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FLORAL-SURFACE MICROMORPHOLOGY OF *CORYBAS SELANGORENSIS* J.DRANSF. & G.SM. AND *CORYBAS HOLTTUMII* J.DRANSF. & G.SM. (ORCHIDACEAE)

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Abstract

An anatomical profiling work on floral micromorphology of two critically endangered and endemic helmet orchids, *Corybas selangorensis* J.Dransf. & G.Sm. and *Corybas holttumii* J.Dransf. & G.Sm., was conducted. The objective of this study was to understand the diversity of the floral-surface micromorphology of the two species for the first time and their taxonomic significance. The micromorphology of the floral parts was observed using Scanning Electron Microscope (SEM); these were photographed, described, and illustrated. In the floral micromorphology of *C. holtumii*, there was an occurrence of anisocytic stoma which were observed on the dorsal sepal, and in contrast, rare type holo-paracytic stomata which were observed on the dorsal sepal and bract of *C. selangorensis*. Sessile and long capitate glandular trichomes were observed on the dorsal sepal of *C. holttumii*, whereas neither glandular trichomes nor papillae were observed on *C. selangorensis*. Presence of stomata, glandular trichomes, and papillae on the dorsal sepal and labellum are the distinguishing characters. Trichomes and stomata offer more values on anatomical adaptions in defence and pollination for such species with inconspicuous habit rather than for release of fragrance. Different types of periclinal and anticlinal walls of the epicuticular ornamentation are useful to delimit and recognize the studied species from a similar subsection.

Introduction

CORYBAS SALISB. (1807: 83) is a peculiar genus belonging to family Orchidaceae, subfamily Orchidoideae, and tribe Diurideae; it comprises of about 153 species distributed throughout the tropics and subtropics of Asia to Pacific including the Subantarctic Islands (Govaerts, 2019). The type species is *Corybas aconitiflorus* Salisb. (1807: 83). All species are small and inconspicuous from a distance; it can be as big as a thumb, and largely terrestrial. *Plants* consist of a single underground *tuber* and a single small heart-shaped *leaf* with its pale green, white or red veins subtending a single white and crimson-purple flower of various shades. The appearance of dorsal *sepal* forms a helmet-like hood over the large labellum that gives rise to its common name “helmet orchid”, or the fascinating unusual spider-like form of sepals and petals morphologies contributes to its another common name “spider orchid” (Go *et al*., 2015). The *labellum* is erect from a tubular base and has two short spurs. *Column* is short and the anther erects on its back. There are two *pollinia* which are two-lobed, ecaudiculate, and granular (Dransfield *et al*., 1986). As noted by Holttum (1964), the *pedicel* of *Corybas* is short during flowering but elongates in *fruit*, which putatively provides the seeds with a better chance of dispersal.

The genus is further divided into two sections (Royen, 1983); Section *Corybas* P. Royen with two spurs at the base of the labellum and Section *Steleocorys* (Endl.) P. Royen with base without spurs but replaced by two open auricles or slits. Section *Corybas* is divisible into two subsections; Subsection *Corybas* P. Royen and Subsection *Gastrosiphon* (Schltr.) P. Royen. All Malaysian species belong to Subsection *Corybas*, except *Corybas fornicatus* (Blume) Rchb.f. (1871: 67) which belongs to Subsection *Gastrosiphon*. In Peninsular Malaysia, *Corybas* was first discovered in 1977. According to Dransfield *et al*. (1986), there are eleven species from Malaysia, of which ten are confined to mountains and growing in thick moss cushion on the ground, rocks or trees basal in the montane forests, and one, *Corybas calcicola* J.Dransf. & G.Sm. (1986: 608) is an endemic species to Peninsular Malaysia and found in the lowlands on limestone hills (Seidenfaden and Wood, 1992). In Malaysia, *Corybas* is considered regionally as a critically endangered species due to many factors, such as habitat loss, logging activities, microclimate changes, and its obscure appearance growing along the hiking trails in the montane forests. Furthermore, there has been no successful *ex situ* conservation effort for *Corybas* until today due to their specificity in abiotic requirement. Thus, conservation and protection against local extinction are unquestionably necessary.

