BIODIVERSITAS
Volume 23, Number 8, August 2022
Pages: 4159-4168

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d230838

Comparative leaf epidermis study in *Habenaria* spp. (Orchidaceae) from Thailand

NOODUAN MUANGSAN[1,♥], PIYAPORN SAENSOUK[2], THOTSAPORN CHANOKKHUN[1], SANTI WATTHANA[1]

[1]School of Biology, Institute of Science, Suranaree University of Technology. Nakhon Ratchasima 30000, Thailand.
Tel./Fax.: +66-44-223-000, ♥email: nooduan@g.sut.ac.th
[2]Diversity of Family Zingiberaceae and Vascular Plants for Its Applications Research Unit, Mahasarakham University. Mahasarakham 44150, Thailand

Manuscript received: 30 May 2022. Revision accepted: 28 July 2022.

Abstract. *Muangsan N, Saensouk P, Chanokkhun T, Watthana S. 2022. Comparative leaf epidermis study in* Habenaria *spp. (Orchidaceae) from Thailand. Biodiversitas 23: 4159-4168.* The variation of epidermal and stomatal characteristics could be used for plant classification, including orchids. This study aimed to investigate leaf epidermis characters of *Habenaria* from Thailand and focused on the *H. rhodocheila* group and related species. Fourteen accessions belonging to 10 species were studied and compared. Epidermal peels were taken from the upper and lower surfaces of the leaves and were observed under a light microscope. The stomata of all accessions studied were distributed only in the lower epidermis, where they were hypostomatic and anomocytic. The epidermal cell density showed significant differences between the two surface sides. The stomatal size is significantly different among all samples. The highest stomatal index was presented in *H. rostrata* and the lowest on *H. rhodocheila* (red). There is no gap variation of epidermis characteristics in *H. rhodocheila* and its closely related species. It is indicated that the leaf epidermal characters are the shared characters of the genus *Habenaria* from Thailand. *H. rostrata* has tentatively separated from other species based on epidermal anatomical characteristics.

Keywords: Anatomy, epidermis, *Habenaria*, Orchidaceae, stomata

INTRODUCTION

Habenaria Willd. (Orchidaceae) is a large terrestrial orchid genus, belonging to the subfamily Orchidoideae. The genus consists of 928 species, distributed throughout the tropical, subtropical, and temperate regions (Batista et al. 2013; Govaerts et al. 2022). Some species are well known for ornamental purposes and their therapeutic properties (Teoh 2016). The phylogenetic relationship between *Habenaria* and its alliance revealed that it is not monophyletic (Jin et al. 2014, 2017). Kränzlin (1893) proposed 32 sections for intrageneric classification, then Pridgeon et al. (2001) modified Kränzlin's system and proposed 37 sections. *Habenaria* is reported 46 species in Thailand (Kurzweil 2011; Kurzweil et al. 2017). Kurzweil (2009) grouped Thai *Habenaria* based on morphological characteristics into 9 groups. The *H. rhodocheila* group consists of *H. rhodocheila* and *H. carnea* (Kurzweil 2009). Judging from morphological characteristic a recently new species, *H. janellehayneana* should be placed in this group.

The leaf shape of the genus *Habenaria* is very diverse, varying from linear to broadly ovate, lanceolate to elliptic. The leaves arise in the rainy season and bloom in the mid-to-late rainy season. Their flowers are visible and recognizable only for one or two months of the year (Kurzweil 2009). Flowers of Habenarias are mainly white, green, or yellow, except in *H. rhodocheila*, *H. carnea* and *H. janellehayneana* with white, pink, red, orange, or yellow (Kurzweil 2009; Sinumporn et al. 2020). They are usually deciduous plants and have underground tubers.

Foliar epidermal characters, such as epidermal cells, stomata, trichomes, cuticles, and wax have been used as taxonomic and phylogenetic characters across the Orchidaceae (Angela et al. 2015; Rindyastuti et al. 2018; Saensouk and Saensouk 2019). Some studies on the foliar epidermis features at the generic level in orchids have been investigated. Singh et al. (2020) reported that stomatal traits, glandular trichome type, distribution, and cuticular thickening pattern were potentially important for the characterization and differentiation of *Bulbophyllum* species, whereas only cuticular wax characteristics distinguished *Holcoglossum* from related genera (Fan et al. 2014). Darmawati et al. (2018) demonstrated that using a squared Euclidean distance of 34.9% similarity, two *Dendrobium* species were distinguished from other groups based on leaf anatomical features. Pradhan and Bajracharya (2018) found the distribution of stomata on the leaf surface, stomata type, and epidermal cell shape in *Dendrobium* were taxonomically significant. Later, Pradhan and Bajracharya (2020) used cluster analysis with anatomical and micromorphological characters to classify 26 *Dendrobium* species of Nepal into 6 sections. Several reports on leaf epidermal characters of *Habenaria* were demonstrated (Stern 1997; Dangat and Gurav 2016; Verma et al. 2018), but no defined characters among the genera have been reached. More members of this genus need to be done to complement the existing taxonomic information.

The *Habenaria rhodocheila* group shows a variety of leaf morphology such as lanceolate with white spots in *H. carnea* or lanceolate - oblong with entire to undulate margin, sometimes with reddish margin in *H. rhodocheila* (Kurzweil 2009, 2011). Thus, this study aimed to investigate leaf epidermis focused on the *H. rhodocheila* group and compared it with the member of other groups as proposed by Kurzweil (2009). Leaf epidermis characteristics shall be part of finding the taxonomic information in the genus *Habenaria.*

MATERIALS AND METHODS

Plant materials

The terrestrial orchids of 10 different *Habenaria* species were collected from different regions of Thailand during the year 2021 (Figure 1; Table 1). *Habenaria* species were identified with the help of available manuals and floras (Kurzweil 2009, 2011). Fourteen accessions were included in this study.

