Research Article

Interspecific and Intersexual Differences in the Chemical Composition of Floral Scent in *Glochidion* Species (Phyllanthaceae) in South China

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Plants of the *Glochidion* (Phyllanthaceae) genus are pollinated exclusively by host-specific *Epicephala* (Gracillariidae) moths. Floral scent has been thought to play key role in the obligate pollination mutualism between *Glochidion* plants and *Epicephala* moths, but few studies have been reported about chemical variation in floral volatiles of *Glochidion* species in China. Floral volatiles of male and female flowers of five *Glochidion* species in south China were collected by dynamic headspace absorption technique and then were chemically analyzed by using gas chromatography-mass spectrometry. A total of 69 compounds were identified from floral scents of five investigated species. *Glochidion hirsutum* and *G. zeylanicum* showed no qualitative differences in floral scent, whereas there were clear variations of floral scent among other species (*G. eriocarpum*, *G. daltonii*, and *G. sphaerogynum*) and also they distinctly differed from these two species. Male flowers emitted significantly more scent than female flowers. *Glochidion* plants exhibited qualitative and quantitative differences in floral scent between two sexes of flowers. The findings suggest that the volatile variation of floral scent among *Glochidion* species reflects adaptations to specific pollinators. Sexual dimorphism in floral scent has evolved to signal alternative rewards provided by each sex to *Epicephala* moths.

1. Introduction

Mutualism is cooperative interactions in which each associated species benefits from its partner [1, 2]. Obligate pollination mutualism between plants and their seed-parasitic pollinators is known as one of the most specialized plant-insect interactions [3–6], where insects usually exclusively pollinate for the host flowers in which they lay eggs; in turn the hosts offer seeds to feed pollinator larvae [7, 8]. The classical examples of such nursery pollination systems are the fig-fig-wasp [4] and yucca-yucca-moth interactions [6]. Moreover, the obligate pollination mutualism between *Epicephala* moths (Gracillariidae) and tribe Phyllanthaeae plants (Phyllanthaceae) has been also documented, including the associations of *Epicephala* moths with *Glochidion*,

Phyllanthus, *Breynia*, *Flueggea*, and *Sauropus* species [5, 9–11]. *Glochidion*, the largest genus in the tribe Phyllanthaeae (Phyllanthaceae), contains around 318 species of monoecious shrubs/trees mainly distributed in tropical Asia, Pacific islands, Australia, and Madagascar [12], a few also in tropical America and Africa, and in China ranging from southwest China to Taiwan [13]. All *Glochidion* plants rely on species-specific female moths of the genus *Epicephala* for obligate pollination [5, 14]. At night, a female moth actively collects pollen grains from the host male flower using unique proboscises equipped with numerous hairs and then deposits them on the female flower and subsequently lays an egg using the specialized acicular ovipositor. The hatched larva consumes only a subset of the maturing seeds within a single fruit to complete the development and drill out of the fruit. The rest

of the seeds ensure that the hosts reproduce their offspring [5].

The most intriguing characteristics among *Glochidion-Epicephala* mutualism are extremely high taxonomic diversity and species specificity of both plants and moths [5, 7, 14]. Pollinators' encounter with host plants is the key step in these specialized pollination interactions. Floral scents are complex blends of volatile organic compounds, which have been thought to be crucial chemical signals guiding obligate nocturnal moths to locate host plants in *Glochidion-Epicephala* associations. Interspecific variations of floral scents among *Glochidion* plants are essential for maintaining species specificity of *Glochidion-Epicephala* mutualism [15–17], and *Epicephala* moths are also capable of discriminating their hosts using floral odor [16, 18].

So far, studies on *Glochidion-Epicephala* mutualism have focused on pollination biology and insect taxonomy in China [19, 20] and Japan [5, 9, 10]. However, little is reported about the chemical composition of floral scents from *Glochidion* plants in China. Recently, two pollination relationships between *Glochidion* and *Epicephala* were found in south China: (1) one *Epicephala* species exclusively pollinates one *Glochidion* species (*G. eriocarpum*, *G. daltonii*, and *G. sphaerogynum*) (unpublished data); (2) one *Epicephala* species jointly pollinates two *Glochidion* species (*G. hirsutum* and *G. zeylanicum*) [12]. For maintaining extremely high species specificity of *Glochidion-Epicephala* mutualism, floral scents may differ among *Glochidion* species pollinated by different *Epicephala* moths, while *Glochidion* species pollinated by the same *Epicephala* moth should produce similar floral signals. Therefore, it is speculated that variations in floral scent among different *Glochidion* species should reflect differences in *Epicephala* moth species with which the plants are associated.

The dioecious plants pollinated by animals often, but not always, transmit similar floral signals between the male and female flowers to attract the same pollinator to complete conspecific pollen transfer [21–23]. An implicit assumption in the fact is that pollinators use similar signals to seek the similar reward from flowers of both sexes (e.g., floral nectar) [21]. Conversely, if the plants offer different rewards to the specific pollinators on the male flowers (e.g., pollen) and female flowers (e.g., ovule), floral characters (e.g., shape, visual, and odor) may be selected to diverge between both sexes of the flowers to signal alternative rewards [23, 24]. Interestingly, in *Epicephala*-pollinated Phyllanthaceae plants, there was sexual dimorphism of floral scents between male and female flowers, which is in contrast to scent from non-*Epicephala*-pollinated Phyllanthaceae [18, 25]. Female *Epicephala* moths pollinate the host plants, involving distinctly different behaviors on male and female flowers: pollen collection from male flowers and then pollen deposition and oviposition on female flowers [5, 9–11]. Therefore, it was thought that sexual dimorphism in floral scent of *Epicephala*-pollinated Phyllanthaceae plants has evolved to signal alternative floral rewards provided by each sex to *Epicephala* moths [18]. In our field observations on *Glochidion-Epicephala* mutualism, *Epicephala* moths also exhibited similar high specific pollination behaviors on male and female flowers as found in previous studies [5, 9–11].

