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Leaf epidermal features in relation to taxonomy of some species of *Bulbophyllum* (Orchidaceae) from Northeast India

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Abstract: Twelve epiphytic species of the genus *Bulbophyllum* Thouars, including one endemic (*B. sunipia* J.J.Verm., Schuit. & de Vogel), collected from Northeast India (Meghalaya) were investigated for their leaf epidermal features using bright field and scanning electron microscopy (SEM) and hand sectioning. Features such as epidermal cell size, stomata type, stomatal complex size and index, trichome type and density, cuticular thickening pattern, number of subsidiary cells, number of stomatal rims and stomatal ledge aperture (SLA) shape were considered to generate data for their qualitative and quantitative assessment. The stomata in *B. striatum* (Griff.) Rchb.f. were found to be of the tetracyclocytic type, with 4–6 subsidiary cells, and a high density (80.52/mm^2) and highest number of stomatal groups (14–16). *Bulbophyllum cherrapunjeense* Barbhuiya & D.Verma exhibited some distinguishing features *viz*. lowest stomatal density (17.61/mm^2), range of subsidiary cell number (3–5), area occupied by stomata (0.017 mm^2), and maximum glandular trichome density (235.27/mm^2) on the adaxial surface per mm^2 of lamina, and smooth cuticular thickening. Characters like occurrence of stomatal rim, group arrangement of stomata (except in *B. affine* Wall. ex Lindl., *B. cherrapunjeense* and *B. cauliflorum* Hook.f.), epicuticular wax deposition on the surface and presence of scales (multicellular sessile glandular trichome) are reported for the first time for this genus. The study is important for species identification and provides some baseline data for future empirical studies on the taxonomic value of the studied leaf characters.

Keywords: Cuticular thickening, Cuticular wax, Epiphytes, Glandular trichomes, Stomatal ledge aperture (SLA).

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Introduction

Angiosperm leaves are supposed to be the most varied anatomical organs (Metcalfe & Chalk, 1950; Carlquist, 1961; Hickey, 1973). A large number of investigations (Baranova, 1972; Cutler, 1979; Barthlott, 1981; Rindiyastuti *et al.,* 2018) have examined the diagnostic value of leaves. The anatomy of leaves holds huge significance in plant identification and is a valuable taxonomic parameter (Aybeke *et al*., 2010). Leaf epidermal characters have proven important in classification (Stace, 1965, 1984), and have been frequently studied for taxonomic purpose in several taxa (Wang & Tao, 1993; Luo & Zhou, 2001; Shi & Li, 2003; Ren *et al*., 2007). Studies on leaf epidermal characters of the families Polygonaceae in West Africa (Ayodele and Olowokundejo, 2006) and Moraceae (Ogunkunle, 2013), and some species of family Fabaceae by Saheed and Illoh (2010) have been found to be of considerable taxonomic significance. There is abundant recent literature on leaf epidermal attributes that illustrate their taxonomic value among dicotyledons and monocotyledons (Tomlinson, 1961, 1969; Ahmad, 1964; Inamdar, 1970; Gilani *et al.,* 2002; Agbagwa & Okoli, 2006; Adedeji & Jewoola, 2008; Moore *et al.,* 2010; Fan *et al.,* 2014).

Orchidaceae is one of the highly evolved and largest families of the order Asparagales with approximately 25,000 to 35,000 species belonging to 750 to 850 genera distributed worldwide (Dressler, 1981; Chase *et al*., 2005; Saito, 2006;

Hossain, 2011; Seberg *et al*., 2012). The members of family Orchidaceae are found in every biome such as the arctic-circle, semi-desert, semi-arid, aquatic, some are temporarily submerged during periodic flooding, on mangrove trees in estuaries and even adapted to salt spray (Hágsater *et al*., 2005; Jones, 1988). Atwood (1986) estimated that 73% of species of Orchidaceae are epiphytic. The diversity of orchids and their habitat lead to anatomical, morphological and ecological variation (Mehra & Vij, 1974; Rao & Khasim, 1986; 1987; Pridgeon, 1986; Arditti, 1992; Stern & Morris, 1992).

The family Orchidaceae has always been of great interest to taxonomists for elucidating its evolutionary status, distribution, habitat, pollination mechanism, foliar micromorphology, floral morphology and anatomy. However, some consistent lacunae in study of vegetative anatomy are evident from the available literature. The earliest records on orchid anatomy were seen in the work carried out by Chatin (1856), Möbius (1887), Sprenger (1904) and Solereder and Meyer (1930). Williams (1974) stressed the usefulness of anatomical data in orchid taxonomy that led to studies on the anatomy of leaf, stem and root. Such studies of *Dresslerella* Luer (Pridgeon & Norris, 1979) and subtribe Pleurothallidinae Lindl. ex G.Don (Pridgeon, 1982), and on the leaf of *Caladenia* R.Br. (Pridgeon, 1993) and *Dendrobium* Sw. (Carlsward *et al*., 1997), the root and leaf of tribe *Habenariinae* Verm. (Stern, 1997a), and on the root of members of subtribe *Orchidinae* Verm. (Stern, 1997b) are worth mentioning. Subsequently other studies appeared on the comparative vegetative anatomy of various tribes and subtribes of family Orchidaceae *viz*., *Calypsoeae* Dressler, and subtribes Laeliinae Benth., Oncidiinae Benth. & Hook. f., and Vanillinae Meisn. for their systematic and taxonomic significance (Stern & Judd, 2000; Stern & Carlsward, 2006, 2008, 2009). During recent years the anatomical features of the members of Orchidaceae have been used to support their identification and classification (Aybeke *et al*., 2010; Fan *et al*., 2014).

In India, leaf epidermal features of orchids were first studied by Vij *et al*. (1991), while Das and Paria (1992) investigated the stomatal structure of some Indian orchids with reference to taxonomy. The leaf anatomy of epiphytic orchids were studied by Oliveira and Sajo (1999), Arevalo *et al.* (2011). Mulgaonkar (2005) studied the epidermal anatomy of three Indian corticolous orchids and noted the relationship between epidermal characters and environmental conditions.

The Pantropical orchid genus *Bulbophyllum* Thouars belongs to the subfamily Epidendroideae Kostel., tribe *Dendrobieae* Lindl. ex Endl., subtribe *Bulbophyllinae* Schltr. It is estimated to contain 1,700 species (Dressler, 1993; Fischer *et al*., 2007; Sieder *et al*., 2007), and as such is considered as one of the largest genera among all vascular plants. It is one of the most important genera of the family for its diversity, different growth forms and morphological and anatomical characters. (Vermeulen, 1991; Dressler, 1993; Smidt *et al.,* 2011). It comprises nearly 5% of all orchids, with about 100 species reported from India of which 63 species are from the Northeast (Augustine *et al.,* 2001; Misra, 2007). Sehgal and Mehra (1984) studied the distribution pattern of orchids in Khasi and Jaintia Hills of Meghalaya state, and Kataki (1986) reported 28 species of *Bulbophyllum* from this state.