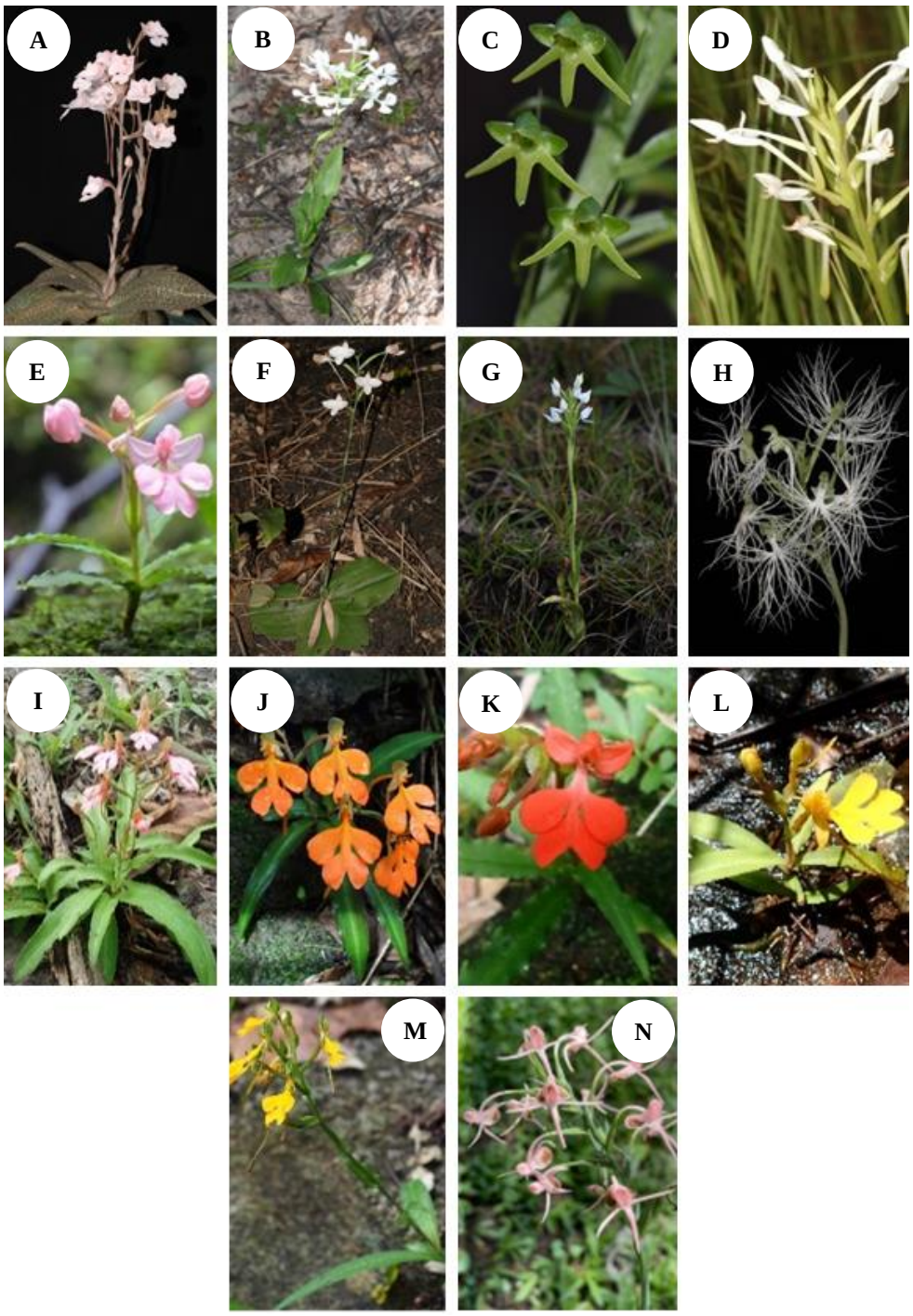

Figure 1. Fourteen accessions of *Habenaria* were used in the study. [A] *H. carnea*, [B] *H. dentata*, [C] *H. furcifera*, [D] *H. hosseusii*, [E] *H. janellehayneana*, [F] *H. lindleyana*, [G] *H. malintana*, [H] *H. myriotrica*, [I] *H. rhodocheila* (pink), [J] *H. rhodocheila* (orange), [K] *H. rhodocheila* (red), [L] *H. rhodocheila* (yellow 1), [M] *H. rhodocheila* (yellow 2), and [N] *H. rostrata*

Table 1. Source of *Habenaria* used in the study

Species	Group*	Locality	Habitat	Alt. (m asl.)	Voucher numbers
H. carnea Weathers, Gard. Chron.	*H. rhodocheila* group	Satun, Manung, Palmpattana	Dry evergreen forest	50	T. Chanokkhun 430
H. dentata (Sw.) Schltr.	*H. dentata* group	Ubon Ratchathani, Sindhorn, Nikhomlamdomnoi	Dry evergreen forest	145	T. Chanokkhun 178
H. furcifera Lindl.	n/a	Ubon Ratchathani Sindhorn, Nikhomlamdomnoi	Dry evergreen forest	145	T. Chanokkhun 195
H. hosseusii Schltr.	*H. hosseusii* group	Sakhon Nakhon, Phu Phan National Park	Mixed deciduous forest	300	T. Chanokkhun 433
H.janellehayneiana Cholteo, Moloney, & Yong Gee	*H. rhodocheila* group	Phitsanulok, Phu Hin Rong Kla National Park	Evergreen forest	1117	T. Chanokkhun 434
H. lindleyana Steud.	n/a	Nakhon Ratchasima, Khao Yai Nation Park	Mixed deciduous forest	250	T. Chanokkhun 435
H. malintana (Blanco) Merr.	*H. dentata* group	Chiang Mai, Mae Rim, Pong Yaeng	Dipterocarp forest	700	T. Chanokkhun 436
H. myriotricha Gagnep.	*H. medioflexa* group	Sakhon Nakhon, Phu Phan National Park	Dipterocarp forest	300	T. Chanokkhun 437
H. rhodocheila Hance)pink(	*H. rhodocheila* group	Sakhon Nakhon, Phu Phan National Park	Mixed deciduous forest	422	T. Chanokkhun 438
H. rhodocheila Hance)orange(	*H. rhodocheila* group	Chaiyaphum, Thep Sathit National Park	Mixed deciduous forest	500	T. Chanokkhun 439
H. rhodocheila Hance)red(	*H. rhodocheila* group	Nakhon Phanom, Phu Lungka National Park	Mixed deciduous forest	150	T. Chanokkhun 440
H. rhodocheila Hance)yellow 1(	*H. rhodocheila* group	Sakhon Nakhon, Phu Phan National Park	Evergreen forest	333	T. Chanokkhun 441
H. rhodocheila Hance)yellow 2(	*H. rhodocheila* group	Chantaburi, Khao Sabab	Evergreen forest	500	T. Chanokkhun 442
H. rostrata Wall. ex Lindl.	*H. rostellifera* group	Amnat Charoen, Phu Sing-Phu Pha Phung	Mixed deciduous forest	200	T. Chanokkhun 196

