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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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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.

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 (Asparagaceae: *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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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], Kirirom 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

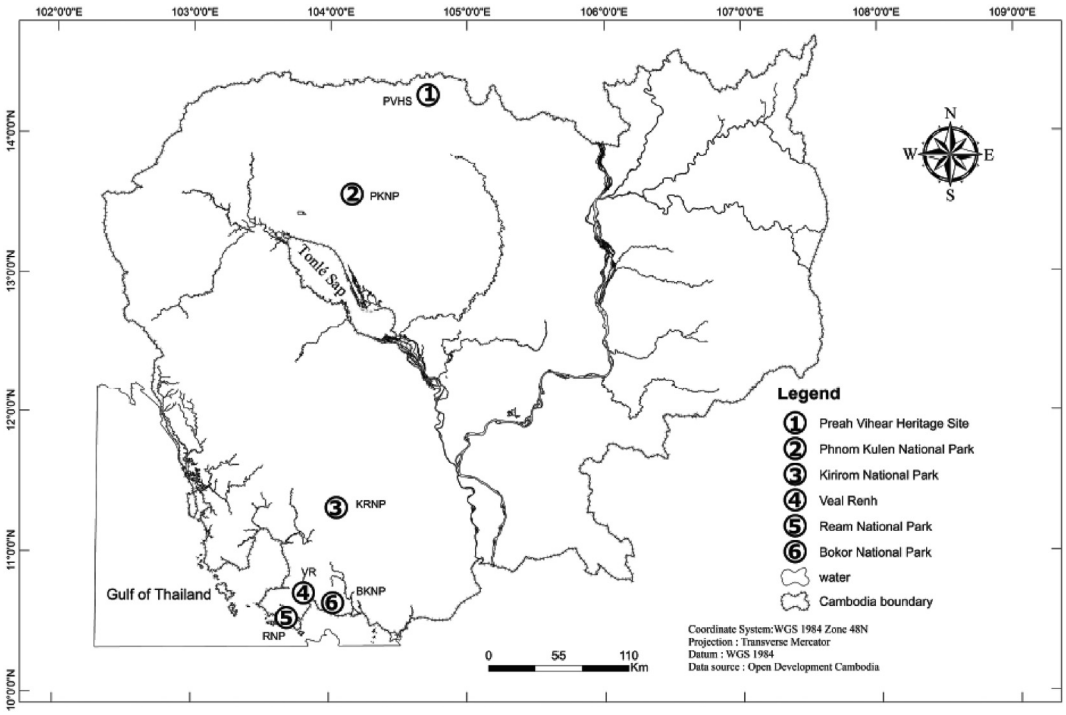

Fig. 1. Map of study sites in Cambodia. The six sites, Preah Vihear Heritage Site (PVHS), Phnom Kulen National Park (PKNP), Kirirom National Park (KRNP), Veal Renh (VR), Ream National Park (RNP), and Bokor National Park (BKNP), are indicated.

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

Glochidion 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)
G. coccineum	✓					
G. littorale				✓	✓	✓
G. sp. 1	✓	✓		✓	✓	✓
G. glomerulatum		✓				
G. rubrum			✓			

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