

COMPARATIVE FLORAL SURFACE MICROMORPHOLOGY HELPS TO DISCRIMINATE BETWEEN SPECIES OF *PAPHIOPEDILUM* (ORCHIDACEAE: CYPRIPEDIOIDEAE) FROM PENINSULAR MALAYSIA

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ABSTRACT. The floral micromorphology of critically endangered *Paphiopedilum* Pfitzer [*P. barbatum* (Lindl.) Pfitzer, *P. callosum* var. *sublaeve* (Rchb.f.) P.J.Cribb and *P. niveum* (Rchb.f.) Stein] were analyzed concerning either infrageneric taxonomy or physioecological demands. The first two species are phylogenetically close and superficially identical but occur with distinct phytogeographical distributions in the region. The third species is a phylogenetically distant congener that inhabits limestone areas in the northern part of Peninsular Malaysia. Using scanning electron microscopy (SEM), we investigated the surface of the dorsal sepal, synsepal, lateral petals, pouch or labellum, and staminode. Amongst the investigated features were epicuticular waxes, epicuticular ornamentation, trichome distribution and type, pustular glands, and papillae. Our study supports the distinction of *P. barbatum* from *P. callosum* var. *sublaeve*, which belong to subgenus *Paphiopedilum*, and from *P. niveum*, a species belonging to subgenus *Brachypetalum*, a separated monophyletic clade. Comparatively, *P. barbatum* has Type III non-glandular trichomes on the margin of its lateral petals, which are absent in *P. callosum* var. *sublaeve*. *Paphiopedilum callosum* var. *sublaeve* and *P. niveum* are distinguishable from *P. barbatum* by a confined distribution of papillae. The epicuticular ornamentation and distribution of trichomes on staminode discriminate between *P. barbatum* and *P. callosum* var. *sublaeve* and differentiates them from *P. niveum*. Compared to *P. barbatum* and *P. niveum*, stomata in *P. callosum* var. *sublaeve* were superficial with prominently raised guard cells. From the physioecological view, the absence of glandular trichomes, and the low occurrence of papillae and stomata on the floral parts explain the unscented flowers of *P. barbatum* and *P. callosum* var. *sublaeve*. A combination of the features examined is taxonomically valuable for delimitation of the species at the infrageneric level, although the diagnostic characters are far inadequate for a generic taxonomic revision. A study with a more extensive sampling from the three subgenera of *Paphiopedilum*, including subgenus *Parvisepalum*, is anticipated to elucidate the level of variation of the analyzed microcharacters.

KEY WORDS: physioecological importance, Peninsular Malaysia, subgenus *Brachypetalum*, subgenus *Paphiopedilum*, scanning electron microscopy, taxonomic delimitation

Introduction. *Paphiopedilum* originates from the Greek word ‘*Paphian*’ an epithet for Aphrodite, the Roman goddess known as Venus, and “*pedilon*” which means slipper (Cash 1991, Cribb 1998). Orchids in this genus are commonly known as slipper orchids because of the unique slipper or shoe-like flowers (Cash 1991, Cribb 1998, McGough *et al.* 2006). The genus *Paphiopedilum* Pfitzer comprises about 167 species, with distribution extending from Southern China to Tropical Asia (Braem 1988, Cribb 1998, Chen *et al.* 2005, Govaerts *et al.* 2021). *Paphiopedilum* gained

its popularity and investment value in the horticulture industry through its exotic appearance and production of large flowers on small plants (Cribb 1998). Most of the species are regarded as endangered and threatened with extinction due to habitat destruction, over-collection and illegal trading. They are amongst the plants listed on the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES – Appendix 1). Within this list, one can find well-known Malaysian species *Paphiopedilum barbatum* (Lindl.) Pfitzer (Bearded *Paphiopedilum*) (Rankou 2015a),

Paphiopedilum callosum (Rchb.f.) Stein (*Callus Paphiopedilum*) (Rankou *et al.* 2015), *Paphiopedilum niveum* (Rchb.f.) Stein (*Snow-White Paphiopedilum*) (Rankou 2015b), *Paphiopedilum rothschildianum* (Rchb.f.) Stein (*Rothschild's Paphiopedilum*) (Rankou 2015c), *Paphiopedilum sanderianum* (Rchb.f.) Stein (*Sander's Paphiopedilum*) (Rankou 2015d) and *Paphiopedilum stonei* (Rchb.f.) Stein (Rchb.f.) Stein (*Stone's Paphiopedilum*) (Rankou & O'Sullivan 2015).

Systematically, *Paphiopedilum* is considered an early branch group due to its geographical distribution and relatively unspecialized floral structures (Rosso 1966). The subfamily Cyripedioideae is unusual amongst the Orchidaceae because of the presence of two fertile stamens, the disposition of these stamens in the inner staminal whorl lateral to the style, and the incomplete adnation of stylar and staminal tissues (Rosso 1966). A saccate labellum is usually present and is responsible for the common name "slipper orchids" so often applied to these plants (Seidenfaden & Wood 1992, Cribb 1998). Taxonomically, *Paphiopedilum* is classified based on morphological, cytological, and molecular phylogenetic data into three subgenera; *Parvisepalum*, *Brachypetalum* and *Paphiopedilum* (Cribb 1998, Chochai *et al.* 2012). Until now, only subgenus *Brachypetalum* and subgenus *Paphiopedilum* are recorded for Peninsular Malaysia. We investigate four aspects to identify an orchid species: general morphology, chromosome numbers, leaf and floral anatomy, and DNA barcoding. Species delimitation based on general floral morphology for *Paphiopedilum* species found in Peninsular Malaysia shows a clear resolution for most of the species, except for the highly resemblant ones, for instance, *P. barbatum* and *P. callosum* var. *sublaeve* (Rchb.f.) P.J.Cribb belong to subgenus *Paphiopedilum* (Seidenfaden & Wood 1992, Cribb 1998, Leong 2014). A work on DNA Barcoding of Endangered *Paphiopedilum* species of Peninsular Malaysia using four DNA barcode loci and their combinations (*rbcl*, *matK*, ITS, *trnH-psbA*) published by Rajaram *et al.* (2019) clusters each species as a monophyletic clade. The *matK* sequences discriminate the closely related *P. barbatum* and *P. callosum* var. *sublaeve*, therefore supporting the species circumscription by Cribb (1998) (Rajaram *et al.* 2019). Nevertheless, slipper orchids are infamously variable, and unusual plants may sometimes be

natural hybrids, especially when the putative parents grow sympatrically (Averyanov *et al.* 2007, Leong 2014, van der Ent *et al.* 2015). Natural hybridizations between two confusable *Paphiopedilum* species occur in Peninsular Malaysia, e.g. in between *P. barbatum* and *P. callosum* var. *sublaeve* – where the chloroplast *matK* sequence matched that of *P. barbatum* and the nuclear ITS sequence matched that of *P. callosum* var. *sublaeve* (Khew *in prep.* cited in Leong 2014).