Note: *: the tentative phylogenetic group was applied by Kurzweil (2009). The species which have not been cited by Kurzweil (2009) are indicated as n/a, except *H. janellehayneana* which can be classified with *H. rhodocheila* group

Procedures

Mature leaves were fixed in FAA fixative (3.7% v/v formaldehyde, 50% ethanol, and 5% acetic acid) and washed with sterile distilled water five times before use. Following Saensouk and Saensouk (2019), epidermal peels were taken from the upper (adaxial) and lower (abaxial) surfaces of the leaves. After that, the specimens were stained with 1% safranin for 10-15 min and dehydrated through an alcohol series: 15%, 30%, 50%, 70%, 95%, and 100%, 5-10 min each, respectively. Then the specimens were submerged in a mixture of pure xylene: absolute ethanol (1:1 v/v) for 10-15 min, followed by pure xylene for 15-20 min, mounted in DePeX, and photographed under the light microscope ZEISS Axiostar plus Lab A1. The number of stomata, the length, and width of the guard cell, type of stomata, epidermal cell shape, number of epidermal cells, crystal type, trichome, gland, and wax ornamentation were recorded.

Data analysis

Ten readings (n=10) were taken per species for correct analysis of the stomatal index (the percentage of the number of stomata to the total number of epidermal cells), the stomatal density (number of stomata per mm^2 observed in all species), and the epidermal cell density (number of epidermal cells per mm^2). All tests were considered significant at $P < 0.05$ and analyses were done using the SPSS package version 20 (SPSS Inc. USA), then performed a post-hoc Duncan's multiple range test (DMRT) to identify specific mean differences. We reported all descriptive statistics as means ± standard error. All specimens examined and slide collections are kept at the Suranaree University of Technology Herbarium, Thailand.

RESULTS AND DISCUSSION

Upper leaf epidermis

The upper epidermal cell shape of 14 accessions of *Habenaria* is polygonal with 4-7 sides on the upper surface (Figure 2). *H. janellehayneana, H. myriotrica,* and *H. rhodocheila* (yellow 1) presented glands on the upper leaf surface (Table 2; Figures 2E, 2H, and 2L). Crystal and trichrome in all accessions were absent. Moreover, two types of cuticular ornamentation, including smooth and striate were recorded (Figure 2; Table 2). The epidermal cell density showed significant differences in most studied species ($P<0.05$), ranging from 33± 2 per mm^2 to 111.90± 27.50 per mm^2 in which *H. janellehayneana* had the maximum density, whereas *H. carnea* (32.80± 2.14 per mm^2) and *H. lindleyana* (34.20±3.25 per mm^2) had the minimum density (Table 2).

Table 2. Upper leaf epidermis of 14 accessions of *Habenaria* was used in the study

Species	Epidermal cell density (number/ mm^2 leaf area(	Crystal	Trichome	Gland	Cuticular ornamentation
H. carnea	32.80±2.14^{h}	-	-	-	St
H. dentata	54.10±15.76^{efg}	-	-	-	St
H. furcifera	91.50±16.79^{bc}	-	-	-	St
H. hosseusii	47.10±10.98^{fg}	-	-	-	St
H. janellehayneana	111.90±27.50^{a}	-	-	+	S, St
H. lindleyana	34.20±3.25^{h}	-	-	-	St
H. malintana	50.10±3.54^{efg}	-	-	-	St
H. myriotrica	58.30±2.49^{def}	-	-	+	S, St
H. rhodocheila)pink(	50.10±6.91^{efg}	-	-	-	St
H. rhodocheila)orange(	66.70±5.59^{d}	-	-	-	St
H. rhodocheila)red(	82.30±11.11^{c}	-	-	-	St
H. rhodocheila)yellow 1(	97.50±9.75^{b}	-	-	+	S
H. rhodocheila)yellow 2(	60.10±12.53^{de}	-	-	-	S
H. rostrata	42.60±4.83^{gh}	-	-	-	S
F-test	*				

Note: S: smooth, St: striate, -: no present, +: present. Means in the same column followed by different letters a-h are significantly different according to Duncan's multiple range test)DMRT(at $P \geq 0.05$, 05; * significant at $P < 0.05$

Lower leaf epidermis

The lower epidermal cell shape of all accessions is polygonal like the upper leaf epidermis)Figures 2 and 3(. *H. furcifera* presents glands on the lower epidermis)Table 3; Figure 3C(. The cuticular membrane ornamentation is smooth and striate, like the upper leaf epidermis. The epidermal cell density of the lower side significantly differed among accessions ($P \leq 0.05$) and ranged from 64.40±3.16 per mm^2 to 240.30±15.22 per mm^2, with *H. rhodocheila*)yellow 1(having the maximum epidermal cell density)240.40 ±15.22 per mm^2(and *H. lindleyana* having the minimum density)64.40 ±3.16 per mm^2( )Table 3(. Like the upper epidermis, there was no crystal or trichome on the lower leaf epidermis.

In all species examined, stomata could only be observed on the lower epidermis of the leaves and have anomocytic type with no evident distinction between the subsidiary and epidermal cells)Table 3; Figure 3(. The length and width of stomata show variations of significant importance in most studied species (significant at $P \leq 0.05$). The stomatal length varied from 45.63±3.16 µm to 67.47±2.13 µm, while stomatal width ranged from 18.43±2.16 µm to 31.46 ±2.37 µm. Maximum stomatal density was observed in *H. rostrata*)56.10±4.97 per mm^2(and the minimum stomatal density was found in *H. carnea*)11.80±1.22 per mm^2(and *H. lindleyana*)13±1 per mm^2(. The stomatal index significantly varied from 10.51±0.82)*H. rhodocheila*)red(to 29.37±1.34)*H. rostrata*( )Table 3(.

