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Morphological and anatomical characteristics and DNA barcode of *Vanilla tiendatii*

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ABSTRACT

Vanilla tiendatii is a rare species and it has only been found in Quy Dat town, Minh Hoa district, Quang Binh Province, Vietnam. In this study, the detailed anatomical characteristics of this species were provided for the first time. Furthermore, five molecular markers, including ITS, *psa*B, *psb*B, *psb*C, and *mat*K were firstly successfully sequenced. The lengths of these regions were ranging from 606 to 730 bps. They were deposited in the NCBI database with the accession numbers of PP696975, PP721190, PP721191, PP721189, and PP721188, respectively. The results also demonstrated that there were differences in these molecular markers between V. *tiendatii* and other *Vanilla* species, V. *yersiniana* and V. *albida*, which were similar in morphological characteristics.

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INTRODUCTION

The *Vanilla* Miller is one of the largest genera within the Orchidaceae family, with over 100 accepted species. These plant species are widely distributed in subtropical and tropical regions, including West Africa, Asia, South, and Central America (Pridgeon *et al.*, 1999; Cameron, 2012). Several commercial *Vanilla* plants were cultivated as the source of the vanilla flavor. The life forms of *Vanilla* species are epiphytic, lithophytic or terrestrial climbers; the stems are thick or thin creep; flowers are short lived (Pridgeon *et al.*, 1999). In Vietnam, six species belonging to *Vanilla* genus have been recorded so far, including V. *aphylla* (Blume, 1825), V. *atropogon* (Schuiteman *et al.*, 2013), V. *cardinalis* (Averyanov *et al.*, 2022), V. *siamensis* (Downie, 1925), V. *tiendatii* (Nguyen *et al.*, 2020), and V. *yersiniana* (Guillaumin, 1964).

Vanilla tiendatii Vuong, V.H.Bui, V.S.Dang & Aver. was described as the new species for the flora of Vietnam in 2020 in which the type specimens were collected from Trung Hoa commune, Minh Hoa district, Quang Binh province, Vietnam. The morphological features of this species are characterized by having: lithophytic habitat; a little branching or simple stem; glossy green, ovate or broadly elliptic leaf blade; the lip apex of the flowers are reddish or pink, and densely covered by a group of fat papillate hairs (Nguyen *et al.*, 2020). To date, the V. *tiendatii* are only found in the type location and studies on this plants are limited. Currently, accurate classification of plant species, especially valuable plants like so many *Vanilla* species is very important. Thus, several supporting methods have been applied to solve the difficulties in taxonomy using the comparative morphological assay in which anatomical and molecular methods are considered to play an important role in standardization and classification of valuable plants (Stern & Judd, 2000; Van *et al.*, 2022). The present study, thus, provides the details of the anatomical characteristics and DNA barcode of V. *tiendatii* for the first time.

MATERIAL AND METHODS

Plant Materials

The specimens of *Vanilla tiendatii* were collected from Quy Dat town, Minh Hoa district, Quang Binh province, Vietnam,

approximate coordinates 17.833203 N, 105.965277 E, on 06 April 2023. The vouchered specimen (NPN-1120) was deposited at the Herbarium of the University of Science, Vietnam National University-HCMC (PHH).

In addition, the molecular regions of two *Vanilla* species, *V. yersiniana* and *V. albida*, from the NCBI database were also used in this study (Table 1).

Methods

Morphological characteristics

The process of collecting samples and determining the scientific name of the studied species is carried out according to the guidelines of the Royal Botanic Gardens, Kew (Bridson and Forman 1999). The vegetative and reproductive characteristics of *V. tiendatii* were compared with those of previous reports (Blume, 1825; Downie, 1925; Guillaumin, 1964; Schuiteman *et al.*, 2013; Nguyen *et al.*, 2020; Averyanov *et al.*, 2022).

Anatomical characteristics

The leaf, stem, and root of *V. tiendatii* were cut into thin slices and then, they were bleached using Javel water. The iodine green-carmine double staining method was used to stain these microscopic specimens. They were washed with water several times and preserved in 10% glycerol (Truong *et al.*, 2007). The specimens were observed and taken the pictures using the Olympus BX53 Digital Upright Microscope.

