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Floral morphology and Potential Pollinator of *Vanilla siamensis* Rolfe ex Downie (Orchidaceae: Vanilloideae) in Thailand

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ABSTRACT

The orchid genus *Vanilla* has a pantropical distribution. It includes notable species, particularly *Vanilla planifolia* and *V. pompona* from the tropical Americas, and the first commercial hybrid *V. tahitensis.* Both species are used in various industries, including food, pharmaceuticals, cosmetics, tobacco, insect repellent, and traditional crafts. Less known is *V. siamensis* from Thailand. Here we investigate the flower morphology, fruit set, and natural pollinator of *V. siamensis*. Flower morphology and pollination experiments indicate that the species is pollinator-limited, thus undergoing pollination via deception. During observations of visiting insects, eight insect groups were collected (Blattodea, Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Mantodea, and Orthoptera), representing twenty-eight insect morpho-species. The most frequent visiting group was the Hymenoptera, which accounted for the largest value of frequency of occurrence with 80% of the total visitations. The main visitor, *Thrinchostoma* sp. 2 is most likely the pollinator as observed at Khao Soi Dao Wildlife Sanctuary in the eastern region of Thailand. This species was observed entering a flower carrying Vanilla-like pollen before exiting with the pollen having been removed. It is therefore suggested that pollination of *V. siamensis* is by the Thrinchostoma bees, under natural conditions; however, visitations are infrequent, resulting in pollinator-limitation when compared to hand pollination experiments.

Keywords: vanilla fruit, vanilla flower, insect pollinator, stingless bee, bee species

INTRODUCTION

The family Orchidaceae, possibly the second largest plant group in the world (Bory *et al*., 2008; Chase *et al.*, 2015), is renowned for its diverse and often remarkable pollination strategies (Micheneau *et al*., 2009). About 27000 species are known, or roughly one out of every 15 plants currently described (Dressler, 1981; Dressler, 1993; Tremblay *et al*., 2005). At the same time, for many orchid species, pollination events have never been recorded. This is especially true for epiphytic species where the lack of access to flowering plants makes observation difficult.

In many tropical areas, species of the pantropical orchid genus *Vanilla* occur as lianas and hemi-epiphytes (Cameron, 2011). The genus is best known for the widely cultivated tropical crop plant *V. planifolia* Jacks. ex Andrews, the main source of natural vanilla flavor. While most of the ca. 120 species of *Vanilla* have relatively large and conspicuous flowers, these are invariably ephemeral, usually lasting a single day, or only a few hours (Cameron, 2011). Four species are native to Thailand, *V. aphylla* Blume, *V. pilifera* Holttum, *V. griffithii* Rchb.F. and *V. siamensis* Rolfe ex Downie, not including the widely cultivated *V. planifolia* that is native to Mexico (Díaz-Bautista *et al*., 2018). They all occur as trailing vines along tree trunks and in some cases on rock. Very little is known about the biology of *V. siamensis* and it was thought, at the beginning of the century, to be an endemic species of Thailand. The first recorded collections of *V. siamensis* (1905, 1907 and 1909) were by the British botanist Dr. Arthur Francis Kerr (1877–1942). He was the first scientist to be sent to, then, Siam, by the British government and catalogued many plants species. All his specimens were then sent to the Herbarium collection at the Royal Botanic Gardens, Kew, UK; although, significant collections are also held in the Herbarium of the University of Aberdeen.

Vanilla siamensis Rolfe ex Downie is a robust species found in Thailand, China (Yunnan) and probably Cambodia (Schuiteman, pers. comm.). Here we describe the results of pollination experiments and observations of this striking orchid species. A few Vanilla species have been suggested to be self-pollinated through stigmatic leakage or reduced rostellum (Soto-Arenas and Cameron, 2003; Householder *et al*., 2010; Soto-Arenas and Dressler, 2010; Lubinsky *et al*., 2006); however, the majority are dependent on pollinators (Peakall, 1994; Proctor *et al.*, 1996; Soto-Arenas and Cameron, 2003; Petersson, 2015). The most common reward for orchid pollinators is nectar (Dressler, 1993; Roubik and Ackerman, 1987); although, no Vanilla species are known to produce a floral reward (Proctor *et al*., 1996). Several Vanilla species in the American tropics are pollinated by Euglossine bees (Dressler, 1981), a tribe that is known to collect fragrance from non-nectar producing orchids (Dodson *et al*., 1969). So far, there is no direct evidence for this scent collecting behavior in any Vanilla species (Proctor *et al.*, 1996; Gigant *et al*., 2011). Many orchids have also developed different types of deceptive systems to attract pollinators (van der Pijl and Dodson, 1966), a strategy known to be used by several Vanilla species in the American tropics; although, the exact form of the deception remains unclear (Soto-Arenas and Cameron, 2003).

Vanilla siamensis is of particular interest as extracts and have been shown to have pharmaceutical properties in the treatment of osteoporosis. It is therefore important to have an understanding of the species pollination biology, not only for the conservation of the species, but also for the exploitation of the species through breeding in relation to its pharmaceutical properties. This study therefore examines the 1) flower morphology of *V. siamensis*, 2) natural pollination, and 3) pollinator behavior observations of *V. siamensis.*

MATERIALS AND METHODS

The study was conducted at Khao Soi Dao Waterfall, located in Khao Soi Dao Wildlife Sanctuary (KSD-WS), Chanthaburi, Thailand (13.1041889°N, 102.1945329 °E), and at a second site in Chiang Mai Province, Thailand (18.7767366°N, 99.2470935 °E). Both study sites are classified as tropical moist evergreen forest and were chosen due to the accessibility of *V. siamensis* at both sites. The study was undertaken in the flowering season, May 2019 to June 2019.