Cytologically, the genus is characterized by significant chromosome variation, ranging from $2n = 26$ to 42 (Duncan & Macleod 1949, Karasawa 1979, Karasawa & Aoyama 1988). Pollen studies and anatomy observations on the leaf, root, stem, and inflorescence for members of subfamily Cyripedioideae are enumerated in Pfitzer (1903), Holm (1904), Cheadle (1942), Rosso (1966) and Atwood (1984). The systematic significance of inner and outer cuticular micromorphology of mottled and xeromorphic leaves of *Paphiopedilum* species is unclear for either taxonomical or ecological purposes (Guan *et al.* 2011). The floral micromorphology of this genus, on the other hand, has not been thoroughly examined, except for pollen morphology. Pollens of some *Paphiopedilum* species, including *P. barbatum*, *P. callosum* and *P. niveum*, were studied under the microscope by Williams & Broome (1976), Newton & Williams (1978), and Burns-Balogh & Hesse (1988), are taxonomically useful at the intergeneric level. The exine of *P. callosum* is formed by isolated sporopollenin particles of the thick, peripherally channelled intine. *Paphiopedilum niveum* differs by having the foveolate exine with small pits. However, studies on the micromorphology of other floral parts of the genus *Paphiopedilum* are lacking. Given the above, we employed scanning electron microscope (SEM) observations to evaluate surface microstructures' applicability in taxonomic delimitation and physioecological functions.

Materials and methods

Species selection.— Three *Paphiopedilum* species from Peninsular Malaysia were selected to predict the congeneric contrasts (Fig.1): *Paphiopedilum barbatum* and *P. callosum* var. *sublaeve* belong to section *Barbata* in subgenus *Paphiopedilum* with mottled

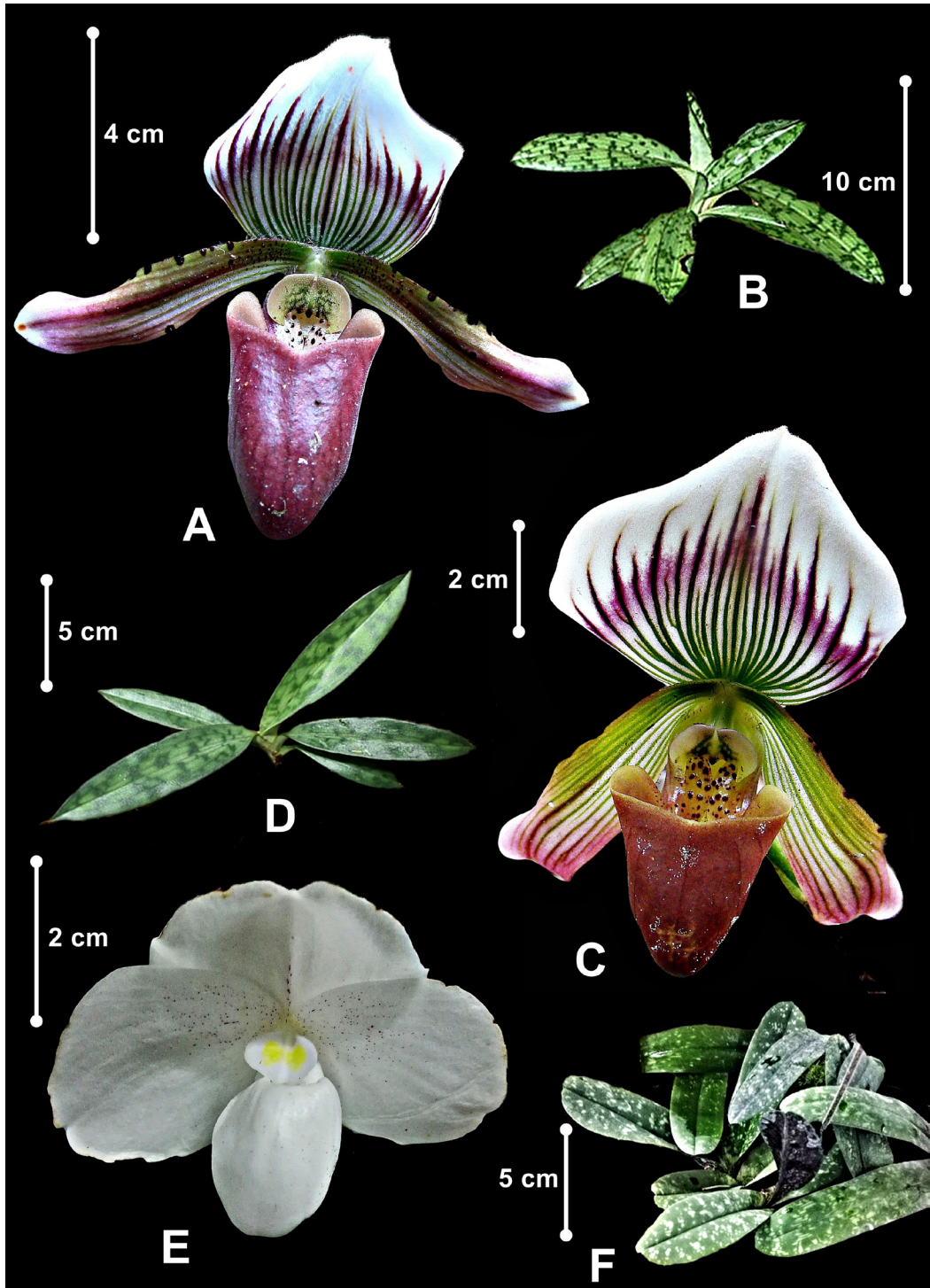


FIGURE 1. Examined species of *Paphiopedilum* from Peninsular Malaysia. A–B. *Paphiopedilum barbatum* flower (A) and leaves (B). C–D. *Paphiopedilum callosum* var. *sublaeve* flower (C) and leaves (D). E–F. *Paphiopedilum niveum* flower (E) and leaves (F). Photographs by Rusea Go and Edward Entalai Besi.