Much work has been done on the floral anatomy of *Bulbophyllum* reflecting their use as diagnostic characters in systematics (Blanco *et al*., 2013; Davies & Stpiczynska, 2014; Nunes *et al*., 2014). However, the literature on the anatomy of vegetative parts such as root, bulb and leaf of the genus *Bulbophyllum* is meager necessitating the need for further exploration. The first record on root anatomy of *Bulbophyllum* including *B. careyanum* (Hook.) Spreng. dates back to 1856 when Chatin examined the structure and function of the root in several orchids. Such earlier investigations reported mostly

the root anatomy – as cited in *Genera Orchidacaerum* by Pridgeon *et al.* (2014) – while the leaf anatomical descriptions of several *Bulbophyllum* species were published by Möbius (1887), followed by leaf anatomical studies on some species of the genus (Tominski, 1905; Oliver, 1930; Pridgeon *et al.,* 2014). Kaushik (1983) investigated the anatomy of petiole, pseudobulb, rhizome and root including leaf blade of *B. reptans* (Lindl.) Lindl. ex Wall.. Rao and Khasim (1987) studied the anatomy of three species of *Bulbophyllum.* Piazza *et al.* (2015) studied the anatomy of leaf, pseudobulb and root of 13 species of *Bulbophyllum* to ascertain the importance of anatomical features in systematics. Muthukumar and Shenbagam (2018) worked on the leaf, root and pseudobulb anatomy of *B. sterile* (Lam.) Suresh.

Seemingly, anatomical and morphological investigations have been carried out on many species of *Bulbophyllum* in different parts of the world. However, study on the anatomy and epidermal micromorphology on *Bulbophyllum* species in India has largely remained unexplored. Therefore, the present investigation was undertaken for developing morpho-anatomical data based on epidermal features to facilitate elucidation of interspecific taxonomic affinities in future studies.

Materials and Methods

Collection and identification of samples

Twelve epiphytic species of *Bulbophyllum* were collected from different localities from their natural environment in Meghalaya State, India (Table 1). The taxonomic identities of the plants were determined with the help of herbarium specimens available at the Botanical Survey of India, Shillong (Meghalaya) and the World Checklist of the Royal Botanic Gardens, Kew (https://wcsp.science.kew.org/qsearch.do). A detailed study of the leaf epidermal micromorphology of each taxon was carried out. The epidermal characters were examined as suggested by Metcalfe (1960) for grasses and Williams (1975) for orchids. The terminology for the description of leaf epidermal characters was adopted from Metcalfe (1961), Stace (1965), Fahn (1979) and Baranova (1992).

Preparation for light microscopic examinations

Leaves of the studied species are simple with a basal pseudobulb, petiolate, sub-sessile or sessile,

Table 1. Taxa, distribution and altitude of species of *Bulbophyllum* used in the present study

S.No.	Taxon	Collection site	Altitude (m)
1.	*B. affine* Wall. ex Lindl.	Mukhaialong Community Reserve, Jaintia Hills, Meghalaya	100–1800
2.	*B. ambrosia* (Hance) Schltr.	Planted at Botanical Survey of India, Eastern Regional Centre Garden, Shillong (collected from Mizoram)	300–1300
3.	*B. bisetum* Lindl.	Cherapunjee, Khasi Hills, Meghalaya	1500–2000
4.	*B. cauliflorum* Hook.f.	Chyrmang Community Reserve, Jaintia Hills, Meghalaya	600–2000
5.	*B. cherrapunjeense* Barbhuiya & D.Verma	Cherapunjee, Khasi Hills, Meghalaya	1460
6.	*B. gymnopus* Hook.f.	Chyrmang Community Reserve, Jaintia Hills, Meghalaya	600–2000
7.	*B. leopardinum* (Wall.) Lindl. ex Wall.	Mukhaialong Community Reserve, Jaintia Hills, Meghalaya	1300–3300
8.	*B. pteroglossum* Schltr.	Upper Shillong, Khasi Hills, Meghalaya	1000–2500
9.	*B. reptans* (Lindl.) Lindl. ex Wall.	Tuber Community Reserve, Jaintia Hills, Meghalaya	300–1600
10.	*B. striatum* (Griff.) Rchb.f.	Tuber Community Reserve, Jaintia Hills, Meghalaya	1500–2330
11.	*B. sunipia* J.J.Verm., Schuit. & de Vogel	Chyrmang Community Reserve, Jaintia Hills, Meghalaya	900–2300
12.	*B. umbellatum* Lindl.	Upper Shillong, Khasi Hills, Meghalaya	1000–2200

succulent or less succulent. They are sessile in *B. cherrapunjeense* and *B. cauliflorum*. The leaf shape varied from oblong to linear oblong, narrowly oblong to elliptic oblong. The leaf apex is mostly obtuse or less frequently acute with a notched tip.

Fresh mature leaves were fixed in FAA prepared with 50% ethanol then preserved in 70% ethanol according to Johansen (1940). The samples for bright field microscopy were taken from the least variable region of the leaf *i.e.*, the mid-lamina as suggested by Wilkinson (1979). Epidermal peels were removed using a razor blade as described by Johansen (1940), thereafter scraped from the inner side to leave only the epidermis by removing undesired cell layers. Glycerine mounts of hand cut leaf transverse sections and epidermal peels were stained with 1% safranin (prepared in 70% alcohol) for micromorphological examinations. Photomicrographs were taken under daylight without using filters under a Nikon-H600L microscope (Tokyo, Japan) at desired magnifications. Ten samples per individual were collected and three individuals were sampled for each species.

Sample preparation for scanning electron microscopic examinations

To examine the ultra-micromorphology of the leaf epidermis, scanning electron microscopy (SEM) was used. A piece of < 0.5 cm^2 leaf lamina from the central lamina region was taken. Sample pieces were washed and shade dried for 60 days. The dried samples were placed on metallic stubs with the help of double-sided adhesive tape keeping exposed the surface to be observed. The samples were coated with a Platinum Sputter Coater (JEOL, JFC 1600, Auto Fine Coater, Tokyo, Japan) and scanned using a JEOL JSM 6490 LV (Tokyo, Japan) scanning electron microscope. Photomicrographs were taken at desired magnifications.

Data generation and analysis

Numerical data were obtained by recording the number of ocular divisions for measuring cellular dimensions (*i.e.*, length and breadth), and for the number / density of epidermal surface structures such as stomata, epidermal cells, trichomes and scales. All numerical length data were converted to measurable units after calibration with a stage micrometer (the smallest count of the stage micrometer was 0.01 mm). At least 10 observations were recorded for each parameter. The results were presented as means ± standard error of the mean (SE). Statistical analyses were carried out using the analysis ToolPak in Microsoft Excel to calculate correlation coefficients (r^2) on the means to deduce interactions between pairs of epidermal characters.

