2004b). Finch et al. (2019) found that 6 of 59 sampled *Breynia oblongifolia* (Müll.Arg.) Müll.Arg. female flowers had been pollinated but appeared to completely lack *Epicephala* eggs and any tissue scarring of oviposition on flowers. In this study, although we did not dissect flowers to check for pollen and moth eggs, we suspect that *Epicephala* moths may not always oviposit in pollinated flowers in *Glochidion* (see Luo et al., 2017), consistent with observations in *Phyllanthus* (Kawakita and Kato, 2004a). One intriguing possibility is that a female *Epicephala* moth may oviposit in only some but not all flowers which it pollinates as a way of concealing larvae from braconid wasp attack, as previously suggested in *Breynia* (Kawakita and Kato, 2004b) and reported for other plant-herbivore-parasitoid interactions (Thompson, 1986, 1987). To vigorously evaluate these hypotheses, more detailed investigations of oviposition and moth pollination behavior, as well as parasitoid searching strategy, are needed.

In these *Glochidion*-*Epicephala* associations studied here, we found that a single *Epicephala* does not consume all the seeds in a fruit (as previously reported; Kato et al., 2003; Goto et al., 2010; Luo et al., 2017), while the presence of more than one *Epicephala* larvae can destroy all seeds (Fig. 4b). This suggests that the number of larvae in a fruit also depends on the number of ovules per fruit, as

