Chapter 8
Species-Specific Floral Scents as Olfactory Cues in Pollinator Moths

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Keywords Active pollination behavior • *Epicephala* • *Glochidion* • Phyllantheae • Sexual dimorphism • Species specificity

8.1 Floral Scents as Pollinator Attractants

Within the diverse taxa in Phyllanthaceae, many species are obligately pollinated by host-specific seed-parasitic moths. There must be some mechanism by which a plant species attracts a pollinator partner because several Phyllanthaceae plants can co-occur in tropical regions.

To attract pollinators, flowers generally use visual and olfactory signals (Proctor et al. 1996; Ollerton et al. 2011). Although diurnal butterflies mainly use visual cues to detect flowers, olfactory cues together with visual cues are used in pollination mutualism by oligolectic bees (Dötterl et al. 2005), pompilid wasps (Shuttleworth and Johnson 2009), bumblebees (Byers et al. 2014), euglossine bees (Ackerman 1989), fungus gnats (Okamoto et al. 2015), and thrips (Terry et al. 2007). Another well-known case in which plants strongly depend on olfactory signals to attract pollinators is the deceptive pollination system, in which flowers emit floral scents mimicking pollinators' brood sites (Stensmyr et al. 2002; Oelschlägel et al. 2014) or virgin females of the pollinator species (Peakall and Whitehead 2014). Chemical mimicry of insect brood sites has been observed in 61 plant species in 11 families (Jürgens et al. 2013), the flowers of which emit floral scents mimicking decaying plant material, fungi, carrion, or dung and are pollinated by saprophagous, fungivorous, or coprophagous beetles and flies (Jürgens et al. 2013).

In contrast to diurnal pollination, some plants are pollinated only during the night when visual cues are essentially unavailable. Due to the darkness, nocturnal animal-pollinated flowers depend on chemical signals, and many nocturnal insect pollinators have developed an olfactory sense, as reported in fig wasps (Chen et al. 2009) and moths (Kato and Inoue 1994; Singer 2002; Raguso et al. 2003; Sugiura and Yamazaki 2005; Okamoto et al. 2008). Plants pollinated by nocturnal moths

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usually have a conspicuous perianth with a white, pale yellow, or light pink color and heavy scents described as "pleasant" or "sweet" (Dobson 2006; Waser 2006). These pale flowers stand out against the background on moonlit and starlit nights, and olfactory signals guide the moth pollinators to the flowers regardless of how dark it is. In *Datura* (Solanaceae) flowers, the pollinator moth, *Manduca sexta*, has been observed feeding on flowers only when both visual and olfactory cues are available, even at night (Raguso and Willis 2005).

As discussed in other chapters, the Phyllanthaceae plants adopting obligate pollination mutualism are pollinated by gracillariid moths only during the night. This suggests that the mutualism is underpinned by chemical communication between the flowers and pollinator moths. This chapter focuses on the floral scent and the olfactory sense of moths, both of which contribute to the high host-specificity of the mutualism.

8.2 Species Specificity Mediated by Floral Scents

The *Epicephala* moth-pollinated plant genus *Glochidion* includes more than 300 species distributed from tropical Asia to Australia and Polynesia (Govaerts et al. 2000). All members of *Glochidion* are thought to be pollinated exclusively by species-specific *Epicephala* moths. Five species commonly occur at forest edges in southern Japan, *G. acuminatum*, *G. lanceolatum*, *G. obovatum*, *G. rubrum*, and *G. zeylanicum*, and a few *Glochidion* species can grow sympatrically. In such populations, flowering periods often overlap and the sympatric *Glochidion* species do not grow in spatial isolation. The inconspicuous flowers and their nocturnal flowering suggest that the flowers attract moth pollinators via scent. Therefore, the high host-specificity of the pollinator moths suggests that (1) the floral scent composition differs among *Glochidion* species, and (2) pollinator *Epicephala* moths can distinguish between the floral scents of their own host and nonhost species.