Results

All observations on micromorphological characters and quantitative assessments of epidermal surface features are listed in Tables 2 & 3 and illustrated in Figs. 1, 2, 3 & 4. The means of quantitative epidermal characters such as epidermal cell density, stomatal index, glandular trichome density and size of the stomatal complex varied greatly (Table 3). A positive correlation was found between stomatal density and epidermal cell density (r^2 = 0.5483, P<0.01), glandular trichome density of both abaxial (r^2 = 0.5828, P<0.01) and adaxial (r^2 = 0.5639, P<0.01) surfaces and the stomatal index (r^2 = 0.5828, P<0.01), and a negative correlation between stomatal complex size and glandular trichome density of the abaxial surface (r^2 = 0.4087, P<0.025).

Epidermal cells: Epidermal cells on both leaf surfaces were polygonal (Figs. 1a-k, 2a-k), except for *B. reptans* where the cells were rectangular to polygonal arranged in a parallel manner (Fig. 1 & 2). The epidermal cells on the adaxial surface were straight walled except in *B. reptans* where the anticlinal wall curved / arched (Fig. 2). Cells on the abaxial surface of *B. reptans* were axially elongated so their length was more than the breadth, while those on the adaxial surface had a virtually equal length and breadth (Fig. 1l). The cell wall of abaxial epidermal cells in all species was non-sinuous to arched and thick walled (Figs. 1 & 2). The epidermal cell walls on both surfaces had pit connections. The ratio of abaxial : adaxial

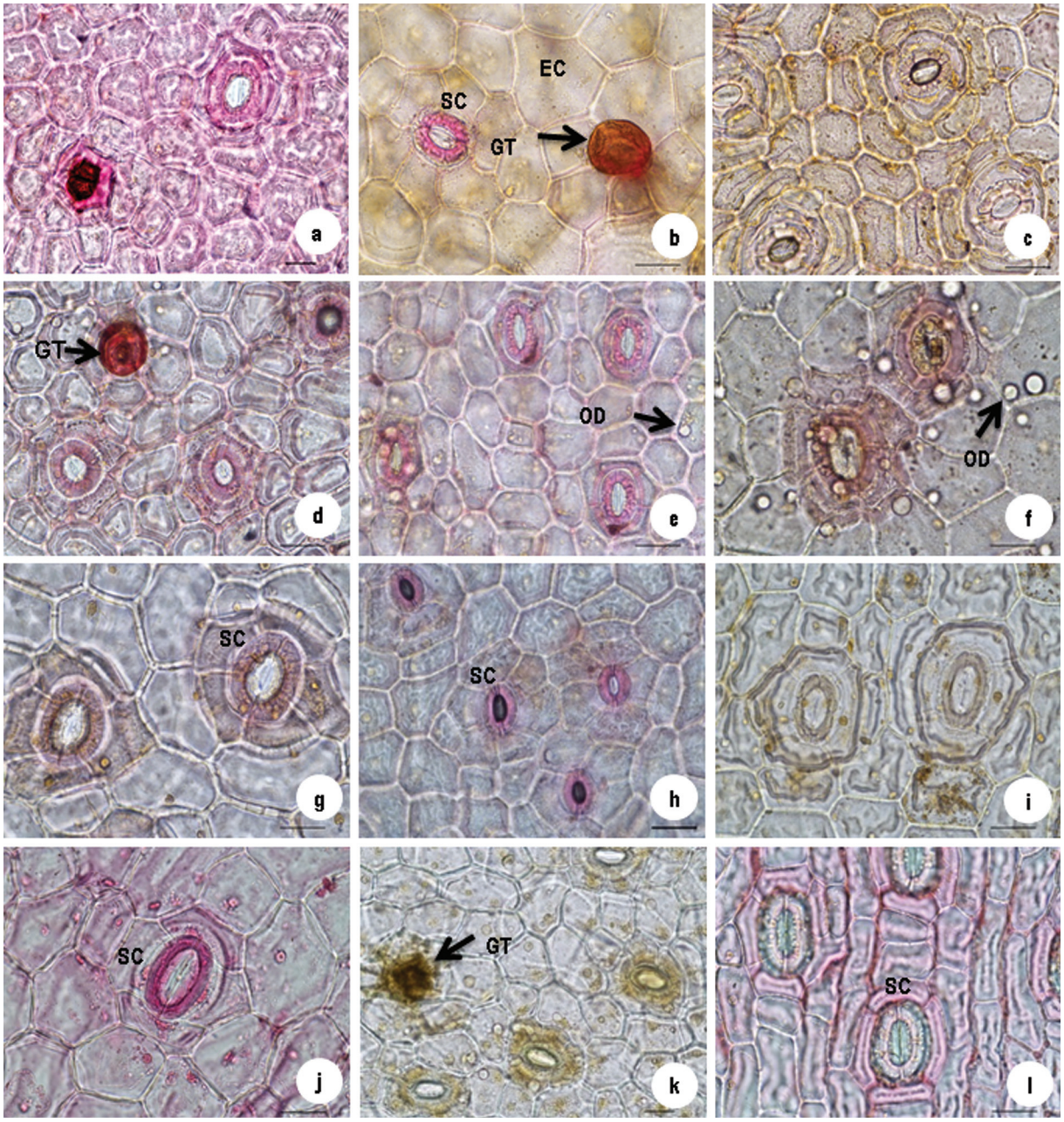

Fig. 1. Photomicrographs of *Bulbophyllum* Thouars. species (at 400×) showing variation in leaf (abaxial) epidermal characters epidermal cell shape and stomatal rim: **a.** Large epidermal cells (EC) with cuticular thickening and stoma of *B. affine* Lindl.; **b.** Small round stoma, subsidiary cell (SC), and glandular trichomes (GT) of *B. cherrapunjeense* Barbhuiya & D.Verma; **c.** EC arrangement pattern and large stomata of *B. cauliflorum* Hook.f.; **d.** Small EC showing cuticular thickening along the inner wall of EC, small round stomata of *B. ambrosia* (Hance) Schltr.; **e.** EC and stoma with rim of *B. gymnopus* Hook.f.; **f.** Thin walled EC and round stoma with 4–5 subsidiary cells (SC) of *B. sunipia* J. Verm., Schuit. & de Vogel; **g.** Large, thick walled EC, large stomatal complex with 4–5 SC of. *B. striatum* (Griff.) Rchb.f.; **h.** Thin walled EC and small round stoma with 4–6 SC and cuticular thickening on the surface of *B. leopardinum* (Wall.) Lindl. ex. Wall.; **i.** Large EC with cuticular thickening and stomata of *B. bisetum*; **j.** Large thin walled EC and stoma with 4 SC of *B. pteroglossum* Schltr.; **k.** EC, stomata and GT of *B. umbellatum* Lindl.; **l.** EC arranged in row and stomata with 4–5 SC. Note the cuticular thickening on EC of *B. reptans* (Lindl.) Lindl. ex. Wall. EC – epidermal cell; GT – glandular trichome; OD – oil droplet; SC – subsidiary cell.