TABLE 1. *Paphiopedilum* species examined including their locality, habitat and voucher.

Species	Type Locality	Habitat	Voucher Deposited
<i>P. barbatum</i>	Terengganu	Peaty areas and rocky boulder in waterfall in lower montane forest	EDW060 (UPM)
<i>P. callosum</i> var. <i>sublaeve</i>	Kedah	Highland heath forest with ground made up of granite, quartzite and sandstone	RG4574 (UPM)
<i>P. niveum</i>	Perlis	Limestone hill forest	WY125 (UPM)

leaves, mostly spotted warty petals and thick-textured labellum, and *P. niveum*, the only representative of subgenus *Brachypetalum* in Peninsular Malaysia, with mottled leaves, concolourous white flowers and thin-textured labellum. *Paphiopedilum barbatum* thrives under deep dark valleys, open areas or rocky boulders covered with humus, leaf litters or carpets of thick moss at streamside from about 200 m to 1200 m a.s.l. Both *P. callosum* var. *sublaeve* and *P. niveum* confined to the northern part of Peninsular Malaysia differ in habitat types. *Paphiopedilum callosum* var. *sublaeve* occurs in mossy forest or open vegetation with the ground covered with sphagnum mosses or coarse white sand, whereas, *P. niveum* is a calcicoles congener inhabiting limestone cliff shaded from direct sunlight at about 300 m a.s.l.

Sample collection and processing.— One individual for each species was obtained through field sampling conducted in three different localities in Peninsular Malaysia, allowed by a permit. A complete specimen for each species was processed as an herbarium specimen following techniques outlined in Bridson & Forman (2000) and deposited in the Herbarium of Universiti Putra Malaysia (UPM). The voucher numbers and attributes are listed in Table 1. Two flowers of each species were used in macro- and micromorphology examinations. The flower specimens were dissected and photographed under AM4113ZT Dino-Lite Digital Microscope. Species identification was accomplished by morphological assessment by referring to the published taxonomic monographs and the botanical illustrations of Seidenfaden & Wood (1992) and Leong

(2014). The currently accepted names of the orchids were validated through the KEW World Checklist of Selected Plant Families (Govaerts *et al.* 2021).

Micromorphology examination.— The microstructural study was carried out in Microscopy Unit (EM) in the Institute of Biological Sciences (IBS), UPM, Malaysia. The floral parts examined were dorsal sepal, synsepal, lateral petals, pouch or labellum and staminode. For SEM, the samples were processed according to a modified protocol by IBS explained in Besi *et al.* (2020): First, fragments about 1 cm × 1 cm were excised from the margin, basal, apex and middle portions of the floral parts, except for the staminode which was used entirely. The excised samples were put into separate vials and soaked in fixative (4% glutaraldehyde) for two days at 4°C. After two days, samples were washed with 0.1 M sodium cacodylate buffer for three changes of 30 min each and post-fixed in 1% osmium tetroxide for 2 h at 4°C. Then, samples were rewashed with 0.1 M sodium cacodylate buffer (three times 30 min each) before dehydration with series of acetone: 35% (30–45 min), 50% (30–45 min), 75% (30–45 min), 95% (30–45 min), and 100% (1 h for three changes). The samples were further dried using the critical dryer Leica EM CPD 030 for about 30 min. Lastly, the samples were mounted on stubs using double-sided carbon adhesive tabs and then sputter-coated with gold in auto fine coater Baltec SCD 005 Sputter Coater. The coated samples were examined under the Jeol JSM 6400 SEM (Beam voltage: 15 kV). The surface of each floral part was observed under various magnifications (15x–4000x). All the stubs prepared are housed in the EM unit in IBS, UPM, Malaysia.

The microstructures observed on the floral parts were trichomes and papillae, pustular glands, stomata, epicuticular ornamentation and waxes. Classification of stomata was according to Wilkinson (1979) and Carpenter (2005) based on shapes and patterns of the stomatal ledges flanking aperture, guard cells and peristomatal striae, and arrangement of the contact cells. Here, we have adopted the term ‘contact cell’ to take the place of the subsidiary cell and neighbouring cell, to refer to any cell, specialized or not, that is adjacent to the stoma (Upchurch 1984). The studied *Paphiopedilum* species have some stomata where

TABLE 2. Trichomes types on the floral parts *Paphiopedilum barbatum*, *P. callosum* var. *sublaeve* and *P. niveum*, including description on the morphology.

Type	Morphology description
I	Simple, uniseriate, non-glandular, unicellular, rugose, ca. 100-200 μm , narrowly clavate
II	Simple, uniseriate, non-glandular, multicellular, long, ca. 200-1,000 μm , moniliform
III	Simple, uniseriate, non-glandular, multicellular, elongated, ca. 200-2,000 μm , moniliform with topmost cell very narrow
IV	Simple, multiseriate, non-glandular, bicellular, multiseriate base, short, ca. 100-400 μm
V	Simple, uniseriate, non-glandular, bicellular or multicellular, short, ca. 100-400 μm
VI	Simple, uniseriate, glandular, unicellular, sessile, ca. 5-20 μm , barrel-shaped
Papillae globular or tall, striated	

contact cells' patterns were not shown clearly in the SEM micrographs. Thus, the stomata type was omitted from the analysis and these stomata were described based on guard cells, stomatal ledges and peristomatal striae. For the individual stomatal parameters, stoma length and width, a magnification of 500x and a measurement method in Savvides *et al.* (2011) were employed in the current study. Stoma width was chosen instead of guard cell width since the latter changes up to 50% as stomata close (Shope & Mott 2006). Meanwhile, trichomes were described and classified based on Theobald *et al.* (1979), Adedeji *et al.* (2007), and Angulo & Dematteis (2014). Comprehensive terminologies of trichome morphology follow Angulo & Dematteis (2014). The parameter measurements were done using a ruler and the values obtained were multiplied with the magnification scales. Surface's cuticular ornamentation was described following Piwowarczyk (2015), Ghimire *et al.* (2018), and Kong & Hong (2018), and description on epicuticular waxes was based on Wilkinson (1979). Assessment of the examined species and the comparative study were conducted following Ghazalli *et al.* (2019).

