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Research Article

Leaf Anatomical Characteristics of *Nepenthes* Species in Western Sarawak, Borneo

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ABSTRACT

The dimorphism and phenotypic plasticity of genus *Nepenthes* is taxonomically challenging. They depicted a variety of pitcher morphological features that often lead to difficulty in the species delineation. However, there is an alternative that could aid to distinguish the *Nepenthes* species which is by observing the anatomical characteristics of their leaves. Despite few researches had reported the comparative study on leaf anatomy of certain *Nepenthes* species, yet, there are very scarce data showing other distinct anatomical characteristics that could be used to differentiate the *Nepenthes* species especially in the western of Sarawak. Hence, this research was performed to identify anatomical characteristics that could be useful taxonomic tools for *Nepenthes* species identification. There are nine species with one variety and three natural hybrids were included in the study namely, *Nepenthes albomarginata* Lobb, *N. ampullaria* Jack, *N. gracilis* Korth, *N. hirsuta* Hook. f., *N. hispida* Beck, *N. mirabilis* (Lour.) Druce, *N. mirabilis* var. *echinostoma* (Hook. f.) J. H. Adam & Wilcock, *N. nazreeana* sp. nov. ined., *N. rafflesiana* Jack, *N. reinwardtiana* Miq., *N.* x *trichocarpa* Miq, *N.* x *hookeriana* Lindl, and *N.* x *kuchingensis* Sh. Kurata. The anatomical data of leaves, i.e stomatal complex type, stomatal size and density, type of anticlinal walls, type of glandular structures and others were analysed, tabulated and discussed.

Key words: Anatomy, carnivorous plants, lowland species, Malaysia, Nepenthaceae, pitcher plants

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INTRODUCTION

Borneo is a hotspot for tropical pitcher plant diversity. Commonly known as monkey cups (Malay: Periuk Kera), this bizzare group of pitcher plant were recognised as genus *Nepenthes* in a monotypic family, Nepenthaceae. Odoardo Beccari, Frederick Burbidge, Hugh Low and Johan Teysmann were among the first to discover *Nepenthes* in Borneo (Phillips *et al.*, 2008). Astonishingly, *Nepenthes* x *hookeriana*, and *N. veitchii* were reported by Phillips *et al.* (2008) as the earliest Borneo species to be introduced. Despite the exact number of *Nepenthes* species is unknown (Thorogood, 2010), Murphy *et al.* (2019) reported that there are approximately 160 – 180 species were distributed and described so far. In Borneo Malaysia, Sarawak has the most *Nepenthes* species with 33 different species distributed at lowland and highland areas (Steiner, 2002; Damit *et al.*, 2018; Tamizi *et al.*, 2020).

Generally, species delineation of genus *Nepenthes* is done primarily based on its morphological characteristics especially the pitcher morphology (Ridley, 1924; Corner, 1988; Clarke, 1997; Jebb & Cheek, 1997; Cheek & Jebb, 2001; Rizqiani *et al.*, 2018; Ghazalli *et al.*, 2021). The pitcher is actually a modified leaf that developed in various shape, size and colour from the tendril which protruded from the apex of the lamina. The pitcher morphology evolved to facilitate the habit of the plant and aid in adaptation and survival in low-nutrients soil, mainly nitrogen (Phillips *et al.*, 2008; Thorogood, 2010). Nonetheless, the genus *Nepenthes* is taxonomically challenging due to their

extreme convergence of morphological features associated with carnivorous syndrome and high intraspecific variability (Thorogood, 2010). So, other alternative has been used to assists in the species identification through anatomical study.

Few anatomical study of *Nepenthes* has been done to identify diagnostic characteristics that can be used to aid in the species identification. The very first study of anatomical characteristics of *Nepenthes* was carried out by Metcalfe and Chalk (1950) by using slides collection preserved in Kew Botanic Gardens. Then, followed by Toma *et al.* (2002), Pavlovic *et al.* (2007), Biati (2012), and Paluvi *et al.* (2015). However, these studies showed lack of data as they are mainly focused on the cultivated *Nepenthes* species which are *N. maxima, N. mirabilis, N. alata,* and *N. gracilis*. Hence, several anatomical studies from other researchers on the wild specimen has been evaluated and identified to provide supporting data. The latest anatomical descriptions have been provided by Ghazalli *et al.* (2021) focusing on 14 *Nepenthes* species distributed at difference habitat in Peninsular Malaysia.