1. Floral morphology of *Vanilla siamensis*

Nine inflorescences were randomly selected at plots 10 m apart for the study. Five flowers were randomly selected on each inflorescence for floral measurements. The floral characters were measured within one hour after collection using a digital caliper to the nearest 0.1 mm. The number of individual flowers were counted for each inflorescence. These data were used to calculate the average number of flowers per inflorescence.

2. Natural pollination and pollinator behavior observations

Experimental design

A combination of two techniques was used for collecting the data on diversity of insect pollinators, which were the sweep-net method, and digital video recording (DVR) devices (McCravy, 2018). Wherever *V. siamensis* flowers were observed, insects which visited the flowers were caught by sweep-net.

Three Brinno 200TLC digital cameras, binocular, and extra supporting long lens Canon were set up by using tripods. The DVR has the potential to greatly benefit the recording of pollinator diversity, activity, and breeding systems in natural ecosystems. Moreover, the DVR can objectively improve the direct data collection of pollinator diversity and activity over direct human observation. All sample points were labelled when insects visited on inflorescence as recorded by video. They were caged with fine muslin cloth to protect the flower from the other visiting insects. Observations were conducted for 6 weeks during the period 06.45-12.00 h from May 2019 to June 2019.

Identification of visitors

All insect specimens were identified to the species or family level. Identifications were performed using taxonomic keys (Triplehorn and Johnson, 2005; Michener, 2000). The Thrinchostoma species was identified by Dr. Alain Pauly at the Royal Belgian Institute of Natural Sciences in Brussels and other species were identified by entomologists at Chiang Mai University. Some insect species unable to collect because of high location and period time of the day. Insects were classified through the help of digital cameras.

3. Data Analysis

A frequency of occurrence (FO) was used to quantify the probability of finding an insect group in study site. Frequency of occurrence of each insect group was calculated by; FO (%) = (number of times in which the insect group is found/ total number of sampling times) ×100.

A t-test was used to compare the means of frequency of occurrence of insect (classified by morphospecies) visits on *V. siamensis* flowers. Before applying parametric tests, we tested for normality and homogeneity of variances. All data were log10(x+1) transformed to meet conditions of normality, homogeneity of variances, and sphericity. Statistical analyses were performed with SPSS Ver. 20.0.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

1. Morphology of *Vanilla siamensis* flower

Inflorescences emerge as a light green protuberance from the leaf axil, measuring 5–8 cm. long, most often as an un-branched (rarely branched) raceme. Anthesis (flower opening) occurs in the early morning. Flowers open acropetalic i.e., from the base of the inflorescence upwards. Each inflorescence has only a single flower open at any one time, with each flower lasting up to three days. Flowers measure 8–9 cm across, are pale green with a cream-colored or yellow and red-pink labellum (Fig. 1A-E). Flowers are pedicellate and each flower is subtended by a small pointed bract. The flower is resupinate, with the labellum being lowermost. Interestingly, we found the "white bearded" *V. siamensis*, which has a white labellum, is the most common (98 %; n = 98 vines of vanilla), and the "red bearded" *V. siamensis*, with a red-pink labellum, is extremely rare (2 %; n = 2 vines of vanilla).

The corolla consists of two upper petals that resemble the sepals but are slightly smaller, and a trumpet shaped labellum. The apex of the labellum bears a tuft of fat, elongate hairlike projections, producing a beard-like appearance, while about halfway the labellum, underneath the anther, there is a patch of backward-pointing lamellae, producing a hump on