Leaf epidermal characters were found to be largely consistent within species or genus. The ranges of the stomatal index (Figure 4C), stomatal density (Figure 4D), and length of guard cell of the lower epidermis (Figure 4F) revealed discontinuous variations that could discriminate *H. rostrata* from other taxa, but other characters showed continuous variation (Figures 4A, 4B, and 4E). Despite flower color and habitat differences, 5 accessions of *H. rhodocheila* group showed continuous variation among various features (Figure 4), except the feature of lower epidermal cell density (Figure 4B) with red and yellow 1 forms having a wide gap.

Discussion

The upper leaf surface possessed lower epidermal cell density less than the lower leaf side in all species due to its smaller-sized cells. The upper epidermis cell density was 1.4-3.1 folds more than the upper leaf surface. This observation of size difference agreed with those of Verma et al. (2018). The larger epidermal cells may help the species adapt to a drier environment better than other species. Similarly, Dangat and Gorav (2016) found a variation in epidermal cell density in 18 species of *Habenaria*, ranging from 66 ± 11 per mm^2 to 194 ± 11 per mm^2. Many orchid species, particularly epiphytic orchids, contain enormous epidermal cells that serve as water storage cells (Angela et al. 2015).

Several types of stomata have been identified in orchids, namely anomocytic, cyclocytic, paracytic, pentacytic and tetracytic (Angela et al. 2015; Yang et al. 2016; Saensouk and Sensouk 2019). Due to their close relatives, our work supports the previous findings that the genus *Habenaria* is mostly hypostomatic and has anomocytic type of stomata (Stern 1997; Dangat and Gurav 2016; Verma et al. 2018; Hegde and Krishnaswamy 2021). *H. digitata*, on the other hand, has amphistomatic leaves with stomata evenly distributed on both sides. This character was found in *Peristylus goodyeroides* (D.Don) Lindl. (synonym *H. goodyeroides* D.Don), a closely related genus to *Habenaria* (Das and Paria 1992). Likewise, there is a record of this occurrence in *Paraphalaenopsis* spp. (Garvita and Wawangningrum 2020). In addition, Verma et al. (2018) discovered the presence of diacytic and twin stomata in *H. plantaginea* and *H. marginata*, respectively. According to Dangat and Gorav (2016), *H. furcifera* in the Western Ghats of India had the highest stomata density (78± 8 per mm^2) and stomatal index (37.0), whereas the stomatal density and stomatal index in our study were 17.90±3.78 per mm^2 and 11.91±1.68%, respectively. This difference in both characters would be due to environmental

variables. Similarly, Hegde and Krishnaswamy (2021) investigated 5 species of *Habenaria*, including *H. crinifera* Lindl., *H. longicorniculata* J. Graham, *H. heyneana* Lindl., *H. elwessi* Hook. f., and *H. multicaudata* Sedgw. found a stomatal density in a range between 36±8 per mm^2 and 57±3 per mm^2, and a stomatal index between 27.1% and 46.8% with *H. elwessi* having the highest indices. According to prior research and our findings, the number of stomata varied from species to species. In many species, the light intensity has been shown to influence the frequency of stomata development on leaves. The stomatal size is related to water deficient conditions. The smaller stomata help to reduce water loss by transpiration (Xu and Zhou 2008).