Study on the leaf anatomical of *Nepenthes* in Sarawak, however never been conducted before. Therefore, this preliminary study was conducted to investigate the usefulness of leaf micro morphology for species identification. Nine lowland wild *Nepenthes* species with one variety, and three natural hybrids were selected for the purpose of the study.

MATERIALS AND METHODS

There are nine lowland *Nepenthes* species with one variety and, three natural hybrids were involved in the study; *Nepenthes albomarginata* Lobb, *N. ampullaria* Jack, *N. gracilis* Korth, *N. hirsuta* Hook. f., *N. hispida* Beck, *N. mirabilis* (Lour.) Druce, *N. mirabilis* var. *echinostoma* (Hook. f.) J. H. Adam & Wilcock, *N. nazreeana* sp. nov. ined., *N. rafflesiana* Jack, *N. reinwardtiana* Miq., *N.* x *trichocarpa* Miq, *N.* x *hookeriana* Lindl, and *N.* x *kuchingensis* Sh. Kurata. All species were collected from selected areas in western Sarawak (Table 1). The collected *Nepenthes* species were observed, described and authenticated beforehand by using several past literatures and dichotomous keys delineated by Clarke (1997), Jebb and Cheek (1997), Cheek and Jebb (2001), Clarke & Lee (2004), and Phillips *et al.* (2008).

Mature leaves were used for examination and preserved in the 70% ethanol. Preparation of cuticular specimen were done according to the standard procedures, adopted from Cutler (1978). The specimens were transverse sectioned at the central region of the lamina using scalpel. The cuticular samples then, were treated chemically in the boiling 15% nitric acid to separate the adaxial and abaxial of leaf surfaces. The sections were stained with Safranin and followed with dehydration by using a series of ethanol; 50%, 60%, 70%, 80%, 90% and 100% with two drops of Hydrochloric acid (HCl) in 70% to balance the colour. The samples then were mounted on the sliding microscope and observed under compound microscope at various magnifications. The images and data; Stomatal complex type, type of anticlinal wall, stomatal size, stomatal density, and glandular structure were analysed with aid of several anatomical literatures and compared with studies performed by Cutler (1978), Cutler *et al.* (2007), Al Farishy *et al.* (2017), Rizqiani *et al*. (2018), and Ghazalli *et al.* (2020; 2021).

RESULTS AND DISCUSSION

Stomatal complex type

Stomatal plays an important role in the gases exchange for plant photosynthesis. A stomatal is a cell structure that enclosed by a pair of parenchyma cells which also known as guard cells. Identification of anatomical characteristics of stomatal can be a taxonomic tool to help in *Nepenthes* species delineation. In this study, nine *Nepenthes* species including a variety, and two hybrids collected from western Sarawak develop a hypostomatic leaves which the stomata present on the abaxial surface only except for a natural hybrid; *N.* x *kuchingensis*. *Nepenthes* x *kuchingensis* develop an amphistomatic leaves as there are stomata present on both surface but rarely present on the adaxial surface (Figure 2d and Figure 4d). The leaves of all *Nepenthes* species studied shown an anomocytic or ranunculaceous stomatal complex type (Figure 1 until Figure 4). Metcalfe and Chalk (1950) discovered that *Nepenthaceae* can be distinguished by the anomocytic stomatal complex type which each stomatal with guard cells is enclosed by five or more subsidiary cells that are not differentiated by the epidermal cells. The finding supported and agreeable with other research by Al Farishy *et al.* (2017) and Ghazalli *et al.* (2020; 2021). However, a study by Rizqiani *et al.*, (2018) found that *Nepenthes* does not always have anomocytic stomatal complex type, as *N. bicalcarata* has actinocytic stomata.

Stomatal size

The *Nepenthes* species studied develop a stomatal size between 24.05 – 33.76 µm × 21.26 – 30.69 µm (Table 1). The shortest length of stomata was observed on the abaxial surface of *N.* x *kuchingensis* with 23.87 µm, followed by *N. mirabilis* and *N. rafflesiana* with 24.05 µm and 24.14 µm respectively. Meanwhile, the longest stomatal was observed in *N. nazreeana* sp. nov. ined. (33.76 µm) followed by *N. reinwardtiana* (32.23 µm), *N. albomarginata* (31.90 µm) and *N hispida* (31.08 µm).