Profiling on floral-surface micromorphology of two endemic and montane species of *Corybas* from Peninsular Malaysia; *Corybas selangorensis* J.Dransf. & G.Sm. (1986: 606) and *Corybas holttumii* J.Dransf. & G.Sm. (1986: 590) was conducted in order to enhance knowledge on floral micromorphology of the respective critically endangered, *Corybas* species. Researches on floral micromorphology of genus *Corybas* are lacking with an earlier study on monads of *C. aconitiflorus* that was

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published by Ackerman and Williams (1981). In addition, *Corybas* has an ephemeral flower and rarely flowering in the wild, hence, a taxonomic study of the floral parts is timely and significant. As pointed out by Dransfield *et al*. (1986), differentiating characters of a fragile flower-type as in *Corybas* are lost and difficult to interpret when specimens are dried or stored in methylated spirit solution. Therefore, an anatomical study of a fragile and inconspicuous flower using electron microscopy works better with a freshly collected specimen. The floral micromorphology study encompassed an observation of the microstructures such as trichomes, stomata, epicuticular ornamentation, and epicuticular wax on the surface of the floral parts.

Material and Methods

Flower material was taken from the plants obtained through convenience field sampling carried out in Peninsular Malaysia. The sample collections were accompanied by a research permit. Herbarium specimens were processed according to the standard herbarium specimen preparation techniques outlined in Bridson and Forman (2000) and deposited in the Herbarium of Universiti Putra Malaysia (UPM). The Voucher and Gene Bank Accession numbers are listed in Table 1. The flowers were dissected and studied based on the illustrations and descriptions from reliable published accounts, including Dransfield *et al*. (1986) and Seidenfaden and Wood (1992) prior to the identification and classification processes. The fresh flower specimens were measured and photographed to assist the botanical drawing as shown in Fig. 1 (H-N). Current accepted names of the orchids were validated through KEW World Checklist of Selected Plant Families (WCSP) (Govaerts, 2019).

Floral-surface micromorphology analysis was carried out using Scanning Electron Microscope (SEM) in Anatomy Lab and Scanning Electroscope Room in the Faculty of Agriculture, UPM, Malaysia. The bracts, spur, dorsal sepal, lateral sepals and petals, labellum, and column were fixed and preserved in Copenhagen solution (70% methylated spirit, 29% water, 1% glycerol) for three days. This was followed by vacuuming in a vacuum chamber at 25ºC in Hg (630 mm) for 3 hr. The samples were post-fixed in 1% aqueous Osmium tetroxide for 2 hr. Dehydration of the samples was carried out in ethanol solution series of 30 min each step in the following concentrations: 50%, 70%, 90%, 95%, and lastly twice in absolute ethanol. Next, the plant samples were subsequently subjected to critical-point drying using liquid Carbon dioxide for 70 min. The dried samples were mounted on stubs using double-sided carbon adhesive tabs and then coated with gold in an auto fine coater. The SEM observation was done under SEM JSM-5610LV. The surface micromorphology of each floral part were then observed under various magnifications and enumerated.

Classification of stomata was made according to Wilkinson (1979) and Carpenter (2005) based on shapes and arrangement of the subsidiary cells. Trichomes were described and classified based on Theobald *et al*. (1979), Adedeji *et al*. (2007), and Angulo and Dematteis (2014). Elaborated terminologies of trichome morphology according to Angulo and Dematteis (2014). Kaushik (1983) has termed them as *Handle cells* because of their peculiar structure. The description and classification steps were made following four steps as per suggested in Theobald *et al*. (1979): 1) examination of the overall surface appearance (indumentum); 2) investigation of the morphology of individual trichomes; 3) study of the trichome complement; and 4) histological description of trichomes (glandular or non-glandular and unicellular or multicellular). The parameters were measured using ruler under a clear magnification and the values obtained were multiplied with the magnification scales. For the individual stomatal parameters, stoma length, and width, a magnification of 350× or higher 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 undergoes changes up to 50% as stomata close (Shope and Mott, 2006). Minimum (Min), Maximum (Max), and Mean values were measured and tabulated in Table 5. Epicuticular ornamentation was described following Piwowarczyk (2015), Ghimire *et al*. (2018), and Kong and Hong (2018), and description on epicuticular waxes was based on Wilkinson (1979).

Results

SEM Analysis on Floral-Surface Micromorphology

Three different features of simple and uniserate (unbranched) trichomes vary in structure, distribution, and number of cells were observed and classified as in Table 2 and illustrated as in Fig. 1 (A-C). Scattered pustular glands and epicuticular waxes were also observed on the floral parts and illustrated as in Fig. 1 (D-G). No non-glandular and branched trichomes were observed on the floral parts of both species, thus, these characters are omitted from further morphological assessment in the current paper. Six features of epicuticular ornamentation were classified as in Table 3.

