

Short Communication

Preliminary Foliar Anatomical Assessment of Four *Vanilla* Species (Orchidaceae) from Perak, Malaysia

Akmal Raffi¹, Nur Ashikin Psyquay Abdullah², Mohd Yunus Noor-Syaheera³ and Rusea Go^{1,4*}

¹Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

²Department of Crop Science, Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Campus, 97008 Bintulu, Sarawak, Malaysia

³Department of Plant Science, Kuliyyah of Science, International Islamic University Malaysia (Kuantan Campus), 25200 Kuantan, Pahang, Malaysia

⁴Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

Assessment of the leaf anatomy among *Vanilla griffithii*, *Vanilla kinabaluensis*, *Vanilla sanguineovenosa* and *Vanilla* sp. 1 from Perak showed generic characterization of the epidermal layer and leaf lamina, suggesting the genus to possess plesiomorphic characters from its progenitor except for the leaf margin outlines. The species were found to differ interspecifically and acknowledged to possess taxonomic value. Leaf margin among the species showed similarity in tapering outline but distinguished in the overall shape and apical curve.

Keywords: Leaf anatomy, marginal outlines, taxonomy, *Vanilla*

INTRODUCTION

The genus of *Vanilla* Plum. ex Mill. characters are diagnosed as hemiepiphytic vinous monopodial growth habit, with roots

produced at each internode and having fleshy fruits and wingless seeds with a hard seed coat (Soto-Arenas & Cribb, 2010). Furthermore, the plant structures can be classified by having membranous to coriaceous leaves, axillary racemose to paniculate inflorescence, spreading sepals and petals, labellum usually fused to the column, column complex consisting of pollen in monads, stigmata and rostellum, articulated ovary to the perianth with dehiscent capsule (Pridgeon et al., 1999).

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E-mail addresses:

akmalraffi@gmail.com (Akmal Raffi)

sayung1972@gmail.com (Nur Ashikin Psyquay Abdullah)

noorsyaheera@iiium.edu.my (Mohd Yunus Noor-Syaheera)

rusea@upm.edu.my (Rusea Go)

* Corresponding author

Recently, Soto-Arenas and Cribb (2010) had updated the classification of the genus into two subgenera *Vanilla* and *Xanata* based on the taxonomic reviews that congruent with the phylogenetic analysis. The subgenera *Xanata* which represents the majority and distributed pantropically is further classified into two sections namely *Xanata* and *Tethya* with the introduction of grouping system within the system.

In Peninsular Malaysia, *Vanilla* is considered as understudied in comparison to the other genera of Orchidaceae. Previous checklists stated that there were five species native to this region namely *Vanilla aphylla*, *Vanilla borneensis*, *Vanilla griffithii*, *Vanilla kinabaluensis* and *Vanilla montana* (Govaerts et al., 2017) and two new taxa to science, *Vanilla sanguineovenosa* R. Go et A. Raffi (Raffi et al., 2017a) and *Vanilla norashikiniana* R. Go et A. Raffi (Raffi et al., 2017b). However, the genus is considered taxonomically difficult due to the high degree in vegetative resemblance and flowers that are scarce and ephemeral (Soto-Arenas & Dressler, 2009). With more species expected to be described from this highly species-rich region, it is important to evaluate different techniques that can aid in the identification process. The investigations to distinguish the species had been conducted via several approaches of which among them anatomical description was rarely used. Anatomical assessment is an important approach to get a better understanding of the cell characters which are valued as secondary data in taxonomic studies. Among the plant parts, leaves

are noted to be widely used in taxonomic descriptions based on their highly variables in shapes, sizes, textures and colours and most importantly, they are most prominent part across terrestrials plant. The leaves anatomy can be defined by the abaxial epidermis, adaxial epidermis, lamina, midrib and margin (Cutler et al., 2008).

