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SYNOMONE OR KAIROMONE? – *Bulbophyllum apertum* FLOWER RELEASES RASPBERRY KETONE TO ATTRACT *Bactrocera* FRUIT FLIES

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Abstract—*Bulbophyllum apertum* flower (Orchidaceae) releases raspberry ketone (RK) in its fragrance, which attracts males of several fruit fly species belonging to the genus *Bactrocera*. Besides RK as a major component, the flower contains smaller amounts of 4-(4-hydroxylphenyl)-2-butanol, plus two minor volatile components, veratryl alcohol and vanillyl alcohol. Within the flower, the lip (labellum) had the highest concentration of RK with much smaller quantities present in petals; other flower parts had no detectable RK. Male fruit flies attracted to the flower belong to RK-sensitive species – such as *Bactrocera albistragata*, *B. caudatus*, *B. cucurbitae* (melon fly), and *B. tau*. Removal and attachment of the pollinarium to a fly's thoracic dorsum occurred when a male of *B. albistragata* was toppled into the floral column cavity, due to an imbalance caused by it shifting its body weight while feeding on the see-saw lip, and then freeing itself after being momentarily trapped between the lip and column. During this process, the stiff hamulus (the pollinia stalk protruding prominently towards the lip) acted as a crowbar when it was brushed downwards by the toppled fly and lifted the pollinia out of the anther. If the fly was big or long for the small triangular lip, it would not be toppled into the column cavity and would just walk across the column, during which time the pollinarium could be accidentally removed by the fly's leg, resulting in a failed transport of the pollinarium. This suggests an unstable situation, where the orchid relies only on a particular pollinator species in the complex ecosystem where many RK-sensitive species inhabit. Wild males of *B. caudatus* (most common visitors) captured on *Bulbophyllum apertum* flowers were found to sequester RK in their bodies as a potential pheromonal and allomonal ingredient. Thus, RK can act either as a floral synomone (pollinarium transported) or kairomone (accidental

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removal of pollinarium leading to total pollen wastage), depending on the body size of the male fruit flies visiting the flowers.

Key Words—*Bulbophyllum apertum* (synonym *Bu. ecornutum*), Orchidaceae, fruit flies, *Bactrocera* species, Tephritidae, floral fragrance, raspberry ketone, synomone, kairomone, pheromone, pollination, coevolution.

INTRODUCTION

Bulbophyllum (Orchidaceae) is probably the largest orchid genus, with over 1,000 species grouped under ca seventy sections, and has flowers that have a specialized hinged lip (labellum) mechanism that tips an attracted fly precisely against the column, thus facilitating and ensuring cross pollination. Most species of *Bulbophyllum* produce foul smelling and carrion-like scent to attract flies (Van der Pijl and Dodson, 1969; Dressler, 1981). Studies of some *Bulbophyllum* species have shown the flowers are pollinated by flies belonging to four dipteran families – Calliphoridae, Lonchaeidae, Milichiidae, as well as Tephritidae-without knowledge of the chemical component(s) responsible for fly attraction (Christensen, 1994). However, "a small and active fly" (probably a tephritid fruit fly) was reported to visit and 'fertilize' two species of *Bulbophyllum – Bu. macranthum* and *Bu. stritellum*, as well as *Dendrobium superbum* (synonym – *D. anosmum*) by Ridley in 1890. Fruit flies belonging to the genus *Strumeta* (currently *Bactrocera* Macquart [Diptera: Tephritidae]) appear to be the exclusive pollinators of *Bu. baileyi* F. Müel, the flowers of which release in the morning a fruity scent that is responsible for attracting flies (Symthe, 1969). A "pleasant odor" was also reported for flowers of *Bu. giellerupii* J. J. Smith and described as attractive to *Dacus* (currently *Bactrocera*) fruit flies for pollination (Howcroft, 1983). However, none of the chemical components of all *Bulbophyllum* floral fragrances were identified until the start of the millennium. Recently, the ginger orchid, *Bu. patens* King, was shown to release a ginger essence – zingerone, as a floral synomone that attracts several fruit fly species (Tan and Nishida, 2000); and the fruit fly orchid, *Bu. cheiri* Lindley, releases several phenylpropanoids, of which methyl eugenol is the major component, that attracts male flies of *Bactrocera papayae* Drew and Hancock (not a distinct species from *B. dorsalis*) that assist in pollination (Tan et al., 2002; Nishida et al., 2004).