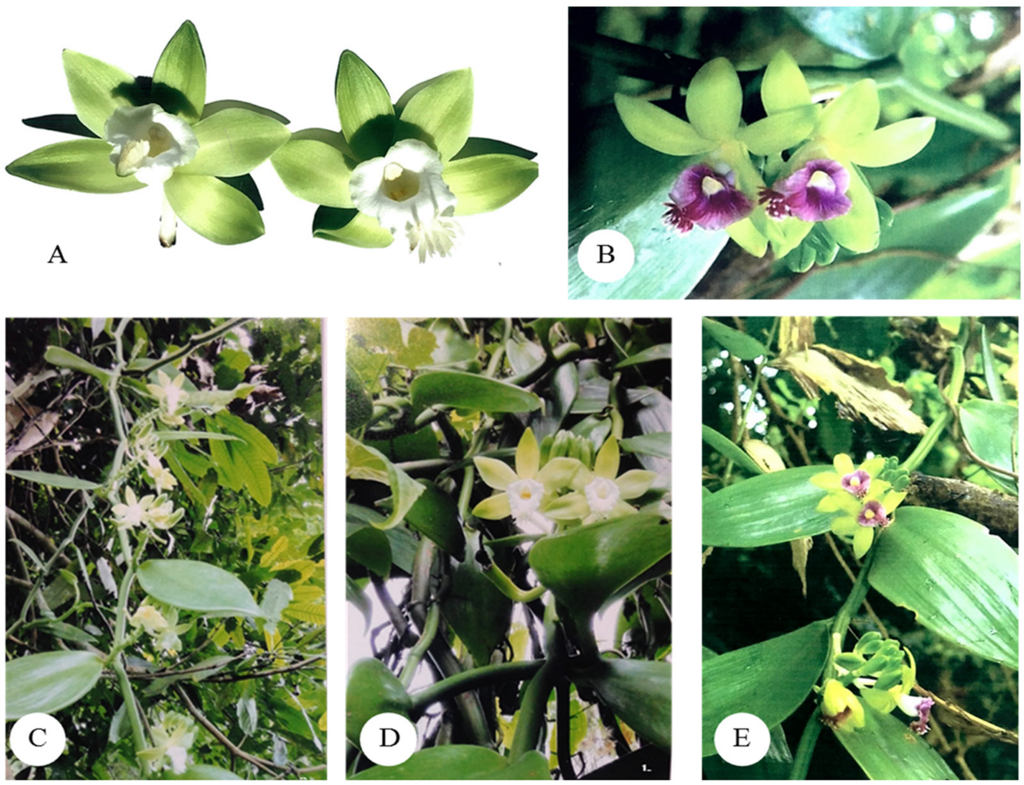

Figure 1. (A), (C) and (D) showing the common "white bearded" *Vanilla siamensis*. (B) and (E) showing the much rarer "red bearded" *V. siamensis*.

the labellum surface. The column, which is adnate to the basal sides of the labellum, has long hairs on its ventral surface (Fig. 2). A single anther is attached to the column, containing four pollen masses (resembling pollinia but of less definite shape and of crumbly texture) covered by a cap/hood. The concave stigma is situated below the anther and is separated from it by a thin flap-like membrane (the rostellum), preventing self-pollination. The ovary is inferior and elongate (Fig. 3).

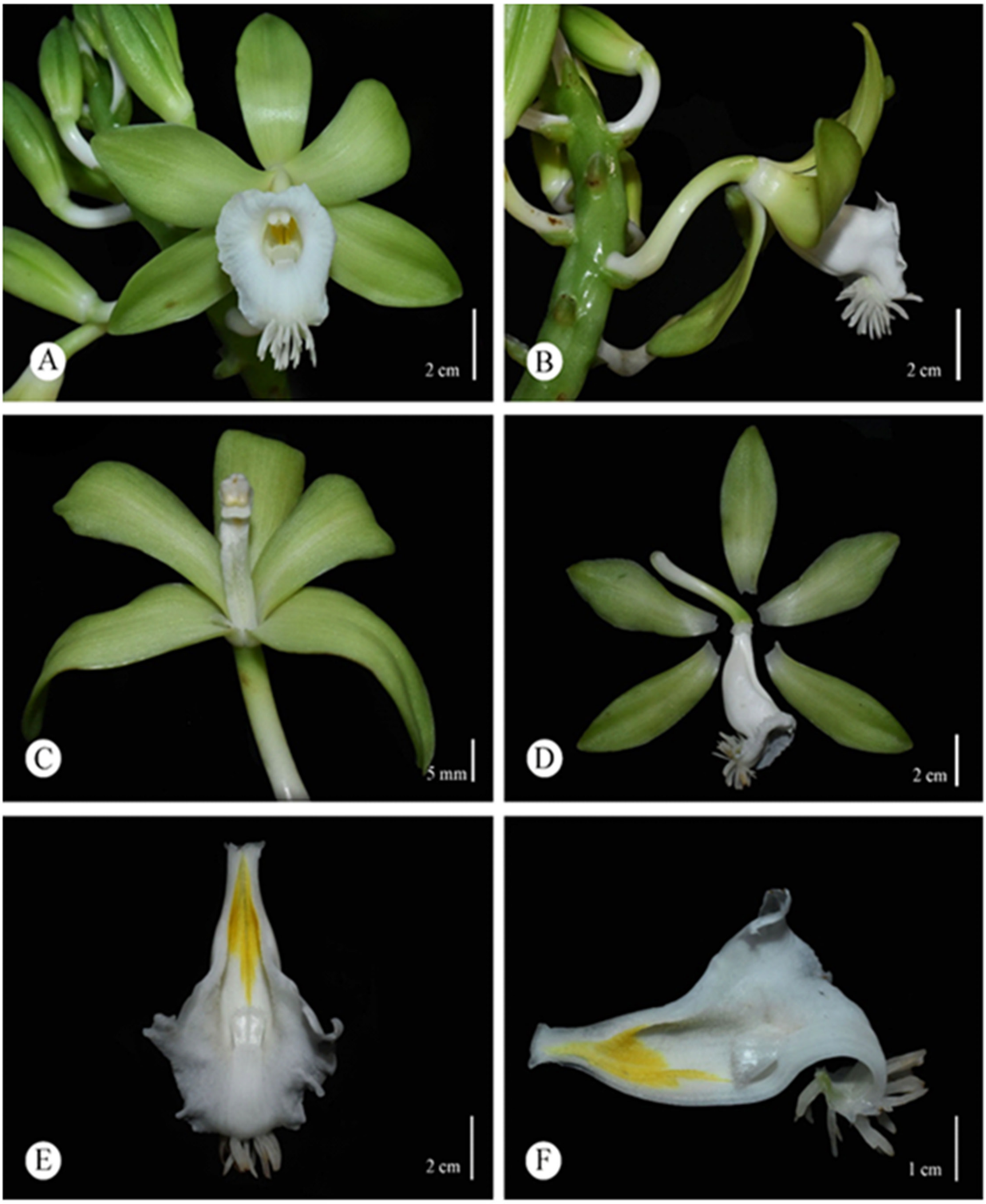

Figure 2. Floral morphology of *Vanilla siamensis*. (A) Flower front view; (B) Flower side view; (C) Flower front view and without labellum; (D) The detail of floral parts showing the sepals, petals, labellum and ovary; (E) Labellum dorsal view; (F) Longitudinal section of the labellum (cont.)