The comparative leaf anatomy of *Vanilla* was previously assessed by Stern and Judd (1999) who also compiled results from previous works on various species. Among the species examined, only two taxa also distributed in Peninsular Malaysia namely *Vanilla pilifera* (synonym to *V. borneensis*) and *V. aphylla* were included. Therefore, this study was conducted to investigate the characters in four leaf parts of four species obtained from different localities in Perak which are *V. griffithii* collected from coastal hill forest (Lumut), *V. kinabaluensis* from lower montane forest with the highest annual precipitation in Malaysia (Bukit Larut), *V. sanguineovenosa* from lower montane forest (Tapah), and the unidentified *Vanilla* sp. 1 from the 130 million years old dipterocarp forest (Sg. Enam). The significance of the cell characters in the systematics of the local genus was evaluated and served as the supplementary data to the previous description.

MATERIALS AND METHODS

Fresh materials were fixed in 70% of formalin acetic acid (FAA). Matured leaf samples of *V. griffithii*, *Vanilla* sp. 1, *V. kinabaluensis* and *V. sanguineovenosa* (Figure 1) were cut into 30 – 50 µm thick



Figure 1. Leaves variation of *Vanilla* species from Perak. a) Elliptic shape: *Vanilla griffithii*; b) Oblong shape: *Vanilla* sp. 1; c) Large leaf, elliptic: *Vanilla sanguineovenosa*; d) Large leaf, narrowly elliptic: *Vanilla kinabaluensis*

slices using a sliding microtome into three main transverse sections; lamina, margin and midrib. They were subjected to bleaching process using 5% sodium hypochlorite (Chlorox®) solution for five minutes. The bleached samples were stained using Safranin and Alcian Blue for five minutes. The samples were then dehydrated twice by series of soaking in 50%, 70%, 95% and 100% alcohol and subsequently mounted onto specimen slides using Canada Balsam. The slides were oven dried at 60°C for one week. Four slides were prepared for each accession. The micrographs of the specimens were captured with an Olympus BX41 compound microscope and Olympus software.

Foliar anatomical characters were described from epidermal peels of adaxial and abaxial surfaces which were stained using Safranin and the three transverse sections. Stomatal density (mm^{-2}) and stomatal index ($s / (s + e) \times 100$; where s = number of stomata per unit area and e = number of epidermal cells in the same unit

area) were calculated in each accession. Stomatal density enumeration was obtained from nine epidermal layers while seven epidermal layers were observed in stomatal index calculation for each accession. Data generated were subjected to statistical analyses using IBM SPSS (Version 22.0, IBM Corp., Chicago, IL, USA). Basic descriptive statistic reported as mean \pm standard deviation was performed. The data were also tested for fitness to a normal distribution by the Shapiro-Wilk test. One way ANOVA and a post-hoc Tukey pair wise comparison were used to test the significant difference among species. Statistical significant difference was set at confidence level of 95% ($\alpha = 0.05$).

RESULTS

Description of Epidermal Layers

Epidermal cells anticlinal walls: straight to curve in adaxial and abaxial epidermis.

Epidermal cells shape: basically isodiametric, polygonal and some elongated

in adaxial and abaxial epidermis (Figure 2). **Stomatal complexes:** hypostomatous, scattered among epidermal cells basically tetracytic with the presence of anisocytic and

anomocytic types in all species, guard cells ovate (Figure 3). Data comprising stomatal size, density and index are summarized in Table 1.