epidermal cell size, as recorded from leaf transverse sections was found to be >1.5 in *B. affine*, *B. ambrosia* and *B. umbellatum*, around 1 in *B. bisetum*, *B. cherrapunjeense*, *B. gymnopus* and *B. sunipia*, <0.77 in *B. cauliflorum*, *B. pteroglossum* and *B. striatum*, and lowest in *B. leopardinum* (0.471) and *B. reptans* (0.534) (Table 3). The epidermal cell density was much higher on the abaxial surface than on the

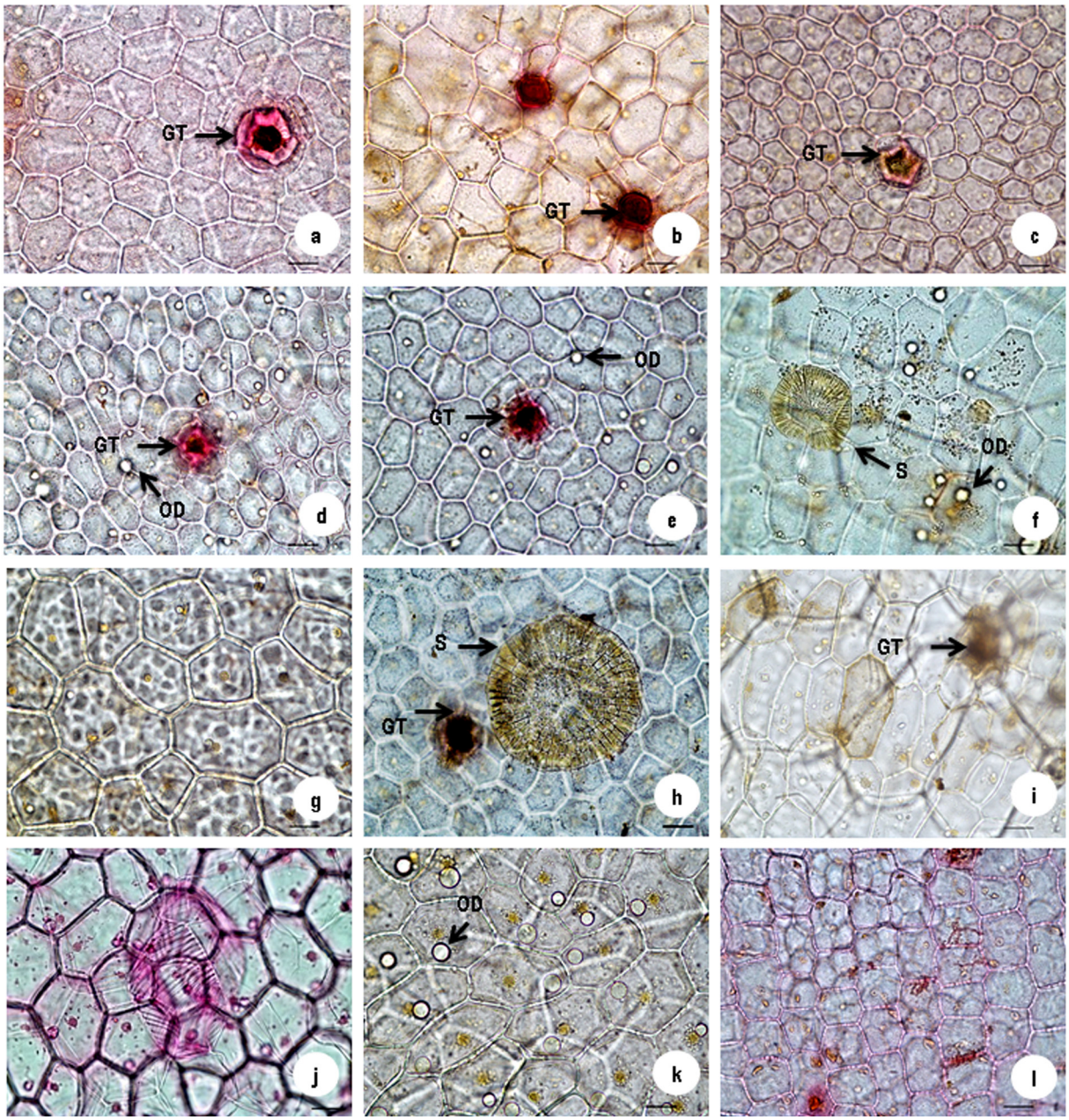

Fig. 2. Photomicrographs of *Bulbophyllum* Thouars. species (at 400×) showing variation in leaf (adaxial) epidermal characters: **a–e, h & i**. Large thick-walled polygonal (epidermal cells) EC and glandular trichomes (GT) of **a.** *B. affine* Wall. ex. Lindl., **b.** *B. cherrapunjeense* Barbhuiya & D.Verma, **c.** *B. cauliflorum* Hook.f., **d.** *B. ambrosia* (Hance) Schltr., **e.** *B. gymnopus* Hook.f., **h.** *B. sunipia* Verm., Schuit. & de Vogel and **i.** *B. leopardinum* (Wall.) Lindl. ex. Wall. respectively. **d-f & h.** Oil droplets (OD) in EC of *B. ambrosia* (Hance) Schltr., *B. gymnopus* Hook.f., *B. sunipia* Verm., Schuit. & de Vogel and *B. umbellatum* Lindl. **f & h.** multicellular sessile GT (scale) of **f.** *B. sunipia* Verm., Schuit. & de Vogel and **h.** *B. leopardinum* (Wall.) Lindl. ex. Wall. respectively. **g, j & k.** Large polygonal EC of *B. striatum* (Griff.) Rchb.f., *B. bisetum* Lindl. and *B. umbellatum* Lindl. respectively. **l.** EC arranged in uniseriate rows square shaped. EC – epidermal cell; GT – glandular trichome; OD – oil droplet; S – scale/ sessile trichome.

adaxial leaf surface, except in *B. ambrosia* (Table 3). Oil droplets were observed in epidermal cells of *B. ambrosia, B. gymnopus, B. sunipia* and *B. umbellatum* (Figs. 1e, f & 2d-f, k).