The first hypothesis was tested by chemical analyses of the floral scents of five *Glochidion* species using gas chromatography-mass spectrometry (GC-MS). The floral scents of *Glochidion* flowers were blends of 42 volatiles, mainly monoterpenes and sesquiterpenes, and were dominated by linalool or β-ocimene. To detect intra- and interspecific variation in the volatile profiles obtained from GC-MS, the profile data were transformed using chord-normalized expected species shared distances (CNESS) and nonmetric multidimensional scaling (NMDS). The CNESS–NMDS analysis of the volatile profile data of the five *Glochidion* species revealed that the floral scents could be clearly distinguished among plant species based on the relative compositions of volatile chemicals, especially the composition of minor compounds (Fig. 8.1a). Although the composition of floral volatiles was also variable among conspecific flowers on different trees, marked interspecific differences were observed in the volatile composition. In CNESS analyses, sensitivity to the abundance of dominant compounds can be adjusted by altering the analysis parameters, that is, by emphasizing the importance of major or minor compounds (Trueblood et al. 1994). Under the parameter set sensitive to both dominant and minor compounds, NMDS scatterplots revealed that the floral samples were divided into three main groups (Fig. 8.1b). *Glochidion lanceolatum*, *G. obovatum*, and *G. rubrum* were not separated from each other in the scatterplot, probably because they shared a dominance of (*R*)-(−)-linalool. In comparison, under the parameter set most sensitive to minor compounds, the five *Glochidion* species were largely distinguished by volatile composition, although there were slight overlaps among the *G. lanceolatum*, *G. obovatum*, and *G. rubrum* samples, suggesting that the presence of minor compounds or their amounts relative to other compounds is also important in attracting specific *Epicephala* moths. Among the three *Glochidion* species, *G. obovatum* and *G. rubrum* are closely related and have allopatric distributions: the former appears on mainland Japan and Okinawa Island and the latter on the Yaeyama Islands. These two *Glochidion* species do not have different scent profiles because they never co-occur (Fig. 8.2). The CNESS/NMDS analysis did not use an equal number of samples from flowers of each plant species, but a similar analysis based on samples from the same numbers of flowers clearly differentiated the floral scent profiles among the five *Glochidion* species.

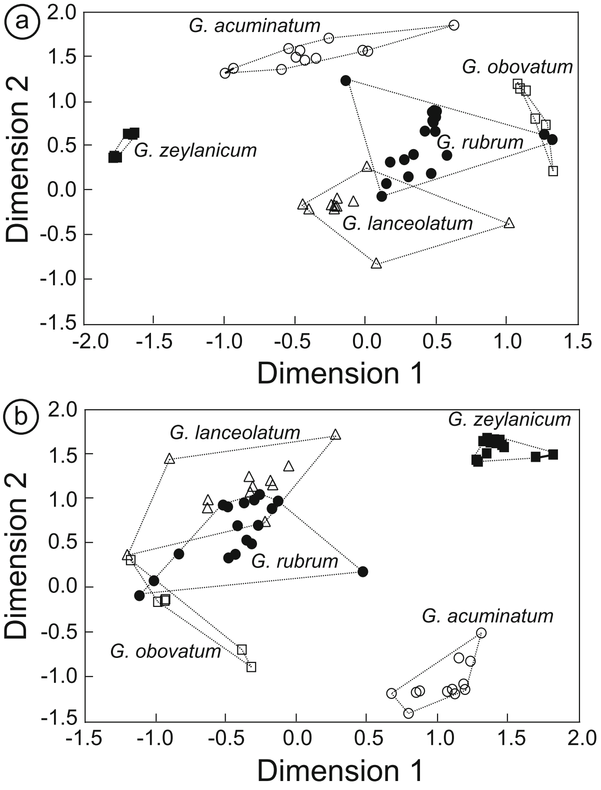

Fig. 8.1 Scatterplots resulting from the NMDS analysis based on CNESS distance index of floral scents in the five Japanese *Glochidion* species. CNESS dissimilarity was calculated by either (**a**) assuming a greater weight on minor compounds and (**b**) weighing all compounds equally