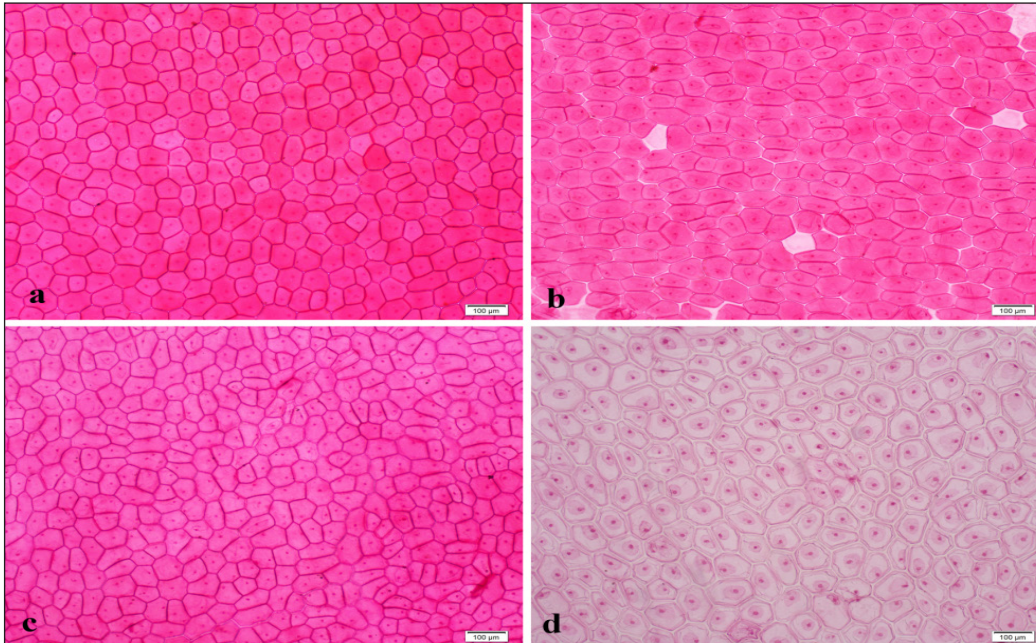


Figure 2. Cells architecture on leaf epidermal layers. a) *Vanilla griffithii*; b) *Vanilla* sp. 1; c) *Vanilla kinabaluensis*; d) *Vanilla sanguineovenosa*

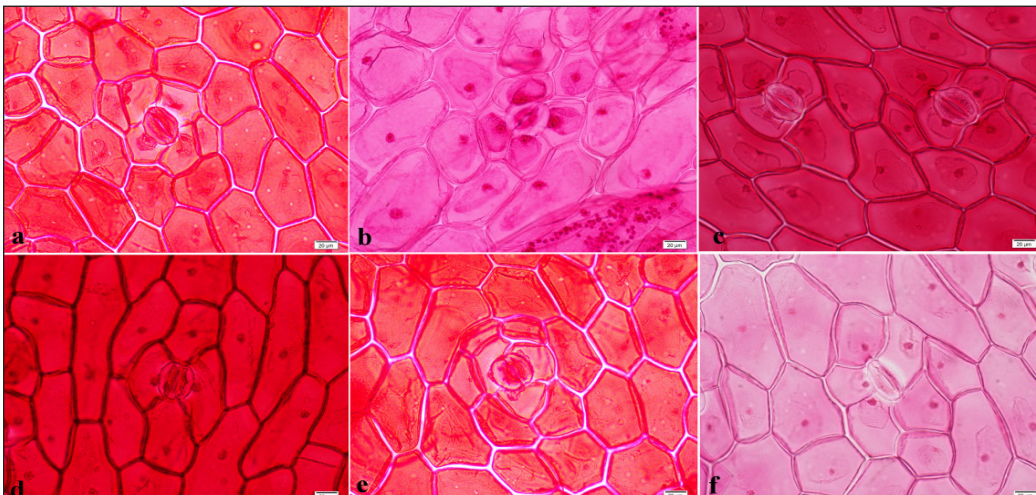


Figure 3. Stomatal types of *Vanilla* species from Perak. a-d) Tetracytic type of stomatal apparatus of (a) *Vanilla griffithii*, (b) *Vanilla kinabaluensis*, (c) *Vanilla sanguineovenosa* and (d) *Vanilla* sp. 1; e) abaxial of *Vanilla griffithii* showing anisotypic stomatal apparatus; f) anomocytic stomata in *Vanilla sanguineovenosa*

Table 1

Summary of the stomatal size, density and index of *Vanilla* species from Perak. Means with the same letters within the same column were not significantly different at $\alpha \leq 0.05$

Species	Stomatal size (Length × width, μm)	Stomatal density (mm^{-2})	Stomatal index (%)
<i>Vanilla griffithii</i>	40.00 – 46.67 x 29.33 – 40.00	14.33 ± 0.726 ab	4.371 ± 0.522 a
<i>Vanilla</i> sp. 1	38.67 – 45.33 x 29.33 – 38.67	12.11 ± 0.512 a	4.15 ± 0.171 a
<i>Vanilla kinabaluensis</i>	42.67 – 64.00 x 32.00 – 58.67	19.44 ± 1.192 d	5.35 ± 0.284 ab
<i>Vanilla sanguineovenosa</i>	41.33 – 50.67 x 32.00 – 40.00	16.22 ± 0.572 c	6.74 ± 0.919 c

Description of Transverse Sections of Leaf Lamina and Margin

Cuticles: present adaxially and abaxially.