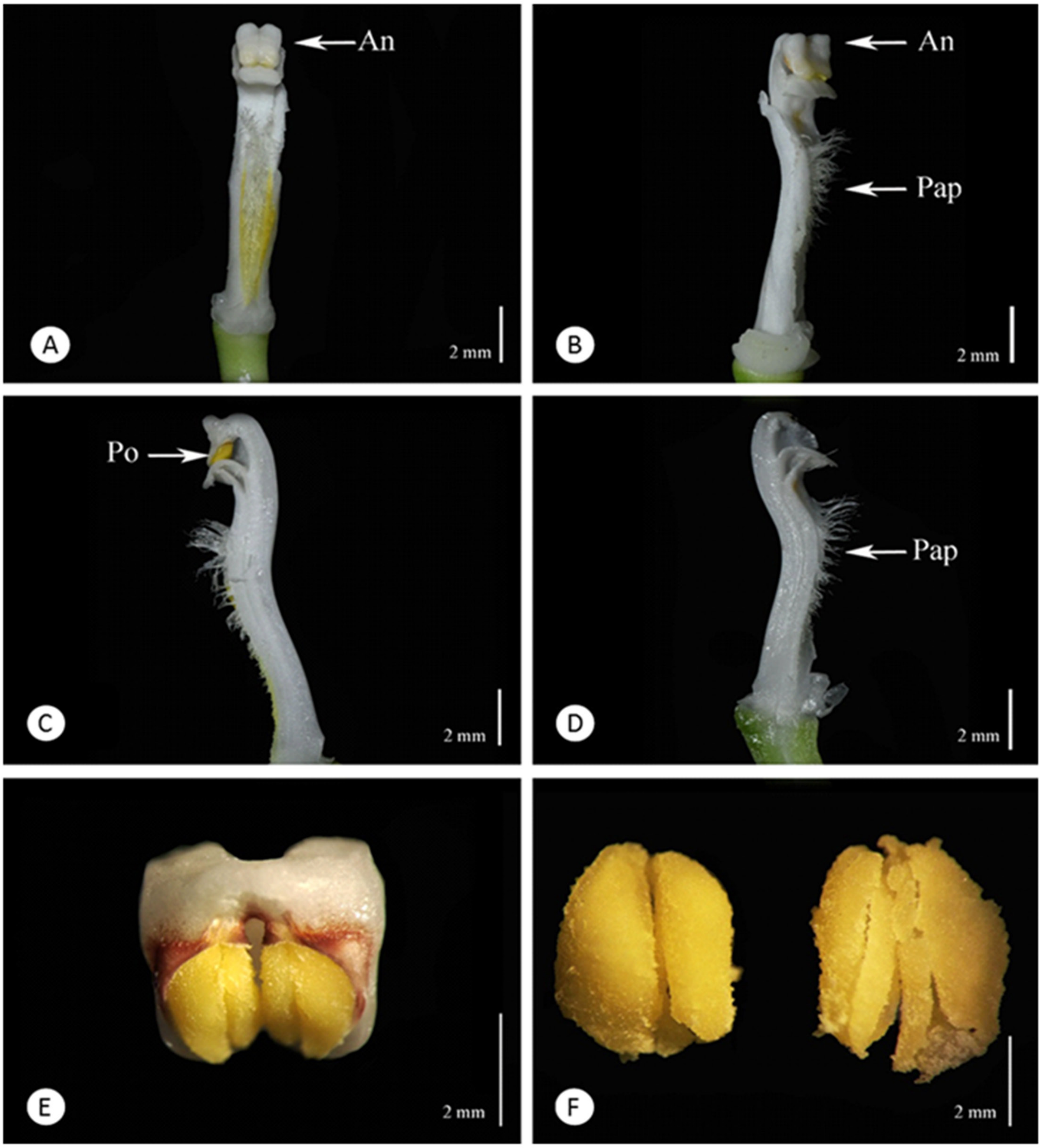

Figure 3. Floral morphology of *Vanilla siamensis*. (A) Column front view; (B) Column side view; (C) Longitudinal section of column with pollen-mass; (D) Longitudinal section of column without pollen-mass; (E) Anther cap and pollinia; (F) Pollinia; An = Anther cap; Pap = Papillae hair; Po = Pollen-mass.

2. Flower visitor diversity

A total of 8 orders, representing 28 morphospecies, were collected during visitation to flowers of *V. siamensis* (Table 1; Fig.4). Hymenoptera made up the majority of the species (80 %), followed by Diptera (50%) and Coleoptera (33%).

Observations were made of insects that visited the flowers of *V. siamensis*, including their activity and frequency of occurrence. Bee species visited *V. siamensis* with significantly higher frequency than all other insect groups combined (Table 1; t-test; F=4.56, P<0.05).