Results. Epicuticular ornamentation was observed on the floral parts of the selected *Paphiopedilum* species. Six different features of simple and uniseriate trichomes, vary in structure, distribution, and number of cells, except branched trichomes. Description of the epicuticular ornamentation and trichomes are in Tables 2 and 3.

TABLE 3. Epicuticular ornamentation on the floral parts *Paphiopedilum barbatum*, *P. callosum* var. *sublaeve* and *P. niveum*, including description on the morphology.

Type	Morphology description
I	Foveate outer periclinal wall; furrowed, straight and rounded anticlinal wall
II	Foveate outer periclinal wall; fibrillary, straight and rounded anticlinal wall
III	Flat outer periclinal wall; reticulate, fibrillary, straight and rounded anticlinal wall
IV	Laevigate and often striated outer periclinal wall; undulate and furrowed anticlinal wall
V	Outer periclinal wall with a complex network of undulate striae; fibrillary, straight and rounded anticlinal wall
VI	Entirely covered by hairs (Type I non-glandular trichomes)
VII	Laevigate-with-seams outer periclinal wall; furrowed, straight and rounded anticlinal wall

Species assessment under SEM.— *Paphiopedilum barbatum* (Fig. 2). *Waxes*: scattered, warty-granulated and flake-like. *Epicuticular ornamentation*: Type III, IV and VII. *Stomata formation*: same level with the epidermal wall, in parallel or random formation. *Stomata distribution*: sparsely occurred on dorsal sepal, synsepal, lateral petals, labellum and staminode. *Stomata ornamentation*: comprise a defined rosette of five to seven contact cells with radial elongation of some cells but not others or characterized by four lateral contact cells. Guard cells and ledges indistinguishable from the neighbouring stomatal apparatus in the staminode. *Stomatal cuticular striation*: smooth or slightly striated. *Stomatal size*: L (11.11–41.67 μm) \times W (4.44–33.33 μm). *Trichome distribution*: present on dorsal sepal, synsepal, lateral petals, labellum and staminode. *Trichome type*: non-glandular—Type I, II, III, and V; glandular—absent. *Pustular glands*: absent. *Papillae*: absent.

Paphiopedilum callosum var. *sublaeve* (Fig. 3). *Waxes*: scattered, warty-granulated and flake-like. *Epicuticular ornamentation*: Type II, III, IV, VI, and VII. *Stomata formation*: superficial, raised from the epidermal wall. *Stomata distribution*: sparsely occurred on dorsal sepal, synsepal and lateral petals. *Stomata ornamentation*: narrowly elliptical outer stomatal ledges with prominent guard