Males of many *Bactrocera* species are attracted to either methyl eugenol (ME) or raspberry ketone (RK), both of which are plant attractants. The RK-sensitive species are also attracted to cue-lure - a synthetic analogue of RK that contains an acetyl group. However, zingerone is the only compound that attracts, though weakly, male flies of both ME- and RK-sensitive species (Tan and Nishida, 2000). Male flies of pest and non-pest species are important pollinators of *Bu. patens* and *Bu. cheiri*, which secrete specific floral fragrance containing zingerone and

ME, respectively. They are grouped under the section Sestochilus that has 60–70 species worldwide (Vermeulen, 1991). As the total number of RK-sensitive fruit fly species (>6) in Malaysia, is higher than that of the ME-sensitive species (3), it is important to search within the section Sestochilus for a *Bulbophyllum* species that secrete RK in order to further understand the coevolution between *Bulbophyllum* orchids and *Bactrocera* fruit flies.

Bulbophyllum apertum Schltr. (name published in 1906 before its currently used synonym *Bu. ecornutum* J. J. Sm. [J. J. Vermeulen, personal communication, 2003]) is an epiphyte with a widespread distribution in the tropics from Thailand to Moluccas, where it is found in podzolic forests and in shrubby forests on limestone hills at 400–1300m elevation. Its inflorescence is one-flowered with pale green, yellowish, or pale pink petals and sepals with or without purple markings; and has a bright orange hamulus – an appendage loosely attached to the pollinia (Vermeulen, 1991). The flower has 'no particular smell,' and the function of the hamulus is unknown (Ramussen, 1985). Nevertheless, the small and non-resupinate flower (Figure 1), with a floral fragrance resembling that of RK, is visited by male fruit flies belonging to several pest and non-pest species (Tan, 2000 a,b). The objectives of this paper are: a) to determine the species of attracted flies and to observe the behavior of fruit fly visitors; b) to observe the role of hamulus in pollinarium removal; c) to analyze the floral fragrance and confirm the presence of RK; and d) to determine the content of RK in various floral organs and fruit fly visitors.

METHODS AND MATERIALS

Plants and Flowers. Observations of the orchid flowers and plants were conducted in the Tenom Orchid Center (on the fringe of a tropical rainforest) Tenom, Sabah, East Malaysia. The *Bu. apertum* (subspecies verrucosum) plants were from the Nabawan population in Sabah. Flowers were individually collected, weighed, and immersed in ethanol (redistilled) for chemical analyses.

Observation of Fruit Flies Attracted to Bu. apertum *Flowers.* Fly attraction to the flowers was observed before and after they bloomed. Pollinarium removal by a fruit fly visitor was carefully observed with special attention paid to the role of the hamulus in this process. After observation, flies visiting the flower were collected in clear plastic bags whenever possible and identified to species.

Extraction of Floral Fragrance. Flowers of *Bu. apertum* were plucked in late morning on the day they bloomed (at 0600–0800 hr), as a day-old (1 d.o.), and on subsequent mornings as 2 and 3 d.o. under natural conditions, where fruit fly feeding could occur. Each flower was immersed in sufficient ethanol in a 5 ml glass vial and used for GC-quantifications. For individual floral organs (column, lip, petals, and lateral and medial sepals), each floral part was carefully removed from a freshly bloomed flower (within 3 hr after it bloomed and not