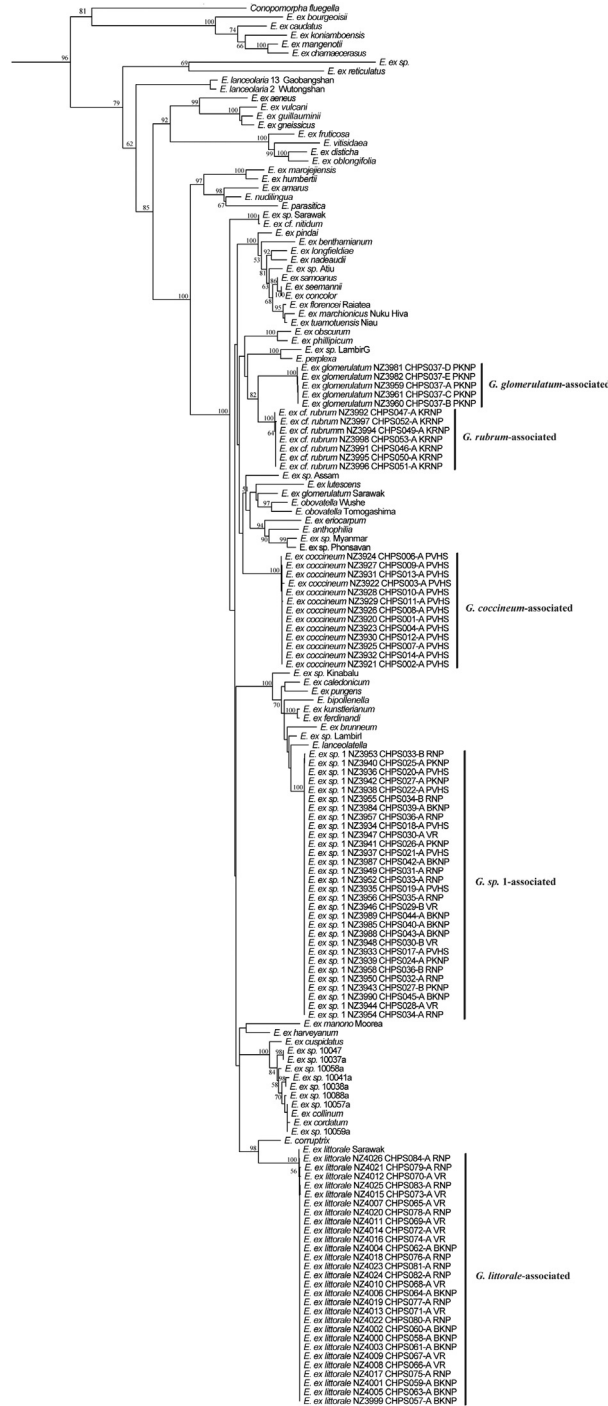


Fig. 5. Phylogenetic hypothesis for *Epicephala* globally recovered using maximum likelihood (ML) and a sequence alignment of 1775 bp comprising three loci (ArgK, COI, and EF1- α). The five clades comprising Cambodian *Epicephala* specimens are indicated by the vertical bars and large-font labels indicating host plants; other *Epicephala* taxa are from previous studies elsewhere in the world (Kawakita et al., 2004; Kawakita and Kato, 2009; Hembry et al., 2013; Luo et al., 2017). ML was implemented in RAxML-HPC2 with 1000 bootstrap replicates. Tip labels indicate the species epithet of the moth species (where names are available) or the species epithet of the host plant from which the specimen was reared. Numbers above branches indicate bootstrap values; bootstrap values ≤ 50 are not shown for ease of reading. The outgroup (*Calybitis phasianipennella*) and two non-*Epicephala* taxa (*Stomphastis labyrinthica* and *Cuphodes diospyrosella*) are pruned from this figure.

we rarely detect more than one *Epicephala* larva inside fruits of those species with fewer (e.g., six) ovules (*Glochidion* sp. 1, *G. glomerulatum*, and *G. rubrum*). In the fruits of *G. sp. 1*, we found that only 3% of fruits had two *Epicephala* larvae ($N = 320$ fruits), whereas *G. glomerulatum* ($N = 50$ fruits) and *G. rubrum* ($N = 25$ fruits) invariably had at most only one *Epicephala* larva per fruit (Fig. 4a). These findings are similar to past reports that indicate that

the number of larvae in a *Glochidion* fruit usually ranges between zero and two, and is often only one (Kato et al., 2003; Goto et al., 2010; but see *G. lanceolarium* (Roxburgh) Voigt: Luo et al., 2017). *G. coccineum* and *G. littorale*, however, have up to three *Epicephala* larvae per fruit, indicating that there is considerable variation in the number of seeds and larvae per fruit among *Glochidion* species, and that seed and larval number may influence each other over the

course of evolution, perhaps by an arms race mechanism in which trees increase seed number per fruit to escape seed predation and moths correspondingly lay more eggs per flower to take advantage of the increased number of seeds. Despite this among-species variation, the frequency with which the entire seed set per fruit is eaten by larvae is very low (Fig. 4b). It is unclear that whether these *Glochidion* species may engage in selective abortion of flowers with high loads of eggs to prevent excessive seed consumption, as reported in yuccas (Pellmyr and Huth, 1994; Richter and Weis, 1995; Wilson and Addicott, 1998; Addicott and Bao, 1999) and *G. acuminatum* (Goto et al., 2010). Our dissections of *G. coccineum* did reveal, however, that *Epicephala* larvae in the fruits of this species appear to be unable to gnaw through the walls of the carpel in which they hatch, suggesting that physical structures in the fruit may limit seed predation in this species, as has been reported in *G. lanceolarium* (Luo et al., 2017) and *Breynia vitis-idaea* (Burman) Fischer (Furukawa and Kawakita 2017).

The association between braconid wasps and *Glochidion* has been previously reported (Hembry et al., 2013b; Kawakita et al., 2015; Henderson et al., 2020). Braconid wasp females probe the fruits and oviposit so that their offspring parasitize and consume moth larvae as their nourishment (Fig. S1e). The presence of braconid wasps may have a significant positive effect on reducing the number of infested seeds caused by *Epicephala* larvae, although we did not conduct a test of this hypothesis here. Indeed, parasitism rates of *Epicephala* can be very high: in one survey 60% of developing *Epicephala* larvae associated with *Phyllanthus bourgeoisii* Baill. were parasitized by braconid wasps (Kawakita and Kato, 2004a). In contrast, Finch et al. (2019) did not find a significant difference in infested seeds between fruit with braconid wasps and without braconid wasps in *B. oblongifolia*. This indicates that the damage to the seeds caused by *Epicephala* larvae may have already occurred before the braconid wasp attacked the *Epicephala* larvae, or braconid wasp parasitism may not immediately stop or reduce feeding behavior by the parasitized *Epicephala* larvae. In this study, we found that seeds infested by *Epicephala* are still found in some fruits from which wasps emerge.