Table 1. The details of the voucher specimens of *Nepenthes* involved in the study (currently deposited in the Herbarium of Universiti Malaysia Sarawak)

Species	Localities	Voucher specimen numbers
N. albomarginata	Malaysia, Borneo, Sarawak, Kuching Division, Kuching, Bako NP.	NH 0017
N. ampullaria	Malaysia, Borneo, Sarawak, Kuching Division, Kuching, Santubong NP, Loop trail.	NH 0008
	Malaysia, Borneo, Sarawak, Kuching Division, Bau, Banjaran Undan, Stenggang Dam.	NH 0034
N. gracilis	Malaysia, Borneo, Sarawak, Samarahan Division, Kota Samarahan, Universiti Malaysia Sarawak, Jalan Kapur roadside.	NH 0005
	Malaysia, Borneo, Sarawak, Kuching Division, Kuching, Bako NP.	NH 0032
N. hirsuta	Malaysia, Borneo, Sarawak, Kuching Division, Bau, Banjaran Undan, Stenggang Dam trail.	NH 0030
N. hispida	Malaysia, Borneo, Sarawak, Kuching Division, Bau, Banjaran Undan, Sepadah Dam trail.	NH 0047
N. mirabilis	Malaysia, Borneo, Sarawak, Samarahan Division, Kota Samarahan, Universiti Malaysia Sarawak, Jalan Kapur Roadside.	NH 0006
	Malaysia, Borneo, Sarawak, Kuching Division, Matang, Matang-Sampadi Roadside.	NH 0022
N. mirabilis var. *echinostoma*	Malaysia, Borneo, Sarawak, Kuching Division, Lundu, Gunung Pueh FR.	NH 0038
N. nazreeana sp. nov. ined.	Malaysia, Borneo, Sarawak, Kuching Division, Matang, Matang-Sampadi Roadside.	NH 0011
N. rafflesiana	Malaysia, Borneo, Sarawak, Kuching Division, Kuching, Bako NP.	NH 0016
	Malaysia, Borneo, Sarawak, Kuching Division, Bau, Banjaran Undan, Sepadah Dam Trail.	NH 0040
N. reinwardtiana	Malaysia, Borneo, Sarawak, Kuching Division, Kuching, Bako NP.	NH 0051
N. x *trichocarpa*	Malaysia, Borneo, Sarawak, Kuching Division, Matang, Matang-Sampadi Roadside	NH 0048
N. x *hookeriana*	Malaysia, Borneo, Sarawak, Kuching Division, Matang, Matang-Sampadi Roadside.	NH 0049
N. x *kuchingensis*	Malaysia, Borneo, Sarawak, Samarahan Division, Kota Samarahan, Universiti Malaysia Sarawak, UNIMAS Arboretum.	NH 0037

*NP (National Park), FR (Forest Reserve)

Table 2. Comparison of stomatal and glandular anatomical characteristic of *Nepenthes* species

Species/variety	Stomatal complex type	Mean of stomatal size on abaxial		Mean of stomatal density (mm^{-2}) on abaxial		Glandular structure (Sessile Glands)	
		Length (µm)	Width (µm)	Abax	Adax	Abax	Adax
N. albomarginata	Anomocytic	31.90	26.56	160.26	-	+	+
N. ampullaria	Anomocytic	30.00	24.19	216.35	-	+	+
N. gracilis	Anomocytic	26.34	21.54	208.33	-	+	+
N. hirsuta	Anomocytic	25.19	23.74	64.10	-	+	+
N. hispida	Anomocytic	31.08	24.19	104.17	-	+	+
N. mirabilis	Anomocytic	24.05	24.72	200.32	-	+	+
N. mirabilis var. *echinostoma*	Anomocytic	30.14	27.22	232.37	-	+	+
N. nazreeana sp. nov. ined.	Anomocytic	33.76	27.28	208.33	-	+	+
N. rafflesiana	Anomocytic	24.14	21.38	160.26	-	+	+
N. reinwardtiana	Anomocytic	32.23	30.69	96.15	-	+	+
N. x *trichocarpa*	Anomocytic	30.26	27.30	296.47	-	+	+
N. x *hookeriana*	Anomocytic	32.16	22.07	488.78	-	+	+
N. x *kuchingensis*	Anomocytic	23.87	21.26	80.13	24.04	+	+