Based on the above discussion, we hypothesized that, in mutualism recently found in China between *Glochidion* and *Epicephala*, (1) variations in floral scent composition among five *Glochidion* species may reflect adaptations to species-specific pollinators with which the host plants are associated and (2) *Glochidion* plants should be selected to produce different fragrance blends between the male and female flowers to induce *Epicephala* moths to get the different rewards from flowers of each sex. Consequently, the floral volatiles of both male and female flowers in five *Glochidion* species were collected, respectively, by the headspace adsorption technique and chemically characterized using gas chromatography-mass spectrometry (GC-MS). Then, non-metric multidimensional scaling (NMDS) was performed to investigate interspecific variations and intersexual differences in floral scent composition of five *Glochidion* species in south China.

2. Methods and Materials

2.1. Plants. Five studied *Glochidion* species, including *G. eriocarpum*, *G. daltonii*, *G. sphaerogynum*, *G. hirsutum*, and *G. zeylanicum*, generally inhabit forest margins of tropical and subtropical forests in south China [13]. Of these five species, *G. hirsutum* and *G. zeylanicum* grow together and occurred in sympatry at the study sites. In contrast, the other three species occurred in other different study sites and so their distributions did not overlap (Figure 1).

Glochidion species investigated in this study are monoecious trees with inconspicuous green female flowers and yellow male flowers which are aggregated on the axils of the leaves (Figure 2). Female flowers are sessile, more or less bud-like, and appear to have specialized morphology: the style fused into a column at the tip with a small opening where the stigma is concealed in the inner surface, which makes it difficult to touch for flower visitors other than *Epicephala* moths. Male flowers are pedunculate with unfolded five or six sepals to expose the stamen. *Glochidion* flowers are small and are fragrant to the human nose only during the evening, and the floral scents of *Glochidion* species may be one of the key signals attracting nocturnal obligate pollinator *Epicephala* moths.

The five *Glochidion* species are associated with four *Epicephala* moth species in our study regions [20, 26]. Among these five species, *G. hirsutum* and *G. zeylanicum* share similar morphology except that the trichomes on the epidermis are produced in the female flowers of *G. zeylanicum* rather than that of *G. hirsutum* (Figures 2(g) and 2(i)) and pollinated by the same *Epicephala* species, *E. bipollenella*. However, the other three species are, respectively, pollinated by other species-specific *Epicephala* moths.

2.2. Floral Scent Collections. To analyze floral scent profiles of both male and female flowers of five *Glochidion* species, odor collections were performed using the dynamic headspace adsorption technique [27]. The details of the study sites, dates, and sample sizes are given in Figure 1 and Table 1. Before scent collection, specimens of each studied species were gathered

TABLE 1: Study sites, dates, and sample sizes of five *Glochidion* species headspace collections.

Species	Study sites	Dates	Samples		Number of flowers	
			Male	Female	Male	Female
<i>G. eriocarpum</i>	Pingxiang, Guangxi	Mar.-Apr. 2013	3	4	300–400	800–1000
<i>G. daltonii</i>	Sichuan, Panzhihua	Apr.-May 2013	7	6	200–500	500–1000
<i>G. sphaerogynum</i>	Wuzhishan, Hainan	Dec. 2013-Jan. 2014	4	4	100–200	300–350
<i>G. hirsutum</i>	Fujian, Xiamen	Sep.-Oct. 2013	3	3	75–150	125–300
<i>G. zeylanicum</i>					80–140	285–400

FIGURE 1: Study sites of five *Glochidion* species from south China in this study.

to save in our lab as a voucher. Female and male flowers of each species were removed from plants and put separately into the polyvinyl acetate bags for floral scent collection (Toppits; Melitta Scandinavia AB, Klippan, Sweden). Air was purified by active charcoal (Sigma-Aldrich, USA) and then introduced into the bag through Teflon tubes (VICI Jour Ltd., Sweden) with a mini air-pump. Air-containing floral volatiles were sucked through the small glass tube (outer diameter

6 mm, length 75 mm) filled with 100 and 50 mg of Tenax-TA (mesh 60/80; Sigma-Aldrich) to adsorb floral scent. Meanwhile, air samples were also collected from empty bags as controls to identify possible background contamination during volatile collections. The flow rate of air in the inlet and outlet of the sample bag was set to 400 mL min^{-1} . Odor collection was done at ambient temperature ($25\text{--}28^\circ\text{C}$) indoors and continued for 3 h during the night. After collection, the filters were sealed with Parafilm and then wrapped with silver paper and stored at -20°C until used for analysis.

Before analysis, the collected floral scents in Tenax-TA filters were eluted with 2 mL of hexane into brown glass vials, and then $10 \mu\text{L}$ of n-Eicosane (1 mg mL^{-1}) was added as the internal standard to calculate release amount of individual scent compounds. The eluate containing the floral volatiles was carefully concentrated down to $100 \mu\text{L}$ under N_2 flow.