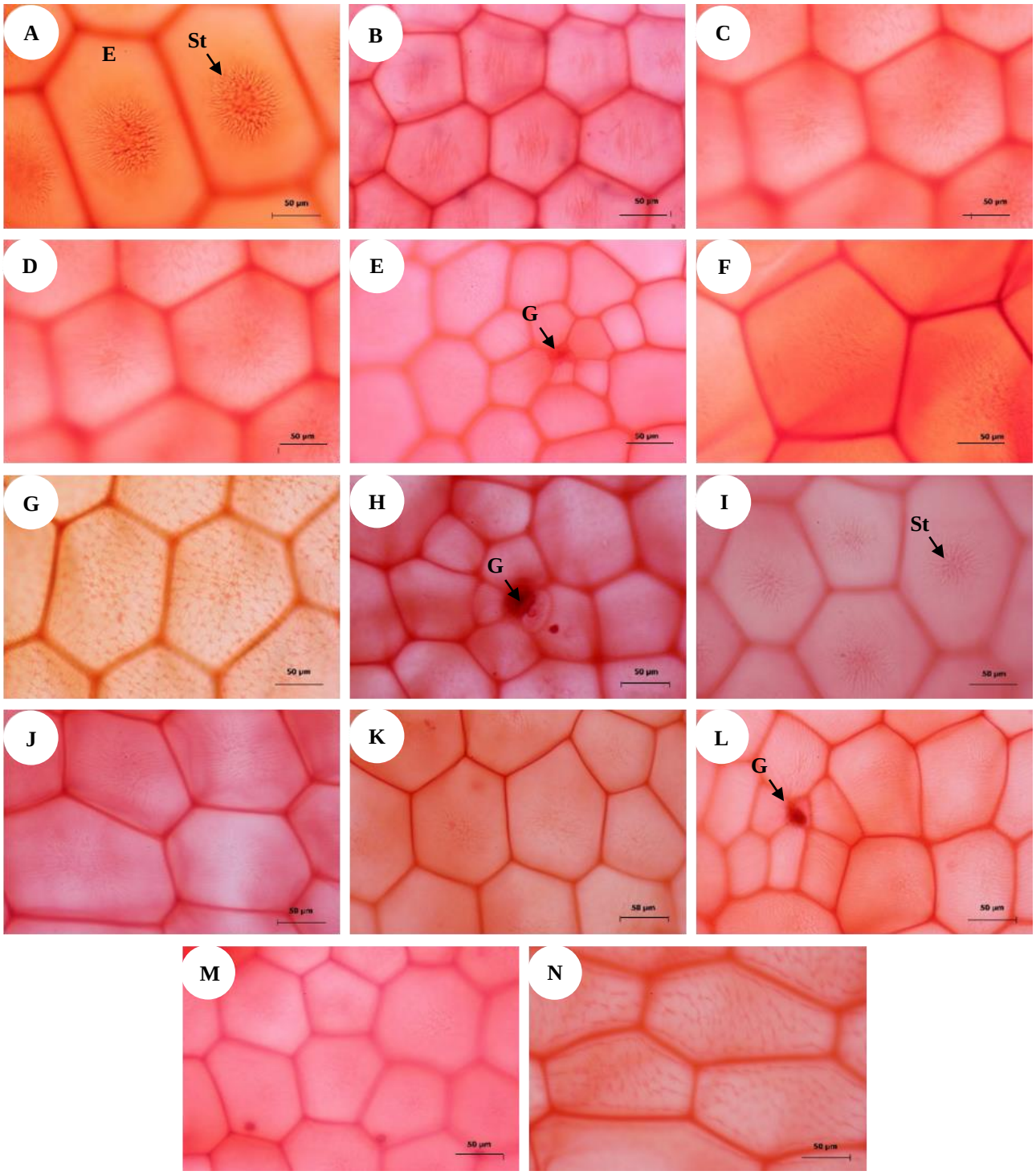

Figure 2. Upper leaf epidermis and stomata of 14 *Habenaria* accessions.]A[*H. carnea*,]B[*H. dentata*,]C[*H. furcifera*,]D[*H. hosseusii*,]E[*H. janellehayneana*,]F[*H. lindleyana*,]G[*H. malintana*,]H[*H. myriotrica*,]I[*H. rhodocheila*)pink(,]J[*H. rhodocheila*)orange(,]K[*H. rhodocheila*)red(,]L[*H. rhodocheila*)yellow 1),]M[*H. rhodocheila*)yellow 2), and]N[*H. rostrata*. E: epidermal cell, G: gland, S: smooth, St: striate, Bar= 50 um

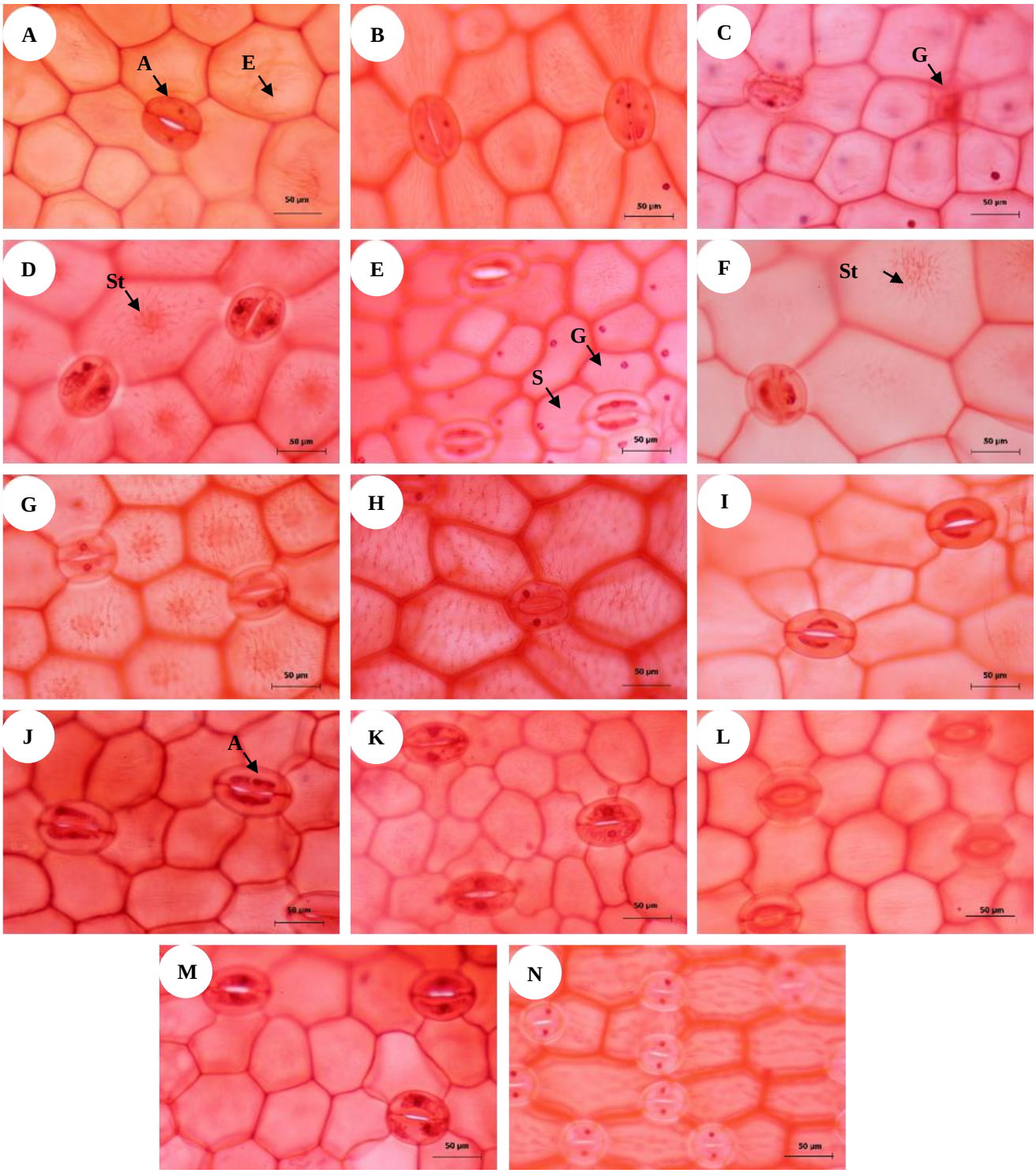

Figure 3. Lower leaf epidermis and stomata of 14 *Habenaria* accessions.]A[*H. carnea*,]B[*H. dentata*,]C[*H. furcifera*,]D[*H. hosseusii*,]E[*H. janellehayneana*,]F[*H. lindleyana*,]G[*H. malintana*,]H[*H. myriotrica*,]I[*H. rhodocheila*)pink(,]J[*H. rhodocheila*)orange(,]K[*H. rhodocheila*)red(,]L[*H. rhodocheila*)yellow 1),]M[*H. rhodocheila*)yellow 2), and]N[*H. rostrata*. A: anomocytic, E: epidermal cell, G: gland, S: smooth cuticle, St: striate cuticle, Bar= 50 um