*Abaxial (Abax), Adaxial (Adax), Present (+), Absent (-)

Table 3. Informal classification of stomatal density for the studied *Nepenthes* species

Group 1 (Stomatal density ≤ 100 mm^{-2})	**Group 2** (Stomatal density 101 ≤ 200 mm^{-2})	**Group 3** (Stomatal density 201 ≤ 300 mm^{-2})	**Group 4** (Stomatal density ≥ 301 mm^{-2})
N. hirsuta *N. reinwardtiana* *N.* x *kuchingensis*	*N. albomarginata* *N. mirabilis* *N. rafflesiana* *N. hispida*	*N. ampullaria* *N. gracilis* *N. mirabilis* var. *echinostoma* *N. nazreeana* sp. nov. ined *N.* x *trichocarpa*.	*N.* x *hookeriana*

Stomatal density

Stomatal density of the included *Nepenthes* in the study were varied. Table 2 shows the average of stomatal density in mm^{-2} on the abaxial surface of leaves. *Nepenthes hirsuta* has the lowest stomatal density with an average of 64.10 mm^{-2}, whereas *N.* x *hookeriana* has the highest stomatal density on the abaxial surface of the leaves with an average 488.78 mm^{-2}. The stomatal density of *N.* x *kuchingensis,* the only amphistomatic species in this study recorded with 24.04 mm^{-2}. Based on the results (Table 3), the studied *Nepenthes* can be divided into four informal groups, consist of **Group 1** (Stomatal density ≤ 100 mm^{-2}), **Group 2** (Stomatal density 101 ≤ 200 mm^{-2}), **Group 3** (Stomatal density 201 ≤ 300 mm^{-2}), and **Group 4** (Stomatal density ≥ 301 mm^{-2}). Khan *et al.* (2014) mentioned that the stomata can vary in size and density due to genetic factors and/or growth under different environmental conditions such as light intensity, humidity, temperature, atmospheric carbon dioxide and nutrient availability.

Type of anticlinal walls

Other than that, the epidermal cells of *Nepenthes* species are formed in polygonal shape with straight or/and sinuous anticlinal walls. *Nepenthes mirabilis, N. mirabilis* var. *echinostoma, N. nazreeana* sp. nov. ined, *N. rafflesiana, N. reindwartiana* and *N.* x *hookeriana* have straight anticlinal wall on both surfaces of leaves. Meanwhile, the sinuous anticlinal wall also can be seen on both surface in *N. gracilis, N. hispida* and *N.* x *kuchingensis*. Other species, such as *N. albomarginata*, *N. ampullaria*, *N. hirsuta*, and *N.* x *trichocarpa*, exhibit different anticlinal wall characters simultaneously on both; sinuous on the abaxial and straight on the adaxial surfaces.

Glandular structure

Besides, there is a glandular structure known as sessile glands present on the leaf surfaces (Table 2). The sessile glands are developed among the polygonal epidermal cells on the adaxial and abaxial surface of all *Nepenthes* studied as shown in Figure 1 until Figure 4. There are two forms of sessile glands can be seen in the studied *Nepenthes* which are orbicular and irregular flower-like shape. These glands are part of external structures that derived from the epidermis which exude variety of secondary plant products or compounds as a defense mechanism or to attract the insects (Cutler *et al.,* 2007; Crang *et al.,* 2018).

