Characterization of *Ceropegia vietnamensis*: a study based on morphological, microanatomical and molecular data

Caracterización de *Ceropegia vietnamensis*: un estudio basado en datos morfológicos, microanatómicos y moleculares

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Highlights

- This study presents the first detailed morphological, anatomical, and molecular data (ITS and *trn*L) for *C. vietnamensis*, supporting its taxonomic delimitation.
- These data strengthen the taxonomy of section *Esculentae* and facilitate the recognition of *C. vietnamensis* among related species in *Ceropegia*.

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Ceropegia vietnamensis; morfología vegetal; microanatomía comparada; *ITS*; intrón *trn*L.

ABSTRACT

Introduction. *Ceropegia vietnamensis* is a recently described, range-restricted species endemic to the Binh Chau–Phuoc Buu Nature Reserve, Vietnam. It closely resembles *C. laotica*, *C. bulbosa*, and *C. murlensis* in general morphology and floral traits, making its distinction based solely on external characters challenging. **Objectives.** This study provides the first integrated dataset for *C. vietnamensis*, combining detailed morphological, anatomical, and molecular evidence to support its recognition and facilitate future taxonomic assessments. **Materials and Methods.** Comparative morphology was used to confirm species identity. Anatomical structures of vegetative organs were examined via transverse sections stained with iodine green–carmine. Total genomic DNA was extracted using the CTAB 2X protocol, and the ITS and *trn*L intron regions were amplified by PCR. **Results.** The anatomical structure of *C. vietnamensis* was described for the first time and compared to the closely related *C. bulbosa* var. *lushii*, revealing diagnostic differences in root, stem, and leaf anatomy. Sequence comparisons showed 93–96% identity in the ITS region and 99% identity in the *trn*L region when aligned with *C. bulbosa* and *C. murlensis*, indicating moderate molecular divergence consistent with species-level distinction. **Conclusions.** The combination of morphological, anatomical, and molecular data strengthens the taxonomic delimitation of *C. vietnamensis* and contributes valuable reference information for future phylogenetic and systematic studies within the genus *Ceropegia*.

RESUMEN

Introducción. *Ceropegia vietnamensis* es una especie recientemente descrita y de distribución restringida, endémica de la Reserva Natural Binh Chau–Phuoc Buu, en Vietnam. Presenta una marcada similitud morfológica con *C. laotica*, *C. bulbosa* y *C. murlensis*, lo que dificulta su delimitación basada únicamente en caracteres externos. **Objetivos.** Este estudio proporciona el primer conjunto de datos integrados sobre *C. vietnamensis*, que incluye evidencia morfológica, anatómica y molecular, con el objetivo de respaldar su reconocimiento taxonómico y facilitar futuras evaluaciones sistemáticas. **Materiales y métodos.** Se realizó un análisis morfológico comparativo para confirmar la identidad de la especie. Las estructuras anatómicas de los órganos vegetativos se examinaron mediante cortes transversales teñidos con verde yodo–carmín. El ADN genómico total se extrajo mediante el protocolo CTAB 2X, y las regiones ITS e intrón *trn*L fueron amplificadas por PCR. **Resultados.** La estructura anatómica de C. vietnamensis se describe por primera vez y se compara con la de *C. bulbosa* var. *lushii*, revelando diferencias diagnósticas en raíz, tallo y hoja. Las comparaciones de secuencias mostraron un 93–96 % de identidad en la región ITS y un 99 % en la región *trn*L frente a *C. bulbosa* y *C. murlensis*, lo que indica una divergencia molecular moderada compatible con separación a nivel de especie. **Conclusiones.** La combinación de datos morfológicos, anatómicos y moleculares refuerza la delimitación taxonómica de *C. vietnamensis* y proporciona una base valiosa para estudios filogenéticos y sistemáticos futuros en el género *Ceropegia*.

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INTRODUCTION

Ceropegia L. sensu stricto comprises approximately 200 species within the subfamily Asclepiadoideae of the family Apocynaceae. These species are widely distributed across China, Madagascar, India, tropical Arabia, the Canary Islands, Africa, northern Australia, New Guinea, and Southeast Asia [1-6]. Loureiro was the first to record three species of *Ceropegia* in the flora of Vietnam: *C. candelabrum*, *C. cordata*, and *C. obtusa* [7]. In 2017, Tran revised the family Asclepiadaceae and documented four species of *Ceropegia*, adding *C. driophila* as a new record for the country [7]. In 2022, *C. vietnamensis* was described as a species new to science [9]. More recently, *C. trichantha* was added to the Vietnamese flora, bringing the total number of *Ceropegia* species recorded in the country to six [10].