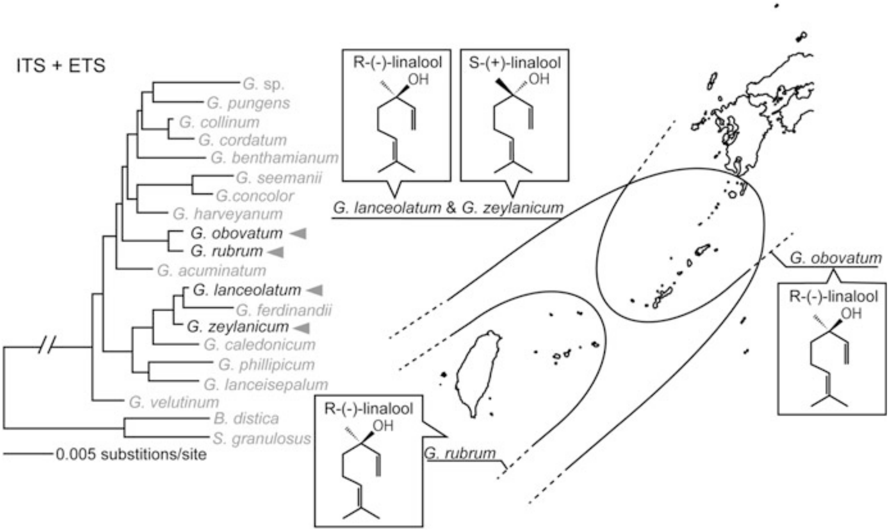

Fig. 8.2 Relationship between dominant floral scent compounds and distribution pattern of four *Glochidion* species. Phylogenetic tree based on ITS and EST shows two pairs of closely-related species: *G. obovatum*/*G. rubrum* and *G. lanceolatum*/*G. zeylanicum*. The former species pair occurs in allopatry and share R-(-)-linalool as the dominant compound. The letter species pair occurs in sympatry, and each has different dominant compound

To test the hypothesis that a pollinator *Epicephala* moth can distinguish between the floral scents of its own host and nonhost species, a bioassay was used to investigate the response of pollinator moths to floral scents. Pollinator moths reared from the seeds of *G. lanceolatum* and *G. rubrum* were used in a preference test using a Y-shaped olfactometer. In this bioassay, an unmated male/female moth introduced into a Y-shaped tube was allowed to select its way in response to airborne stimuli of floral volatiles of the two *Glochidion* species. The preference test revealed that *Epicephala* moths could detect their host plants by floral scents alone (Okamoto et al. 2007). Interestingly, the fact that both males and females responded to the floral scent suggests that the moths may use the floral scents as cues to visit flowers not only for pollination but also for mating.

8.3 Not a Private Channel, But a Blend

A “private channel” involves exclusive, system-specific chemicals that mediate species-specific interactions between plants and insects and between fungi and spore dispersers (Steinebrunner et al. 2008; Chen et al. 2009; Franke et al. 2009; Soler et al. 2010). Well-known examples of private channels are found in sexually deceptive orchids: the floral scents of *Ophrys* and *Chiloglottis* species mimic the sex pheromones produced by female pollinator insects (Ayasse et al. 2003; Schiestl and Peakall 2005). Species-specific olfactory signals also mediate the association between *Ficus* plants and fig wasps, and the chemical that attracts single pollinator species has recently been identified in *Ficus semicordata* (Chen et al. 2009). The receptive male and female syconia of *F. semicordata* emit scents consisting primarily of 4-methylanisole, which is attractive to its sole pollinator wasp, *Ceratosolen gravelyi*. Although 4-methylanisole is not rare as a floral scent compound, and has been detected in flowers of other angiosperms, it has not been found in flowers of other *Ficus* species (Proffit et al. 2008, 2009).

To detect the presence or absence of private channels in the obligate pollination mutualism in Phyllanthaceae, chemical analyses of floral scents and electrophysiological analyses of the pollinator's olfactory receptors in the *Breynia–Epicephala* obligate pollination mutualism were conducted. In contrast to *Glochidion*, *Breynia* is characterized by shrubby habitat, saccate male flowers, discal or clavate female flowers with connate sepals and bifid stigmas, red/black fleshy fruit, and nocturnal leaf-folding behavior. In *Breynia vitis-idaea* from Ishigaki Island, a mixture of two floral volatiles, 2-phenylethyl alcohol and 2-phenylacetonitril, was as attractive as real flowers to the females of specific *Epicephala* moth species, although each compound alone was less attractive (Svensson et al. 2010). The two volatiles are common floral compounds frequently found in various angiosperms (Knudsen et al. 2006), including male flowers of *Glochidion lanceolatum* (Okamoto et al. 2013).