Adaxial epidermal layers: single epidermis layer of 1:2 ratio (length: width) except 1:3 ratio in *V. kinabaluensis*. **Abaxial epidermal layers:** single epidermis layer of 1:2 ratio except 1:1 ratio in *V. kinabaluensis*.

Hypodermis: uniseriate at adaxial and abaxial epidermal layer. **Mesophyll:** homogenous, isodiametric in shape, section width.

Vanilla griffithii = 16 – 18 cells, *Vanilla* sp. 1 = 14 – 17 cells, *Vanilla kinabaluensis* = 10 - 13 cells and *Vanilla sanguineovenosa* = 15 – 16 cells, cells smaller and compact at both polar region but largest and loose towards the centre (Figure 4).

Cell inclusions: mucilaginous idioblast present and scattered. **Vascular bundles:** collateral, arranged in one row, alternately between small and large bundle, centrally in the mesophylls of *Vanilla griffithii* and *Vanilla sanguineovenosa* and close the adaxial layer in *Vanilla* sp. 1 and *Vanilla kinabaluensis*, sclerenchyma cells ensheathing the vascular bundles, more on the phloic side. **Bundle sheaths:** basically isodiametric, some polygonal. **Outline of leaf midrib:** obscure. **Outline of leaf margin:** *Vanilla griffithii*, rounded with

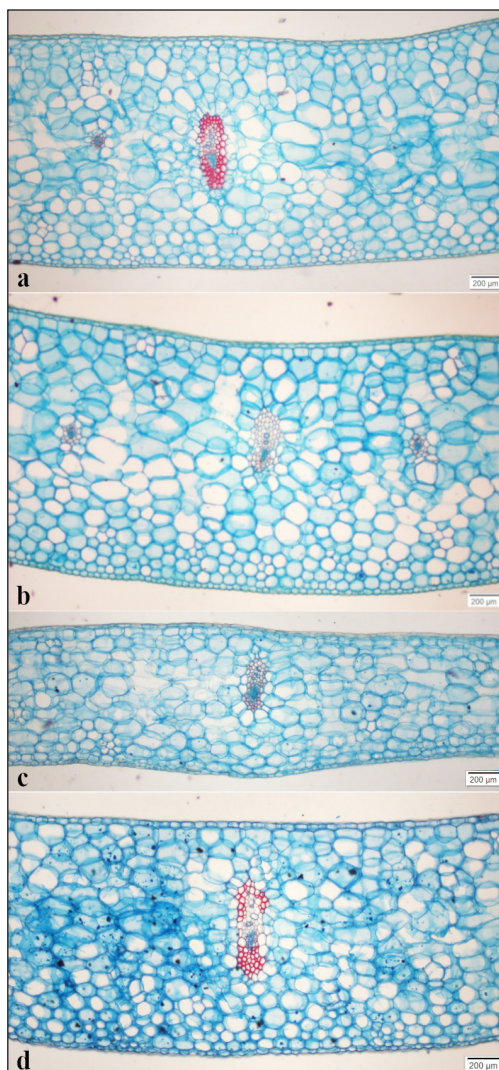


Figure 4. Lamina transverse sections of *Vanilla* species from Perak. a) *Vanilla griffithii*; b) *Vanilla* sp. 1; c) *Vanilla kinabaluensis*; d) *Vanilla sanguineovenosa*

convex-shaped outline, apex acute, recurved 10° towards the abaxial layer, no dent present; *Vanilla sp. 1*, rounded with convex-shaped outline, apex acute, dent present; *Vanilla kinabaluensis*, pointed with blunt outline, apex obtuse, recurved 20° towards the abaxial layer, no dent present; *Vanilla sanguineovenosa*, pointed with needle-shaped outline, apex acute, recurved 45° towards the abaxial layer, no dent present (Figure 5).