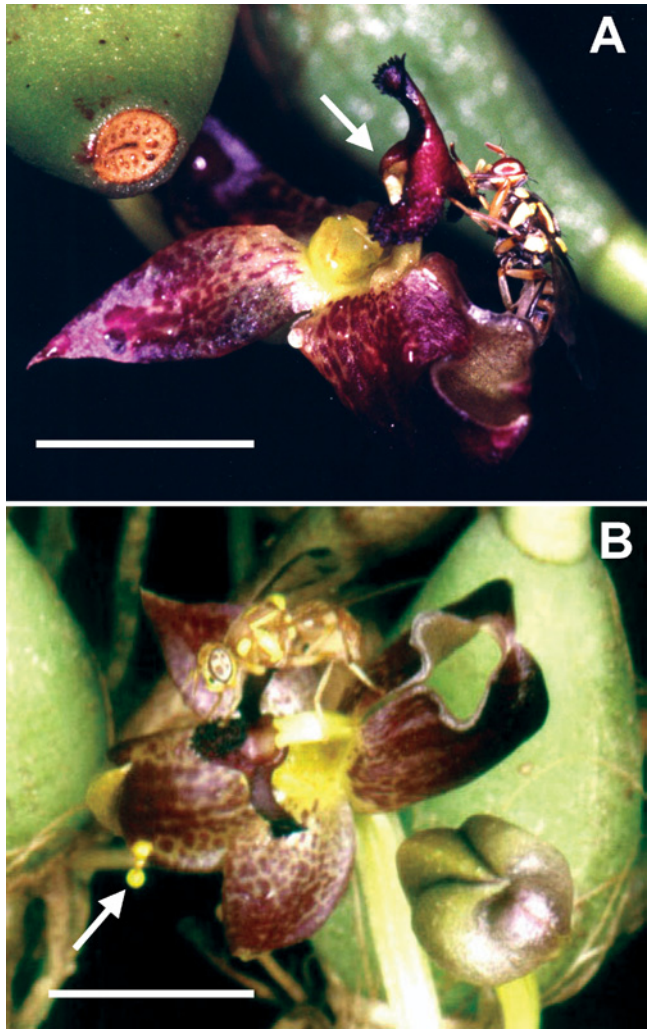

FIG. 1. (A) Flower of *Bulbophyllum apertum* with a male fruit fly of *Bactrocera caudatus* feeding on the triangular lip, which is in an open position and twisted by the fly [bar = 1 cm]. Note: a) Arrow points to hinge between the lip and column-foot, and b) the fruit fly is longer than the floral lip. (B) Flower of *Bulbophyllum apertum* with a misplaced pollinarium on the medial sepal, and a male melon fly (*Bactrocera cucurbitae*) feeding on the triangular purple lip, which is in a closed position, tilted towards the column [bar = 1 cm]. Note: a) arrow points to a pollinarium (pollinia + hamulus) freshly deposited after being removed accidentally by the fly's leg, b) the melon fly is longer than floral lip, and c) a floral bud at bottom right.

exposed to fruit flies), weighed and soaked in 0.5 ml ethanol in a 1 ml glass vial for quantification of RK. For GC-MS analysis, a combined extract of four flowers was concentrated under reduced pressure (ca. 20 mmHg, below 35°C) and partitioned between a mixture of hexane, benzene, and methyl acetate (1:1:2 v/v/v), and 1% sodium bicarbonate in water. The organic layer was washed with saturated sodium chloride in water and dried over anhydrous sodium sulfate and concentrated in partial vacuum, and a portion was used for GC-MS.

Extraction of Volatiles from Fruit Flies. Two *B. caudatus* male flies (main visitors) were captured after feeding for over 20 min on *Bu. apertum* flowers. The whole body was soaked in 0.5 ml ethanol in a 1 ml glass vial.

Chemical Analysis. GC-MS analyses were performed on an Hewlett Packard 5989B mass spectrometer coupled with an HP 5890 series II plus gas chromatograph, using an HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness) and programmed from 60° (2 min holding) to 290°C at a rate of 10°C/min; the GC was equipped with a total ion monitor. GC-quantification of volatile chemicals was performed on an HP 4890A gas chromatograph using HP-1MS and HP-5MS capillary column (15 m × 0.25 mm, 0.25 μm film thickness) and programmed from 60° (1 min holding) to 280°C at a rate of 10°C/min; the GC was equipped with a total ion monitor and a flame ionization detection.