Vanilla-like pollen was observed on the thorax of *Thrinchostoma* sp. 1 and *Thrinchostoma* sp. 2 as they visited the flowers of *V. siamensis* (Fig. 4E, 4F,

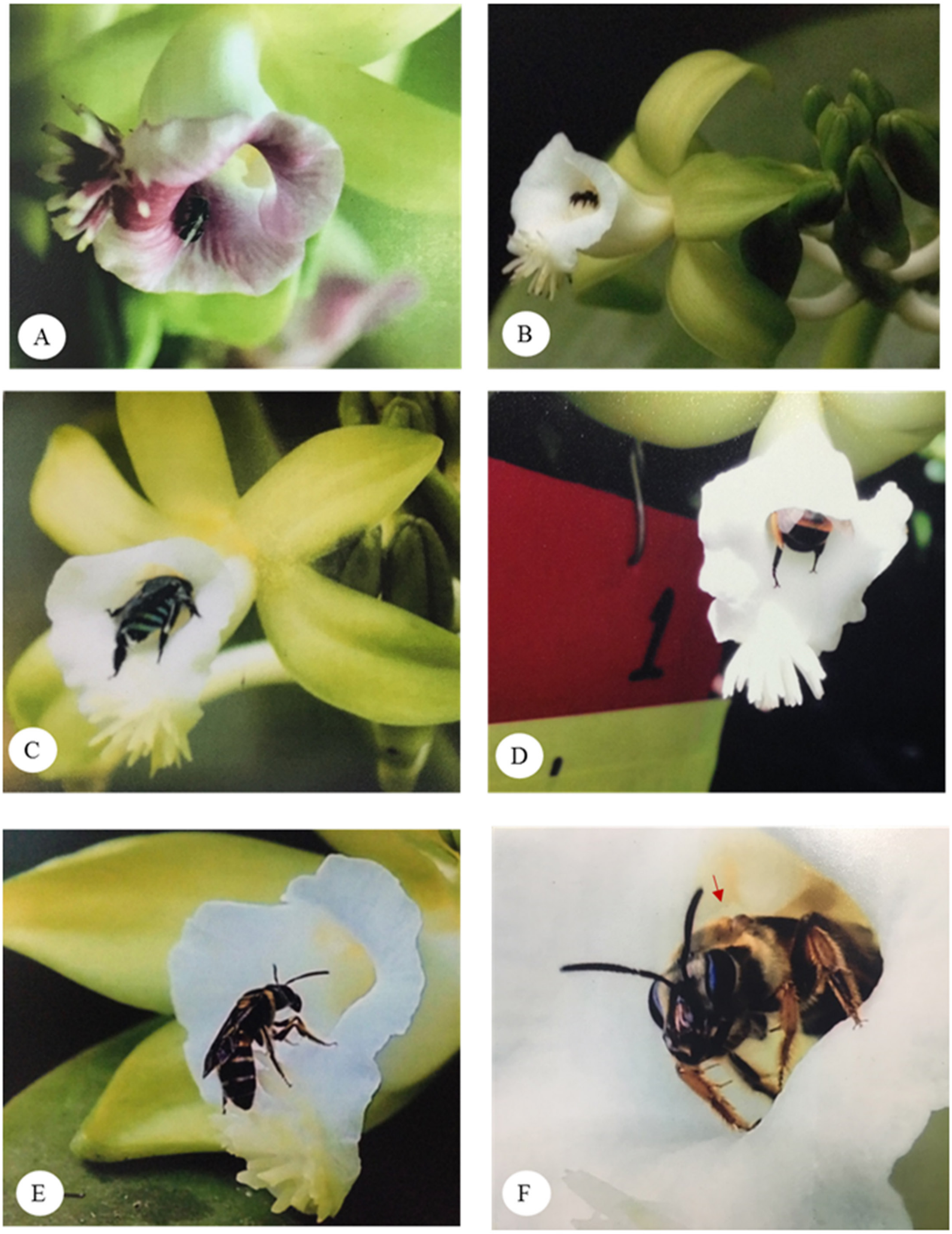

Figure 4. Bee species of Hymenoptera visiting the Vanilla flower, captured on digital video recording (DVR) devices; (A-B) the *Trigona* sp. (C) *Nomia* sp. 1, (D) *Nomia* sp. 2, (E) *Thrinchostoma* sp. 1. and (F) Red arrow indicating pollen deposited on the thorax of *Thrinchostoma* sp. 2. The captured pollinator specimens have been lodged at The Royal Botanic Gardens in Chiang Mai, Mae Rim.

Fig. 5). Thrinchostoma bees caught on *V. siamensis* were measured. Morphological measurements of the operative size of *Thrinchostoma* sp. 1 showed these bees to have a functional height of 5 mm. They had a total body length of 13 mm, including a thorax length of 3.9 mm, with a thorax width of 3.2 mm and a thorax height of 2.9 mm (Fig. 6). *Thrinchostoma* sp. 2 also had a functional height of 5 mm. They had a total body length of 12 mm, of which the thorax length is 4 mm, with a thorax width of 3 mm and a thorax height of 2 mm (Fig. 7).

Following visitation of *V. siamensis* flowers by a *Thrinchostoma* sp.1 and *Thrinchostoma* sp.2 that was carrying Vanilla-like pollen, the ovary developed rapidly, doubling in length in a few weeks. It was observed that when pollination did not occur, then within one to three days, the flower would detach from the ovary, and later drop off entirely. The sizes of *V. siamensis* fruit by insect visitors were recorded. Fruit development resulted in the elongation of the pedicel, with the pedicel changing color from white to light yellow and eventually to green, showing the presence of photosynthesis (Fig. 8). Only one flower out of 28 flowers (3.6%) was pollinated by *Thrinchostoma* sp. 1 and *Thrinchostoma* sp.2 and developed into a fruit.