DISCUSSION

Variations in Leaf Anatomy of Four Species from Perak

Information on the architecture of the cell has a significant role in plant systematics (Silva et al., 2014) since these cytological components displayed traits that are genetically controlled (Carlsbecker & Augstein, 2018). The inclusion of anatomical evaluation with the data in morphology is vital in plant conservation as it allows reliable identification of the conservation units (Heywood & Iriondo, 2003). As for the *Vanilla* species from Perak, their characterization of the cells composition in both epidermal surfaces and lamina transverse sections were generically congruent with the descriptions by Stern and Judd (1999) on the American and African species suggesting the genus to share plesiomorphic characters from its progenitor. However, crystalliferous idioblast documented in that study were absence among the assessed species but in the form of mucilage suspected to result from genetic and environment differentiation caused

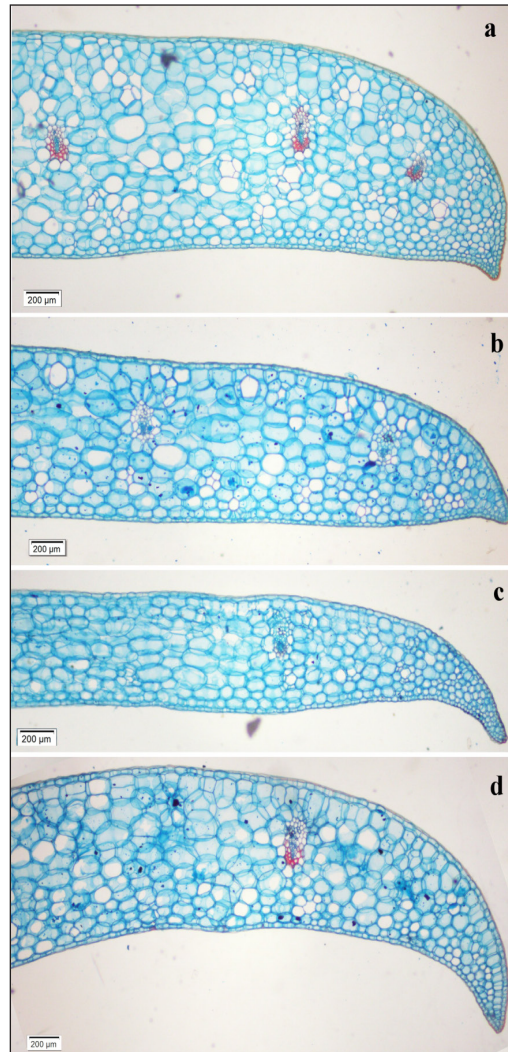


Figure 5. Margin transverse section of *Vanilla* species from Perak. a) *Vanilla griffithii*; b) *Vanilla sp. 1*; c) *Vanilla kinabaluensis*; d) *Vanilla sanguineovenosa*

by biogeographical divergence (Konyar et al., 2014). Stomatal characteristics enumeration in the epidermal layer showed that the length, density and frequency were in accordance with the respective leaf sizes; lesser in smaller leaves and larger in broader ones. Since the stomatal size of plant species were reported to show significant positive

correlation to its genomic size (Hodgson et al., 2010) while the density and index were found to aid in the identification of *Ficus* species (Ogunkule & Oladele, 2008), it implies that these data could serve as discriminating factors. Furthermore, the stomatal size in angiosperms was noted as key to plant survival as species with smaller stomata would be able to adapt faster to the environmental shifts (Drake et al., 2013). This was in agreement with the stomatal characteristics depicted by the widely distributed *V. griffithii* as the species used in this study was collected in the uncharacteristic locality of xeric condition. Another eminent variation was noted in the mesophyll layer thickness of the leaf lamina. Each species displayed different range in the number of layers which *V. griffithii* appeared to have the thickest. This characteristic should be treated as a promising identification tool as it was demonstrated to be one of the delimitating characters in *Cussonia* (De Villiers et al., 2010). The species examined also depicted two types of vascular bundle arrangements which were centrally and adaxially and grouped them into two respective groups sharing similarity in overall leaf shape; a) elliptic (*V. griffithii* and *V. sanguineovenosa*) and b) oblong (*Vanilla* sp. 1 and *V. kinabaluensis*). The importance of vascular bundles orientation and arrangement in providing species clustering were shown in the taxonomic study on *Passerina* species by Bredenkamp and Van-Wyk (2001). However, the assessment of these characteristics is only useful in the lamina