Table 3. Lower leaf epidermis and stomata of 14 *Habenaria* accessions

Species	Epidermal cell density (number/ mm^2(	Width of guard cell)µm(	Length of guard cell)µm(	Stomatal density number/ mm^2 leaf area(	Stomatal index)%(	Type of stomata	Crystal	Trichome	Gland	Cuticular ornamentation
H. carnea	84.90±13.21^{j}	31.46±2.37^{a}	64.90±5.51^{abc}	11.80±1.22^{h}	12.32±1.20^{g}	A	-	-	-	St
H. dentata	121.70±14.15^{g}	25.53±1.69^{cde}	58.70±4.74^{ef}	29.90±2.84^{bc}	19.80±1.80^{d}	A	-	-	-	St
H. furcifera	132.60±19.95^{ef}	27.39±1.42^{bc}	63.12±3.99^{cd}	17.90±3.78^{g}	11.91±1.68^{gh}	A	-	-	+	St
H. hosseusii	93.90±6.34^{ij}	24.86±2.07ef	57.73±3.97^{f}	32.00±3.97^{b}	25.36±1.73^{b}	A	-	-	-	St
H. janellehayneana	229.20±13.97^{b}	24.44±1.91^{f}	67.19±3.50^{ab}	30.30±2.45^{bc}	11.68±0.75^{ghi}	A	-	-	-	S, St
H. lindleyana	64.40±3.16 ^{k}	30.73±2.04^{a}	63.38±3.94^{bcd}	12.90±1.3^{h}	16.70±1.72^{e}	A	-	-	-	St
H. malintana	109.90±6.65^{h}	25.39±1.83d^{ef}	62.29±4.36^{cde}	27.50±1.71^{cd}	20.04±1.23^{d}	A	-	-	-	St
H. myriotrica	96.30±6.99^{i}	24.04±2.37^{f}	59.66±4.05^{def}	29.50±2.50^{bc}	23.47±1.65^{c}	A	-	-	-	St
H. rhodocheila)pink(	123.80±4.87^{fg}	26.48±2.40^{bcde}	58.42±2.43^{ef}	19.60±2.63^{fg}	13.67±1.73^{f}	A	-	-	-	S, St
H. rhodocheila)orange(	161.20±6.69^{d}	26.90±2.05^{bcd}	63.67±4.57^{abc}	21.40±1.71^{ef}	11.72±0.75^{g}	A	-	-	-	St
H. rhodocheila)red(	225.90±12.87^{b}	22.18±1.76^{g}	67.47±2.13^{ab}	26.60±3.33^{d}	10.51±0.82^{i}	A	-	-	-	St
H. rhodocheila)yellow 1(	240.30±15.22^{a}	18.43±2.16^{h}	59.72±5.11^{def}	31.90±4.22^{b}	11.71±1.27^{ghi}	A	-	-	-	St
H .rhodocheila)yellow 2(	152.00±9.20^{d}	27.64±1.83^{b}	65.78±2.57^{abc}	21.60±2.31^{ef}	12.43±1.07^{g}	A	-	-	-	St
H. rostrata	134.70±7.58^{e}	19.53±2.21^{h}	45.63±3.16^{g}	56.10±4.97^{a}	29.37±1.34^{a}	A	-	-	-	S
F-Test	*	*	*	*	*					

Note: A: anomocytic, S: smooth cuticle, St: striate cuticle, -: no present, +: present. Means in the same column followed by different letters a-k are significantly different according to Duncan's multiple range test)DMRT(at $P \geq 0.05$, * significant at $P < 0.05$