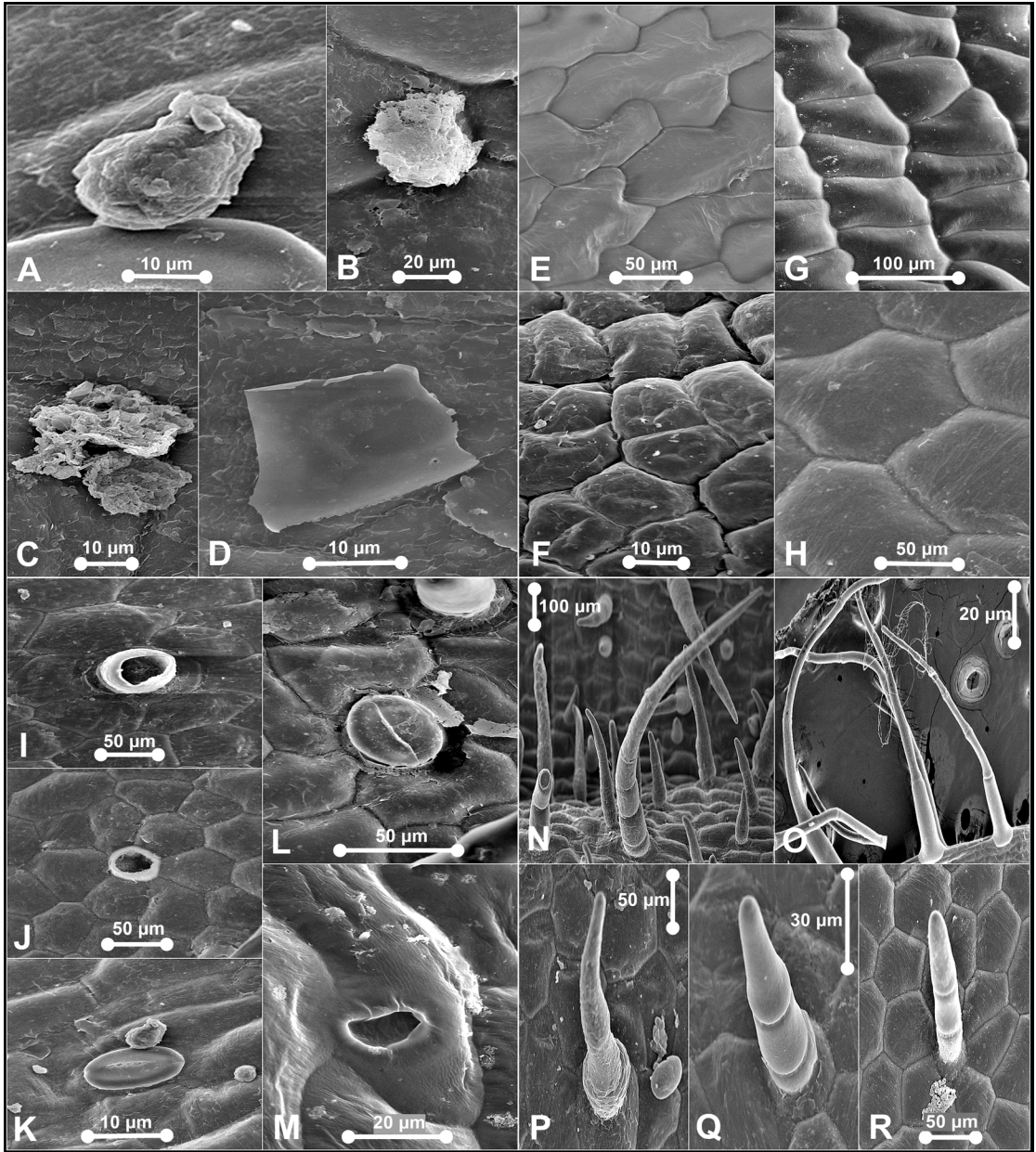


FIGURE 2. SEM observations of epicuticular waxes (A–D), epicuticular ornamentation (E–H), stomata (I–M) and trichomes (N–R) on floral parts of *Paphiopedilum barbatum*. **A.** Warty-granulated wax. **B.** Warty-granulated wax. **C.** Warty-granulated wax. **D.** Flake-like wax. **E.** Type III epicuticular ornamentation. **F.** Type IV epicuticular ornamentation. **G.** Type VII epicuticular ornamentation. **H.** Type VII epicuticular ornamentation. **I.** Aperture from by detachment of trichome on dorsal sepal. **J.** Aperture from by detachment of trichome on synsepal. **K.** Stoma on synsepal – characterized by four lateral contact cells. **L.** Stoma on synsepal – comprise a defined rosette of five to six contact cells. **M.** Nectarostoma on staminode. **N.** Trichomes on dorsal sepal – Type II. **O.** Trichomes on petal – Type II. **P.** Trichomes on dorsal sepal and staminode – Type II. **Q.** Trichomes on labellum – Type II. **R.** Trichomes on petal and labellum – Type II. Photographs by Edward Entalai Besi.

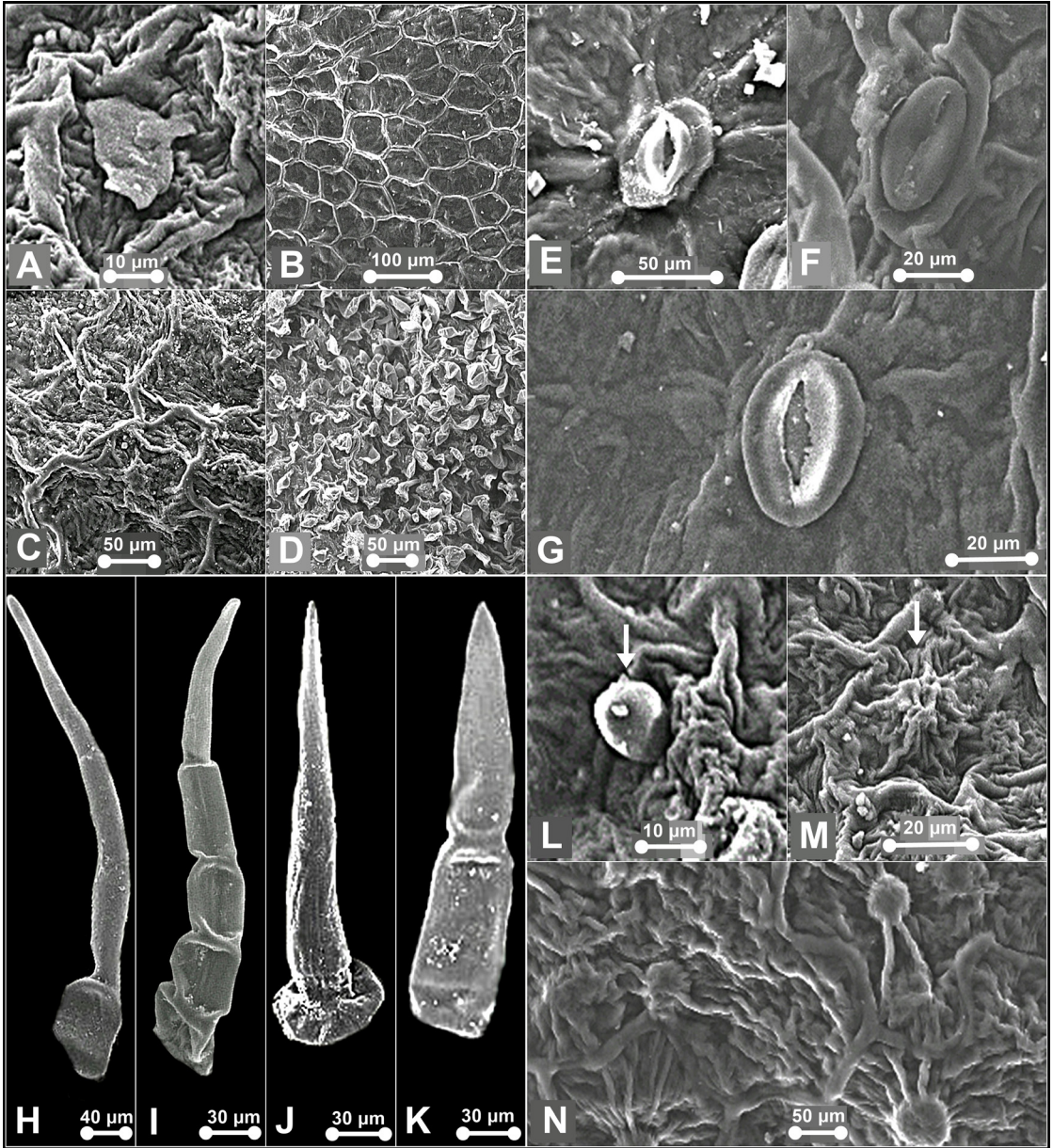


FIGURE 3. SEM observations of epicuticular waxes (A), epicuticular ornamentation (B–D), stomata (E–G), trichomes (H–L) and papillae (M–N) of *Paphiopedilum callosum* var. *sublaeve* and *P. niveum*. **A.** Flake-like wax on the pouch of *P. niveum*. **B.** Type II epicuticular ornamentation. **C.** Type V epicuticular ornamentation. **D.** Type VI epicuticular ornamentation. **E.** Stoma on dorsal sepal of *P. callosum* var. *sublaeve*. **F.** Stoma on lateral sepals of *P. niveum*. **G.** Stoma on dorsal sepal of *P. niveum*. **H.** Trichomes on synsepal of *P. callosum* var. *sublaeve*. **I.** Trichome on dorsal sepal of *P. callosum* var. *sublaeve*. **J.** Trichome on dorsal sepal of *P. niveum* – Type IV. **K.** Trichome on synsepal of *P. callosum* var. *sublaeve* – Type IV. **L.** Trichome on the pouch of *P. niveum* – Type VII. **M.** Papillae on the pouch of *P. niveum* – tall and striated. **N.** papillae on the pouch of *P. callosum* var. *sublaeve* – globular and striated. Photographs by Edward Entalai Besi and Lam Shun Jia.