DNA extraction, PCR reaction and sequencing data analysis

The total DNA of *Vanilla tiendatii* was extracted from the fresh leaves using the CTAB 2X method with some modification (Aboul-Maaty & Oraby, 2019). One nuclear ribosomal RNA gene, internal transcribed spacer (ITS) region, and four chloroplast genes, including psaB, psbB, psbC and matK from the studied species were amplified using a Mastercycler PCR machine (Eppendorf, Germany) with the PCR components following: 12.5 µL master mix (Phu Sa Company, Vietnam), 1.25 µL of each forward and reverse primer (Table 2) at concentration of 10 µM; 9.0 µL deionized water and 1.0 µL DNA sample. The PCR cycle consists of 5 minutes at 95 °C; 35 cycles including, denaturation (1 minute at 94 °C), primer annealing (1 minute 30 seconds at 55 °C) and a final extension (1 minute 30 seconds at 72 °C). PCR products were purified and sequenced at the LOCI Institute of Molecular Biology (Thu Duc City, Ho Chi Minh City, Vietnam) using an ABI 3500: DNA analysis system 3500 Series Genetic Analyzer (Applied Biosystems™ 3500 XL Genetic Analyzer). The five studied sequences of *V. tiendatii* were processed using FinchTV and Bioedit softwares. The Basic Local Alignment Search Tool (BLAST) on NCBI (National Center Biotechnology Information) database was used to align studied sequences with those of closely related species from NCBI database.

Table 1: The sequences on the NCBI database used in this study

	Accession numbers				
Molecular regions	*psa*B	*psb*B	*psb*C	*mat*K	ITS
V. yersiniana	KF835666	KF835678	KF835692	-	-
V. albida	FN545390	FN545437	FN545484	MW828232	MW829668

RESULTS AND DISCUSSION

Taxonomic Treatment

Vanilla tiendatii Vuong, V.H. Bui, V.S. Dang & Aver. Taiwania 65: 438-442, 2020 (Figure 1)

Type: Truong Ba Vuong, Bui Van Huong, BV 355 (holotype: VNM 00023882), Trung Hoa commune, Minh Hoa district, Quang Binh province, Vietnam.

Studied specimens: NPN-1120 (PHH!), Quy Dat town, Minh Hoa district, Quang Binh province, Vietnam, approximate coordinates 17.833203 N, 105.965277 E, 06 April 2023.

Distribution: *V. tiendatii* was only found in the type location (Minh Hoa district, Quang Binh province, Vietnam).

Ecology: the plant is found in the dry limestone forests and has a climbing form, grows with some climbing species belonging to Araceae family like *Rhaphidophora* spp.

Figure 1: *Vanilla tiendatii* Vuong, V.H. Bui, V.S. Dang & Aver. a) Habit, b) Leaves, c) Stem, d) Inflorescence, e) Flower, side view, f) Flower, frontal view, g) Column, frontal view and h) Apical part of column and anther cap with pollinia. Photos Nga Nguyen-Phi

Anatomical Characteristics

Roots (Figures 2 & 3)

The cross-section of root is nearly circular and divided into 2 distinct regions in which 2/3 of the radius belongs to the cortical area while the remaining part is a pith area. *Cortex*: the piliferous layer includes many root hairs at the underground part while the above part remains the traces with a layer of distorted polygonal cells, irregular in size, and curved walls impregnated with phellem. The exodermis layer consists of 1-3 layers of polygonal cells, closely arranged, the outermost cell layer has walls impregnated with phellem and 3-4 times larger than the inner cell layers. The cortical parenchyma contains the globose or polygonal cells with cellulose walls, irregular in size, haphazardly arranged; the endodermis with its casparian strips are clear. *Stele*: the pericycle consists of 2-4 layers of polygonal cells, cellulose walls or impregnated with walls impregnated with lignin, irregular in size, interspersed with endodermic cells. The phloem and xylem are arranged below the pericycle, including 10-11 bundles of phloem and 10-11 bundles of protoxylem arranged alternately in a circle. Phloem bundles are oval; cells are polygonal, irregular in size, haphazardly and radially arranged. The protoxylem bundle is triangular, consisting of 3-5 polygonal vessels, with radial differentiation. There are 12-13 large metaxylem vessels that connected or not connected with the protoxylem bundle. The medullary rays consist of 3-5 rows of polygonal cells, irregular in size, walls impregnated with lignin, 2-3 layers around the phloem bundle, thicker walls, clearly seen in underground roots. The medullary parenchyma includes nearly globose cells with walls impregnated with lignin or cellulose, irregular in size, and haphazardly arranged.