2.3. Chemical Analysis of Floral Scents. To analyze chemical composition of floral scents, the headspace samples of *Glochidion* species were subjected to GC-MS, using an HP 6890 gas chromatograph (Agilent Technologies Inc., USA), and coupled with an HP 5975 mass spectrometer (Agilent Technologies Inc.). The GC was equipped with HP-5MS capillary column ($30 \text{ m} \times 0.25 \text{ mm} \times 250 \mu\text{m}$ film thickness; Agilent Technologies Inc.). Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1} . For each sample, $2 \mu\text{L}$ was injected in a split mode (the split ratio was 1:1) for 1 min with an injector temperature of 250°C . After injection, the oven temperature was programmed to be kept at 40°C for 5 min, followed by an increase of 3°C min^{-1} to 150°C and $10^\circ\text{C min}^{-1}$ to 200°C , where it was held for 5 min. Mass spectra were recorded at 70 eV in the electron impact ionization mode, and the ionic source temperature was 230°C .

Compound identification was carried out using the NIST 08 mass spectral libraries and further confirmed by comparison of retention indices with those reported in the literature [28, 29], the NIST Chemistry WebBook (<http://webbook.nist.gov/chemistry/>) [30], and The Pherobase (<http://www.pherobase.com/>) [31]. Compounds in the samples presented at higher or similar percentages in controls were considered as contaminants and excluded from the analysis. For the quantities of compounds, the amount of each volatile compound was calculated as its peak area relative to the total peak area on gas chromatograms.

2.4. Statistical Analyses. Unpaired *t*-test was used to determine if there were significant differences in the total amounts

FIGURE 2: Flowers of the five *Glochidion* species in this study. Female of *G. eriocarpum* (a), *G. daltonii* (c), *G. sphaerogynum* (e), *G. hirsutum* (g), and *G. zeylanicum* (i). Male flowers of *G. eriocarpum* (b), *G. daltonii* (d), *G. sphaerogynum* (f), *G. hirsutum* (h), and *G. zeylanicum* (j).

of floral scents emitted from single male and female flower of each species; one-way ANOVA was used to test the significant differences between relative content of four chemical classes compounds with each species; NMDS is a nonparametric dimension reduction technique that carries on without the assumption of linear response and escapes many of

the distortions of eigenvector techniques [32]. In order to analyze the interspecific variation and sexual dimorphism of floral scent composition in *Glochidion* species, NMDS was first performed to visualize dissimilarities among the headspace samples of floral scents using Canoco 5.0 for Windows and then the Mann-Whitney *U* test was used to

test for significant differences among them. All figures were plotted with Graphpad Prism 5.0.

3. Results

3.1. Chemical Composition of Floral Volatiles. The chemical compositions of floral volatiles of both male and female flowers of five investigated *Glochidion* plants are summarized in Table 2. The compounds were categorized into four chemical classes by biosynthetic pathways [33, 34]: aliphatics, monoterpenes, sesquiterpenes, and aromatics.

A total of 69 volatile compounds were detected and identified from the five species, comprising 35 terpenoids (12 monoterpenes and 23 sesquiterpenes), 21 aliphatics, and 13 aromatics. Dominant compound classes were monoterpenes and sesquiterpenes. Of the 69 detected compounds, 46 were found in at least two species, while only 10 were commonly shared by all five species. Notably, two compounds, (Z)-3-hexen-1-ol (compound 2) and (E)- β -ocimene (compound 25), occurred frequently in both male and female flowers of all species; and 23 (*G. zeylanicum*) to 48 (*G. daltonii*) compounds were found in each species. There was a high degree of overlap in floral compounds produced between *G. hirsutum* and *G. zeylanicum*, and only one compound, (E)-linalool oxide (furanoid) (compound 27), was found only in *G. zeylanicum*; thus the two species were thought to have similar composition pattern in floral volatiles. If *G. hirsutum* and *G. zeylanicum* were considered as one species, 5-6 compounds were unique to a single species. For instance, butyl butyrate (compound 5), (Z,Z,Z)-1,5,9,9-tetramethyl-1,4,7-cycloundecatriene (compound 47), γ -elemene (compound 38), β -humulene (compound 42), and mellein (compound 69) were species-specific compounds for *G. sphaerogynum*, while (E,E)-2,6-dimethyl-1,3,5,7-octatetraene (compound 30), α,β -copaene (compounds 39, 34), benzeneacetaldehyde (compound 58), and methyl benzoate (compound 59) were only produced in both *G. hirsutum* and *G. zeylanicum* and not in any other species. Most of the compounds were detected in relatively small amounts (between trace amounts and <10%) in flowers of each sex; the floral scent of each species studied was dominated by only 1-4 compounds in relatively high amounts as follows. In male flowers, *G. eriocarpum* was dominated by β -elemene (compound 35, 28.22%), α -selinene (compound 52, 16.09%), β -selinene (compound 49, 14.54%), and longiborneol (compound 56, 14.16%); *G. daltonii* was dominated by (E,E)- α -farnesene (compound 54, 40.3%) and β -elemene (compound 35, 27.13%); *G. sphaerogynum* was dominated by linalool (compound 28, 59.75%); *G. hirsutum* and *G. zeylanicum* were dominated by (E)-2-nonenal (compound 11, 30.6 and 23.5%, resp.), (E,Z)-2,6-nonadienal (compound 31, 19.19 and 17.46%, resp.), and (E)-2-nonen-1-ol (compound 12, 14.14 and 11.17%, resp.). In female flowers, *G. eriocarpum* was dominated by (E)- β -ocimene (compound 25, 42.6%) and (E)-2-hexen-1-ol (compound 3, 11.55%); *G. daltonii* was dominated by linalool (compound 28, 44.85%); *G. sphaerogynum* was dominated by linalool (compound 28, 23.61%), β -elemene (compound 35, 22.64%), α -selinene (compound 52, 12.03%), and β -selinene

(compound 49, 11.87%); *G. hirsutum* and *G. zeylanicum* were dominated by (E)- β -ocimene (compound 25, 59.4 and 62.74%, resp.) and linalool (compound 28, 12.43 and 12.26%, resp.).