Ceropegia vietnamensis Nguyen-Phi & Luu was described based on type specimens collected from the Binh Chau–Phuoc Buu Nature Reserve, Xuyen Moc District, Ba Ria–Vung Tau Province, Vietnam [9]. To date, it remains a rare and narrowly distributed species, known only from its type locality.

Previous studies have shown that species within the genus *Ceropegia* often exhibit highly similar morphological features [7-10], making accurate identification challenging, particularly in the absence of reproductive structures. Consequently, integrative taxonomic approaches are essential for accurate species delimitation in the genus.

Among these, molecular markers and anatomical traits are considered particularly valuable and have been widely applied to distinguish morphologically similar *Ceropegia* species [11-17]. DNA barcoding, which relies on sequence variation within short, standardized regions of the genome, has proven to be a powerful and efficient method for species identification, especially when morphological data are insufficient [18-19]. To date, several DNA barcode regions, particularly *ITS* and *trn*L, have been used to support phylogenetic and taxonomic studies within *Ceropegia* [12-17]. The present study provides, for the first time, DNA barcoding data and anatomical descriptions for *C. vietnamensis*.

MATERIALS AND METHODS

Plant Materials

Specimens of *Ceropegia vietnamensis* were collected from the Binh Chau–Phuoc Buu Nature Reserve, located in Xuyen Moc District, Ba Ria–Vung Tau Province, Vietnam (approximate coordinates: 10°36'27.28"N, 107°33'20.56"E). Two voucher specimens, designated as BC-0102 and BC-0103, were deposited in the Herbarium of the University of Science, Vietnam National University–Ho Chi Minh City (PHH).

Morphological Characterization

Comparative morphological analysis was conducted to confirm the taxonomic identity of the collected specimens [20]. Both vegetative and reproductive traits were assessed and compared against published descriptions of related species within the genus *Ceropegia* [1-6,8-10,21]. Key morphological features,

including stem structure, leaf shape, corolla morphology, and gynostegial architecture, were documented. High-resolution images of floral and vegetative structures were captured using a Canon EOS 90D digital camera fitted with a macro lens to support detailed morphological comparison.

Anatomical Analysis

Freehand transverse sections of the stem, root, and leaf were prepared using a sterilized razor blade. The sections were bleached using sodium hypochlorite (NaOCl) and subsequently stained with a dual stain consisting of iodine green and carmine. This differential staining highlights tissue composition: carmine stains cellulose-based cell walls pink, whereas iodine green stains lignified and suberized cell walls blue, enabling precise tissue differentiation under microscopic examination. After thorough rinsing with distilled water, the sections were mounted and preserved in 10% glycerol[22]. Microscopic observations and image documentation were performed using an Olympus BX53 Digital Upright Microscope.

DNA Extraction, PCR Amplification, and Sequence Analysis

Genomic DNA was extracted from fresh leaf tissue using a modified CTAB 2X protocol [(23)]. The internal transcribed spacer (*ITS*) region of nuclear ribosomal DNA, using ITS1 and ITS4 primers after White et al., [(24)]; and the chloroplast *trn*L intron, using c and d primers after Taberlet et al., [(25)], were selected as DNA barcode regions for amplification. PCR reactions were carried out using a Mastercycler PCR system (Eppendorf, Germany). Each 25 µL reaction contained: (1) 12.5 µL of PCR master mix, (2) 1.25 µL of each forward and reverse primer, (3) 9.0 µL of nuclease-free water, and (4) 1.0 µL of DNA template.

Thermal cycling conditions were as follows: initial denaturation at 95 °C for 5 minutes; 35 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, and extension at 72 °C for 1 minute and 30 seconds; followed by a final extension at 72 °C for 10 minutes. The amplified products were purified and sequenced at the LOCI Institute of Molecular Biology (Ho Chi Minh City, Vietnam) using an ABI 3500 XL Genetic Analyzer (Applied Biosystems™ 3500 Series). Sequence chromatograms were edited and aligned using FinchTV and Seaview software. The *ITS* and *trn*L sequences obtained from *C. vietnamensis* were compared with those of closely related species—*Ceropegia bulbosa* (*ITS* and *trn*L) and *C. murlensis* (*ITS*)—previously published in molecular phylogenetic studies[(12–13)]. Sequence similarity and species-level identity were verified using the Basic Local Alignment Search Tool (BLAST) available through the NCBI database.