Stomata: The leaves of all species studied were hypostomatic, *i.e.* stomata were confined to the abaxial surface (Fig. 1a-l). The stomata were of the tetracytic type in all species except in *B. striatum* where they were of the tetracyclocytic type (Table 2). The number of subsidiary cells was variable

Table 2. Qualitative characters of leaf stomata and other surface features in 12 species of *Bulbophyllum* from Northeast India

S. No.	Taxon	Stomata type	Number of SC	No. of stomatal groups in FoV	Twin stomata in FoV	Number of SR+PSR	SLA shape	Sub-stomatal chamber	Scale (multicellular sessile GT)	CT pattern
1.	*B. affine*	Tetracytic	4	1-2	-	1+1	elliptical	very large	-	papillose
2.	*B. ambrosia*	Tetracytic	4-6	-	1-2	1+1	round[a]	small	+	papillose
3.	*B. bisetum*	Tetracytic	4	7-11	1-2	2+1	elliptical[a]	small	-	papillose$_{ab}$
3.	*B. cauliflorum*	Tetracytic	4-5	9-13	-	1+1	elliptical[a]	large	-	papillose$_{ab}$
5.	*B. cherrapunjeense*	Tetracytic	3-5	-	-	1+0	round	large	-	smooth
6.	*B. gymnopus*	Tetracytic	4-6	2-3	1-3	1+2	elliptical[a]	large	-	papillose$_{ab}$
7.	*B. leopardinum*	Tetracytic	4-6	11-14	1-2	1+1	round[a]	small	++	papillose
8.	*B. pteroglossum*	Tetracytic	4-5	1-2	1-3	1+1	elliptical	large	-	papillose$_{ab}$
9.	*B. reptans*	Tetracytic	4-5	7-15	1-2	1+1	elliptical	very small	++	papillose$_{ab}$
10	*B. striatum*	Tetracyclocytic	4-6	14-16	2-7	1+1	elliptical	very large	-	papillose
11.	*B. sunipia*	Tetracytic	4-5	0-1	2-3	1+1	round	small	++	papillose$_{ab}$
12.	*B. umbellatum*	Tetracytic	4-5	3-5	1	1+1	elliptical	large	-	papillose$_{ab}$

a – narrow; + – present in low frequency; ++ – present in high frequency; - – absent; ab – abaxial, CT – cuticular thickening; FoV – objective's field of view at 100× magnification; GT – glandular trichome; PSR – peristomatal rim; SC – subsidiary cells; SLA – stomatal ledge aperture shape; SR – stomatal rim; the number of observations was between 5 to 10 for each parameter, except for 'number of SR+PSR' and 'SLA shape' where it was 1 or 2.

Table 3. Quantitative characters of leaf surface in 12 species of *Bulbophyllum* from Northeast India as means (* with standard errors)

S. No.	Taxon	Stomata density* (St/mm^2)± SE	Stomatal complex size* (µm^2) ± SE	Stomatal index	Area occupied by St/mm^2 of leaf	EC size ratio Ab: Ad	EC$_{ab}$ density* (EC/mm^2)	EC$_{ad}$ density* (EC/mm^2)	GT$_{ad}$ density* (GT/mm^2)	GT$_{ab}$ density* (GT/mm^2)
1.	*B. affine*	23.9 ± 2.78	961.54 ± 22.6	3.3	0.022	1.524	708.33 ± 16.8	636.6 ± 11.5	113.23 ± 2.3	216.40 ± 2.9
2.	*B. ambrosia*	51.58 ± 2.14	616.05 ± 28.8	3.55	0.031	1.756	1406.6 ± 47.17	1427.98 ± 19.84	192.49 ± 2.3	281.82 ± 2.3
3.	*B. bisetum*	54.10 ± 5.05	1106.15 ± 44.8	5.845	0.059	1.097	873.15 ± 19.11	460.47 ± 9.11	12.58 ± 1.9	26.42 ± 1.9
4.	*B. cauliflorum*	88.07 ± 4.36	1003.47 ± 28.1	5.18	0.088	0.719	1611.68 ± 28.62	1562.60 ± 17.86	148.46 ± 2.3	184.94 ± 3.1
5.	*B. cherrapunjeense*	17.61 ± 3.18	965.14 ± 25.8	2.422	0.017	0.904	695.75 ± 17.35	574.96 ± 12.40	235.27 ± 1.9	211.38 ± 3.4
6.	*B. gymnopus*	76.74 ± 4.52	534.45 ± 30.4	4.07	0.041	0.966	1804.17 ± 27.99	1024.12 ± 26.30	142.17 ± 3.1	362.34 ± 2.5
7.	*B. leopardinum*	84.29 ± 4.00	436.71 ± 15.3	5.33	0.036	0.471	1499.70 ± 37.04	714.62 ± 11.37	65.42 ± 2.5	122.03 ± 1.9
8.	*B. pteroglossum*	33.96 ± 3.10	1277.27 ± 53.3	5.33	0.043	0.767	598.87 ± 8.93	352.27 ± 7.34	44.03 ± 2.3	119.52 ± 1.9
9.	*B. reptans*	74.23 ± 2.78	1329.29 ± 83.1	5.36	0.098	0.534	1309.72 ± 11.32	700.78 ± 17.98	12.58 ± 2.5	152.23 ± 2.5
10.	*B. striatum*	80.52 ± 6.94	1379.95 ± 26.6	9.31	0.110	0.632	783.82 ± 20.53	261.68 ± 9.04	15.09 ± 1.9	37.74 ± 1.8
11.	*B. sunipia*	41.51 ± 3.10	1397.74 ± 77.6	6.02	0.058	1.026	647.94 ± 9.46	412.66 ± 12.69	47.80 ± 1.9	123.29 ± 1.9
12.	*B. umbellatum*	49.06 ± 3.30	1327.93 ± 28.7	5.98	0.065	1.871	773.75 ± 15.54	335.56 ± 7.02	25.16 ± 3.0	101.90 ± 1.8

EC – epidermal cell; EC$_{ab}$ – epidermal cell abaxial; EC$_{ad}$ – epidermal cell adaxial; GT$_{ab}$ – glandular trichome abaxial; GT$_{ad}$ – glandular trichome adaxial; St – stomata; the number of observations was 10 for each parameter.

ranging from 3-6. Four subsidiary cells were observed in *B. affine* and *B. bisetum.* In others their number was variable. *B. cherrapunjeense* had 3–5 subsidiary cells and 4–5 were observed in *B. cauliflorum, B. sunipia, B. pteroglossum, B. umbellatum* and *B. reptans*, while for the rest of the taxa *viz. B. ambrosia*, *B. gymnopus, B. striatum* and *B. leopardinum* the number of subsidiary cells was 4–6 (Table 2).