The relative contents of four chemical classes of compounds from male and female flowers among the respective five species are shown in Figure 3. For male flowers, the floral scents emitted by the five species could be divided into four groups: *G. eriocarpum* (86.65%) and *G. daltonii* (94.76%) dominated by sesquiterpenoids; *G. sphaerogynum* (67.87%) dominated by monoterpenoids; aliphatics with monoterpenoids in *G. hirsutum* (56.46 and 23.33%, resp.); aliphatics with aromatics in *G. zeylanicum* (38.56 and 29.85%, resp.). Likewise, the floral scents produced by female flowers of the five species were also divided into four groups: *G. eriocarpum* (51.73 and 32.68%, resp.) dominated by monoterpenoids with aromatics; *G. sphaerogynum* (67.95%) dominated by sesquiterpenoids; *G. hirsutum* (73.71%) and *G. zeylanicum* (78.02%) dominated by monoterpenoids. The findings indicated degrees of difference in chemical composition of floral scents among the five species.

In addition, there were clear differences in scent composition pattern of four different chemical classes between the male and female flowers of the five species. For example, in *G. daltonii*, the major components of male flowers were sesquiterpenoids, whereas those of the female flowers were monoterpenoids.

3.2. Interspecific Variation of Floral Scents. The NMDS analysis of floral scent profiles of both male and female flowers of the five *Glochidion* species is shown in Figure 4. For male flowers, the floral samples were divided into four groups: *G. daltonii*; *G. eriocarpum*; *G. sphaerogynum*; and *G. hirsutum* and *G. zeylanicum*. The floral samples of *G. hirsutum* and *G. zeylanicum* greatly overlapped ($\chi^2 = 0.048$, $P = 0.827$), whereas those of the other three species were evidently distinguished from each other, as well as being obviously separated from *G. hirsutum* and *G. zeylanicum* ($\chi^2 = 17.50$, $P = 0.001$). A similar separation pattern was also observed in the floral samples of female flowers (*G. hirsutum* and *G. zeylanicum*: $\chi^2 = 1.19$, $P = 0.275$; *G. daltonii*, *G. eriocarpum*, *G. sphaerogynum* and *G. hirsutum*, or *G. zeylanicum*: $\chi^2 = 12.752$, $P = 0.005$). Altogether, the results suggested that *G. hirsutum* and *G. zeylanicum* had no difference in floral scent composition, whereas there were clear variations in floral scent among the other three species and it greatly differed from these two species.

3.3. Emission of Floral Scents. Total emissions of floral scents emitted by male and female flowers of the five *Glochidion* species are shown in Figure 5. In all investigated species, a male flower produced on average significantly greater amounts of scents than a female flower (*G. daltonii*: $t = 9.18$, $P < 0.001$; *G. eriocarpum*: $t = 11.61$, $P < 0.001$; *G. sphaerogynum*: $t = 3.63$, $P < 0.05$; *G. hirsutum*: $t = 7.88$, $P < 0.001$; *G. zeylanicum*: $t = 12.35$, $P < 0.001$). In addition, regardless of male or female flower, *G. hirsutum* and *G. zeylanicum* produced similar amounts of floral odor. Altogether,

TABLE 2: Chemical composition of floral scents of both male flowers and female flowers of five *Glochidion* species.