Stem (Figure 4)

The micrograph stem cross section is nearly globose. The epidermis consists of a layer of polygonal cells, cellulose wall, flat cuticle, scattered with stomata. There is no distinction between the cortical parenchyma and medullary parenchyma; the medullary parenchyma includes many layers of globose or polygonal cells, haphazardly arranged, scattered with large air cavities. There are many vascular bundles, the larger they get into the micrograph, scattered in the parenchyma area, including phloem above, xylem below, surrounded by sclerenchyma sheath. The xylem consists of 1-2 the large metaxylem vessels and 0-2 small protoxylem vessels, irregular, surrounded by polygonal parenchyma cells, wall impregnated thin lignin. The phloem cells are polygonal, have wavy walls,

Table 2: The sequences of five PCR primers used in this study

Primers	5′-3′	References
psaB49L/psaB848R	CCGTCGCAAGGAAAACTATAA/TTCGGGATTGGTCACAGTAT	Bouetard *et al.*, 2010
psbB434L/psbB1212R	TGGTCCTGGAATATGGGTGT/CCTAATTGGGCACGTCTAGC	Bouetard et al., 2010
psbC25L/psbC786R	GGTCTGGCTCTGAACCTACG/GGGCTAAGGGTCAARTTGGT	Bouetard et al., 2010
KIM 3-F/KIM 1-R	CGTACAGTACTTTTGTGTTTACGAG/ ACCCAGTCCATCTGGAAATCTTGGTTC	Taberlet *et al.*, 1991
ITS1/ITS4	TCCGTAGGTGAACCTGCGG/TCCTCCGCTTATTGATATGC	White *et al.*, 1990

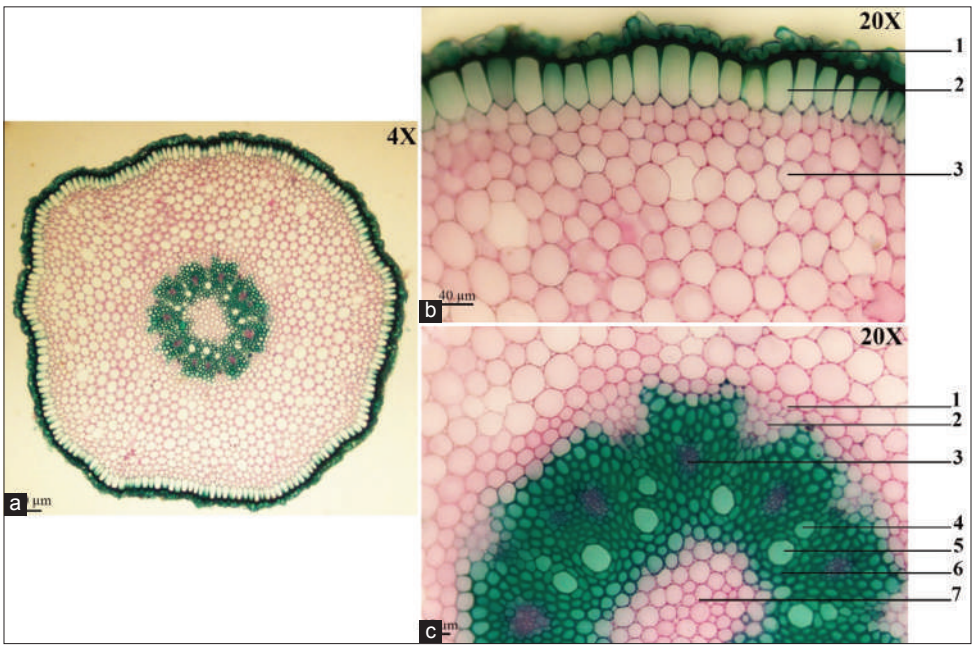

Figure 2: The cross-section of aerial roots of *V. tiendatii*. a) the whole cross-section, b) cortex (1: piliferous layer, 2: exodermis, 3: cortical parenchyma) and c) stele: (1: endodermis, 2: pericycle, 3: primary phloem, 4: primary xylem 1, 5: metaxylem, 6: sclerenchymatous conjunctive tissues, 7: parenchymatous pith)