Quantification of Volatiles. 1) Flowers. Quantification of the volatile components in a whole flower and each floral organ was performed using GC under the conditions described above by comparing the FID-intensities with those of the standard samples.

2) Flies. Portions of the ethanol fly extract were subjected to GC-MS analysis and GC-quantification as described above for flowers.

3) Authentic Samples. Raspberry ketone (4-(4-hydroxyphenyl)-2-butanone) (**1**), vanillyl alcohol (**3**), and veratryl alcohol (**4**) were purchased from Tokyo Chemical Industries Co. Ltd. Rhododendrol (racemic, 4-(4-hydroxyphenyl)-2-butanol; synonymous with frambinol or betuligenol) (**2**) was synthesized by reduction of **1** using lithium aluminium hydride in ether.

RESULTS

Fruit Fly Species. Species of male fruit fly visitors to *Bu. apertum* flowers were identified as *B. albistragata* (de Meijere), *B. caudatus* (Fabricus), *B. cucurbitae* (Coquillett) (the melon fly), and *B. tau* (Walker), hereafter collectively referred to as RK-sensitive species. These fruit flies respond to cue-lure baited traps (Tan and Lee, 1982). Usually a fruit fly, rarely two, was seen on a flower at any one time under natural conditions. Except for *B. albistragata*, most male visitors appeared to be too big for the relatively small and moveable floral lip of the *Bu. apertum* flower (Figure 1A). Some of the larger flies were observed to feed on the lip until satiation while standing on a lateral sepal (Figure 1A). No other

insects were observed on the orchid flowers, with the exception of an occasional ant walking across a petal or sepal.

Pollinarium Removal and Pollination. In one instance, a male *B. albistragata* effectively removed a pollinarium. The fly first landed near a flower, climbed onto it, and then began to probe and lap with its proboscis the surface of a petal. It eventually climbed on top of the see-saw lip (normally on a higher plane than the column - a characteristic of a non-resupinate flower). The fly continued to feed and move slowly along the short lip, and suddenly, it was toppled into the column cavity by the lip due to an imbalance during shifting of the fly's weight from one side of the floral hinge to the other (facing the column). The fly became momentarily trapped between the closed lip and column. While the fly struggled to free itself from a tight situation, the pollinarium was dislodged. During this process, the viscidium (a sticky area at the base of the hamulus) was touched, and pushed downwards (during tilting of the fly), then upwards (as the fly moved backward to free itself), sticking to the fly's thoracic dorsum. The fly with the pollinarium stuck onto its thorax immediately took off. The stiff hamulus (the bulbous end that has a sticky viscidium protruded prominently in the direction of the floral lip from the anther) acted like a crowbar to lift the pollinia out of the anther. In several other cases, where the fly was too big for the lip mechanism to work effectively, the fly either just lapped on the lip surface while standing on one of the lateral sepals without alighting on the lip (Figure 1A), or if it did go onto the lip, it simply walked across the column during an imbalance. During these visits, mainly by *B. caudatus*, the pollinarium was either not removed or was accidentally removed. In the latter case, one of the fly's legs either trampled on or brushed downward onto the hamulus, thereby removing the pollinarium (four instances of eleven observed). Figure 1B shows a pollinarium freshly deposited on the medial sepal after being removed accidentally by a melon fly's leg.

One or two relatively large seedpods were observed in the study site on several occasions over a period of seven years among two clumps of *Bu. apertum* pseudobulbs on a tree trunk and side branches over 3 m above ground. The seedpods were positive evidence that successful pollination of flowers had occurred in the previous season.