3. Pollinator behavior observations

The behavior of the Thrinchostoma sp. 1 and *Thrinchostoma* sp. 2 was observed in as much detail as possible, including their approach entering and leaving the flower, and the time it spent in the flower (Fig. 9). This can be seen from various still picture frames. The observation for pollinators began on 14 May and continued until 5 June 2017. Every day equipment was set up at 06.15 before sunrise and stayed in place until late afternoon. On a few occasions, it was not possible to observe any pollination taking place due to constant rain and morning mist. On the ninth day, perfect conditions appeared. Few visitors approached the flower when it was fully open and did not make any attempt to enter the flower. At 07.45 on 22 May, approximately 3±0.4 (SD ; N=30) Thrinchostoma bee specimens per day visited the flowers

As the first Thrinchostoma bee approached a white-flowered *V. siamensis*, it spent over two minutes hovering round the open flower, before landing on the tuft of white hairs onto the labellum. The function of the white hairs on the labellum was possibly to act as a supporting pad as well as guiding line the insect into the labellum (Vargas *et al*., 2019; Johnson *et al*., 1998; Johnson *et al*., 2003). Within a few seconds, the bee started to move along the labellum and into the flower. On high magnification of the video, pollen masses can be clearly seen attached to its back. The bee stayed for ninety seconds inside before turning its body around.

Table 1. Frequency of occurrence of insect visitation to the flowers of *V. siamensis* in this study

Order (Family; species)	Morphospecies	Frequency of Occurrence (%; N=30 days)
1.Blattodea (Blatellidae; *Blatella* sp. 1, *Chrysocoris* sp. 1) – cockroaches	2	10
2.Coleoptera (Chysomellidae; *Chrysomyia* spp.) – beetles	4	33
3.Diptera (*Chryops* spp.) i.e. flies	3	50
4. Hemiptera (Pantamiidae; *Chrysocoris* sp., *Callidea* sp.) – true bugs	2	6
5. Hymenoptera: (Apidae; *Apis cerana*, *Trigona* sp., *Thrichostoma* sp. 1, *Thrichostoma* sp. 2 Halictudae; *Nomia* sp. 1, *Nomia* sp. 2) – bees; (*Dolichoderus* sp. 1, *Camponotus* sp. 1, *Camponotus* sp. 2, *Camponotus* sp. 3, *Polyrachis* sp. 1, *Crematogaster* sp. 1, *Crematogaster* sp. 2, *Crematogaster* sp. 3) – ants	14	80
6. Lepidoptera (Papilionidae; *Graphium* sp.) – butterflies	1	10
7. Mantodea (*Mantidae* sp.) – mantises	1	3
8. Orthoptera (*Acrididae* sp.) – grasshoppers	1	3
Total	**28**	