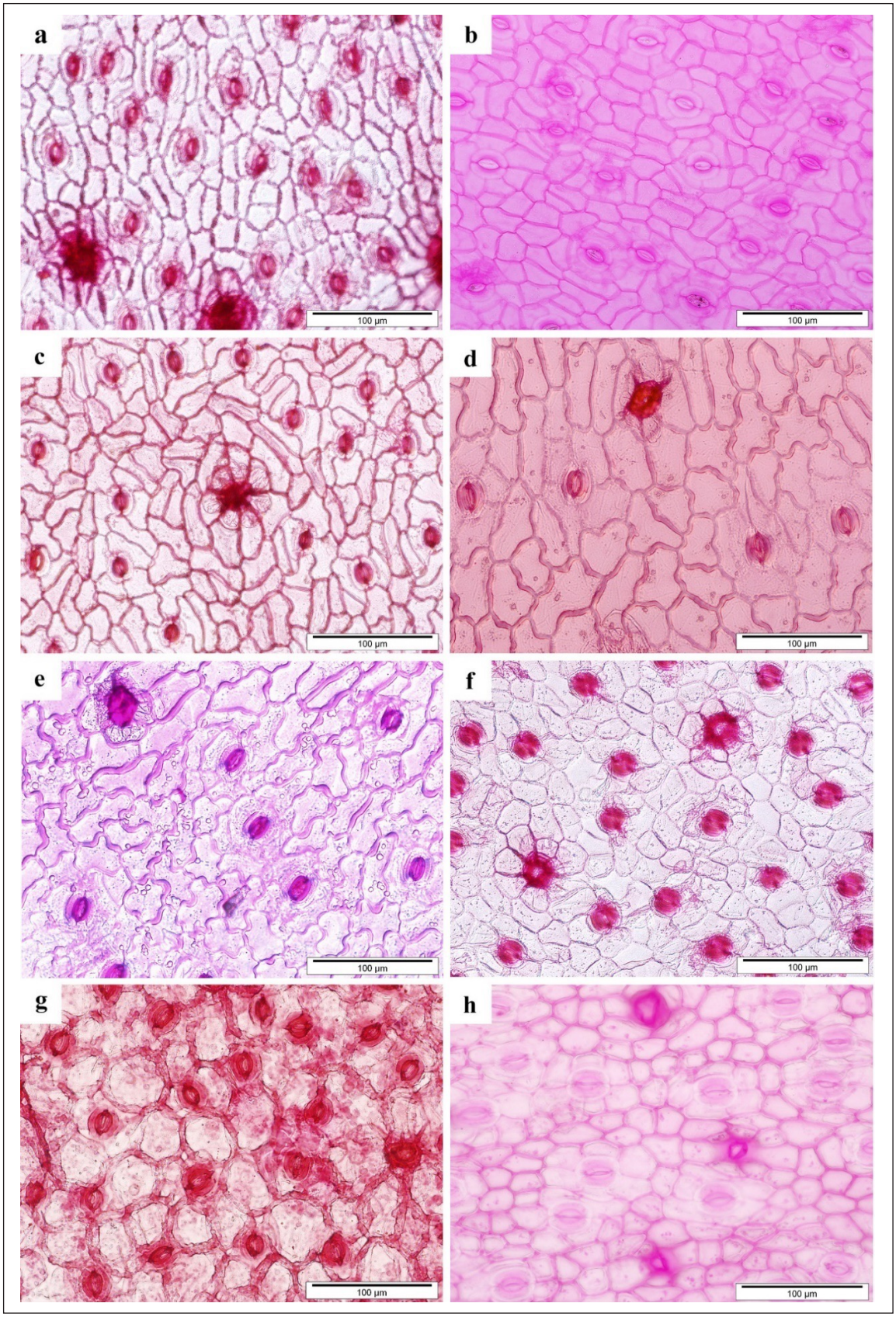

Fig. 1. Abaxial surface of leaves in *Nepenthes* species. (a) *N. albomarginata,* (b) *N. ampullaria,* (c) *N. gracilis,* (d) *N. hirsuta,* (e) *N. hispida,* (f) *N. mirabilis,* (g) *N. mirabilis* var. *echinostoma*, (h) *N. nazreeana* sp. nov. ined. Magnification 400×, scale bar = 100 µm