Floral Components of Bu. apertum. Figure 2 shows the GC trace of an extract of the floral lip of *Bu. apertum* where the attractant chemicals were concentrated. The major volatile component **1** was identified as raspberry ketone (RK, 4-(4-hydroxyphenyl)-2-butanone). RK was accompanied by its corresponding alcohol - rhododendrol (**2**). Vanillyl alcohol (**3**) and veratryl alcohol (**4**) were also detected as minor constituents. The identification was done by comparing their mass spectra and GC-retention times (on HP-1 and HP-5) with those of the authentic samples:

Compound **1** (raspberry ketone). MS: *m/z*(%) 164(71, M^+), 149(9), 121(15), 107(100), 91(13), 77(14), 43(19). Compound **2** (rhododendrol). MS: *m/z*(%) 166(67, M^+), 148(36), 133(100), 121(7), 107(95), 94(7), 77(19), 45(12).

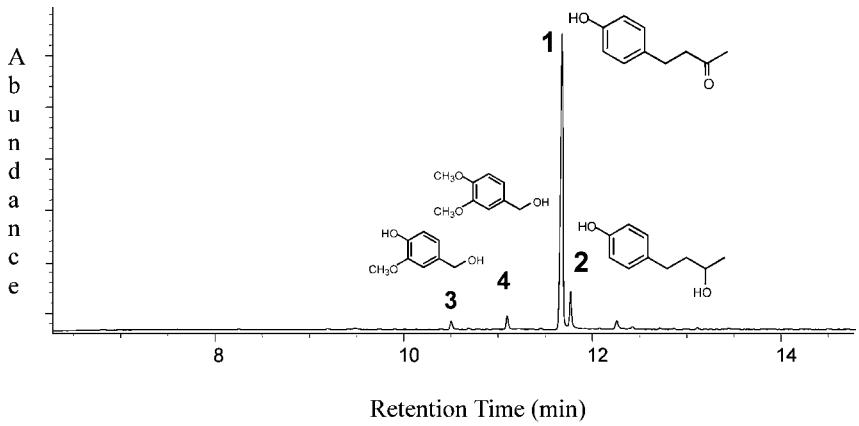

FIG. 2. Gas chromatogram of an extract from the lip of the *Bulbophyllum apertum* and structure of compounds. 1: Raspberry ketone, 2: Rhododendrol, 3: Vanillyl alcohol, 4: Veratryl alcohol.

Compound **3** (vanillyl alcohol). MS: *m/z*(%) 154(100, M^+), 137(38), 125(37), 122(26), 93(38), 65(33). Compound **4** (veratryl alcohol). MS: *m/z*(%) 168(100, M^+), 151(31), 139(45), 137(30), 109(20), 97(12), 93(13), 65(17).

Quantities of Raspberry Ketone in Floral Parts and Male Fruit Fly Bodies. Figure 3 shows the GC quantification and fresh weight of floral parts – column, lip, petals, and lateral and medial sepals. RK was almost exclusively in the lip, with small quantities (<0.5 μg) in the petals, and none or undetectable amounts in the other three floral parts. The quantity of rhododendrol in the lip was more or less proportional to that of RK (approx. 15% of **1**). Both vanillyl and veratryl alcohols were detected in low quantities (less than 1 μg/lip) in two of the ten lip specimens.

The mean ($\pm$SD) floral RK content for 1 d.o. was 7.8 $\pm$ 5.0 μg, 2 d.o. 9.2 $\pm$ 6.2 μg, and 3 d.o. 5.9 $\pm$ 3.8 μg. Although a slight decrease in RK was noted for the 3 d.o. flowers, there was no significant difference in RK content of the three different ages of flowers.

The total RK sequestered in the body tissue of *B. caudatus* after feeding on a *Bu. apertum* flower was 5.0 μg per fly.

DISCUSSION

Bu. apertum flowers show a large variation in their average RK content both within and among flower groups of different ages. Besides possible varietal differences, RK may be dependent on environmental factors such as temperature