TABLE 4. Features and distribution of floral-surface micromorphology characteristics of *Paphiopedilum barbatum*, *P. callosum* var. *sublaeve* and *P. niveum* (epidermal, stomata).

Species	Floral parts	Epicuticular ornamentation	Anticlinal wall features	Epicuticular striation	Stomata contact and epidermal cells ornamentation	Peristomatal rim ornamentation	Pattern of stomatal distribution
<i>P. barbatum</i>	Dorsal Sepal	III, IV	Furrowed	Smooth	Present	Present	Parallel
	Synsepal	III, VII	Furrowed	Smooth	Present	Present	Random
	Lateral Petals	IV, VII	Fibrillary, furrowed	Smooth	Present	Present	Random
	Labellum	IV	Undulate, furrowed	Smooth	Present	Present	Parallel
	Staminode	VII	Furrowed	Smooth	Present	Present	Parallel
<i>P. callosum</i> var. <i>sublaeve</i>	Dorsal Sepal	II	Fibrillary	Rugulate	Present	Present	Parallel
	Synsepal	II, VII	Fibrillary, furrowed	Rugulate	Present	Present	Parallel
	Lateral Petals	II, III	Fibrillary	Rugulate	Present	Present	Parallel
	Labellum	IV	Undulate, furrowed	Rugulate, striated	Absent	Absent	Absent
	Staminode	VI	Unclear	Unclear	Absent	Absent	Absent
<i>P. niveum</i>	Dorsal Sepal	III	Fibrillary	Densely rugulate	Present	Present	Parallel
	Synsepal	III	Fibrillary	Striated	Present	Present	Parallel
	Lateral Petals	II	Fibrillary	Rugulate, striated	Absent	Absent	Absent
	Labellum	II, V	Fibrillary	Rugulate, striated	Absent	Absent	Absent
	Staminode	VI	Unclear	Unclear	Absent	Absent	Absent

cells. Contact cells indistinguishable. *Stomatal cuticular striation*: radiating peristomatal striae in irregular orientation from ledge cells. *Stomatal size*: L (41.38–52.38 μm) \times W (22.79–31.21 μm). *Trichome distribution*: present on dorsal sepal, synsepal, lateral petals and labellum. Two major groups of trichomes were observed on the dorsal sepal; non-glandular and glandular trichomes. The long and non-glandular trichomes were mostly located marginally and glandular trichomes on the dorsal sepal. *Trichome type*: non-glandular—Type I, II, III, and V. *Pustular glands*: sessile, widely-scattered on sepals. *Papillae*: congregated on labellum, globular, striated and connected by radiating striae.

Paphiopedilum niveum (Fig. 3). *Waxes*: scattered, warty-granulated and flake-like. *Epicuticular*

ornamentation: Type II, III, V and VI. *Stomata formation*: parafacial, semi-raised from the epidermal wall. *Stomata distribution*: sparsely occurred on dorsal sepals and synsepal. *Stomata ornamentation*: narrowly elliptical outer ledges and distinct irregular quadrilateral guard cells. Contact cells indistinguishable from the neighbouring stomatal apparatus. *Stomatal cuticular striation*: long radiating buttressed striae. *Stomatal size*: L (37.98–45.45 μm) \times W (30.32–38.66 μm). *Trichome distribution*: presence on dorsal sepal, synsepal, lateral sepals, labellum and staminode. Non-glandular trichomes were dense in petals and sepals. Glandular trichomes occasionally occur on the labellum. *Trichome type*: non-glandular—Type I, II, III, IV, and V; glandular—Type VI. *Pustular glands*: occur sparsely on sepals, sessile to

subsessile, resemble subsessile trichomes, except the former commonly striated or connected by striae, or resemble papillae, except the former not prominently protruding. *Papillae*: congregated on labellum, tall striated.

Comparative study on the floral-surface micromorphology.— Prominent cuticular sculpturing was clearly observed on the epidermal surface of the selected species and varied significantly in anticlinal and periclinal wall characteristics. Stomata were present in floral parts of *P. barbatum* but only occurred occasionally for *P. callosum* var. *sublaeve* and *P. niveum*. Trichomes were observed in all studied species. All had diverse types of trichomes on their floral parts. The features and occurrence of each micromorphology are shown in Table 4 and 5.

Discussion. Questions have arisen over the usefulness of floral-surface micromorphology in the recircumscription of confusable *Paphiopedilum* species found in Peninsular Malaysia, *P. barbatum* and *P. callosum* var. *sublaeve*. At first, we discuss the taxonomic significance and then the physioecological importance of epicuticular ornamentation, stomata and trichomes. Non-glandular trichomes are classified as non-glandular for not functioned as secretory structures (Peterson & Vermeer 1984). The non-glandular trichomes occur on various floral parts (Ko *et al.* 2007, Baran *et al.* 2010). Glandular trichomes, papillae and floral stomata play essential roles in fragrance and metabolite release which offers food to ensure pollinators revisit (Davies & Turner 2004, Choi & Kim 2013, Stpiczyńska *et al.* 2018).

Taxonomic aspects.— The invariable presence of warty-granulated and flake-like epicuticular waxes without any unique types on each floral part suggests no significance systematics value for the studied species. Contrariwise, the multi-pattern epicuticular ornamentation on the floral parts offers a significant taxonomic value to discriminate the infrasubgeneric *P. barbatum* and *P. callosum* var. *sublaeve*. The epicuticular sculptures are also consistent to differentiate them from their congener *P. niveum* (Table 4).

Stomata were found in all three studied

TABLE 5. Features and distribution of floral-surface micromorphology characteristics of *Paphiopedilum barbatum*, *P. callosum* var. *sublaeve* and *P. niveum* (trichome).