Gas chromatography with electroantennographic detection (GC-EAD), coupled GC-MS, and olfactometer bioassays were used to determine which compounds elicit responses from the antennal olfactory receptors of the pollinator *Epicephala* moths. The results revealed that five compounds, including the two above-mentioned volatiles, elicited responses from the antennal olfactory receptors of both males and females of the pollinator *Epicephala* moths (Fig. 8.3). These results

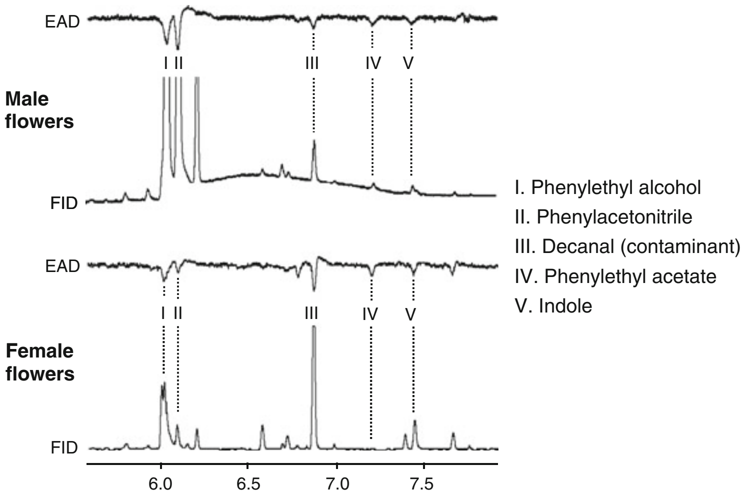

Fig. 8.3 Simultaneous responses of the flame ionization detector (*FID*) and electroantennographic detection (*EAD*) using the antennae of female *Epicephala* moths to male and female floral scents of *Breynia vitis-idaea*

indicate that *B. vitis-idaea* uses a blend of conventional floral scent compounds as an attractant for its exclusive *Epicephala* pollinator, and that system-specific chemistry is not a necessity for efficient host location by the moth partners.

8.4 Evolution of Sexual Dimorphism of Floral Scent

Like some members of *Ficus*, but unlike *Yucca*, most members of Phyllanthaceae are monoecious; that is, male and female flowers grow on a single plant. In *Epicephala* moth-pollinated species, male and female flowers are visited by the same female moth, although with different behavior on each: pollen collection from male flowers and active pollination and oviposition on female flowers. This begs the question, do the floral scents differ between male and female flowers?

Most angiosperms are hermaphrodites bearing bisexual flowers, although about 30% of angiosperm species are monoecious or dioecious, producing unisexual flowers (Lloyd and Webb 1977). Among these monoecious and dioecious plants, wind- and water-pollinated unisexual flowers exhibit extensive sexual dimorphism in floral traits, which are specialized for pollen release in males and pollen reception in females. However, because animal-pollinated plants must attract the same pollinator species to both male and female flowers to secure conspecific pollen transfer, the flowers of both sexes tend to resemble each other in morphological and olfactory traits. Consequently, many animal-pollinated unisexual flowers exhibit low sexual divergence in floral signals, even if they have different floral rewards (Willson and Ågren 1989; Fenster et al. 2004). However, this is not the case in the obligately moth-pollinated plants in Phyllanthaceae because the pollinator moths execute different missions on flowers of different sexes.