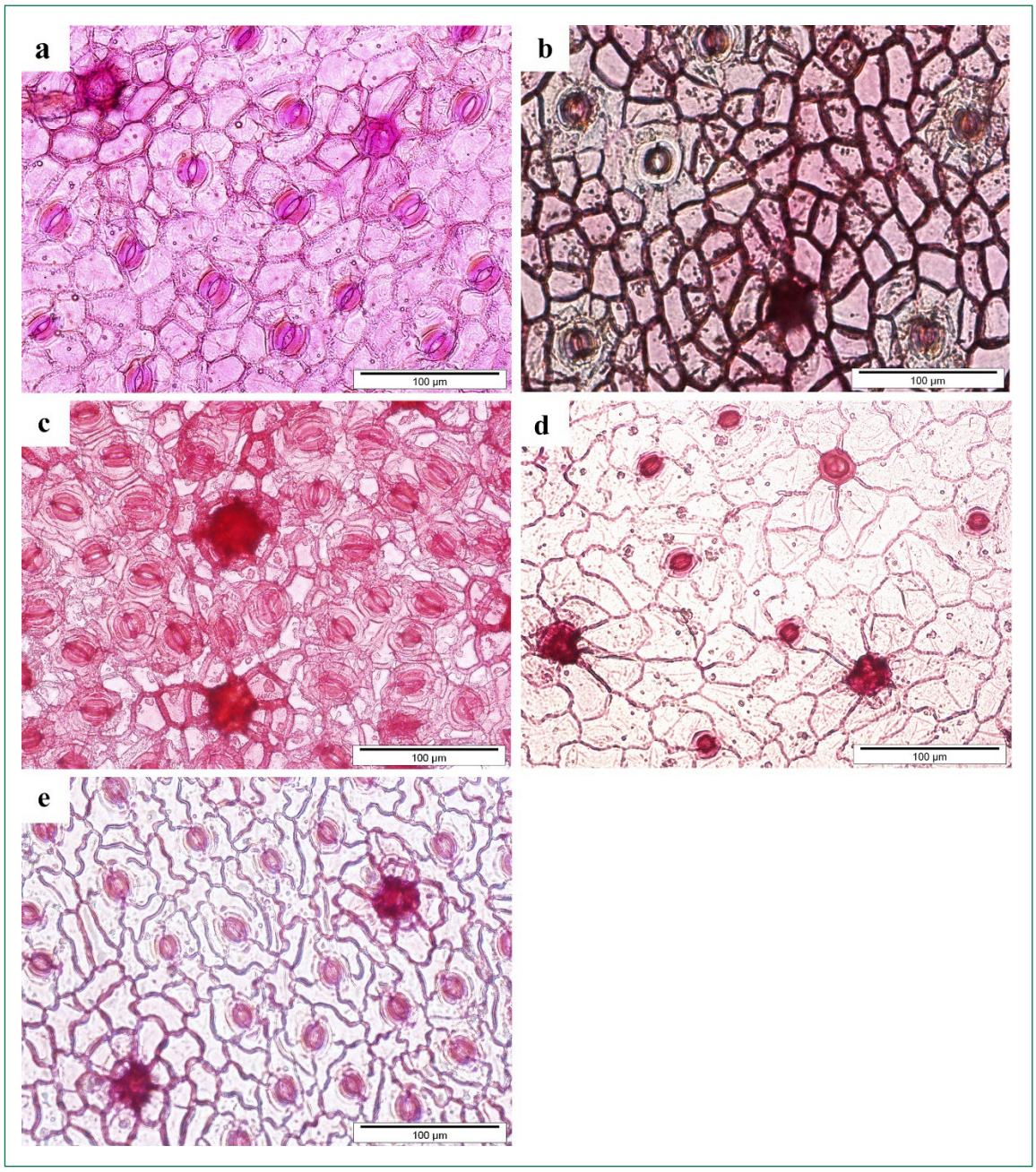

Fig. 2. Abaxial surface of leaves in *Nepenthes* species. (a) *N. rafflesiana,* (b) *N. reinwardtiana,* (c) *N.* x *hookeriana,* (d) *N.* x *kuchingensis*, (e) *N.* x *trichocarpa*. Magnification 400×, scale bar = 100 µm.