Corybas selangorensis [Fig. 1 (H-J)]

Neither trichomes nor papillae were observed on the floral parts. Ornamentation of surface of the dorsal sepal

is Type I and V with warty-granulated and variegated flake-like epicuticular waxes and rare type holo-paracytic stomata occurred on the dorsal sepal and bract. The stomata occurred with two different forms: 1) slightly narrow and raised rim with striae and flat guard and subsidiary cells [Fig. 2 (A)]; 2) round, grossly developed and raised rim, raised guard and subsidiary cells, and narrow aperture [Fig. 2 (B)]. No stomata were observed on the lateral petals and sepals, labellum, column, and spur. The surface marking on lateral petals and lateral

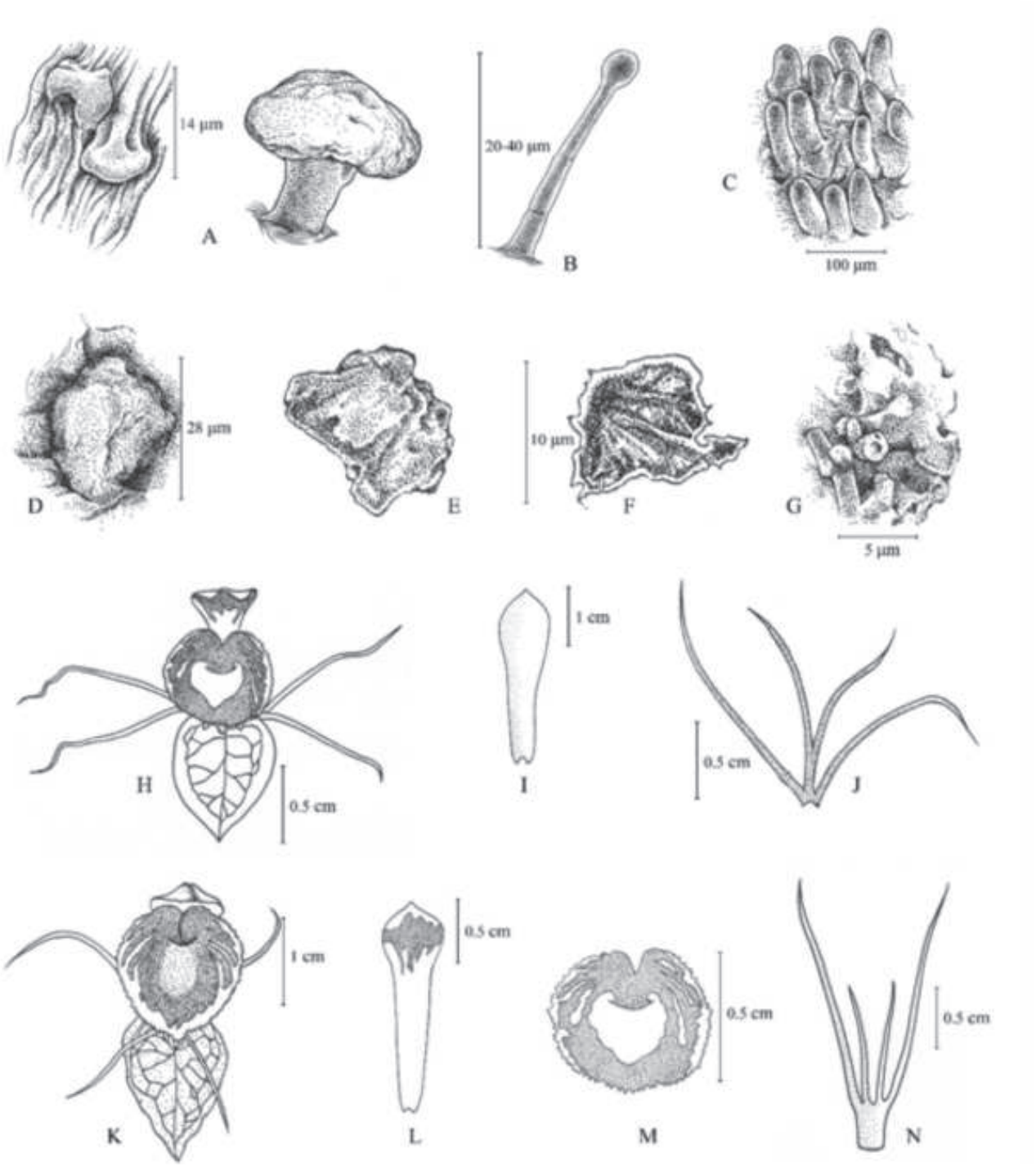

Fig. 1. Botanical illustration of *C. selangorensis* (H-J) and *C. holttumii* (K-N) and the micromorphological characters on the floral's surface (A-G): A, Type I trichomes; B, Type II trichomes; C, Papillae; D, Pustular glands; E, Warty-granulated waxes; F, Flake-like epicuticular waxes; G, Cylindric epicuticular waxes; H, Plant; I, Dorsal sepal; J, Lateral petals and sepals, K, Plant, L, Dorsal sepal, M, Labellum, N, Lateral petals and sepals. (Drawn by Khalid Hashim).

Table 1. List of *Corybas* species from Peninsular Malaysia examined.

Species	Distribution	Type locality	Source	Voucher deposited	Gene bank accession aumber
C. selangorensis	Peninsular Malaysia	Pahang	UPM	LYE002	KC627273.1
C. holttumii	Peninsular Malaysia	Pahang	UPM	LYE003	KC627269.1

sepals is Type VI that made of coarsely cuticular striation and strongly undulate and sinuate anticlinal walls along with warty-granulated waxes [Fig. 2 (F)]. The labellum has Type VI ornamentation [Fig. 2 (J)] on its surface along with several forms of epicuticular waxes like warty-granulated, variegated flake-like and cube-shaped waxes that aggregated into groups. In comparison with *C. holttumii*, the labellum does not possess papillae. The column has Type I [Fig. 2 (H)], V, and IV [Fig. 2 (G)] surface's ornamentation with warty-granulated and variegated flake-like waxes. The spur has also Type VI surface's marking with well-developed cuticular striation as in lateral petals and sepals, labellum and bract, except with warty-granulated and cube-shaped waxes [Fig. 2 (J)]. The ornamentation of bract consists of holo-paracytic stomata with raised peristomatal rims and surrounded by mixed of warty and short-cylindric granulate, and variegated flake-like waxes. Pustular glands [as illustrated in Fig. 1 (D)] were scattered occasionally on dorsal sepal and labellum.