Female *Epicephala* moths deliver pollen grains from male to female flowers using their proboscis, and then lay eggs in ovaries because the larvae consume part of the developing seed until pupation. Therefore, active pollination by *Epicephala* moths is a specialized trait to secure food for larvae. Because active pollination behavior involves the collection of pollen from male flowers and then depositing it on female flowers, *Epicephala* moths must be able to distinguish male and female flowers and to visit male flowers before visiting female flowers. A Y-tube preference test indicated that mated female *Epicephala* moths without prior experience of pollen collection preferred the scent of male flowers over that of female flowers (Okamoto et al. 2013). Virgin female moths first mate before visiting flowers; the mating enhances their motivation to visit male flowers to collect pollen. Having collected pollen, the moths are attracted by female flowers, which they pollinate and oviposit.

In *Epicephala* moth-pollinated plants of the genera *Glochidion*, *Breynia*, and *Phyllanthus* (section *Anisonema*), the floral scents of the male and female flowers of each plant species are composed of similar compounds, although the scent profiles clearly differ between male and female flowers. In contrast, in the Phyllanthaceae

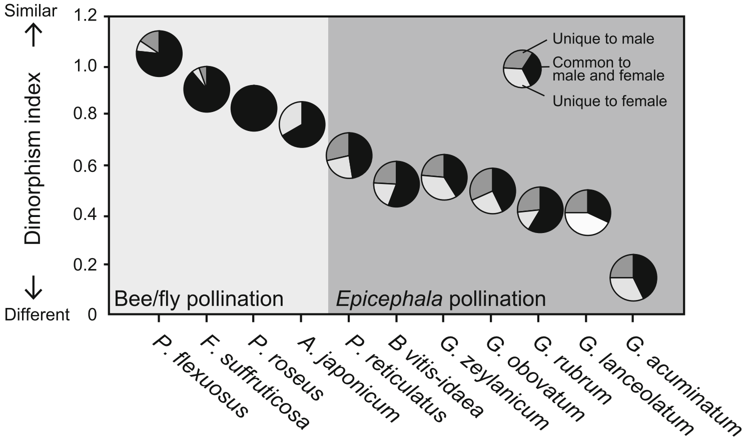

Fig. 8.4 Pattern of sexual dimorphism in floral scent of *Epicephala* moth-pollinated and bee/fly-pollinated species. Dimorphism index approaches 1 as the male and female floral scents become more similar. Pie graphs indicate the proportion of compounds unique to either male or female floral scents. Black color indicate compounds shared between male and female flowers, dark gray indicates compounds unique to male flowers, and light gray indicates compounds unique to female flowers

generally pollinated by diurnal bees and flies, for example, *Antidesma*, *Flueggea*, and *Phyllanthus* (subgenera *Kirganelia* and *Phyllanthodendron*), the floral scent profiles are very similar between male and female flowers (Fig. 8.4). Sexual dimorphism of the floral scent profiles was investigated using a dimorphism index (*D*) based on the Bray–Curtis dissimilarity index (Bray and Curtis 1957), which is a positive value that approaches 0 as the floral scents become more sexually dimorphic, and approaches 1 as male and female floral scents become more similar. The *D* values of 11 Phyllanthaceae species (Fig. 8.4) suggest that the obligately moth-pollinated plants have low *D* indices, indicating sexual dimorphism in floral scents. Marked sexual dimorphism was observed in floral scents between male and female flowers in multiple Phyllanthaceae lineages that have independently evolved via *Epicephala* pollination, strongly indicating that the sexual dimorphism of floral scent is associated with *Epicephala* pollination (Fig. 8.5).

Interestingly, the difference between male and female floral scents involves major qualitative differences in volatile blends. For example, the dominant compounds are derived from different biosynthetic pathways: in *Glochidion zeylanicum* and *G. lanceolatum* the dominant compounds of the female floral scent are terpenoids synthesized by the 2-*C*-methyl-ᴅ-erythritol 4-phosphate/1-deoxy-ᴅ-xylulose 5-phosphate pathway (MEP/DOXP pathway), whereas those of the male floral scent are simple aromatic compounds synthesized by the shikimic acid pathway (Fig. 8.6). In another case, male and female flowers emit different enantiomers of the same compound as dominants from each sex: in *G. obovatum* and *G. rubrum*,