part as its arrangement in all species is more adaxially positioned when moving towards the margin. Besides that, the studied species showed no prominent midrib outline confirming the foliar architecture of *Vanilla* orchids by Cameron and Dickison (1998). However, margin outlines were found to differ interspecifically and acknowledged to possess taxonomic value.

Taxonomic Significance of Transverse Section of Leaf Marginal Outlines

In general, the leaf margin outline among the species can be described as tapering, apex consisting of mesophyll cells flanked by hypodermal layers of which cells division at the adaxial layer determined the part orientation, vascular bundles present towards the marginal edge and presence of scattered mucilaginous inclusion. This study proposed the marginal outline to serve as a delimiting character in the leaf parts of the examined *Vanilla* (Figure 6). This is concordance with the anatomical assessment of different plant habits, from shrubs to woody, which pinpointed the incorporation of leaf marginal outline to be beneficial in plant identification (Hussin et al., 2000; Kantachot et al., 2007; Srinual & Thammathaworn, 2008; Talip et al., 2003, 2012). However, comparative anatomy investigations on the marginal outline were not aware to be reported in *Vanilla* or other genera in Orchidaceae except for the characterization of its cells composition in certain species (Stern, 2014). The character of marginal outline is usually neglected compare to petiole and midrib anatomical

characteristics (Talip et al., 2003). Hence, this study should serve as a preliminary data on the utilization of the distinguishing character. A proposed dichotomous key depicting the classification of each accession using leaf marginal outlines are provided as follows:

Tentative Key to the Species from Perak, Malaysia

- 1 Marginal outline rounded with convex-shaped 2
- 2 Marginal outline pointed with various shapes 3
- 2 Apex straight, dented on abaxial *Vanilla sp. 1*
- Apex recurved, smooth (no dent present) on abaxial *Vanilla griffithii*
- 3 Apex acute, recurved 45° towards abaxial..... *Vanilla sanguineovenosa*
- Apex obtuse, recurved 20° towards abaxial *Vanilla kinabaluensis*

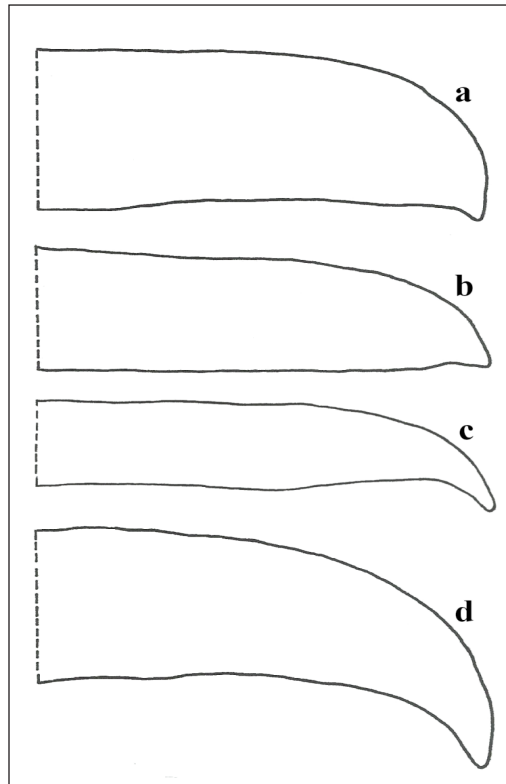


Figure 6. Schematic diagram of the marginal outlines of *Vanilla* species from Perak. a) *Vanilla griffithii*; b) *Vanilla sp. 1*; c) *Vanilla kinabaluensis*; d) *Vanilla sanguineovenosa*

CONCLUSION

Leaf marginal outlines were acknowledged to be one of the distinguishing anatomical characters among *Vanilla* species from Perak.

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