Table 2. Types of simple and uniseriate trichomes on the floral parts *C. selangorensis* and *C. holttumii*, including description on the morphology.

Type	Morphology
I	glandular, unicellular, and sessile stalk, multicellular head, capitate
I I	glandular, multicellular, and long stalk, unicellular head, capitate, papillae pustular to elongated

Corybas holttumii [Fig. 1 (K-N)]

The dorsal sepal has Type I and II capitate glandular trichomes with wrinkled heads [Figs. 1 (A-B), 3 (A)], while the surface cuticle was observed with Type IV ornamentation at the apex, basal, and margin areas [Fig. 3 (B)], and Type V ornamentation occurred conspicuously along the midrib with weakly cuticular striation [Fig. 3 (C)]. Scattered warty-granulated waxes were also observed on the epicuticular surface. There is also an anisocytic stoma surrounded by three cells, one of which is usually smaller than the other two, and partly hidden by surrounding epidermal cells [Fig. 3 (D)]. On the contrary, there are no stomata nor trichomes were observed on the lateral petals and sepals, column, and spur. The epicuticular ornamentation is Type V with warty-granulated and flake-like waxes occurred occasionally on the lateral petals and sepals [Fig. 2 (E-F)]. Type I with warty-granulated waxes and Type V with cube-shaped waxes were observed on the column [Fig. 2 (K)] and on the spur [Fig. 2 (L)], respectively. The bract has Type I capitate glandular trichomes on its surface with Type III ornamentation [Fig. 2 (G)]. In comparison to the other studied floral parts, only labellum has papillae with variegated shapes and irregular from pustular to elongated and tapering forms [Figs. 1 (C), 3 (J)]. The papillae located at the mouth of throat were shorter (30-60 µm), and elongated (80-130 µm) and higher density at the distal part of the labellum. Similarly, its epicuticular ornamentation, where the labellum has Type II, distinctively differs from the other parts [Fig. 3

Table 3. Types of epicuticular ornamentation on the floral parts *C. selangorensis* and *C. holttumii*, including description on the morphology.