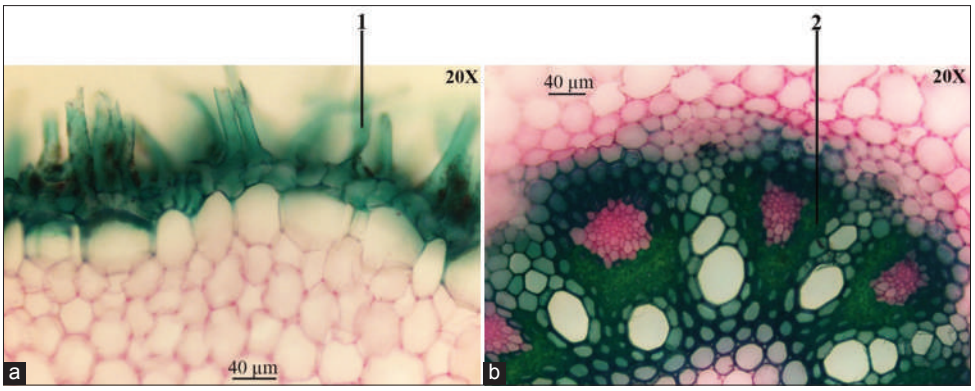

Figure 3: The cross-section of underground roots of *V. tiendatii.* a) cortex (1: root hairs) and b) stele (2: Medullary ray)

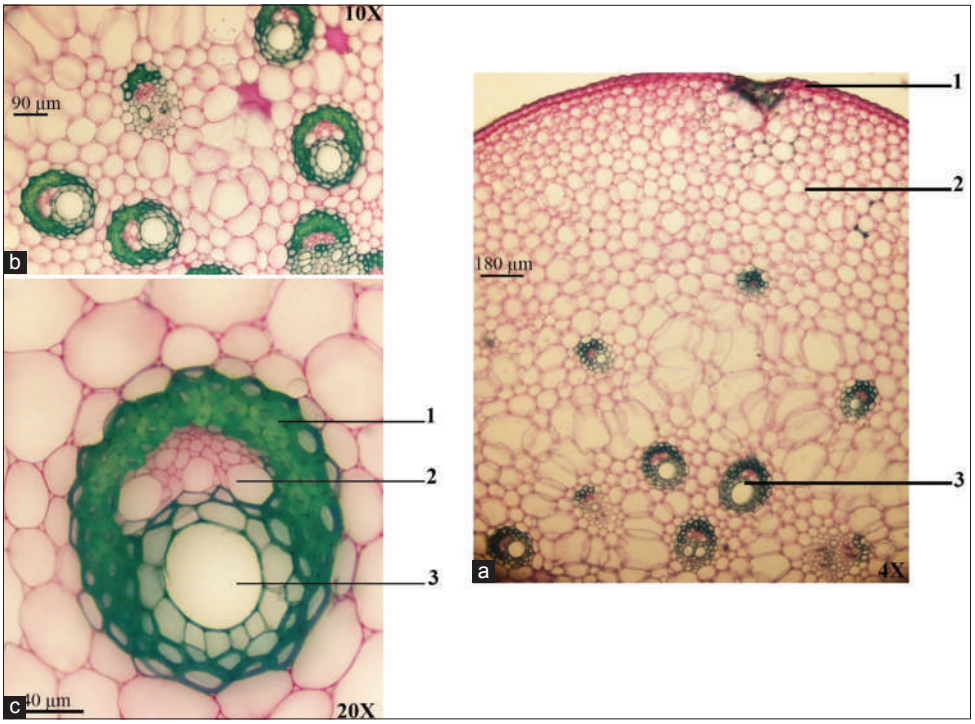

Figure 4: The cross-section of stem of *V. tiendatii.* a) view of cross-section (1: epidermis, 2: parenchyma cell, 3: vascular bundle), b and c) vascular bundles (1: sclerenchymatous bundle sheath, 2: primary phloem, 3: primary xylem)

irregular, haphazardly arranged; sclerenchyma sheath includes 1-3 layers of polygonal cells, wall impregnated thin lignin.

Leaves (Figure 5)

The micrograph leaf cross section has a slightly concave upper side, convex lower side. The midrib and leaf blade have the same structure.

The upper epidermal cells are rectangular while the lower epidermal cells are polygonal, irregular in size, with cellulose walls; flat cuticles. The parenchyma cell consists of globose or polygonal cells with thin cellulose walls, haphazardly arranged, increasing size towards the inside of the micrograph. Needle-shaped calcium oxalate crystals and starch granules are scattered in the parenchyma area. The vascular bundles are narrow and long, arranged in a row; xylem above, phloem below, surrounded by sclerenchyma sheath. The xylem includes 1-2 nearly globose vessels, irregular in size located in the parenchyma area; the xylem consists of polygonal cells, thin cellulose walls, haphazardly arranged. The phloem includes polygonal cells with curved walls, irregular in sizes, and haphazardly arranged. The sclerenchyma sheath consists of 1-3 layers of polygonal cells, wall impregnated thin lignin, closely arranged.