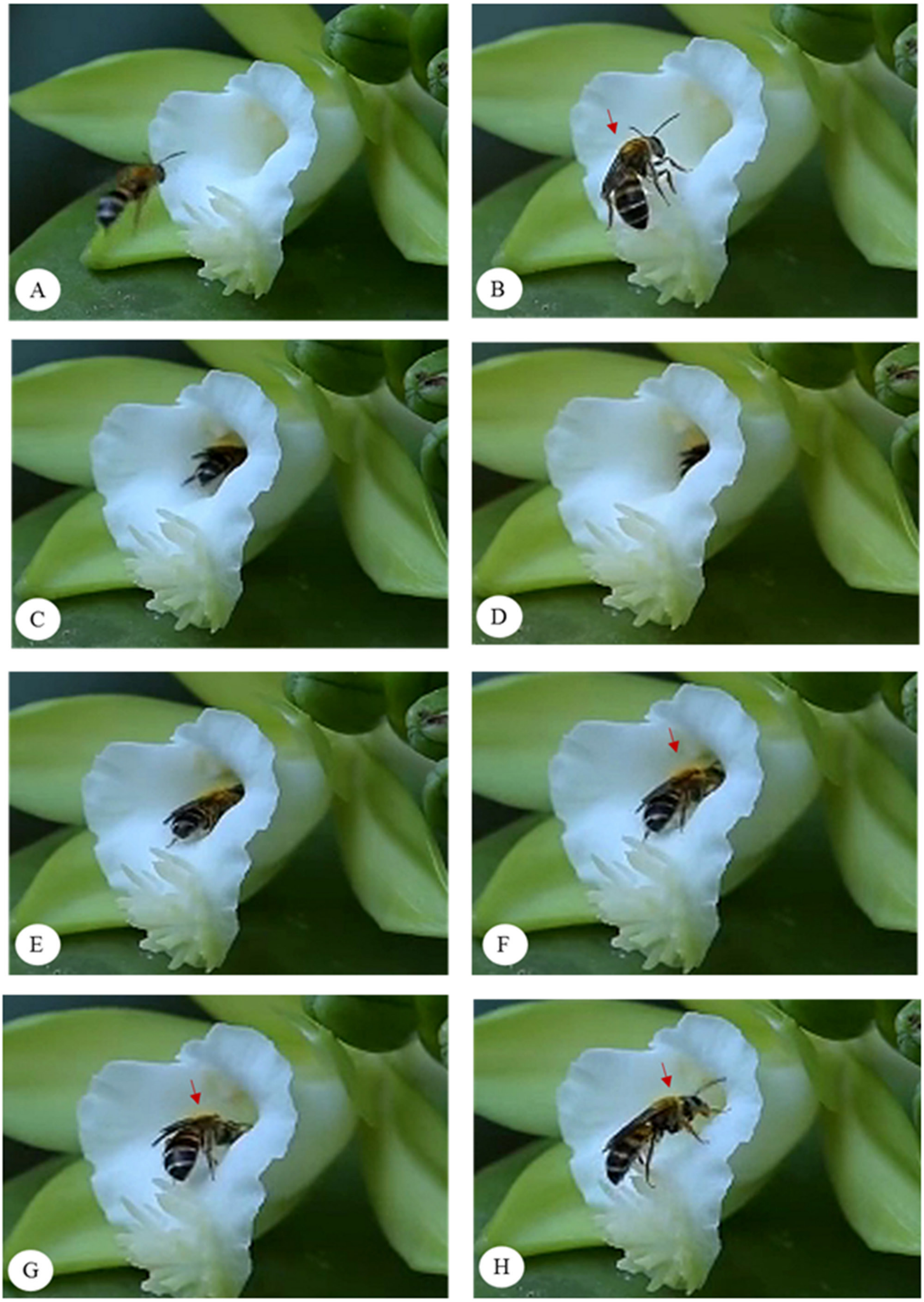

Figure 5. A series of video stills (A-H) showing *Thrinchostoma* sp. 1 visiting the flower of *Vanilla siamensis*; (B) Red arrow indicates the absence of the pollen grains on the thorax of *Thrinchostoma* sp. 1, before entering the Vanilla flower. (F-H) The red arrows indicate the pollen grains on the thorax of *Thrinchostoma* sp. 1, after leaving the Vanilla flower.

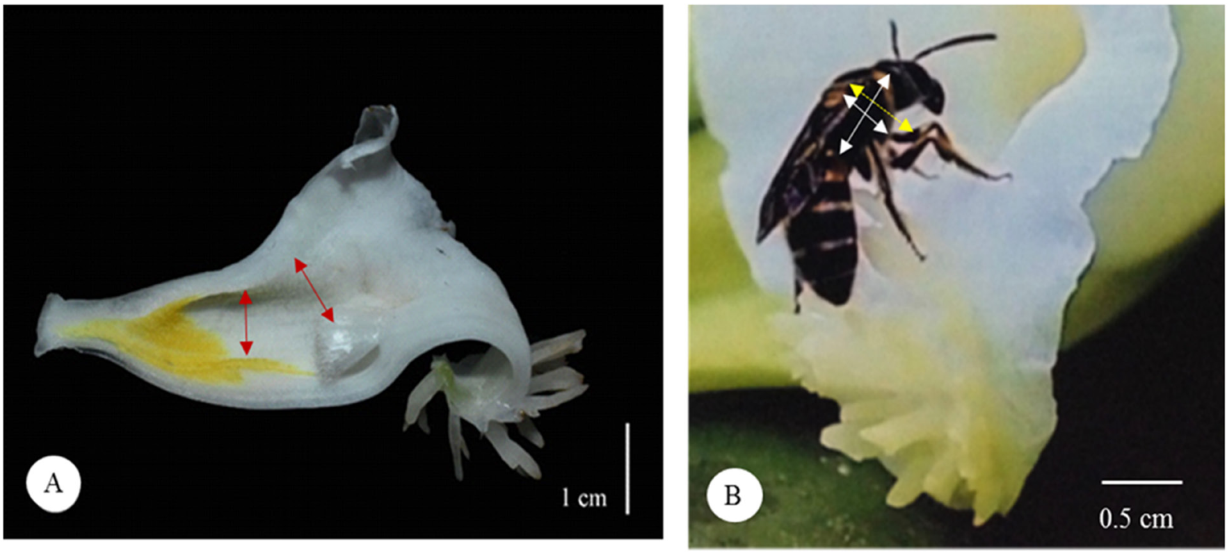

Figure 6. Morphological measurements of (A) longitudinal section of labellum, and (B) *Thrinchostoma* sp. 1 with morphological measurements of thorax length (TL) and thorax height (TH) indicated with white arrows, and the functional height approximated by a yellow arrow.

Figure 7. *Thrinchostoma* sp. 2 with morphological measurements of thorax length (TL) and thorax height (TH) indicated with arrows, and the functional height approximated by a dotted arrow.

The distance between the anther and the nearest part of the labellum was at least 3.2±0.2 (SD ; N=30) mm (Fig. 3A and 3B), which was related to the functional height of the bee (Fig 7).

It proved difficult to observe the behavior once the bee was inside the labellum tube, particularly because the flower was situated more than 2.5 m above ground level, and the observation was made facing a bright sky. The bee crawled into the labellum and spent the majority of the time crawling on and around the central crest. Before leaving, it turned around the labellum with its head lifted. No direct field observation could be made on mechanical interactions between the bee and the anther or stigma. The interpretation by Rasmussen (1985) suggested the act in the genus as follows "The flower is gullet-shaped and often provided with a tuft of retrorse hairs or crests, forcing visiting insects, when retreating, to raise their body and thereby touch the versatile anther." The effective pollinator is expected to have a functional height that is sufficient to be able to activate the pollination mechanism.