Compounds	RI ^a	G. eriocarpum		G. daltonii		G. sphaerogynum		G. hirsutum		G. zeylanicum	
		Male flowers (N ^b = 3)	Female flowers (N = 4)	Male flowers (N = 7)	Female flowers (N = 6)	Male flowers (N = 4)	Female flowers (N = 4)	Male flowers (N = 3)	Female flowers (N = 3)	Male flowers (N = 3)	Female flowers (N = 3)
Aliphatics											
1	(E)-2-Hexenal	854	0.04 ± 0.01	0.39 ± 0.18	0.04 ± 0.02	0.25 ± 0.24	1.23 ± 0.21	0.46 ± 0.17	—	—	—
2	(Z)-3-Hexen-1-ol	857	0.12 ± 0.13	5.77 ± 3.43	0.03 ± 0.04	0.19 ± 0.15	0.19 ± 0.16	0.58 ± 0.33	0.60 ± 0.42	0.52 ± 0.22	0.25 ± 0.10
3	(E)-2-Hexen-1-ol	862	0.05 ± 0.06	11.55 ± 3.49	—	—	7.76 ± 5.59	—	—	—	—
4	1-Hexanol	867	—	—	—	0.19 ± 0.11	2.28 ± 1.07	0.64 ± 0.52	4.06 ± 2.15	1.51 ± 0.72	0.12 ± 0.04
5	Butyl butyrate	994	—	—	—	—	—	0.19 ± 0.11	—	—	—
6	(Z)-3-Hexenyl acetate	1007	0.23 ± 0.18	—	0.12 ± 0.08	1.30 ± 0.62	0.16 ± 0.05	0.60 ± 0.36	—	—	—
7	Hexyl butyrate	1011	0.02 ± 0.02	—	0.14 ± 0.18	0.49 ± 0.33	0.13 ± 0.05	0.07 ± 0.02	—	—	—
8	(Z)-2-Hexenyl acetate	1012	0.03 ± 0.05	—	0.39 ± 0.19	1.09 ± 0.84	0.52 ± 0.10	0.08 ± 0.03	—	—	—
9	(Z)-6-Nonenal	1099	—	—	0.10 ± 0.09	—	—	—	—	—	—
10	(Z)-3-Nonen-1-ol	1158	—	—	—	0.18 ± 0.08	1.32 ± 0.46	0.12 ± 0.09	1.07 ± 0.07	1.86 ± 0.41	—
11	(E)-2-Nonenal	1167	0.05 ± 0.03	—	0.22 ± 0.19	0.98 ± 0.65	—	0.22 ± 0.06	36.60 ± 3.04	23.50 ± 0.99	—
12	(E)-2-Nonen-1-ol	1170	0.05 ± 0.04	—	0.16 ± 0.15	0.70 ± 0.85	1.27 ± 0.42	0.17 ± 0.07	14.14 ± 2.04	11.17 ± 3.83	—
13	(Z)-6-Nonen-1-ol	1171	0.03 ± 0.01	—	0.13 ± 0.06	—	—	—	—	—	—
14	(Z)-3-Hexenyl butyrate	1185	0.02 ± 0.01	—	0.04 ± 0.04	0.92 ± 0.56	—	1.11 ± 0.40	—	—	—
15	Hexyl butyrate	1192	0.01 ± 0.01	—	0.08 ± 0.06	7.43 ± 6.55	—	—	—	—	—
16	(E)-2-Hexenyl butyrate	1195	0.02 ± 0.01	—	0.25 ± 0.25	0.71 ± 0.22	1.35 ± 0.45	0.52 ± 0.23	—	—	—
17	(Z)-3-Hexenyl isovalerate	1238	—	—	—	1.00 ± 1.27	—	0.04 ± 0.03	—	—	—
18	(Z)-3-Hexenyl valerate	1239	—	—	—	0.41 ± 0.32	—	0.08 ± 0.03	—	—	—
19	Tetradecane	1400	—	3.61 ± 3.27	—	—	—	—	—	—	—
20	Selinane	1464	—	4.42 ± 3.46	—	—	—	—	—	—	—
21	Pentadecane	1500	—	6.92 ± 5.81	—	—	—	—	—	—	—
Monoterpenes											
22	α-Pinene	939	—	0.70 ± 0.47	—	—	—	—	—	—	—
23	2-Pentylfuran	992	—	—	0.04 ± 0.07	0.50 ± 0.27	—	—	—	—	—
24	(Z)-β-Ocimene	1038	0.06 ± 0.03	0.88 ± 0.09	—	0.08 ± 0.05	0.03 ± 0.01	0.07 ± 0.10	—	0.77 ± 0.20	0.75 ± 0.17
25	(E)-β-Ocimene	1048	6.58 ± 3.67	42.6 ± 23.82	0.50 ± 0.47	3.47 ± 1.64	0.2 ± 0.03	1.49 ± 1.61	2.14 ± 1.53	1.60 ± 0.97	62.74 ± 2.50
26	(Z)-Linalool oxide(furanoid)	1076	0.14 ± 0.15	—	0.11 ± 0.08	0.13 ± 0.05	0.67 ± 0.20	0.19 ± 0.17	—	—	—
27	(E)-Linalool oxide(furanoid)	1088	0.35 ± 0.12	2.93 ± 1.14	0.95 ± 0.82	0.73 ± 0.30	2.86 ± 0.99	0.59 ± 0.45	—	—	1.23 ± 0.72
28	Linalool	1098	4.86 ± 5.05	4.61 ± 2.36	1.02 ± 1.09	44.85 ± 9.15	59.76 ± 11.44	23.61 ± 9.68	—	12.43 ± 0.69	12.26 ± 2.23
29	Hotrienol	1109	—	—	0.13 ± 0.12	3.07 ± 1.19	—	—	—	—	—
30	(E,E)-2,6-Dimethyl-1,3,5,7- octatetraene	1130	—	—	—	—	—	—	—	0.46 ± 0.08	0.40 ± 0.04
31	(E,Z)-2,6-Nonadienal	1155	0.14 ± 0.07	—	0.14 ± 0.07	0.37 ± 0.18	1.64 ± 0.57	0.14 ± 0.04	19.19 ± 2.86	17.46 ± 1.21	—
32	2,5-Dimethyl-1,6-heptadien	1161	—	—	—	—	2.6 ± 1.46	—	—	—	—
33	α-Terpineol	1186	0.01 ± 0.01	—	—	0.10 ± 0.08	—	—	0.65 ± 0.21	—	0.63 ± 0.19

TABLE 2: Continued.