The stomata and epidermal cells were at the same level, the sub-stomatal chambers were irregular in shape and variable in size in most species. The sub-stomatal chamber in *B. affine* and *B. striatum* was very large. *Bulbophyllum cherrapunjeense, B. cauliflorum, B. pteroglossum, B. gymnopus* and *B. umbellatum* had chamber sizes almost equal or slightly larger than the adjacent hypodermal cells. They were small in *B. leopardinum* while smallest in *B. reptans*. The distribution of stomata was non-uniform over the lamina and often distributed in groups (clusters) while they were distributed evenly in *B. cherrapunjeense* and *B. ambrosia*. The density of such stomatal groups per 0.079 mm^2 of leaf area (*i.e.* the objective's field of view) ranged from 0–1 in *B. sunipia,* 1–2 in *B. affine*, *B. pteroglossum*, and 2–3 in *B. gymnopus*. The frequency of stomatal groups was distinctly high in *B. striatum* (14–16), *B. leopardinum* (11–14), *B. cauliflorum* (9–13) and *B. reptans* (7–15). Stomata that occurred in pairs, termed 'twin stomata', were not seen in *B. affine*, *B. cherrapunjeense* and *B cauliflorum.* The average range of twin stomata in an objective's field of view was smallest (1) in *B. umbellatum* and larget (2–7) in *B. striatum*, followed by *B. sunipia* (2–3). In an objective's field of view, species like *B. ambrosia*, *B. leopardinum, B. bisetum* and *B. reptans* showed 1–2 twin stomata, but *B. gymnopus* and *B. pteroglossum* had 1–3 (Table 2). The stomatal density ranged between 17.61 and 88.07/mm^2, and was lowest in *B. cherrapunjeense* and highest in *B. cauliflorum*, followed by *B. leopardinum* (84.29/mm^2) and *B. striatum* (80.52/mm^2) (Table 3).

The correlation coefficient values between the pairs of different surface features like the density of stomata with epidermal cell (r^2 = 0.5483, P<0.01), density of stomata with area occupied by the stomata per unit area of leaf (r^2 = 0.4114, P<0.025), and stomatal index with trichome density (adaxial and abaxial respectively r^2 = 0.5639, r^2 = 0.5828, P<0.01) indicated significant correlations between these characters across the species. The stomatal index (SI) of *B. striatum* was highest (9.31) followed by *B. sunipia* (6.02), and *B. umbellatum* (5.98), whereas this value was lowest in *B. cherrapunjeense* (2.42) (Fig. 4, Table 3). The size of stomatal complexes showed a large variation ranging between 436.71 μm^2 and 1397.74 μm^2 among all species. Small complex sizes were noted in *B. leopardinum* (436.71 μm^2), *B. gymnopus* (534.45 μm^2) and *B. ambrosia* (616.05 μm^2), while medium sizes were noted in *B. cherrapunjeense* (965.14 μm^2) and *B. affine* (961.54 μm^2) (Table 3). The stomatal ledges were present in all species but the shape of the stomatal ledge aperture (SLA) was variable *i.e.* round, narrow round, elliptical and narrow elliptical (Table 2, Fig. 3a–j).

Trichomes: Two types of multicelular glandular trichomes *i.e.* sessile (Fig. 2f,h), and sunken unbranched (Figs. 1 & 3m) were observed. Whereas, the former was present in all species, the latter was observed only in four species, namely *B. ambrosia*, *B. leopardinum*, *B. reptans* and *B. sunipia* (Fig. 2). The density of sunken trichomes on both surfaces showed vast variation between the species, from 12.58 to 235.27 per mm^2 of leaf lamina area on the adaxial, and 26.42 to 362.34 on the abaxial surface. As such, the density of these glandular trichomes was nearly double on the abaxial surface (Table 3).

Theobald et al (1979) identified these trichomes as multicellular sessile glandular trichomes (scales). These were flattened or shield-like circular structures made up of a considerable number of ray cells arising from the centre. The frequency of sessile trichomes was very low in *B. ambrosia, B. reptans*, while *B. sunipia* and *B. leopardinum* exhibited a high frequency (Table 2).