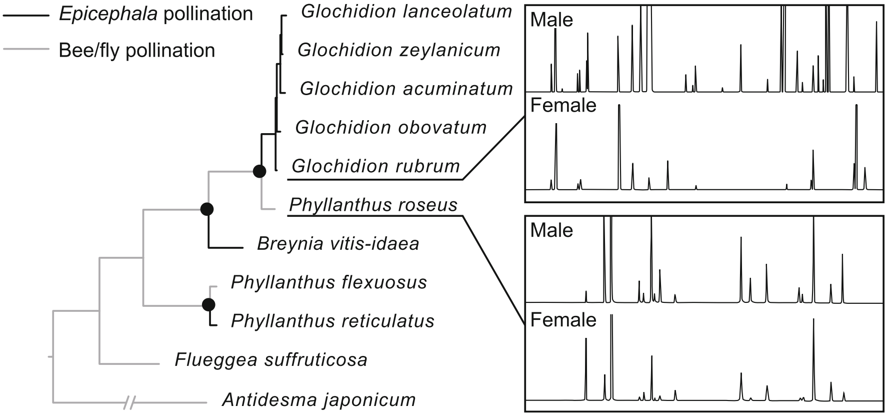

Fig. 8.5 Evolution of floral scent sexual dimorphism in Phyllantheae. Phylogenetic relationships of the 11 Phyllanthaceae species are based on the maximum-likelihood tree of Kawakita and Kato (2009). Black circles on the tree indicate evolutionary transitions from bee/fly pollination to *Epicephala* moth-pollination

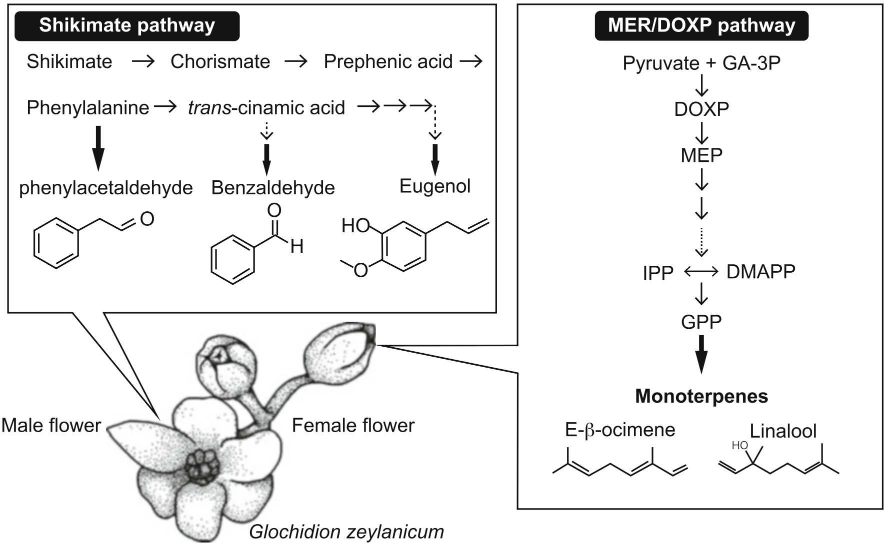

Fig. 8.6 Compounds dominating in the male and female floral scent of *G. zeylanicum* and their synthetic pathways. Simple aromatic compounds are synthesized via the shikimate pathway in the chloroplast. Monoterpenes are synthesized by the *MEP/DOXP* pathway in the chloroplast

(R)-(−)-linalool in male flowers and (S)-(+)-linalool in female flowers. Overall, the average proportion of compounds unique to one sex is 36.5% in *Epicephala*-moth pollinated species, whereas it is only 8.9% in bee/fly-pollinated species (Fig. 8.4).

The sexual dimorphism of floral scents is uncommon in animal-pollinated plants, especially in plants whose female flowers attract pollinator animals without offering rewards. Soler et al. (2012) demonstrated evidence for intersexual mimics of floral scents in dioecious *Ficus* species. Almost one half of *Ficus* species are gynodioecious, producing "functionally male" (hermaphrodite) trees and female trees (Cook and Rasplus 2003). Pollinating fig wasps use only the "female" flowers of male syconia for their brood site, whereas the wasps entering female syconia cannot produce offspring because the long-styled female flowers prevent wasp oviposition. Therefore, it is thought that the pollinator wasps must select male syconia to leave offspring, and are selected to distinguish between male and female syconia. However, selection for male and female syconia to resemble each other chemically is particularly strong for producing seeds of dioecious figs. A chemical analysis of volatiles emitted from syconia revealed that female syconia chemically mimic functionally male syconia (Soler et al. 2012). In contrast with the intersexual chemical mimicry in fig–fig wasp mutualism, the overall similarity and compositional dimorphism in floral scents between male and female flowers of *Epicephala* moth-pollinated plants in Phyllanthaceae must facilitate the delicate nocturnal pollination.