Sclerenchyma may or may not be associated with the vascular bundles; in larger bundles it surrounds the entire bundle always being stronger on the phloic side than on the xylic or lateral sides and in smaller bundles occurring only on the phloic side.

Pairwise Sequence Alignment of Studied Regions Among *V. tiendatii* and Other Related Species

The lengths of 5 sequences of V. *tiendatii*, including ITS, *psa*B, *psb*B, *psb*C, and *mat*K regions were ranging from 606 to 730 bps. All these regions were deposited in NCBI database with the accession numbers of PP696975, PP721190, PP721191, PP721189, and PP721188, respectively. V. *tiendatii* has been recently described as a new species for the flora of Vietnam by Nguyen *et al.* (2020) and the authors of this report also provided that the morphological characteristics of V. *tiendatii* were similar to V. *yersiniana* and V. *albida*. In this paper, thus, the BLAST on NCBI database was used to align five DNA regions of V. *tiendatii* with those of V. *yersiniana* (*psa*B, *psb*B, *psb*C) and V. *albida* (ITS, *psa*B, *psb*B, *psb*C, *mat*K) from NCBI database. The results are shown in the Figures 6 and 7. Accordingly, the pairwise alignment of the *psa*B region between V. *tiendatii* and V. *yersiniana* showed two non-homologous positions per 704 base pairs of the entire aligned length (Figure 6a). Meanwhile, on the entire aligned length of *psb*B (610 base pairs) and *psb*C (620 base pairs) regions, there were one and three non-homologous positions, respectively were recorded (Figures 6b & c). Similarly, Figure 7 showed the pairwise sequence alignment of *psa*B, *psa*B, *psb*C, *mat*K, and ITS regions between V. *tiendatii* and V. *albida*. Accordingly, one non-homologous position was found in the *psa*B region (Figure 7a) while *psb*B and *psb*C regions contained two non-homologous positions (Figures 7b & c). Notably, there were the significant differences in the *mat*k and ITS regions between V. *tiendatii* and V. *albida*. Accordingly, five non-homologous positions were recorded per a total alignment length of 498 base pairs (Figure 7d). Meanwhile, in the ITS region, this difference included twenty non-homologous positions and one gap per a total alignment length of 602 base pairs (Figure 7e).

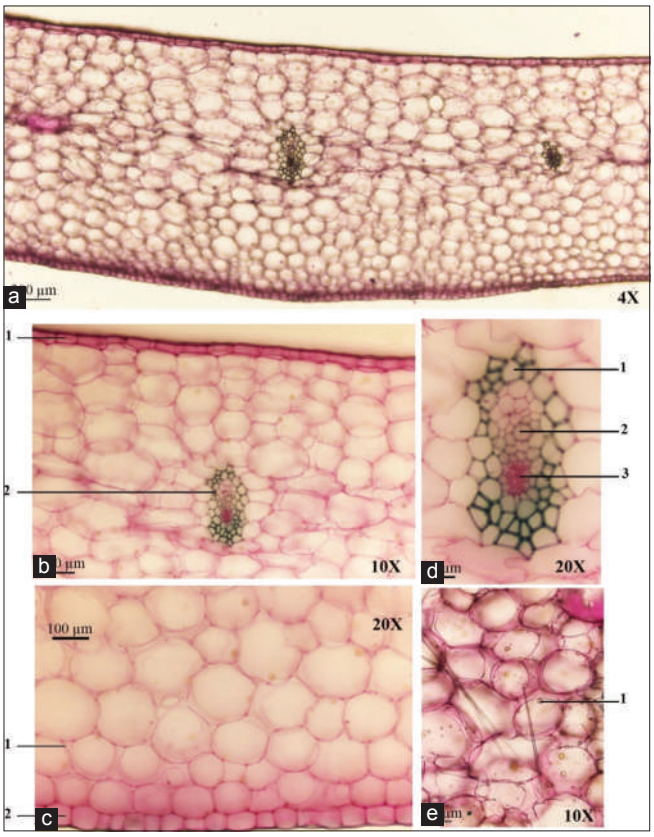

Figure 5: The cross-section of leaves of *V. tiendatii*. a) view of cross-section, b1) uppper epidermis, b2) vascular bundle, c1) parenchyma cell, c2) lower epidermis, d) vascular bundle (1: sclerenchymatous bundle sheath, 2: primary xylem 1, 3: primary phloem) and e) needle-shaped calcium oxalate crystals (1: starch)