Besides *Epicephala* and braconid wasps, we also detected another unknown moth species associated with *G. coccineum*. Interestingly, this undescribed moth species pupates inside empty spaces in the carpels of the fruit of this species (Fig. S1d). In two *Glochidion-Epicephala* species pairs, *G. lanceolarium-E. lanceolaria* from China (Luo et al., 2017) and *Glochidion ferdinandi* (Müll.Arg.) Bailey-*Epicephala colymbetella* from Australia (Henderson et al., 2020) it has been previously reported that *Epicephala* moths pupate inside empty sinuses in the carpels of the mature host fruit. In this study, after the larva of this unknown moth destroys all seeds inside the *G. coccineum* fruit, it creates empty space which may provide a site for itself to pupate in; however, in these spaces the only pupae we found were of this unidentified species of moth. Due to our limited observations, we still don't know exactly why this phenomenon occurs. Regarding this gap, a more detailed study of the associations with this species is needed.

4.3. Phylogenetic relationships of *Epicephala*

The multilocus molecular phylogeny showed that *Epicephala* associated with these five *Glochidion* species belonged to five clades with high bootstrap support values (>99) (Fig. 5). Each of these *Epicephala* clades is uniquely associated with a single *Glochidion* species as its host. *G. littorale* and *G. sp. 1* are each pollinated by their unique *Epicephala* species across multiple sites in Cambodia. At several of these sites, multiple *Glochidion* co-occur, and these findings are consistent with the hypothesis that they are each pollinated by a single unique *Epicephala* species at these locations.

Available data thus suggests that across these sampled localities, the relationship between *Glochidion* and *Epicephala* may even be one-to-one as has been reported in subtropical Japan using the same loci to assess species-specificity (Kawakita and Kato, 2006) and differing from species-specificity on oceanic islands (Hembry et al., 2018). However, very few *Epicephala* were sampled from *G. glomerulatum* ($N = 5$ moths) and *G. rubrum* ($N = 7$ moths), and sampling of additional moths from additional sites for these hosts and *G. coccineum* is necessary to make a determination about species specificity in this mutualism in Cambodia. Regardless, these results indicate that *Glochidion-Epicephala* associations in tropical continental regions may be extremely specialized.

5. Conclusions

The brood pollination mutualism between leafflower trees in the genus *Glochidion* and leafflower moths in the genus *Epicephala* has been widely reported from subtropical and temperate Asia as well as from Australia and the Pacific islands. Our study provides the first detailed evidence that in the center of diversity of *Glochidion* in tropical Asia, this interaction is mutualistic—with female moths actively pollinating host flowers, and moth larvae consuming a subset of the developing seeds—as previously reported at the global margins of its distribution. Furthermore, our study highlights the diversity of this mutualism in the Asian tropics. Although each of the *Glochidion* species here has a mutualistic relationship with leafflower moths, there is considerable among-species pair variation in the number of seeds and larvae per fruit, and at least one species of *Glochidion* (*G. coccineum*) uses physical structures within the fruit to limit seed damage from the larvae of its pollinator, suggesting a range of cost-benefit outcomes in this mutualism. There is also very high species-specificity in this mutualism, with each *Glochidion* appearing to depend on a single unique pollinating moth species for its reproduction. The mutualism between *Glochidion* and leafflower moths in Cambodia and elsewhere in tropical Asia provides an example to understand the role of coevolution in promoting species diversification and the range of mechanisms underlying mutualism stability. For a deeper understanding of the evolution of this species-rich and specialized mutualism, we recommend further research on the diversity of *Glochidion*-leafflower associations in the Asian tropics.

Author contributions

PC and S-XL designed research; PC performed research; GY, S-XL, and DHH conducted species identification; PC and GY formatted and uploaded data to GenBank; PC, S-XL, and DHH analyzed data and wrote manuscript.

Data availability statement

The data that support the findings of this study are openly available in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>), accession numbers MZ393203–MZ393363, MZ393691–MZ393770.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgements

We express our deep thanks to Mr. Khou Eanghourt for his advice and facilitation of fieldwork. We also thank Mr. Nung Mao, Pisal Chheang's family, and many friends for their kind assistance with the field and laboratory work, and Peter van Welzen for discussing the

identification of Cambodian *Glochidion* with us. We also thank the Ministry of Environment (Cambodia), Preah Vihear Heritage Site, Phnom Kulen National Park, Bokor National Park, Kirirom National Park, and Ream National Park for allowing us access to field sites and permission to conduct fieldwork. This project was supported by the National Natural Science Foundation of China (grants no. 31170217 and 31370268 to S.-X. Luo), and the Chinese Academy of Sciences' "The Belt and Road" Master Fellowship Programme for providing P. Chheang a fully funded master's scholarship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pld.2021.07.001>.

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