8.5 Floral Scent Profiles of *Epicephala* Moth-Pollinated and Bee/Fly-Pollinated Plants

The floral scents of 11 Phyllanthaceae plants are composed of 85 volatile compounds in total. The floral profiles of *Epicephala* moth-pollinated plants differ from those of bee/fly-pollinated plants (Fig. 8.4), but the difference is difficult to characterize. Although only one compound, 6-methyl-5-hepten-2-one, is shared by all *Epicephala*-pollinated species, it may not contribute to the attraction of *Epicephala* moths or repellence of other noneffective insect visitors because it occurs in the flowers of more than 50% of the angiosperm families investigated to date (Knudsen et al. 2006). Although 11 Phyllanthaceae species emitted linalool from flowers, (*R*)-(−)-linalool can only be detected in four *Epicephala* moth-pollinated species, *G. acuminatum*, *G. obovatum*, *G. rubrum*, and *G. lanceolatum*, and the remaining species include (*S*)-(+)-linalool in the floral scents, suggesting that the ability to synthesize (*R*)-(−)-linalool evolved after the early diversification of *Glochidion*. Therefore, there is probably no single compound that clearly distinguishes the scents of *Epicephala* moth-pollinated and bee/fly-pollinated species.

The floral scents of seven *Epicephala* moth-pollinated species are dominated by monoterpenes, (*R*)-(−)-linalool, (*S*)-(+)-linalool, and (*E*)-β-ocimene, combined with some simple aromatic compounds (e.g., benzaldehyde, phenylacetaldehyde, and eugenol) and nitrogen-bearing compounds (e.g., indole, methyl anthranilate, geranyl nitrile, and phenylacetonitrile; Okamoto et al. 2007, 2013). In general, moth-pollinated flowers produce large amounts of acyclic terpene alcohols (e.g., linalool and nerolidol), simple aromatic compounds (e.g., methyl benzoate and methyl salicylate), and nitrogen-bearing compounds (e.g., indole and methyl anthranilate; Dobson 2006). Hawk moth- and settling moth-pollinated plants differ in the volatile profile of floral scents, characterized by the dominance of methyl benzoate and scarcity of lilac compounds in the former, and by the dominance of phenylacetaldehyde or benzaldehyde and the scarcity of sesquiterpenoids and nitrogen-bearing compounds in the latter (Patt et al. 1988; Tollsten and Bergström 1993; Knudsen and Tollsten 2004; Dötterl et al. 2006). Most *Epicephala* moth-pollinated flowers have scent profiles similar to those of settling moth-pollinated flowers, although lacking lilac compounds. Furthermore, the scent profile of male flowers of *Phyllanthus reticulatus* is dominated by not only monoterpenes, (*S*)-(+)-linalool, and carene, but also a unique sulfur compound, methional (4-thiapentanal), which has not been found in the floral scents of angiosperms. It is still not known whether these unique compounds function to elicit special moth behavior on male flowers.

The floral scents of bee/fly-pollinated Phyllanthaceae plants are dominated by monoterpenoids, for example, limonene, (*E*)-β-ocimene, and (*E*)-linalool oxide furanoid, combined with a few sesquiterpenoids, for example, geranyl acetone, β-caryophyllene, humulene, elemene, and *trans*-α-farnesene. Unlike *Epicephala* moth-pollinated flowers, simple aromatic compounds tend to be infrequent in bee/fly-pollinated flowers. Such characteristics of the floral scents fall under a generalist pollination syndrome, where the plants are pollinated by a variety of nectar-seeking insects (Dobson 2006).