Type	Morphology
I	reticulate, concave periclinal wall, channeled, and pitted anticlinal wall
II	reticulate, concave periclinal wall, channeled, and sunken anticlinal wall
III	reticulate, flat periclinal wall, fibrillar, and network-veined anticlinal wall
IV	reticulate, laevigate, and convex periclinal wall, channeled, and pitted anticlinal wall
V	reticulate, elongated rectangular, and convex periclinal wall, channeled, and sunken anticlinal wall
VI	reticulate, elongated rectangular and flat periclinal wall, fibrillar, and striated anticlinal wall

Table 4. Features and distribution of floral-surface micromorphology of *C. selangorensis* and *C. holttumii* observed using SEM.

Species	Floral parts	Trichomes (simple/unbranched) Glandular	Papillae	Stomata	Epicuticular ornamentation	Epicuticular wax			
						Warty	Flake	Cylindric	Cubic
C. selangorensis	Dorsal Sepal	-	-	Holo-paracytic	I, V	+	+	-	-
	Lateral Petals and Sepals	-	-	-	VI	+	-	-	-
	Labellum	-	-	-	VI	+	+	-	+
	Column	-	-	-	I, V, IV	+	+	-	-
	Spur	-	-	-	VI	+		-	+
	Bract	-	-	Holo-paracytic	VI	+	+	+	-
C. holttumii	Dorsal Sepal	I, II	-	Anisocytic	IV, V	+	-	-	-
	Lateral Petals and Sepals	-	-	-	V	+	-	-	-
	Labellum	-	Pustular to elongated and tapering	-	II	+	+	-	-
	Column	-	-	-	I	+	-	-	-
	Spur	-	-	-	V	-	-	-	+
	Bract	I	-	-	III	+	-	-	-

-, absent; +, present.

(H)]. The papillae (48 µm > 115 µm) were also longer than the glandular trichomes (21 µm > 39 µm). Pustular glands [Fig. 1 (D)] were also present sporadically on the sepals and petals, spur, and bract.

Table 4 shows that trichomes including papillae were only observed on *C. holttumii*. Distinguishing common and rare types of stomata occurred on *C. selangorensis* and *C. holttumii*, respectively. Also, different types of prominent cuticular ornamentation were clearly observed on the epidermal surface of all of the studied floral parts of both species. Table 5 and 6 show the measurement values of the trichomes and stomata.

1959; Stevenson *et al*., 2017; Whittaker and Feeny, 1971). The *Handle cells* the term used by Kaushik (1983) for trichomes of the Orchidaceae comprises of an epidermal column cell with debris at its outer end and a *pedestal* at its inner end. They have been regarded to absorb water and nutrients. In *Dendrobium formosum,* the handle cells also bear deciduous unicellular black hair at their outer end. In general, the observations showed a resemblance on the presence of epicuticular waxes and pustular glands.

Nevertheless, throughout the observation using SEM on floral parts of *C. selangorensis* and *C. holttumii*, the

Table 5. Minimum (Min), maximum (Max) and mean values of trichome length or diameter in floral parts of *C. holttumii.*

Floral parts	Type of trichomes	Length (µm)		
		Min	Max	Mean
Dorsal Sepal	Glandular	21.67	38.75	30.21
Labellum	Papillae	48.55	115.30	81.93
Bract	Glandular	16.67	20.00	18.34

Table 6. Length and breadth of stomata in floral parts of *C. selangorensis* and *C. holttumii.*

Species	Floral parts	Type of Stomata	Size of Stomata	
			Length (µm)	Breadth (µm)
C. selangorensis	Dorsal Sepal	Holo-paracytic	39.59	41.25
	Bract	Holo-paracytic	43.28	40.02
C. holttumii	Dorsal Sepal	Anisocytic	30.00	16.67