RESULTS

Morphological Characteristics of *Ceropegia vietnamensis*

Perennial, climbing herb, up to 2.5 m long. Tuber subglobose, 1.5–4.5 cm in diameter. Leaves simple, opposite, petiolate; lamina lanceolate to linear-lanceolate, 4–12 × 0.6–4.5 cm, base attenuate, apex acute; adaxial surface puberulent across the blade and along the margins, abaxial surface puberulent only along the midvein. Inflorescences extra-axillary, umbellate cymes bearing 3 to 30 flowers, with up to 2 flowers open simultaneously. Calyx composed of 5 sepals, greenish at base; lobes linear-lanceolate, 6–8 × 0.8–1.1 mm,

apex acuminate, reddish, glabrous. Corolla 45–57 mm in total length, glabrous at the base externally, sparsely minutely hairy toward the apex; lobes 5, folded, 24–30.5 mm long. Gynostegium stipitate, pinkish-white, glabrous. Corona biseriate: outer series with 5 deeply bifid lobes, reddish-maroon, ciliate along the margins and inner surface; inner series with 5 erect, flattened, linear lobes. Pollinaria ovoid, yellow. Follicles paired, linear, slightly curved, up to 15 cm long, equal in size (**Figure 1**).

Type: Nguyen Phi Nga & Le Van Son 829 (holotype SGN!; isotypes SGN!, PHH!), Binh Chau–Phuoc Buu Nature Reserve, Xuyen Moc District, Ba Ria–Vung Tau Province, Vietnam. **Distribution:** *C. vietnamensis* is known only from the type locality. **Studied specimens**: BC-0102 and BC-0103, collected from Binh Chau–Phuoc Buu Nature Reserve, Xuyen Moc District, Ba Ria–Vung Tau Province, Vietnam (PHH!).