Figure 8. (A-B) A fruit of *Vanilla siamensis* after visitation by *Thrinchostoma* sp. 2

DISCUSSION

Observation of natural pollination including the pollinator event by a Thrinchostoma species bee was captured on DVR. This resulted in the formation of a fruit suggesting the species was potentially pollinated by a *Thrinchostoma* bee species. This was the only case of pollination observed out of 28 flowers, giving a fruit set of 3.6%. This indicates that the species is pollen-limited, possibly due to low pollinator visitation. As a very low fruit set at 3.6% in this study by Thrinchostoma sp. 1 and *Thrinchostoma* sp. 2 compared with the non-fruit set for controlled flower pollination by the muslin covered. The low fruit set might be explained by three possible causes for pollen limitation: 1) low pollinator abundance, resulting in some flowers never being visited; 2) low amounts of pollen reaching the stigma, even when the pollinator visits are frequent; 3) low pollen quality, when the weather is too hot or dry, or even in wet conditions, the pollen may lose its viability (Tremblay *et al*., 2005). However, this may not always be the case, as on occasion unusually high fruit set can be seen in the wild. Autogamy has been suggested to occur in a few Vanilla species, all of which have unusually high natural fruit sets (Householder *et al*., 2010; Soto-Arenas and Dressler, 2000; Lubinsky *et al*., 2006), but there is no evidence that *V. siamensis* is autogamous; flowers that are shielded from insect visitors do not set seed (pers. obs.).

Results presented here suggest that two species of Hymenoptera, *Thrinchostoma* sp. 1 and *Thrinchostoma* sp. 2 are pollinators of *V. siamensis.*

Figure 9. Entering Stage. Showing different stages in slow motion in various frames of pollination by Thrinchostoma bee. (A, B) Upon landing on the labellum, the bee moves toward the center of the flower, (C, D) The bee lowers its body, as the inner flower is becoming narrower. (E, F) Moving slowly with yellow-orange lines inside the flower as guideline. (G, H) The bee turns itself around inside the flower. Moving gradually towards the exit and positioning its body and ready to fly away.

Morphological measurements of the operative size of *Thrinchostoma* sp. 2 filmed on *V. siamensis*, with functional height, which is the approximate height at which the insect passes by the column, were estimated based on the leg length and overall size of the insect. Observations of visitors and the floral morphology of *V. siamensis* indicate that the species is bee pollinated. This is consistent with the present knowledge of pollinators for other Vanilla species, all of which are pollinated by different bee species and genera (Ackerman, 1983; Ackerman, 1986; Johnson and Steiner, 1997; Soto-Arenas and Cameron, 2003; Soto-Arenas and Dressler, 2010). From the observations of the floral morphology, an effective pollination of *V. siamensis* would be a bee that is of a specific size, such as *Thrinchostoma* sp. 2. No other smaller insects were observed as potential pollinators, as they would be too small to activate the pollination mechanism (Roubik and Ackerman, 1987). The species produces extra-floral nectaries as an incentive for ants to patrol the flowers as a defense from herbivores. Similar to previous research, the most frequent group of non-pollinating insects found interacting with the buds of *V. siamensis* was ants, which is also true for other orchid species, (Johnson *et al.*, 1998; Johnson and Steiner, 2000; Jersáková *et al*., 2006; Schiestl and Johnson, 2013).

False nectar guides such as fine spots and lines can stimulate visiting and food searching reaction by potential pollinators (Nilsson, 1980). It is possible that the white and red tufts of hairs on the surface of the labellum contribute to the flower's attraction in a similar manner. However, floral fragrance has been shown to be more important than visual cues for bees when orientating at short distances to adjust their approach and landing (Free, 1970). The Vanilla genus is known for its fragrant flowers, with no species lacking a fragrance currently known. Fragrance seems to play an important part in attracting pollinators for many Vanilla species (Soto-Arenas and Cameron, 2003; Lubinsky *et al*., 2006 Householder *et al*., 2010; Soto-Arenas and Dressler, 2010; Pansarin, 2016). For example, the unusual high fruit set produced by *V. chamissonis* Klobschis was attributed to the strong floral fragrance (Gigant *et al*., 2011). Householder *et al*. (2010) described how particularly fragrant flowers of *V. pompona grandiflora* (Lindl.) attract a greater number of pollinators than individual flowers with a faint fragrance. To humans, the faint fragrance of *V. siamensis* is only perceptible at close distance. Most likely, the pollinating bees are also attracted to the flowers of *V. siamensis* because of its conspicuous size, color and placement in the canopy (Roubik and Ackerman, 1987). The orange-yellow inner surface of the labellum of *V. siamensis* creates a color contrast from the rest of the flower that likely contributes to the close-up orientation of the pollinator.

There was a clear indication of the pollinia attached to its back before the bee took off. As soon as the bee left, the flower was immediately covered with a muslin cloth to prevent other insects going in. After ten days, the presence of an abscission zone between the ovary and the remainder of the flower appeared. It is at this point, that the flower fell away and the ovary started to swell within a few days. However, unsuccessful pollination results in the whole flower falling off much sooner, leaving a scar (Soto-Arenas and Dressler, 2010).

CONCLUSIONS

This study suggests that bees of the genus Thrinchostoma are the pollinators of *V. siamensis.* The pollination mechanism is quite straightforward. Upon leaving the flower, the bee passes the central bump on the labellum and is forced to raise its body, activating the pollination process by coming into contact with the rostellum before the anther, and later with the stigma in another flower. However, pollination of *V. siamensis* by the Thrinchostoma bee, under natural conditions, appears to be less favorable when compared to artificial pollination techniques such as hand pollination (Johnson, 2010).

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