Species	Floral Parts	Trichome type	Glandular trichomes	Papillae
<i>P. barbatum</i>	Dorsal Sepal	III	Absent	Absent
	Synsepal	III	Absent	Absent
	Lateral Petals	II, III	Absent	Absent
	Labellum	II, III, V	Absent	Absent
	Staminode	I, II, III	Absent	Absent
<i>P. callosum</i> var. <i>sublaeve</i>	Dorsal Sepal	I, III, V, VI	Absent	Absent
	Synsepal	I, II, III, V	Absent	Absent
	Lateral Petals	II, V	Absent	Absent
	Labellum	I, III, V	Absent	Present
	Staminode	V	Absent	Absent
<i>P. niveum</i>	Dorsal Sepal	I, IV, V	Absent	Absent
	Synsepal	I, IV, V	Absent	Absent
	Lateral Petals	II	Absent	Absent
	Labellum	II, III, V, VI	Present	Present
	Staminode	V	Absent	Absent

Paphiopedilum species. The contact cells obscurity could be a characteristic of a genus. Nevertheless, the stomata can be clearly distinguished based on the prominence of the guard cells and their shape. Solereder (1908) and Carpenter (2005) strongly emphasized the diagnostic importance of the morphology of the guard cells and their cuticular ledges. The outline of the pair of guard cells as seen in surface view is usually constant in the examined specimens and is also possible a characteristic of a genus. Also, stomata in *P. barbatum* differs significantly from *P. callosum* var. *sublaeve* and *P. niveum* by having clear and noticeably contact cells, epidermal cells and peristomatal rim but rather obscure guard cells. Here, we can also deliberately compare between *P. barbatum* and *P. callosum* var. *sublaeve* based on the stomata and stomatal formation when observed from the top view. In comparison, stomata in *P. callosum* var. *sublaeve* were superficial and stand out distinctly with prominently raised guard cells. *P. niveum* had stomata slightly raised and irregular-shaped guard

cells that may provide a unique diagnostic character at the species level. Based on the general designation of the stomatal size provided in Wilkinson (1979), the stomata present on the slipper orchids are termed as 'large', similar to *Corybas holtumii* and *Corybas selangorensis* (see Besi *et al.* 2019).

Dominance of simple non-glandular trichomes and occurrence of variegated stomata on the floral surface of the *Paphiopedilum* species may separate genus *Paphiopedilum* from other genera within the Cypripedioideae subfamily. In many cases, such trichomes were living cells whereas in others they were dead, and the protoplasm was replaced by air spaces (Fahn 1988) and easily distorted or torn as observed on the labellum of *P. callosum* var. *sublaeve*. Different types of trichomes possess varies morphological characteristics were distinctively occurred on these floral surfaces of *Paphiopedilum* species (Table 5). The simple non-glandular trichomes were dominant on the floral surface. In contrast, the papillae were scarce, localized and only occurred on the labellum of *P. callosum* var. *sublaeve* (globular and striated) and *P. niveum* (tall and striated). This suggests the presence of papillae with varied morphology on the labellum of *Paphiopedilum* are of systematic significance and can be used as a diagnostic character to distinguish them further morphologically. There were pustular glands observed on the sepals and petals that resemble either sessile trichomes or papillae. Short and rugose non-glandular trichomes were formed by two to five cell tiers. The trichomes occurred at different length ranged from 61.11 μm to 1533.3 μm for *P. barbatum*, 48 μm to 190 μm for *P. callosum* var. *sublaeve* and 100 μm to 240 μm for *P. niveum*.

The presence of different types of simple non-glandular trichomes on the floral parts of the studied *Paphiopedilum* species denotes species specificity. It provides a piece of useful evidence for delineation of the confusable *P. barbatum* and *P. callosum* var. *sublaeve*. Morphologically, *P. barbatum* differs only by having dorsal sepal broadly ovate, petals with warts on upper or both margins and sometimes on the petals blades too, whereas *P. callosum* var. *sublaeve* has dorsal sepal broadly ovate to suborbicular and petals with warts on upper margin only (Seidenfaden & Wood 1992, Leong 2014). Clearly, these diagnostic characters are inconspicuous

without a definite boundary to discriminate and sometimes misleading. Therefore, here, floral-surface micromorphology serves as a steadfast advanced technique for the taxonomic circumscription of the confusable *P. barbatum* and *P. callosum* var. *sublaeve*. Micromorphologically, *P. barbatum* varies in the diversity of non-glandular trichomes on its floral parts compared to its complex, *P. callosum* var. *laeve* (Table 5). Conspicuously, the former species has the longest Type III non-glandular trichomes (1233.3–1533.3 μm) on the margin of its lateral petals, noticeably elongated and moniliform with topmost cell very narrow, which such trichomes were absent in the latter species. Also, glandular trichomes occurred in *P. callosum* var. *sublaeve* but lacking in *P. barbatum*. Variability of the micromorphology observed on the staminode is systematically insignificant at infrasubgeneric level. Notwithstanding, a combination of the micromorphological characteristics on staminode separates *P. barbatum* and *P. callosum* var. *sublaeve* in subgenus *Paphiopedilum*.

The existence of certain trichome types allows differentiation of the *Paphiopedilum* species from different subgenera. Unlike *P. barbatum* and *P. callosum* var. *sublaeve*, *P. niveum* contrasts by having dense hairs (Type I non-glandular trichomes) along the margin of the staminode (Fig. 4). Besides having distinctive diversity of non-glandular trichomes, the confined distribution of different types of papillae found only on the labellum for *P. callosum* var. *sublaeve* and *P. niveum* are also distinguishing. The trichomes' length and papillae' diameters were not much diverse between the studied species.

Overall, the present research suggests floral-surface features to be very useful in delimitation of the infrageneric taxa from different subgenera of the genus by epicuticular ornamentation, stomata and trichomes. The data from this study laid evidence for delimiting two confusable *Paphiopedilum* species. It provides conclusive proof to support the molecular phylogenetic analyses and validates the possibility of natural hybridization occurrence in between *P. barbatum* and *P. callosum* var. *sublaeve*. Moreover, it demonstrates that the former is indeed distinct from the latter. The floral-surface characteristics differentiate species from two different subgenera to some extent based on the presence of different types