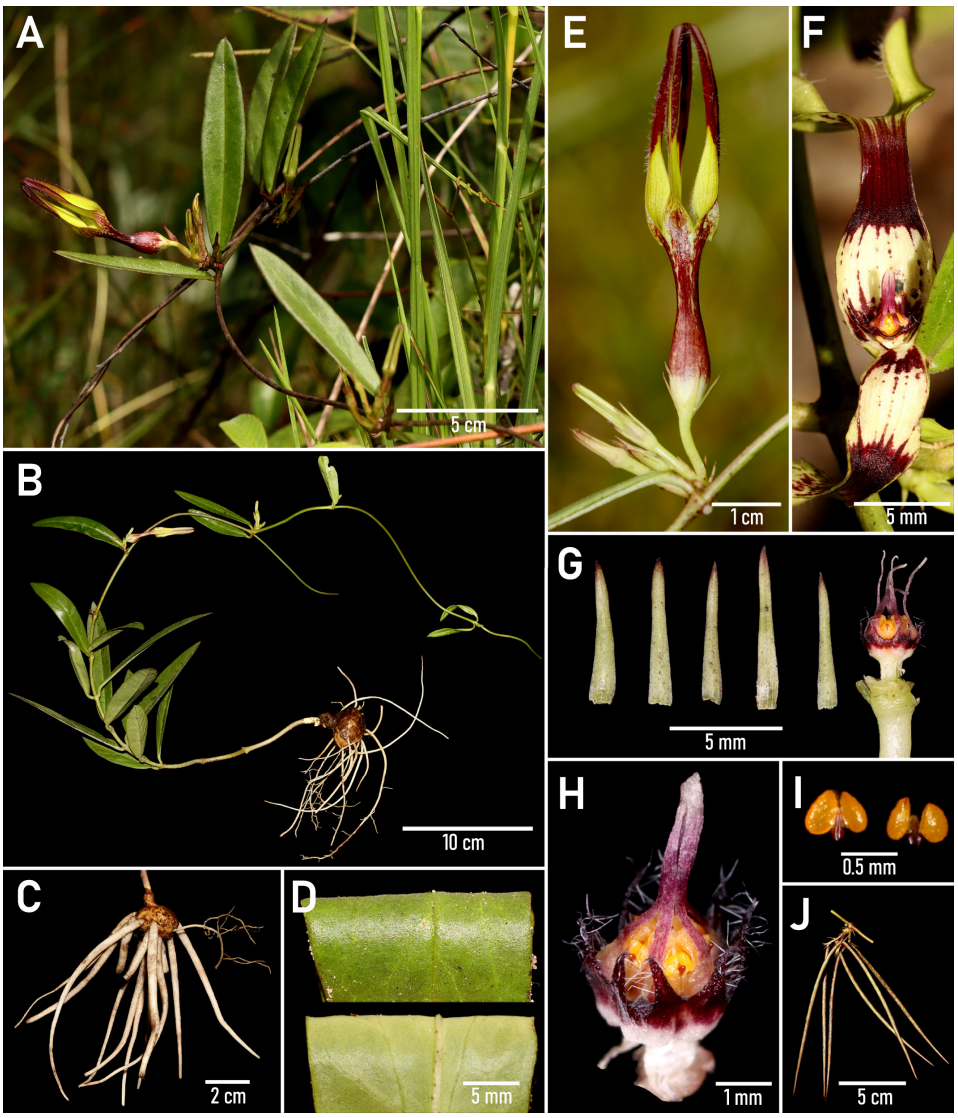

Figure 1. *Ceropegia vietnamensis* Nguyen-Phi & Luu. **A**. Habit. **B.** Whole plant. **C.** Tuber with stout roots. **D.** Adaxial and abaxial surfaces of a leaf. **E.** Inflorescence detail. **F.** Longitudinal section showing floral structure. **G.** Calyx and corona. **H.** Side view of corona. **I.** Pollinaria. **J.** Follicles.

Anatomical Characteristics

Double staining revealed distinct tissue differentiation: primary cell wall tissues (parenchyma, collenchyma, and phloem) appeared red or pink, while secondary cell wall tissues containing lignin or suberin stained blue. Accurate identification of lignified versus suberized tissues also required evaluation of their position, organization, and histological context. Suberized tissues were typically observed at the periphery of roots and stems, while lignified tissues comprised the xylem and sclerenchyma.

Leaf

Midrib: concave on the adaxial side, convex on the abaxial side. The upper and lower epidermis consist of a single layer of rectangular cells with cellulose walls and a thin cuticle on the outer surface, uniform in size and closely packed. Collenchyma forms 2–4 layers beneath the upper epidermis and 1–2 layers above the lower epidermis; cells are polygonal, with cellulose walls, irregular in size, and randomly arranged. Cortical parenchyma consists of polygonal to round cells, irregular in size, with cellulose walls and scattered arrangement. The primary vascular bundle is arc-shaped. The xylem contains 8–11 rows of polygonal vessels with lignified walls; xylem parenchyma has 1–2 layers of rectangular cells with cellulose walls. Phloem occurs in small clusters above and below the xylem. One to two secondary vascular bundles were also observed (**Figure 2**).

Lamina: the upper and lower epidermis are composed of rectangular cells with cellulose walls, and a thin cutin layer on the outer surface. Upper epidermal cells are larger than lower ones. The mesophyll has an asymmetrically differentiated structure. The palisade parenchyma (chlorenchyma) comprises 2–4 layers of elongated rectangular cells arranged parallel to each other and perpendicular to the upper epidermis. The spongy parenchyma includes 2–5 layers of irregular polygonal cells, forming intercellular spaces (**Figure 2**).