Previous studies demonstrated that molecular markers were one of the effective tools in plant taxonomy. For instance, the *psb*A-*trn*H and ITS regions were used to distinguish *Paris vietnamemsis* from other *Paris* species (Nguyen *et al.*, 2018). Two morphologically similar species, *Camellia tamdaoensis* and *Camellia petelotii*, were also distinguished by *mat*K region (Ha & Nguyen, 2015). Moreover, matK and ITS sequence regions also were used to distinguish two *Rothmannia* species such

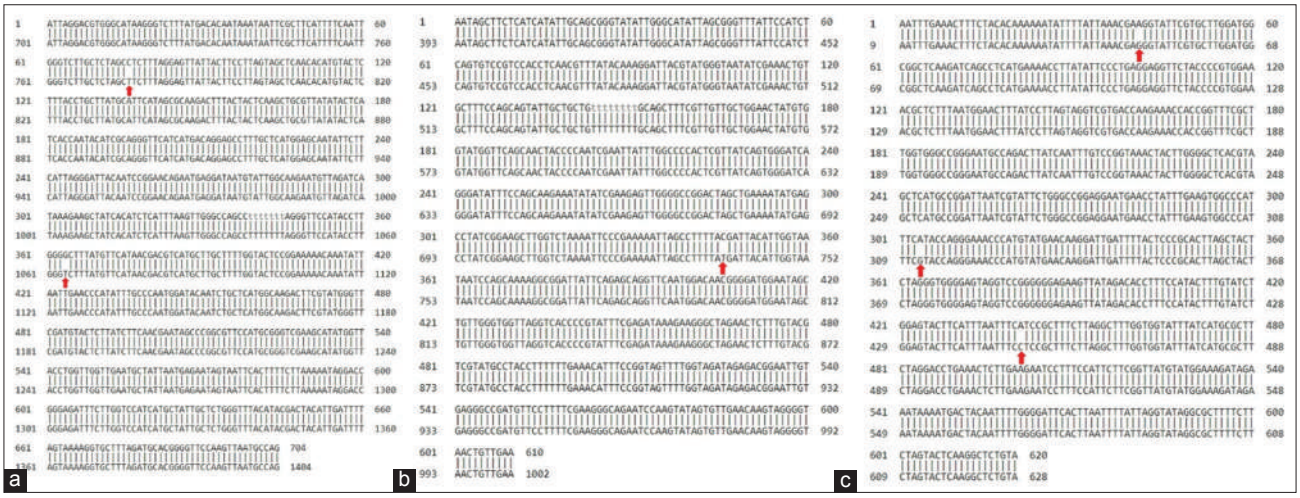

Figure 6: Pairwise sequence alignment of three studied regions between *V. tiendatii* and *V. yersiniana*. Note: a) *psa*B, b) *psb*B and c) *psb*C; the upper rows belong to V. tiendatii while lower rows are included to *V. yersiniana*.

Figure 7: Pairwise sequence alignment of five studied regions between *V. tiendatii* and *V. albida*. Note: a) *psa*B, b) *psb*B, c) *psb*C, d) matK and e) ITS; the upper rows belong to *V. tiendatii* while lower rows are included to *V. albida*

as *R. wittii* and *R. daweishanensis* (Ton *et al.*, 2019). Apart from that, three molecular markers, including *mat*K, *trn*L-F, and ITS were used to distinguish two new species, *Curcuma xanthella* and *Curcuma cotuana* with other *Curcuma* species such as *C. rhomba*, *C. vitelline*, *C. flaviflora*, and *C. singularis* (Van *et al.*, 2022). Similarly, Van *et al.* (2017) demonstrated that *Arisaema condaoense* and *A. roxburghii*, two similar morphological characteristics, were distinct species using *mat*K region. Based on the *trn*L intron and *trn*L-F IGS regions, Van *et al.* (2020) proposed that *Aglaodorum griffithii* should be transferred to *Aglaonema* genus. Claudel *et al.* (2017) transferred all *Pseudodracontium* species to the genus *Amorphophallus* using some molecular markers, including the *mat*K, *rbc*L, *Flint*2, and ITS1. Ngoc-Sam *et al.* (2017) established a new genus, *Vietnamocasia*, with only one species, *Vietnamocasia dauae*, based on the morphological characteristics and molecular markers such as *phy*C, *rpl*20-*rps*12, *trn*K/*mat*K, *trn*L-F.

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