Discussion

Trichomes are classified as being glandular or non-glandular, based on whether or not they function as secretory structures (Peterson and Vermeer, 1984). Glandular trichomes and stomata function as precursors of volatile components of fragrance (Choi and Kim, 2013; Kesselmeier and Staudt, 1999; Kolalite, 1998), but we postulated the presence of the trichomes and stomata more likely not for fragrance release in the unscented flower of *C. selangorensis* and *C. holttumii*. Presumably, the trichomes and stomata provide a food-reward to ensure pollinators revisit (Stpiczyñska *et al*., 2018). The presence of a particular type of trichomes, on the other hand, can frequently delimit species, genera, or even whole families (Metacalfe and Chalk, 1950). The secretion of secondary metabolites may also regulate defence systems in their interactions with insect herbivores, especially as toxins or repellents, and this has been well established through decades of research (Agrawal and Weber, 2015; Berenbaum, 1995; Fraenkel,

glandular trichomes and papillae were distinctively only occurred on the floral surface of *C. holttumii* but not in *C. selangorensis*. Kaushik (1983) has also given a photomicrographic account of *epidermal papillae* of club shaped cells in the leaf of the orchid *Gastrochilus calceolaris* which also occur in W. Malaysia. There were pustular glands observed on the floral parts resemble subsessile glandular trichomes. The variegated cuticular marking on the floral surfaces offer a diagnostic value to distinguish the studied species, but the degree of use at intergeneric level was not considered further in the current research. The variation of epicuticular waxes distinguish the studied species on the presence of different types of waxes on each floral part, especially the presence of cube-shaped waxes on the labellum and presence of short-cylindric granulate waxes surrounding stomata on the bract of *C. selangorensis*, which were absent in *C. holttumii*. Stomata were only found on the dorsal sepal of *C. holttumii* with a common type; anisocytic, and in comparison, holo-paracytic stomata were observed on the dorsal sepals and