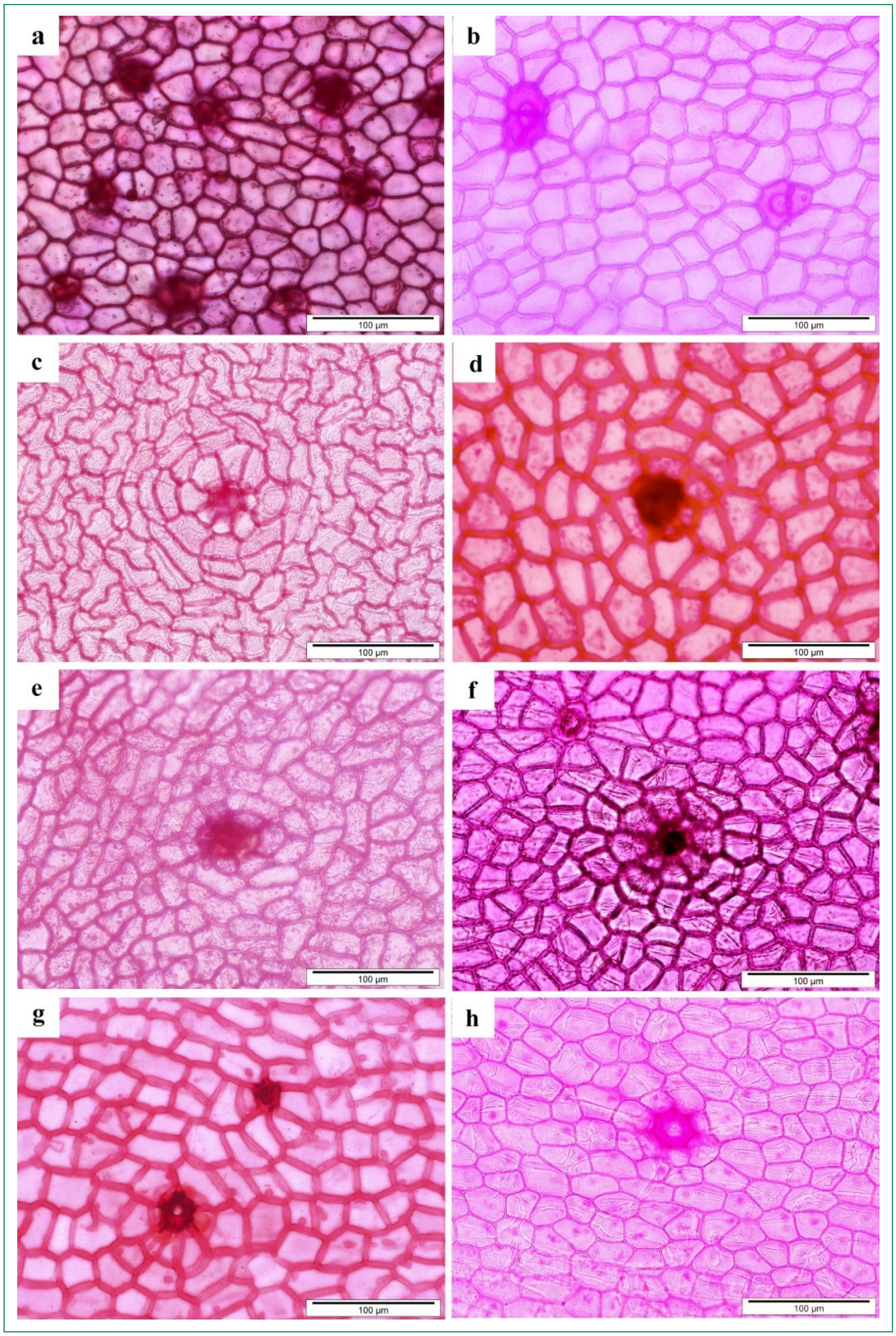

Fig. 3. Adaxial surface of leaves in *Nepenthes* species. (a) *N. albomarginata,* (b) *N. ampullaria,* (c) *N. gracilis,* (d) *N. hirsuta,* (e) *N. hispida,* (f) *N. mirabilis,* (g) *N. mirabilis* var. *echinostoma*, (h) *N. nazreeana* sp. nov. ined. Magnification 400×, scale bar = 100 µm