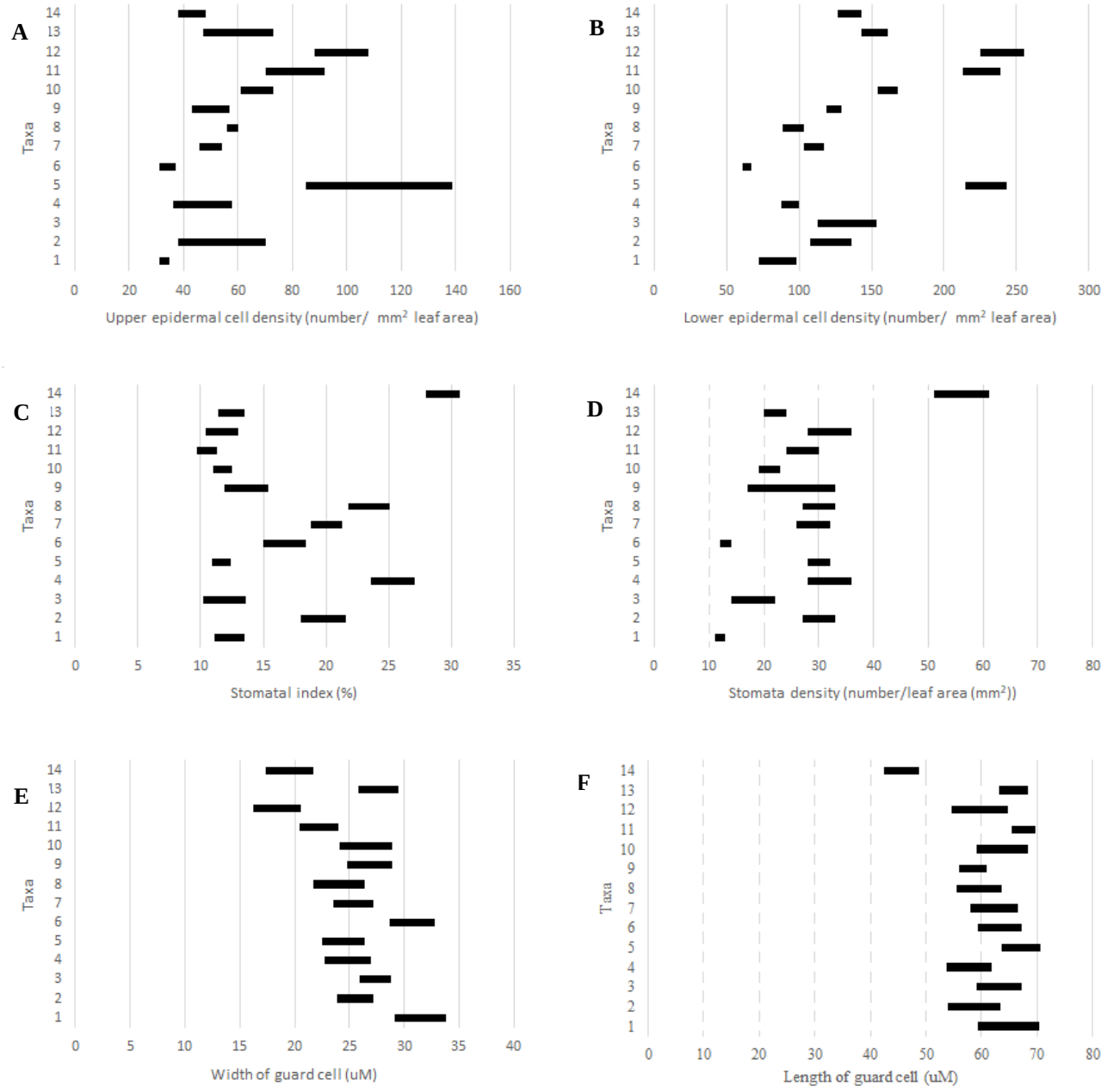

Figure 4. Ranges Summary of leaf epidermis characters of *Habenaria* used in the study: (A) upper epidermal cell density, (B) lower epidermal cell density, (C) stomatal index, (D) stomatal density, (E) width of guard cell, and (F) length of guard cell.]1[*H. carnea*,]2[*H. dentata*,]3[*H. furcifera*,]4[*H. hosseusii*,]5[*H. janellehayneana*,]6[*H. lindleyana*,]7[*H. malintana*,]8[*H. myriotrica*,]9[*H. rhodocheila*)pink(,]10[*H. rhodocheila*)orange(,]11[*H. rhodocheila*)red(,]12[*H. rhodocheila*)yellow 1(,]13[*H. rhodocheila*)yellow 2(, and]14[*H. Rostrata*

Moreira et al. (2013) demonstrated stomatal frequency and cuticle thickness characters coherent with their distinct pattern of the exhibition to sunlight. The leaves of *Epidendrum secundum*grows in more luminous conditions had thicker cuticles and a higher number of stomata per area than those of *Dichaea cogniauxiana* grows in shade and high humidity conditions. The adaption of higher stomatal density was also observed in *Paraphalaenopsis* spp. grown in the greenhouse condition compared to those grown in in vitro condition, regarded as an adaptation to avoid excessive water loss in dry conditions (Garvita and Wawangningrum 2020). Although Verna et al. (2018) found that stomatal density and stomatal index reflected no relationship with plant habitat.

Despite flower color and habitat differences, *H. rhodocheila* showed significantly different among five accessions in most characteristics (Tables 2 and 3) but had continuous variation among various traits (Figure 4). Only *H. rhodocheila* (yellow 1) had glands on the upper leaf surface (Table 2; Figure 2L). As compared to the related group, *H. carnea* and all *H. rhodocheila* groups have greater stomatal density, as well as higher upper and lower

epidermal cell density (Tables 2 and 3). These differences are important in the response of functions of the leaf epidermis to external stimuli.

Habenaria janellehayneana was earlier treated as the same species as *H. rhodochilla* until Choltco et al. (2017) named it a new species. It is distinguished by stigmas that are parallel basally, with convergent apices and the tips touching or nearly touching. In terms of the vegetative part, both *H. rhodocheila* and *H. janellehayneana* have thin leaves and tend to habitat near a waterfall on moss-covered rocks. We observed that *H. janellehayneana* had the highest upper epidermal cell density (111.90±27.50 per mm^2). However, there was no gap variation between these two species in other characters (Figure 4).

Leaf epidermal characters were found to be largely consistent within species or genus. The characteristics of the stomatal index (Figure 4C), stomatal density (Figure 4D), and length of guard cell of the lower epidermis (Figure 4F) revealed discontinuous variations that could discriminate *H. rostrata* from other taxa, while other characters showed continuous variation (Figures 4A, 4B, and 4E). These characters could be supplemented with the data on anatomy to help delimit different *Habenaria* species. Verma et al. (2018) have found a marked variation in stomata frequency and stomata index in 12 species of *Habenaria* sensu lato in Western Himalayan and proposed *H. ensifolia* as a distinct species rather than a synonym of *H. pectinata* based on morphological and foliar micromorphological differences.

All studied specimens were collected in different tentative habitat types, deciduous and evergreen forests (Table 1). There are no separated characters related to habitat types among *H. rhodocheila*, *H. carnea* and *H. janellehayneana* as well as other species which were included in this study (Tables 2 and 3; Figure 4). There is also no unique characteristic of epidermal anatomy related to the elevation. Thus, the leaf epidermis characters may be due to genetic factor rather than environmental factors.

In conclusion, eight of ten *Habenaria* species, namely *H. carnea*, *H. dentata*, *H. hosseusii*, *H. janellehayneana*, *H. lindleyana*, *H. malintana*, *H. myriotrica*, and *H. rostrata* had their leaf epidermal anatomical characteristics recorded for the first time in this study. The stomata of these *Habenaria* species were usually hypostomatic and anomocytic. *H. rhodochiela* and its allied species showed no significant unique character to separate species. Some epidermal characteristics, such as stomatal index, stomatal density, and length of guard cell of lower epidermis could be useful for discriminating *H. rostrata* from other species due to distinct discontinuous variation.

ACKNOWLEDGEMENTS

We thank Suranaree University of Technology for its financial support. We also thank Pornpun Kajornjit and Chuthapond Musimun for their experimental and technical help, Mahasarakham University for providing laboratory facilities, and the Plant Genetic Conservation Project under the Royal Initiation of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG) for cooperation and experimental support. The authors do not have any conflict of interest.

REFERENCES

Angela N, Sharma BH, Chowlu K, Nageswara Rao N. 2015. Foliar anatomy in some species of *Bulbophyllum* Thou. J Orchid Soc India 29: 67-74.

Batista JA, Borges KS, de Faria MW, Proite K, Ramalho AJ, Salazar GA, van den Berg C. 2013. Molecular phylogenetics of the species rich genus *Habenaria* (Orchidaceae) in the New World based on nuclear and plastid DNA sequences. Mol Phylogenet Evol 67 (1): 95-109. DOI: 10.1016/j.ympev.2013.01.008.

Choltco TC, Moloney B, Yong Gee G. 2017. A new *Habenaria* from northern Thailand (Subfamily: Orchidoideae Tribe: Orchideae Subtribe: Orchidinae). Orchideen J 4-5: 1-4.