Compounds	RI ^a	<i>G. eriocarpum</i>		<i>G. daltonii</i>		<i>G. sphaerogynum</i>		<i>G. hirsutum</i>		<i>G. zeylanicum</i>	
		Male flowers (N ^b = 3)	Female flowers (N = 4)	Male flowers (N = 7)	Female flowers (N = 6)	Male flowers (N = 4)	Female flowers (N = 4)	Male flowers (N = 3)	Female flowers (N = 3)	Male flowers (N = 3)	Female flowers (N = 3)
<i>Sesquiterpenes</i>											
34 α -Copaene	1376	—	—	—	—	—	—	—	0.62 $\pm$ 0.19	—	0.68 $\pm$ 0.32
35 β-Elemene	1391	28.22 $\pm$ 3.26	—	27.13 $\pm$ 5.62	7.54 $\pm$ 4.77	—	22.64 $\pm$ 1.89	—	1.36 $\pm$ 1.11	1.95 $\pm$ 1.90	—
36 (Z)-Jasmone	1393	—	—	—	0.16 $\pm$ 0.06	5.63 $\pm$ 0.98	3.26 $\pm$ 2.61	—	—	—	—
37 β -Caryophyllene	1418	1.32 $\pm$ 0.31	—	0.44 $\pm$ 0.12	3.28 $\pm$ 1.41	1.4 $\pm$ 0.44	3.26 $\pm$ 2.61	1.13 $\pm$ 0.61	7.344 $\pm$ 2.37	3.31 $\pm$ 2.16	6.90 $\pm$ 2.34
38 γ -Elemene	1430	—	—	—	—	0.04 $\pm$ 0.03	1.21 $\pm$ 0.47	—	—	—	—
39 β -Copaene	1433	—	—	—	—	—	—	—	0.43 $\pm$ 0.31	0.87 $\pm$ 0.93	0.30 $\pm$ 0.14
40 (E)- α -Bergamotene	1439	—	—	0.04 $\pm$ 0.02	—	—	—	—	—	—	—
41 α -Caryophyllene	1451	0.12 $\pm$ 0.08	—	0.06 $\pm$ 0.06	1.10 $\pm$ 0.35	0.13 $\pm$ 0.11	0.18 $\pm$ 0.08	—	3.53 $\pm$ 0.98	1.45 $\pm$ 0.97	1.71 $\pm$ 0.64
42 β -Humulene	1454	—	—	—	—	—	0.33 $\pm$ 0.12	—	—	—	—
Z,Z,Z-1,5,9,9-Tetramethyl- 1,4,7-cycloundecatriene	1456	—	—	—	—	0.37 $\pm$ 0.37	0.21 $\pm$ 0.20	—	—	—	—
44 α -Patchoulene	1457	0.49 $\pm$ 0.26	—	1.42 $\pm$ 0.62	—	—	2.93 $\pm$ 0.83	—	—	—	—
45 (E)- β -Farnesene	1458	—	—	0.08 $\pm$ 0.09	—	—	—	—	—	—	—
46 4,5-Di-epi-aristolochene	1467	0.14 $\pm$ 0.03	—	0.06 $\pm$ 0.06	—	—	—	—	—	—	—
47 γ -Selinene	1470	8.97 $\pm$ 1.64	—	4.78 $\pm$ 0.72	2.13 $\pm$ 0.85	—	5.64 $\pm$ 1.21	—	—	—	—
48 Germacrene D	1481	0.08 $\pm$ 0.03	—	—	—	—	—	—	—	—	—
49 β-Selinene	1485	14.54 $\pm$ 4.13	—	7.04 $\pm$ 1.00	4.37 $\pm$ 1.71	—	12.03 $\pm$ 2.85	—	—	—	—
50 δ -Selinene	1487	0.20 $\pm$ 0.12	—	0.18 $\pm$ 0.10	—	—	0.38 $\pm$ 0.22	—	—	—	—
51 α -Muurolene	1493	—	—	—	4.59 $\pm$ 2.85	—	—	—	—	—	—
52 α-Selinene	1498	16.09 $\pm$ 3.37	—	7.70 $\pm$ 1.31	4.84 $\pm$ 2.18	—	11.87 $\pm$ 2.98	—	—	—	—
53 Eremophilene	1502	—	—	—	1.24 $\pm$ 0.72	—	—	—	—	—	—
54 (E,E)- α -Farnesene	1506	1.47 $\pm$ 1.15	5.1 $\pm$ 5.96	40.30 $\pm$ 9.92	1.66 $\pm$ 1.03	0.78 $\pm$ 1.34	0.14 $\pm$ 0.04	—	—	—	—
55 α -Panasinene	1518	0.75 $\pm$ 0.15	—	0.31 $\pm$ 0.10	0.43 $\pm$ 0.73	—	0.38 $\pm$ 0.10	—	1.64 $\pm$ 2.05	—	2.50 $\pm$ 1.31
56 Longiborneol	1592	14.16 $\pm$ 2.09	—	3.99 $\pm$ 1.92	—	—	6.98 $\pm$ 2.63	—	—	—	—

TABLE 2: Continued.

Compounds	Rt ^a	G. eriocarpum		G. daltonii		G. sphaerogynum		G. hirsutum		G. zeylanicum	
		Male flowers (N ^b = 3)	Female flowers (N = 4)	Male flowers (N = 7)	Female flowers (N = 6)	Male flowers (N = 4)	Female flowers (N = 4)	Male flowers (N = 3)	Female flowers (N = 3)	Male flowers (N = 3)	Female flowers (N = 3)
Aromatics											
57 Benzaldehyde	962	0.07 ± 0.04	0.51 ± 0.33	0.03 ± 0.02	—	—	—	—	—	—	—
58 Benzeneacetaldehyde	1043	—	—	—	—	—	—	0.58 ± 0.29	—	1.08 ± 0.56	—
59 Methyl benzoate	1090	—	—	—	—	—	—	—	0.42 ± 0.07	—	0.61 ± 0.29
60 Benzoyl cyanide	1094	—	—	—	0.14 ± 0.15	—	—	—	—	—	—
61 Phenylethyl alcohol	1116	0.10 ± 0.07	—	0.06 ± 0.08	—	—	—	10.04 ± 4.05	1.10 ± 0.26	20.76 ± 3.56	0.09 ± 0.05
62 Phenylacetoneitrile	1143	0.17 ± 0.18	—	0.23 ± 0.33	—	—	—	1.92 ± 0.72	—	3.28 ± 0.54	—
63 Methyl salicylate	1190	0.04 ± 0.02	—	—	—	—	0.72 ± 0.16	1.02 ± 0.15	0.92 ± 0.35	3.34 ± 2.82	0.42 ± 0.04
64 Benzothiazole	1227	—	1.48 ± 0.72	—	—	—	—	—	—	—	—
65 2-Phenylethyl acetate	1256	—	—	0.07 ± 0.10	—	—	—	—	—	—	—
66 Indole	1287	—	—	—	—	—	—	1.54 ± 1.75	3.51 ± 0.7	1.38 ± 0.17	2.96 ± 1.73
67 β-Phenylethyl butyrate	1447	—	—	0.06 ± 0.06	—	—	—	—	—	—	—
68 Butylated hydroxytoluene	1512	—	4.61 ± 4.84	—	—	—	—	—	—	—	—
69 Mellein	1546	—	—	—	—	4.73 ± 2.03	0.63 ± 0.17	—	—	—	—
Unknown											
		0.17 ± 0.04	3.93 ± 3.51	0.64 ± 0.60	0.25 ± 0.17	2.2 ± 1.13	0.36 ± 0.08	6.08 ± 0.81	4.68 ± 1.47	5.12 ± 0.44	5.22 ± 1.8

^aRetention index.^bThe number of headspace samples.