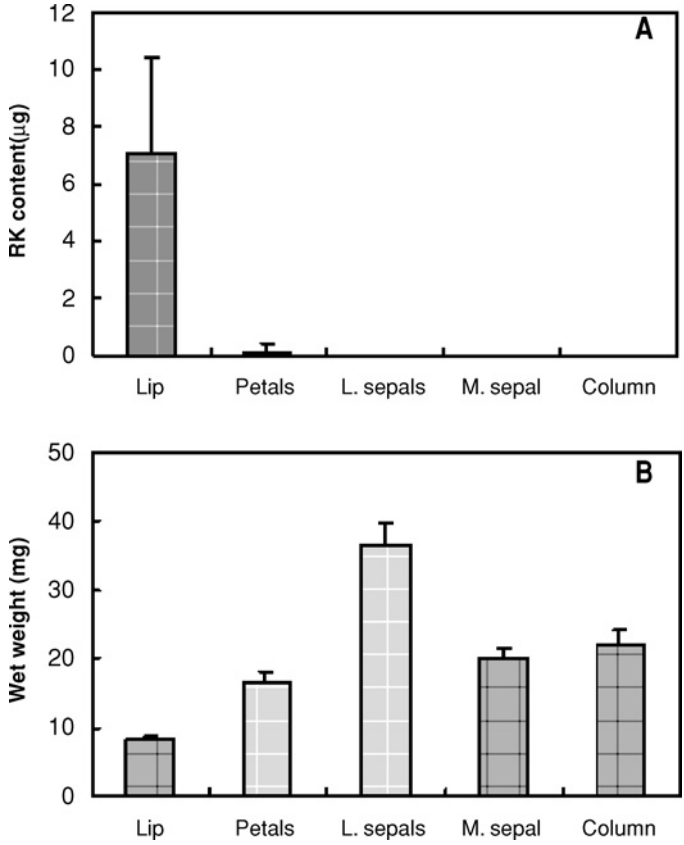

FIG. 3. Quantities of raspberry ketone in each floral organ (lip, lateral sepals, median sepal, petal, and column) ($N = 11$) (A), and fresh weight of floral organs ($N = 5$) (B) in each part of a *Bulbophyllum apertum* flower.

and light intensity that affect the synthesis/production of RK by individual flowers, as well as on the natural release rate by each flower and whether RK has been taken up by flies. These factors warrant further investigation.

The fact that RK is found almost exclusively in the lip of *Bu. apertum* explains why an attracted fly will eventually be led to feed on the lip surface. The see-saw lip seems designed to topple an attracted fly into the column cavity, and the precise removal of the pollinarium, which sticks onto the fly's thoracic dorsum, depends on the efficiency of this see-saw lip mechanism, and plays a vital role in pollination. However, this mechanism works only on flies with certain body sizes. If a fruit fly has a relatively large body, it can stand on one of the lateral

sepals and feed on the lip surface until satiation without climbing onto the lip, thus avoiding being toppled into the column cavity. Furthermore, if a large fly alights on the lip, it can just walk across the column during an imbalance of the see-saw lip; sometimes in this process it accidentally removes the pollinarium with its leg and deposits it on another part of the flower (Figure 1B). Although more cases of accidental removal of pollinarium were observed, leading to total pollen wastage, successful pollination must have also occurred as indicated by the presence of seedpods among some clusters of pseudobulbs.

The function of the hamulus as pollinia stalk is unclear (Rasmussen, 1985; J. J. Vermeulen, 2003, personal communication). The hamulus tends to break just below the bulbous swelling when touched after removal from the anther (Rasmussen, 1985). We propose that the hamulus, being stiff, acts like a crowbar that forces the pollinia out of the anther when the opposite end (bulbous swelling/viscidium) is pushed downwards by the toppled fly. When the pollinarium sticks to the dorsum of a fly, the pollinia on the hamulus protrude like small dumb-bells standing on one end. This is a different mechanism from *Bu. patens* and *Bu. cheiri*, where the pollinarium has no hamulus, but a flexible and soft pollinia stalk. When removed and transported during pollination, the pollinia become attached closely on the thoracic dorsum of a fruit fly (Tan and Nishida, 2000; Tan et al., 2002).