Dangat BT, Gurav RV. 2016. Stomatal studies in the genus *Habenari*a (Orchidaceae). Richardiana 16: 232-240.

Darmawati I, Nyoman R, Dwiyani R, Astarini I. 2018. Analysis on relationship among *Dendrobium* spp. Bali based on characteristics of leaves anatomy. Intl J Biosci Biotech 5: 111. DOI: 10.24843/IJBB.2018.v05.i02.p03.

Das SJ, Paria N. 1992. Stomatal structure of some Indian orchids with reference to taxonomy. Bangladesh J Bot 21 (1): 65-72.

Fan J, He R, Zhang Y, Jin X. 2014. Systematic significance of leaf epidermal features in *Holcoglossum* (Orchidaceae). PLoS ONE 9 (7): e101557. DOI: 10.1371/journal.pone.0101557.

Garvita RV, Wawangningrum H. 2020. Stomata cells studies of *Paraphalaenopsis* spp. from in vitro and greenhouse condition. Biodiversitas 21: 1116-1121. DOI: 10.13057/biodiv/d210335.

Govaerts R, Bernet P, Kratochvil K, Gerlach G, Carr G, Alrich P, Pridgeon AM, Pfahl J, Campacci MA, Holland Baptista D, Tigges H, Shaw J, Cribb P, George A, Kreuz K, Wood J. 2022. World checklist of Orchidaceae. Facilitated by the Royal Botanic Gardens, Kew. http://wcsp.science.kew.org/.

Hegde SM, Krishnaswamy K. 2021. Measurement of stomatal density stomatal index in some species of terrestrial orchids. Ann Agri Bio Res 26 (2): 173-177.

Jin WT, Jin XH, Schuiteman A, Li DZ, Xiang XG, Huang WC, Li JW, Huang LQ. 2014. Molecular systematic of subtribe Orchidinae and Asian taxa of Habenariinae (Orchideae, Orchidaceae) based on plastid *matK*, *rbcL* and nuclear ITS. Mol Phylogenet Evol 77: 41-53. DOI: 10.1016/j.ympev.2014.04.004 pmid:24747003.

Jin WT, Schuiteman A, Chase MW, Li JW, Chung SW, Hsu TC, Jin XH. 2017. Phylogenetics of subtribe Orchidinae s.l. (Orchidaceae; Orchidoideae) based on seven markers (plastid *matK*, *psaB*, *rbcL*, *trnL-F*, *trnH-psba*, and nuclear nrITS, *Xdh*): implications for generic delimitation. BMC Plant Biol 17 (1): 222. DOI: 10.1186/s12870-017-1160-x pmid:29178835.

Kränzlin F. 1893. Beiträge zu einer Monographie der Gattung *Habenaria* Willd. Bot Jahrb Syst 16: 52-223.

Kurzweil H. 2009. The genus *Habenaria* (Orchidaceae) in Thailand. Thai For Bull, Special issue 37: 7-105.

Kurzweil H. 2011. *Habenaria* Willd. In: Santisuk T, Larsen K. Flora of Thailand. Thailand Prachachon Co. Ltd., Bangkok.

Kurzweil H, Raskoti BN, Watthana S, Pingyot T. 2017. *Habenaria iyoensis* (Orchidaceae), a new record for Thailand and Nepal. Thai For Bull Bot 45 (2): 108-112. DOI: 10.20531/tfb.2017.45.2.05.

Moreira ASFP, de Filho JP, dos Isaias RM. 2013. Structural adaptations of two sympatric epiphytic orchids (Orchidaceae) to a cloudy forest environment in rocky outcrops of Southeast Brazil. Rev Biol Trop 61: 1053-1065. PMID: 24027907.

Pradhan B, Bajracharya D. 2018. Taxonomic significance of stomatal complex in fifteen species of *Dendrobium* Swartz (Orchidaceae) of Nepal. J Nat Hist Mus 30: 28-38. DOI: 10.3126/jnhm.v30i0.27400.

Pradhan B, Bajracharya D. 2020. Anatomical study of *Dendrobium* (Orchidaceae) of Nepal. Ann Plant Sci 9 (7): 3916-3948. DOI: 10.5281/aps.2020.9.7.1.

Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN. 2001. Genera Orchidacearum vol. 2, Orchiddoideae, part 1. Oxford University Press Inc., New York.

Rindyastuti R, Nurfadilah S, Rahadiantoro A, Hapsari L, Abywijaya IK. 2018. Leaf anatomical characters of four epiphytic orchids of Sempu Island, East Java, Indonesia: The importance in identification and ecological adaptation. Biodiversitas 19: 1906-1918. DOI: 13057/biodiv/d190543.

Saensouk S, Saensouk P. 2019. Comparative leaf surfaces of Orchidaceae species from Thailand. Suranaree J Sci Technol 27 (3): 030032(1-8).

Singh M, Verma D, Lavania S, Yerramilli V. 2020. Leaf epidermal features in relation to taxonomy of some species of *Bulbophyllum* (Orchidaceae) from Northeast India. Rheedea 4: 270-277. DOI: .22244/rheedea.2020.30.02.02.

Sinumporn P, NarumiKawasaki T, Fukai S. 2020. Development of interspecific hybrids between *Habenaria radiata* and *Habenaria rhodocheila* complex. Adv Hort Sci 34 (1): 3-10. DOI: 10.13128/ahsc8251.

Stern WL. 1997. Vegetative anatomy of subtribe Habenariinae (Orchidaceae). Bot J Linn Soc 125: 211-27. DOI: 10.1006/bojl.1997.0114.

Teoh ES. 2016. Medicinal Orchids of Asia. Springer International Publishing AG, Cham. DOI: 10.1007/978-3-319-24274-3.

Verma J, Thakur K, Sembi JK, Pathak P. 2018. Leaf micromorphology of some *Habenaria* Willd. sensu lato (Orchidaceae) species from Western Himalaya. J Orchid Soc India 32: 103-112.

Xu Z, Zhou G. 2008. Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. J Exp Bot 59 (12): 3317-3325. DOI:10.1093/jxb/ern185.

Yang SJ, Sun M, Yang QY, Ma RY, Zhang JL, Zhang SB. 2016. Two strategies by epiphytic orchids for maintaining water balance: thick cuticles in leaves and water storage in pseudobulbs. AoB Plants 8: DOI: 10.1093/aobpla/plw046.