“—” presents that the compound was not detected.

The boldface compounds present the main compounds of each species (relative amounts >10%).

FIGURE 3: Relative amounts (%) of four chemical classes volatile compound of both male and female flowers from five *Glochidion* species. (a) *G. eriocarpum*; (b) *G. daltonii*; (c) *G. sphaerogynum*; (d) *G. hirsutum*; (e) *G. zeylanicum* (one-way ANOVA: the different lowercase letters on the bars indicate significant difference at $P = 0.05$ level).

FIGURE 4: Score plots from nonmetric multidimensional scaling (NMDS) of floral scents in male and female flowers of five *Glochidion* species.

the results showed evident quantitative differences in floral scents between male and female flowers of the *Glochidion* species.

3.4. Sexual Dimorphism of Floral Scents. The NMDS analysis of floral scents in male and female flowers of each *Glochidion* species is shown in Figure 6. In each investigated *Glochidion* species, the headspace samples of male flowers were clearly separated from those of female flowers (*G. daltonii*: $\chi^2 = 6$, $P = 0.034$; *G. eriocarpum*: $\chi^2 = 9$, $P = 0.03$; *G. sphaerogynum*: $\chi^2 = 5.33$, $P = 0.021$; *G. hirsutum* and *G. zeylanicum*: $\chi^2 = 3.857$, $P = 0.05$). The results indicated a qualitative difference between floral scents produced by male and female *Glochidion* flowers, which is so-called sexual dimorphism of floral scents.

FIGURE 5: Total emission (Mean $\pm$ SD) of scent compounds from male and female flowers of five *Glochidion* species (unpaired *t*-test: “*” and “***” on the bars indicate significant difference at $P = 0.05$ and 0.001 level).

4. Discussion

In total, 69 volatiles are detected from the floral scents of the five *Glochidion* species. Most of these reported volatiles are quite common in floral odors of many flowering plants [33, 34]. In particular, 26 compounds also occur in floral fragrances of other Phyllanthaceae species, close relatives of the investigated *Glochidion* species, which are also exclusively pollinated by *Epicephala* moths [16, 18, 35]. For instance, (*E*)- β -ocimene (compound 25) and linalool (compound 28) occurred together in the five studied *Glochidion* species and are also produced by the flowers of four other reported *Glochidion* species [16, 18]. In addition, these two compounds can attract a wide range of pollinators of flowering plants, such as moths [35, 36], butterflies [37], and bees [38]. Therefore, our results suggest that these interactions between *Glochidion* species and *Epicephala* moths may be mediated by conventional floral compounds. This is similar to findings in most obligate insect-plant mutualism, where identified floral volatiles are also regarded as general floral volatiles [16, 25, 39]. However, Chen et al. [40] showed that an unusual compound, 4-methylanisole, mediated the species specificity in *Ficus semicordata* and *Ceratosolen graveleyi* wasp mutualism through a “private channel.”

In highly specific plant-insect interactions, the floral scent profile of host plants is sometimes dominated by one or few compounds [16, 39, 41, 42], and these dominant compounds may account for variation in floral scents among species [16]. Similarly, our GC-MS showed the floral odor of each of the five species was also dominated by one compound at least, such as, for male flowers, β -elemene (compound 35, 28.22%) in *G. eriocarpum*, (*E,E*)- α -farnesene (compound 54, 40.3%) in *G. daltonii*, linalool (compound 28, 59.75%) in *G. sphaerogynum*, and (*E*)-2-nonenal in *G. hirsutum* and *G. zeylanicum* (compound 11, 36.6 and 23.5%, resp.). The PCA

FIGURE 6: Score plots from NMDS of floral scents in male and female flowers of five *Glochidion* species. The headspace samples with each species are represented by different numbers sharp symbols as shown. The headspace samples within each species are boxed and coloured white for male flowers but grey for female flowers.

results revealed that both male and female flowers of the five species could be divided into four distinct groups: *G. zeylanicum* and *G. hirsutum* clustered together and the other species were spaced with each other. Thus, floral odors of investigated *Glochidion* species could be clearly discriminated mainly due to the dominant compounds. This is in contrast to findings for the odor profile of five other *Glochidion* species that were distinguished by minor compounds [16]. These results suggest that the presence of dominant compounds in floral scents of *Glochidion* species may be crucial for attracting specific *Epicephala* pollinators. However, electroantennographic detection analysis and behavioral tests with authentic chemicals are needed for further screening of specific compounds responsible for this host specificity.