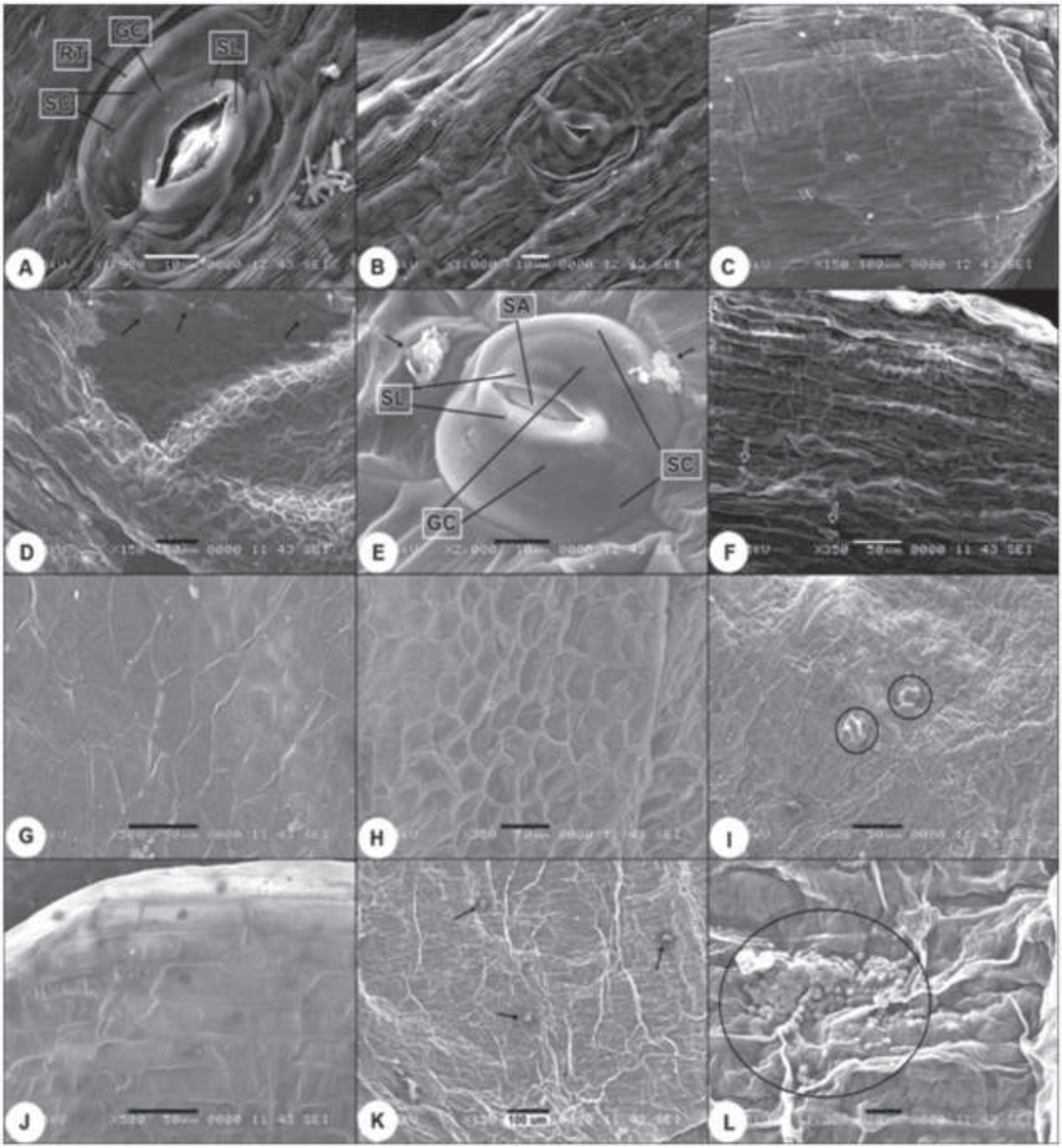

Fig. 2. SEM analysis for bract (A-B), labellum (C-E), lateral petals and sepals (F), column (G-H), labellum (I-J), and spur (K-L) for *C. selangorensis*: A, holo-paracytic (HPS) stoma and short cylindric granulate waxes (arrow); B, distorted holo-paracytic stoma (HPS); C, Type V ornamentation; D, Type I ornamentation; E, another form of holo-paracytic stoma surrounded by warty-granulated waxes (arrows); F, Type VI ornamentation; G, Type IV ornamentation; H, Type I ornamentation; I, pustular glands (circled); J, Type VI ornamentation; K, warty-granulated waxes (arrows); L, clump of cube-shaped waxes (circled). (GC, Guard cell; SL, Stomatal ledge/rim; SC, Subsidiary cell; RT, Radial tangential/walls of subsidiary cells).

bract of *C. selangorensis*. In term of sizes, the stomata on the former species (30 µm × 16. 67 µm) were smaller than the ones observed on the later species (43.28 µm × 40.02 µm) (Table 6) and both types were arranged parallel to the main veins. Based on the general designation of stomatal size provided in

Wilkinson (1979), the stomata of both species are grouped as 'large'. Solereder (1908) strongly emphasized the diagnostic importance of the morphology of the guard cells and of their cuticular ledges. The general appearance of the anisocytic stomata on *C. holttumii* was very similar to