In two RK-sensitive species, *B. cucurbitae* and *B. tryoni* (Nishida et al., 1993; Tan and Nishida, 1995), RK is consumed and positively sequestered into the rectal (pheromonal) gland. We also found that it is sequestered into the body of wild *B. caudatus* after feeding on the *Bu. apertum* flower. The quantity of RK sequestered by the wild male (5.0 μg/male) was sufficient to provoke an aversive effect against a predatory Asian house gecko, *Hemidactylus frenatus*, when topically applied to houseflies (5.1 μg/fly) (Tan, 2000c). Thus, *Bu. apertum* seems to effectively endow a defensive benefit to its potential pollinator.

An attractant to monitor and control RK-sensitive fruit fly pest species has been developed commercially in the form of 'Cue lure' an analog of RK. This lure is not found in nature. It is spontaneously converted to RK in the presence of moisture (Metcalf, 1990). Males of the melon fly that were fed cue lure sequestered RK into the rectal glands (Nishida et al., 1990); wild male melon flies fed on cue lure mated more frequently than wild males not exposed to cue lure; and mass-reared flies that fed on cue lure were more successful in mating competition than control flies (Shelly and Villalobos, 1995). However, the advantage of feeding on cue lure is temporary - lasting less than three days (Shelly and Villalobos, 1995). Therefore, in order to maintain the sexual advantage, wild and polygamous flies may need to visit and feed on a natural RK source regularly. In the tropics, natural floral sources of RK have been previously detected in *Dendrobium superbum* flowers, which have fixed lips and where attracted *B. cucurbitae* male flies were observed to feed only on petals and sepals (i.e., there was no pollinarium removal and, thus, the flies played no role in pollination) (Nishida et al., 1993). Here,

we report the first case of a *Bulbophyllum* species emitting RK that potentially mediates a mutualistic association, in which the orchid flower gets its pollinarium transported during cross-pollination, and the fruit fly benefits by improving its reproductive performance as well as defense. Another species, *Bu. emiliorum*, also has a distinct scent of RK in the morning, to the human nose and its floral fragrance is currently under investigation.

There has been no information on the pollination of *Bu. apertum* by natural pollinators. Self-pollination of *Bu. apertum* has never been observed and appears unlikely (Ramussen, 1985). RK-sensitive fruit fly species seem to act as pollinators in the cross-pollination of the *Bu. apertum* flowers. However, flower visitation by species of male fruit flies whose bodies are apparently too long for effective pollinarium pickup may lead to wastage of pollinia via accidental removal, and the flower thereby loses all its pollen contained in the pollinia without gaining any ecological benefit. Only the smaller male fruit flies are effective pollinators for *Bu. apertum*. Consequently, the floral fragrance containing RK acts as a kairomone, which is a chemical signal where the perceiving organism, here the male fruit fly benefits, and the orchid emitter loses. This may create a situation that selects for *Bu. apertum* to evolve a more efficient lip mechanism -i.e., to produce larger flowers with longer lips to accommodate the long body length of other male fruit flies. Conversely, pollinarium removal by a male *B. albistragata* showed that the *Bu. apertum* flower is well adapted to attracting a relatively small-sized fruit fly that effectively pollinates the flowers, leading ultimately to the formation of seedpods. In this latter orchid-fruit fly association, both organisms benefit and the RK in the floral fragrance acts as a synomone.

From an overall behavioral and chemoecological viewpoint, RK in the floral fragrance of *Bu. apertum* can act as either a kairomone or synomone depending on the body size of the fruit fly visiting this orchid. In contrast, the floral fragrances of *Bu. patens* and *Bu. cheiri*, which contain zingerone and methyl eugenol, respectively, always act as synomones regardless of the fruit fly species attracted (Tan and Nishida, 2000; Tan et al., 2002). In the wild, the other three described species of tephritid fruit flies, *B. caudatus*, *B. cucurbitae*, and *B. tau* that have relatively longer body lengths than *B. albistragata* are more abundant. Therefore, it remains to be seen whether *Bu. apertum* will eventually adapt to the more abundant male fruit flies that have body lengths that are longer than the orchid's lip or maintain the *status quo* and remain dependant on the less abundant, smaller sized fruit flies such as *B. albistragata*, with consequently a lower chance of successful pollination in the tropical rain forest ecosystem.

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