Highly interspecific variation in floral scent has been observed in many plant species [16, 39, 43–50]. It is likely that variation in floral odor composition between species is due to adaptation to host-specific pollinators [16, 50]. The species specificity in the *Glochidion*-*Epicephala* mutualism is extremely high, where each host plant is usually exclusively pollinated by specific pollinator species. Thus, to facilitate host specificity in these interactions, the floral scent should be selected to be strongly divergent among species. Interspecific variation in floral scent profile of *Glochidion* species observed in this study could contribute to the maintenance

of the species specificity of the mutualism and may reflect adaptation to specific *Epicephala* moths. Pollinators can also find and locate their hosts by discriminating the difference in floral odor among species [16, 40]. Our NMDS analysis showed a similar floral scent profile between *G. hirsutum* and *G. zeylanicum*. In *Glochidion*-*Epicephala* mutualism, *E. bipollenella* moths jointly pollinated *G. hirsutum* and *G. zeylanicum* [20]. Similarly, *G. obovatum* and *G. rubrum*, two parapatric species pollinated jointly by the same *Epicephala* species, produced similar floral scents, more similar than those emitted by other cooccurring *Glochidion* species pollinated by other *Epicephala* species [16]. In addition, *Ficus natalensis* and *F. burkei* jointly sharing the same pollinator wasp species emitted similar floral scent profiles, which are different from those produced by other African *Ficus* species [51]. When different plant species are pollinated by the same group of pollinators and especially by closely related pollinator taxa, they may be expected to have floral volatiles of similar chemical composition [43]. This implies that the similarity in odor of different *Glochidion* species in this study may suggest evolutionary convergence to attract the same *Epicephala* pollinators [51].

Generally, but not always, the animal-pollinated dioecious plants produce similar floral signals between male and female flowers. This is because attraction of the same

pollinator to flowers of both sexes is essential for successful conspecific pollen transfer; thus the hosts should be selected to transmit such similar floral scents in the flowers of both sexes [21–23]. Underlying the fact above is that pollinators use similar cues to find the same floral rewards (e.g., floral nectar) from male and female flowers [21, 24]. Selection for similarity in signals from male and female flowers is thought to be particularly strong when one sex (often the female) does not produce any reward and effectively mimics flowers of the other sex, so deceptive pollination occurs in the female [52]. For instance, chemical mimicry in floral scent occurs in fig-fig-wasp highly species-specific pollination systems. Although fig wasps are deceived into pollinating female figs but do not obtain any reward from female figs, the pollinated female figs can produce seeds to ensure the successful reproduction of fig species. Therefore, for fig-fig-wasp attraction, selection for chemical mimicry between sexes is suggested to be particularly critical to maintain the stability of obligate pollination mutualism between fig and fig wasp [40, 52, 53]. In addition, chemical mimicry between sexes of the flowers has also been documented in cycad species [50, 54–57]. Hemborg and Bond [24] proposed that different rewards offered by male and female flowers of some plant species to pollinators could promote the evolution of sexually dimorphic floral signals of flowers. Svensson et al. [25] and Okamoto et al. [18] found that *Epicephala*-pollinated Phyllanthaceae plants consistently showed clear differences in scent between male and female flowers. Similarly, our NMDS results also revealed in *Glochidion-Epicephala* a remarkable sexual dimorphism in floral scent between sexes of the five investigated *Glochidion* species. In *Epicephala*-pollinated Phyllanthaceae, when *Epicephala* moths pollinate for the host, male flowers can offer pollen to pollinators as food, whereas female flowers can provide the ovule for *Epicephala* adults as egg-laying sites and seeds for *Epicephala* larvae as foods [5, 9–12], and it was thought that *Epicephala* moths could get access the different and essential rewards from the flowers of both sexes of hosts; thus sexual dimorphism in floral scent of two sexes in *Epicephala*-pollinated Phyllanthaceae is selected to signal an alternative reward provided by each sex to *Epicephala* moths. *Epicephala* can also distinguish male and female flowers by odor and is crucial for successful reproduction of the moths and plants [18]. Our results also provide strong support that different rewards in male and female flowers result in the sexual dimorphism in floral scent of flowers of *Glochidion* species in this study [24].

Our results showed quantitative differences in floral scent emission between the sexes in each studied *Glochidion* species. In addition, there was a similar finding for *B. vitis-idaea*, a close relative of *Glochidion*, which is also obligately pollinated by species-specific *Epicephala* moths [25]. Okamoto et al. [18] showed that *Epicephala* moths preferred the scent of male flowers over that of female flowers in *G. zeylanicum*. According to sexual selection theory, the males are limited in their reproductive success by access to mates, while females are mostly limited by available resources [58]. In animal-pollinated plants, attraction of pollinators and successful pollination are crucial for reproductive success; thus males in monoecious plants are expected to be under

selection to increase their attractiveness to pollinators by investing more than females in floral traits that enhance pollinator visitation. Generally, male plants should produce more floral scent than the females to increase pollinator attraction. Waelti et al. [59] suggested that, in the *Silene latifolia-Hadena bicruris* nursery pollination system, male flowers emitted significantly more fragrance, especially of the pollinator-attracting compounds, than female flowers, and pollinator-moths preferred male over female flowers in behavioral tests, implying that evolution of the divergence in the floral signal of *S. latifolia* is shaped by sexual selection. Ashman et al. [60] also showed that the smaller hermaphroditic flowers in the gynodioecious strawberry *Fragaria virginiana* produced larger amounts of foliage volatiles, which resulted in attraction of more pollinators compared to female flowers. Therefore, more probably, the observed quantitative difference in floral scent of flowers between the sexes in the five species of *Glochidion* may be an outcome of sexual selection. It is possible that the stronger odor emission of male flowers in *Glochidion* species resulted in increasing attractiveness for *Epicephala* moths compared with female flowers.

5. Conclusions

Our findings suggest that the volatile variation of floral scent among *Glochidion* species reflects adaptations to specific pollinators. Sexual dimorphism in floral scent has evolved to signal alternative rewards provided by each sex to *Epicephala* moths. It is concluded that floral scent has been thought to play key role in the obligate pollination mutualism between *Glochidion* plants and *Epicephala* moths.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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