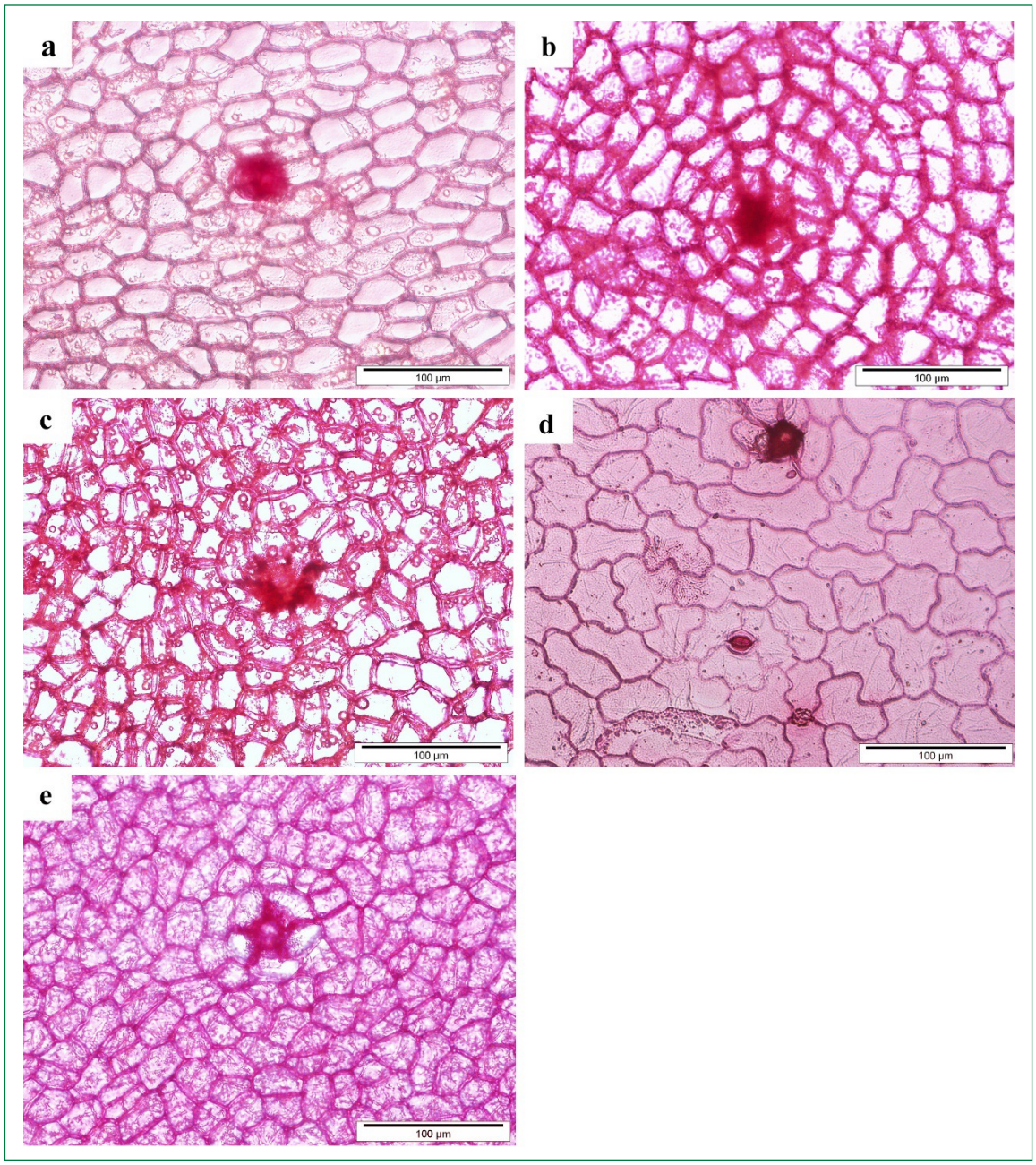

Fig. 4. Adaxial surface of leaves in *Nepenthes* species. (a) *N. rafflesiana,* (b) *N. reinwardtiana,* (c) *N.* x *hookeriana,* (d) *N.* x *kuchingensis*, (e) *N.* x *trichocarpa*. Magnification 400×, scale bar = 100 µm.

CONCLUSION

There are five leaf anatomical characteristics identified in this study which are stomatal complex type, type of anticlinal wall of epidermal cells, stomatal size, stomatal density, and glandular structure. Those characteristics can be used as supportive information for species identification. Some of the characters, such as stomatal size and stomatal density are varying within interspecific and intraspecific, as both are possibly exaggerated by the environmental conditions that could change the results.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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