As discussed, the floral scents of *Epicephala* moth-pollinated and bee/fly-pollinated species cannot easily be distinguished by the presence/absence of individual components. However, the flowers of bee/fly-pollinated taxa, such as *Antidesma*, *Flueggea*, and *Phyllanthus* (subgenera *Kirganelia* and *Phyllanthodendron*), produced fewer volatile compounds (6–18) than *Epicephala* moth-pollinated flowers (17–35; Fig. 8.4; Okamoto et al. 2013), so the greater diversity of constituent chemicals may be a key to the specific attraction of *Epicephala* moths to flowers. Because diurnal pollinators such as bees and flies use mainly visual cues and olfactory cues subsidiarily to detect flowers, these bee/fly-pollinated plants may not need to produce diverse volatile compounds. The floral scents of *Epicephala* moth-pollinated plants are also less variable within species than those of bee/fly-pollinated plants (Fig. 8.7). Among the bee/fly-pollinated plant species, *Phyllanthus roseus* and *Antidesma japonicum* have unique floral scents clearly distinguished from those of other Phyllanthaceae species. Another two species, *Phyllanthus flexuosus* and *Flueggea suffruticosa*, have similar scent profiles that overlap on NMDS plots, and the intraspecific variation in the floral scent profiles is greater than those of other Phyllanthaceae plants (Fig. 8.7), suggesting that natural selection for floral scent specialization has weakened in these generalist-pollinated plants. These chemical analyses of floral scents suggest that the high host-specificity of the obligate pollination mutualism in Phyllanthaceae is underpinned by unique species-specific blends of diverse floral volatiles and the moth's high antennal sensitivity to olfactory cues. In the

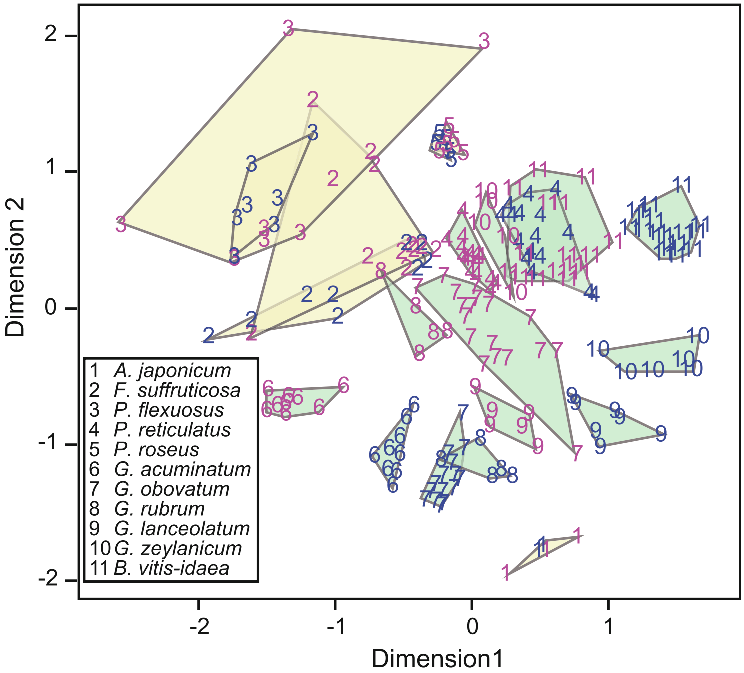

Fig. 8.7 Scatterplot resulting from the NMDS analysis based on the Bray–Curtis index of floral scents. Numbers indicate species. Male and female scent samples are indicated by blue and red coloration, respectively. Pollination system is indicated by green and yellow coloration: green for *Epicephala* moth-pollinated species and yellow for bee/fly-pollinated species

evolutionary process from generalist-pollination to specialized moth-pollination, the plants have developed species-specific blends of floral volatiles and sexual dimorphism of the floral scent to facilitate pollinator moths' missions on male and female flowers. Although the flowers of these plants are small and inconspicuous, the diversity and uniqueness of their olfactory signals are remarkable. The finely tuned flower–moth chemical communication system is an important contributor to the conspicuous diversification of moth-pollinated lineages in Phyllanthaceae.