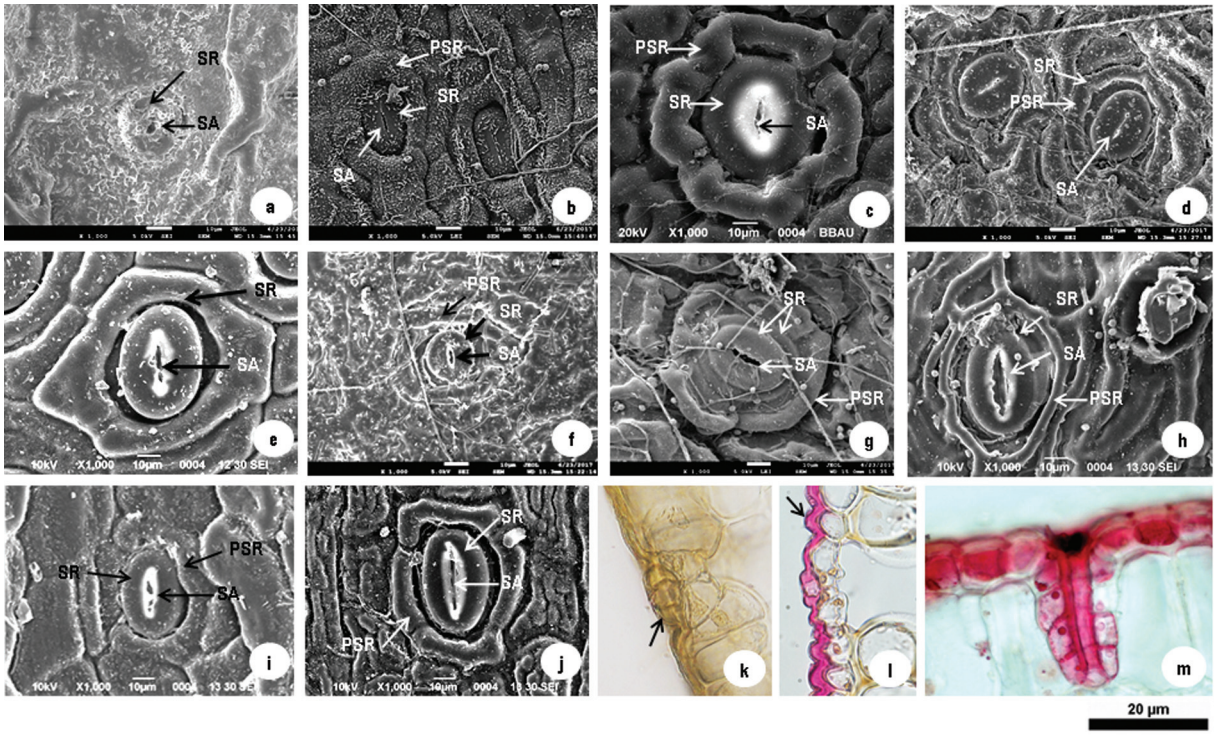

Fig. 3. Scanning electron micrographs **(a-j)** and transmission light microscope images **(k-m)** showing variation in stomatal micromorphology on the leaf surface (abaxial), cuticular pattern and sunken glandular trichome of *Bulbophyllum* Thouars species: **a.** SR and round SA in *B. cherrapunjeense* Barbhuiya & D.Verma; **b.** *B. cauliflorum* Hook.f. showing single SR, PSR and narrow elliptical SA; **c.** *B. ambrosia* (Hance) Schltr. showing single SR, peristomatal rim (PSR) with narrow elliptical SA; **d.** *B. gymnopus* Hook.f. with a very narrow, tiny elliptical SA with one SR and double PSR; **e.** *B. striatum* (Griff.) Rchb.f. with single SR and PSR and narrow elliptical SA; **f.** *B. leopardinum* Schltr. showing single SR and PSR and narrow small elliptical SA; **g.** *B. bisetum* Lindl. showing two SR and one PSR; **h.** *B. pteroglossum* Schltr. showing narrow elliptical SA, one SR and PSR; **i.** *B. umbellatum* Lindl. showing wide elliptical SA, one SR and PSR; **j.** *B. reptans* (Lindl.) Lindl. ex. Wall. showing long narrow elliptical SA, single SR and PSR; **k.** Smooth cuticular thickening and stomatal ledge in leaf transverse section (T.S.) of *B. cherrapunjeense* Barbhuiya & D.Verma; **l.** Papillose cuticular thickening and stomatal ledge in leaf T.S. of *B. gymnopus* Hook. f. **m.** Glandular trichome of *B. cauliflorum* Hook.f. in leaf T.S. 20µm scale bar is the same for k,l and m. PSR – peristomatal rim; SA – stomatal aperture; SR –stomatal rim.

Cuticle and cuticular patterns: The leaf abaxial surfaces of the 10 species observed under SEM revealed ultrastructural details *viz*. cuticular thickening patterns, epicuticular wax, stomatal ledges, stomatal ledge apertures (SLA) and stomatal rims. Both surfaces of the leaves were covered with thick cuticles. The cuticular thickening pattern was papillose in *B. affine*, *B. ambrosia, B. striatum* and *B. leopardinum* on both surfaces, whereas in *B. gymnopus* (Fig. 3l)*, B. cauliflorum, B. sunipia*, and *B. bisetum*, *B. pteroglossum*, *B. umbellatum* and *B. reptans* it was papillose only on the abaxial surface and smooth in *B. cherrapunjeense* (Table 2). Epicuticular wax deposition was observed on the abaxial surface of all species except *B. affine* and *B. sunipia* (Fig. 3a–j).

The cuticular folding outlined around stomata were like a rim or raised ridge known as stomatal rim. Similar folding of the cuticle was observed around stomata (Fig. 3a-j, Table 2). There was one stomatal rim in ten of the examined species with the exception of *B. bisetum* where there were two (Fig. 3g). Similarly, there was just one peristomatal rim in all species examined, except *B. gymnopus* which had two (Fig. 3d). In transverse sections, the leaf cuticular folding exhibited a raised appearance above the guard cells like an incomplete roofed dome termed stomatal ledge (Fig. 3k). The stomatal rim arched over the pore by forming an aperture or slit which confers a specific shape and opened into the outer stomatal ledge, named 'stomatal ledge aperture' (SLA). The SLA displayed either elliptical or round shape with slight variation (Table 2). The

SLA shape was elliptical in *B. affine*, *B. striatum*, *B. pteroglossum*, *B. umbellatum* and *B. reptans* while narrow elliptical in *B. cauliflorum*, *B. gymnopus* and *B. bisetum* and round in *B. cherrapunjeense* and *B. sunipia* while narrowly round in *B. ambrosia* and *B. leopardinum* (Fig. 3a–j).

Discussion

Reports on morphology and vegetative anatomy of more than a dozen species of *Bulbophyllum* from other parts of the world are available, but studies on leaf epidermal features of species of this genus from India has largely remained elusive. The present study fills this gap to some extent and underscore the significance of leaf surface features as potential adjunct taxonomical parameters for species identification and delineation of inter-specific affinities.

The detailed account on qualitative and quantitative leaf epidermal features of the twelve epiphytic species of *Bulbophyllum* here could be taken as useful primer for their use to aid species identification. The leaf of *B. affine*, *B. cherrapunjeense*, *B. ambrosia* and *B. striatum* are succulent and thick, but in other species the leaves are thin, although succulent to some extent. Leaf succulence is reported as an adaptation for dry condition (Hsiao, 1973; Lack & Evans, 2001; Metusala *et al.,* 2017). The range of stomatal density obtained in the present study (17.61–88.07/mm^2) was in line with the range obtained for diverse other orchids by Singh and Singh (1974; *i.e.*, 40–110/mm^2), and Avadhani *et al.* (1982; *i.e.*, 8–180/mm^2). Leaf succulence and stomatal density are inversely related in orchids (Goh *et al.*, 1977). This is consistent with our observations on *B. cherrapunjeense* and *B. affine*, both have comparatively low stomatal density and succulent leaf therefore may be better adapted to xerophytic condition than other species. Piazza *et al.* (2015) based on the examination of cuticle thickness in 13 species suggested that this character may be useful in sectional delimitation of the species in the genus *Bulbobhyllum*.

The leaves of all species examined are hypostomatic (Table 2); similar to 13 other *Bulbophyllum* species analyzed by Piazza *et al.* (2015).

This is found to be a predominant condition mostly in mesophytic orchids and most other plants. It is suggested that this condition conserves water in epiphytes by significantly decreasing water loss through the aerial parts whereas in non-epiphytic plants supply of water is not intermittent (Möbius, 1887; Lavarack, 1971; Williams, 1979). In the present study two types of stomata, *i.e.*, tetracytic and tetracyclocytic, were identified. The latter type was observed only in *B. striatum*. The tetracytic type was reported in *B. odoratissimum* (Sm.) Lindl. *ex* Wall. by Singh and Singh (1974) and for the one species of *Bulbophyllum* studied by Williams (1979). The cyclocytic type of stomata were reported in few species of *Bulbophyllum* by Kaushik (1983) and Rao and Khasim (1987). Likewise, occurrence of variation in number of subsidiary cells between the species has the potential for taxonomic differentiation and deducing interspecific evolutionary relationship, *e.g.*, *B. cherrapunjeense*, *B. affine* and *B. bisetum* can be identified based on the number of subsidiary cells. Rao and Khasim (1987) reported 4–7 subsidiary cells in three species of *Bulbophyllum* including *B. leopardinum* with 4–6 subsidiary cells. Although, Stebbins and Khush (1961) and Withner *et al.* (1974) reported that Orchidaceae lacked subsidiary cells. However, some authors on the other hand reported the presence of subsidiary cells in *Vanilla* (Roux, 1954; Rosso, 1966) of in subfamily Cypripedioideae. The species *B. leopardinum*, *B. gymnopus* and *B. ambrosia* may be comparatively better adapted to draught conditions, since Guan *et al.* (2011) revealed an inverse relationship between stomata size and adaptation to drought condition in other orchid genera. However, empirical studies are needed to confirm whether this hypothesis holds for *Bulbophyllum*. Species like *B. leopardinum, B. cherrapunjeense, B. sunipia* and *B. striatum* have large epidermal cells on both the surfaces that play roles in water storage in other orchid genera (Guan *et al.,* 2011). Santhosh