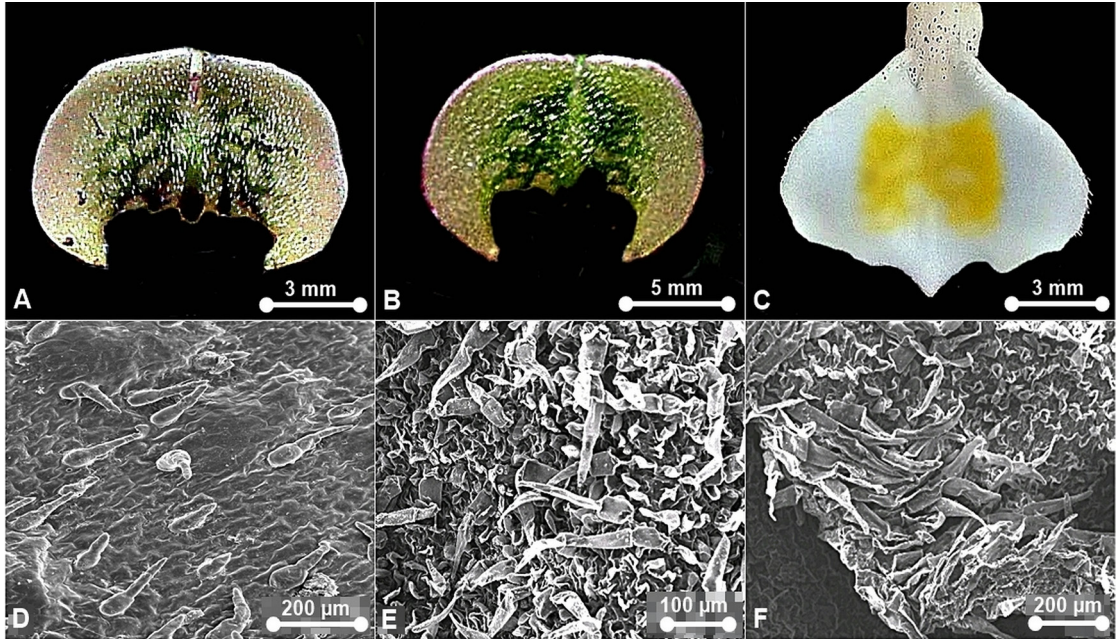


FIGURE 4. Staminode of *Paphiopedilum* and the epicuticular surface. **A, D.** *Paphiopedilum barbatum*. **B, E.** *Paphiopedilum callosum* var. *sublaeve*. **C, F.** *Paphiopedilum niveum*. Photographs by Edward Entalai Besi and Lam Shun Jia.

of epicuticular ornamentation and papillae on the labellum, and the diverse variation and distribution of the non-glandular trichomes on sepals and petals. Also, the occurrence of different formation, cuticular striation and ornamentation of stomata is of taxonomic interest in this study and can be used to identify the species.

Physioecological aspects.— The presence of dense epicuticular waxes on the floral surface of the selected Peninsular Malaysian *Paphiopedilum* species raises questions. One clear role of waxes is to protect the plant from desiccation and herbivorous insects (Davies & Turner 2004). It may or may not offer food rewards. In *Maxillaria*, one of the important ways insect attraction is achieved involves the secretion of wax-like material rich in lipids and protein (van der Pijl & Dodson 1966, Davies *et al.* 2003). It is also reported that wasps may also collect wax from the labella of *Maxillaria* (Dressler 1993). Dense waxes on the labellum of *P. callosum* var. *sublaeve* and *P. niveum* may attract potential pollinators. Male *Bactrocera* fruit flies are often observed to probe the labellum, sepals and petals of *Bulbophyllum* species. The probing and licking behaviours displayed by the flies suggests that

the pollinators' reward may be compounds released by the flower (Ong *et al.* 2011).

Orchid floral stomata are non-functional and practically closed in orchid flowers (Hew *et al.* 1980). Our finding supports this claim as the stomata found in the studied species were closed (Fig. 2K,L and Fig. 3F), or opened with a small aperture (Fig. 3E,G). Also, there were nectarostomata without a presence of guard cells (Fig. 2M), which might indicate modified stomata, cavities where the waxes are exuded through on the cuticular surface, known to occur and are of great diagnostic value in some plant species (Pant & Mehra 1965, Wilkinson 1979, Chattopadhyay *et al.* 2014, Prashanta Kumar & Krishnaswamy 2014, Baruah 2017, Verma *et al.* 2018, Besi *et al.* 2019, Besi *et al.* 2020). Notably, apertures formed by the detachment of the trichomes which could have been mistakenly identified as stomata in plant specimens (Fig. 2I,J). Waxes observed on floral surface indicates an active function of the unspecialized osmophores on the floral parts of orchid species, the regular epidermal cells secreting volatile oils (Toh *et al.* 2017). Identical to our previous finding on *Corybas* anatomical profiling work, the trichomes and stomata of the *Paphiopedilum* species offer more values on anatomical adaptations

in defence and pollination rather than for the release of fragrance (Bési *et al.* 2019). The densely hairy staminode may mimic an aphid mimicry as aphidophagous hoverflies lay eggs on false brood sites on their flowers (Bänziger *et al.* 2012, Jin *et al.* 2014). *Paphiopedilum* flowers are postulated rewardless or nectarless to the pollinators and luring hoverflies or bees by deceit (Bänziger 1996, 2011, Bänziger *et al.* 2012). This is supported by the lack of glandular trichomes, papillae and stomata occurring on the labellum and reproductive parts. However, thorough observations are lacking for Malaysian species (Leong 2014). The low occurrence of glandular trichomes, papillae and stomata on the floral parts explains the unscented flowers of *P. barbatum* and *P. callosum* var. *sublaeve*. Except, the labellum of *P. callosum* var. *sublaeve* and *P. niveum*, although lacking trichomes, are heavily clothed with papillae. Though no odour is detectable to the human nose in *P. niveum*, when a live flower is wrapped in a plastic bag for a couple of hours, *P. niveum* release a faint, pleasant fragrance (Bänziger *et al.* 2012). Therefore, the papillae may function as osmophores for *P. niveum*.

Conclusions. Features of floral parts surfaces, such as epicuticular ornamentation, stomata (formation, distribution, ornamentation and size), trichome (distribution and type) are recognized as useful to differentiate highly confusable species and delimit species from different subgenera of *Paphiopedilum*. SEM analysis of floral-surface micromorphology supports a segregation of a narrowly distributed *P. callosum* var. *sublaeve* from *P. barbatum*, a widespread species in Peninsular Malaysia. The latter species is known to produce a wide range of flower morphology

and colouration along the elevation gradients. All these diagnostic characters based on floral-surface morphology of these selected species should be used with care at intergeneric and intersubgeneric levels. It should be noted that these characters are far from being enough at this time to fully discriminate *Paphiopedilum* species in Peninsular Malaysia. A larger sampling is required to know the level of variation of the analyzed characters and to be able to make stronger conclusions. The usefulness of these floral microcharacters in biological and ecological aspects is difficult to predict based on the current preliminary finding. A further investigation on chemical compound released by *Paphiopedilum* flowers in relation to pollination mechanism is highly recommended.

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