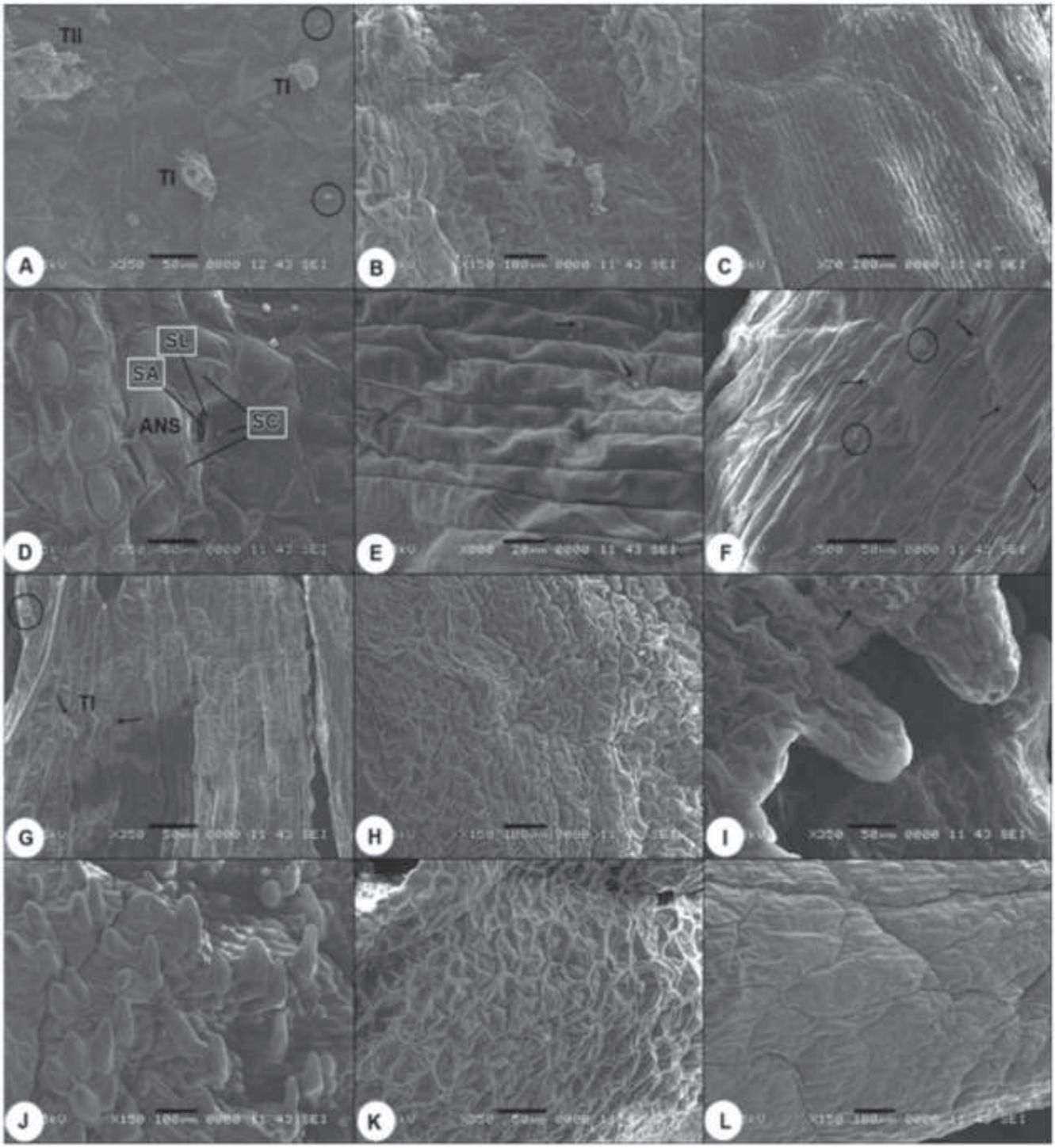

Fig. 3. SEM analysis for dorsal sepal (A-D), lateral petals and sepals (E-F), bract (G), labellum (H-J), column (K), and spur (L) for *C. holttumii*: A, Type I (TI) and II (TII) glandular trichomes, pustular glands (circled); B, Type IV epicuticular ornamentation; C, Type V epicuticular ornamentation; D, Anisocytic stoma (ANS); E -F, Type V ornamentation with warty-granulated (arrows) and flake-like (circled) waxes of the lateral petals and lateral sepals; G, Type III ornamentation with warty-granulated (circled) and Type I (TI) trichomes; H, Type II ornamentation; I, Tooth at the margin with variegated flake-like waxes; J, Pustulate to elongated and tapering papillae at the apex; K, Type I ornamentation; L, Wrinkled surface of the spur with Type V ornamentation. (SL, Stomatal ledges; SA, Stomatal aperture; SC, Subsidiary cells).

nectarostomata. Nectarostomata are cavities where the waxes are exuded through on the cuticular surface. Also, its general outline without a presence of guard cells might indicate abnormal stomata which are known to occur sporadically but widely and are of great diagnostic value in some plant species (Baruah (2017); Chattopadhyay (2014); Pant and Mehra, 1965; Prashanta Kumar and Krishnaswamy, 2014; Verma *et al.,* 2018; Wilkinson, 1979). Anisocytic and holo-paracytic (generally called paracytic) stomata clearly own a distinctive arrangement of subsidiary cells and striae when observed from the top view, especially the rare type holo-paracytic stomata observed in *C. selangorensis* were prominently raised and narrow to round peristomatal rims that provide a unique delimitation key at the species level.

Decisively, the data from this study provided evidence for species-level delimitation of two *Corybas* species belong to the same subsection to an extent based on the presence of glandular trichomes and papillae, and different types of stomata and epicuticular ornamentation. Only *C. holttumii* bears glandular trichomes and papillae on its floral parts and has distinctively different cuticular ornamentation of its surface. Non-glandular trichomes were absent in both the studied species. Also, the presence of abnormal and rare types of stomata is of taxonomic interest in this study which can be used to discriminate the species.

Conclusion

Presence of the glandular trichomes, papillae, and diverse types of stomata on the floral parts were strongly acknowledged to be the distinguishing anatomical characters to differentiate *C. selangorensis* and *C. holttumii*. The glandular trichomes and papillae have clearly not functioned for the fragrance release but may rather be useful for either as defence system or pollination. The presence of rare type holo-paracytic stomata on the dorsal sepal and bract of the *C. selangorensis* may be useful as diagnostic characters for future comparative anatomy study of *Corybas* species in Peninsular Malaysia.

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