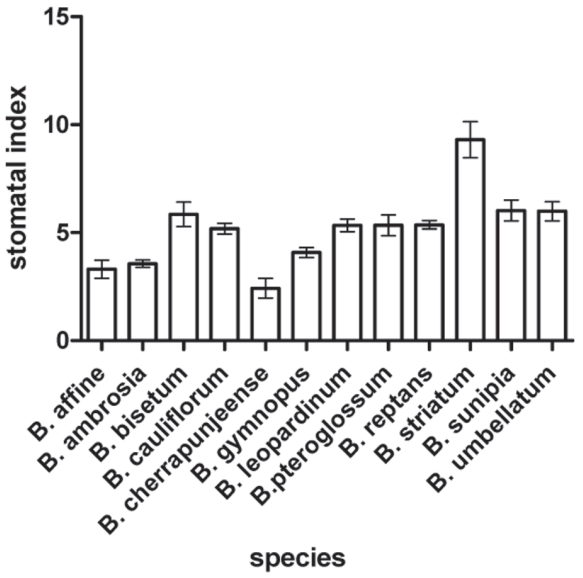

Figure 4. Stomatal index (means ± SE) of *Bulbophyllum* species, *i.e.* the percentage of stomata in relation to epidermal cells on the abaxial epidermal surface.

et al. (2015) reported on the dependence of stomatal characters on environmental conditions rather than genetic factors. However, some stomatal features observed in *Bulbophyllum* species *viz*. stomatal aperture shape, stomatal rim number and sub-stomatal chamber size may be used to understand their taxonomic significance. Stomata have significant roles in the physiology, evolution and ecology of plants (Hetherington & Woodward, 2003). A positive correlation between stomatal index (SI) and glandular trichomes density on the abaxial ($r^2 = 0.5828$, $P<0.01$) and adaxial ($r^2 = 0.5639$, $P<0.01$) surface was found in the present study.

The presence of a leaf cuticle is a very common feature of epiphytic orchids. The cuticular thickening pattern was smooth in *B. cherrapunjeense* whereas in other species papillose cuticles were observed which is an important diagnostic feature (Table 2) (Pridgeon, 1993). According to Haworth and McElwain (2008) and Yang *et al*. (2016) thick cuticles in epiphytic orchid leaves revealed the adaptation to tolerate extreme arid conditions. Stomatal ledges form an opening on the surfaces of leaves which can have variable shapes in all species. The stomatal ledge was previously reported in some species of *Bulbophyllum* by Sprenger (1904) and in orchids by Rasmussen (1987). *B. gymnopus* and *B. affine* exhibited characteristic numbers of stomatal rims, *i.e*., 1–3 and 2 respectively (Moore *et al*., 2010). The leaf epidermal characters examined in this study *viz*. thick and papillose cuticular thickening, smaller stomata, sub-stomatal chamber, stomatal ledge, large epidermal cells, mucilage secreting glandular trichomes and stomatal rim indicate that the species appears to be variably adapted to their ecological niche. Rasmussen (1987) recognized them as xeromorphic characters. A fairly high number of stomata per unit area and their occurrence in groups (clusters) could be considered as an attribute to their micro-environment. Rao and Khasim (1987) observed twin stomata in *B. leopardinum*. The stomatal ledges were present in all species. Yukawa *et al*. (1992) discussed the stomatal ledge in *Dendrobium* and reported the shape of stomatal ledge as important taxonomic marker. The presence of large sub-stomatal chambers in *B. affine*, *B. striatum*, *B. pteroglossum* and *B. umbellatum* is the water conserving attribute in these species. The sunken glandular trichomes are found in abundance on both leaf surfaces. Rao and Khasim (1987) identified them as 'absorbing trichomes' and Oliveira and Sajo (1999) also observed absorbing trichomes in orchid species. The occurrence of two types of glandular trichomes, variation in their morphological forms and density are important for providing taxonomic relationships.

The trichome types and their structure could be used as parameter for species identification (Seithe, 1979; Lavania, 1990). The diversity in glandular trichomes indicated adaptation towards their microclimate (Pridgeon, 1982; Pridgeon & Stern, 1982; Kaushik, 1983; Rao & Khasim, 1987; Isaiah & Rao, 1992). Occurrence of oil droplets in abaxial and adaxial epidermal cells could be another micro-taxonomic parameter. Pridgeon *et al.* (2014) reported taxonomic significance of oil droplets in epidermal cells of *Bulbophyllum* species.

Beside epidermal features, here we provide useful information on the deposition of epicuticular wax

on the abaxial surface of leaf as examined by scanning electron microscopy, and the due identification of epicuticular wax according to Barthlott *et al.* (1998). This is the first report for the genus *Bulbophyllum* that can be used to supplement species characterization since wax morphotypes have significance in species delineation (Barthlott *et al*., 1998).

In the present study eight dermal characters were considered as taxonomic marker for species differentiation in *Bulbophyllum*: (1) hypostomaty; (2) number of subsidiary cells; (3) stomatal rim; (4) shape of stomatal ledge; (5) cuticular thickening pattern of leaf abaxial surface; (6) the type of epicuticular wax; (7) glandular trichomes (sunken trichomes and scales) and (8) stomata type. There were nine putative adaptive features observed in leaf dermal micromorphology of this genus: (1) leaf succulence, (2) reduced stomatal density, (3) low stomatal index (Fig. 4), (4) reduced stomata size, (5) high trichome density, (6) presence of stomatal ledge, (7) large sub-stomatal chamber, (8) hypostomaty, and (9) large epidermal cells. The presence of these features in the *Bulbophyllum* species studied here may support their ecological adaptation to the harsh conditions they are exposed to since these plants grow on the bark of trees or rocks. However, empirical studies are required to confirm their significance in this hypothesis.

Conclusion

The leaf dermal characters studied in the present study of 12 *Bulbophyllum* species were found to have potential taxonomic significance. The stomatal traits, glandular trichome type, their distribution and cuticular thickening pattern were potentially valuable for characterization and differentiation of the species. However, the tetracytic type of stomata is the most common type in the genus, and thus is of limited use for the taxonomy of the genus. Interspecific variations in these dermal features could help to identify species like *B. cherrapunjeense*, *B. striatum*, *B. leopardinum*, *B. gymnopus* and *B. bisetum*. The distinct arrangement of epidermal cells in *Bulbophyllum reptans* is useful for this species's characterization. Such an outcome from the present study would be useful as taxonomic marker between the species in the genus and genera within the family.

The epicuticular wax deposition, presence of multicellular sessile glandular trichomes (scales) on the dermal surfaces of leaves are reported for the first time for the genus *Bulbophyllum*. Further studies on the morphological variations of these epicuticular waxes would be important to understand the role in species characterization. Structural biochemical studies of wax components would help to understand its physiological functions, potential role in adaptation towards the varied environmental conditions the plants encounter. Further studies involving more species could provide more reliable and forceful criteria to ascertain the reliability of leaf epidermal characters as diagnostic features, and to develop micro-morphological taxonomic keys for the genus.

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