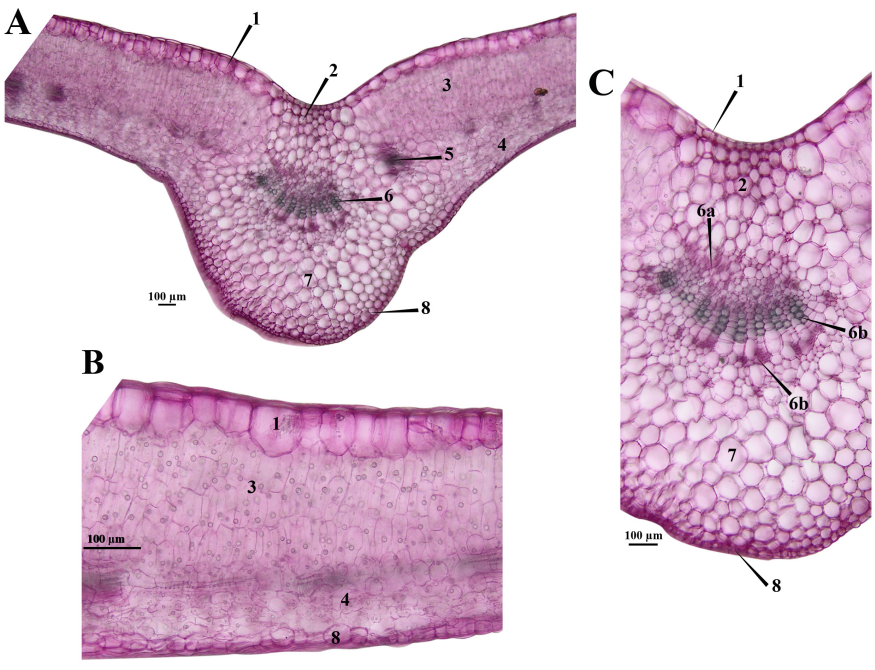

Figure 2. Transverse section of leaf of *C. vietnamensis*. **A.** Whole leaf view. B. Lamina. **C.** Midrib. **1:** upper epidermis, **2**: angular collenchyma, **3:** palisade parenchyma, **4:** spongy parenchyma, **5:** secondary vascular bundle, **6:** primary vascular bundle (6a: inner phloem, 6b: phloem, 6c: xylem), **7:** parenchyma cell, **8:** lower epidermis.

Petiole

Transverse section of the petiole is concave on the upper surface and convex on the lower. Both epidermal layers consist of a single row of rectangular cells with cellulose walls, covered by a thin cuticle. Angular collenchyma consists of 2–3 cell layers beneath the upper epidermis and 1–2 layers above the lower, polygonal, with cellulose walls, irregular in size and randomly arranged. Parenchyma cells are polygonal or round, with cellulose walls and variable dimensions. The primary vascular bundle is arc-shaped. The xylem consists of 9–14 rows of polygonal vessels with lignified walls. Phloem is arranged in small groups above and below the xylem. One or two secondary vascular bundles are present (**Figure 3**).

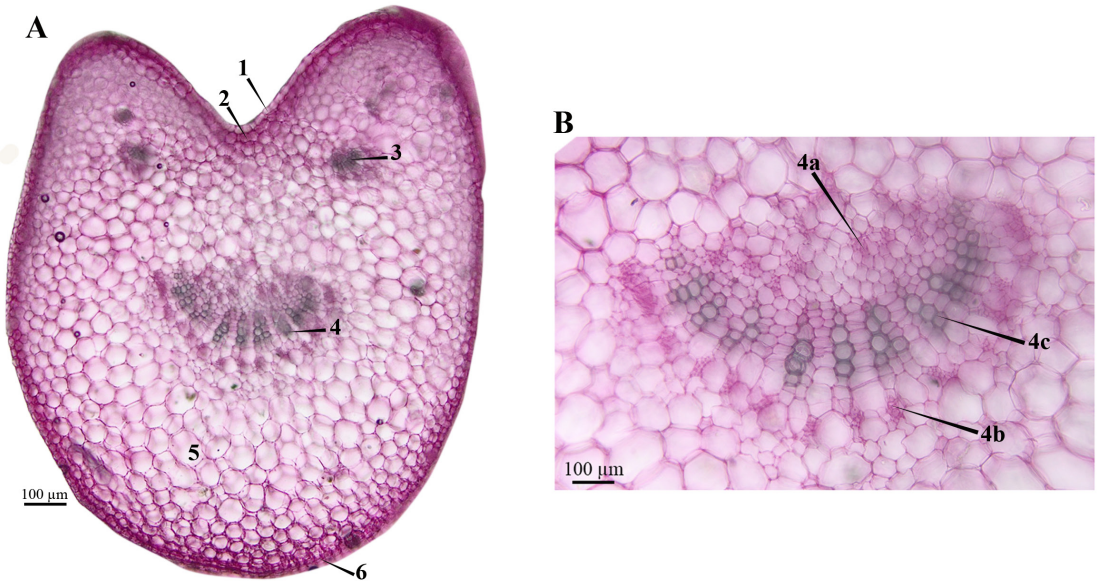

Figure 3. Transverse section of the petiole. **A:** Whole view. **B:** Vascular bundle. **1:** upper epidermis, **2:** angular collenchyma, **3:** secondary vascular bundle, **4:** primary vascular bundle, **5:** parenchyma cell, **6:** lower epidermis. E. Primary vascular bundle (**4a:** inner phloem, **4b:** phloem, **4c:** xylem).

Stem

The stem is circular in cross-section and organized into two main regions: the ***cortex***, occupying one-third of the radius, and the stele, occupying the remaining two-thirds. The epidermis is a single layer of rectangular cells with a thin cuticle. Angular collenchyma forms 1–2 layers of polygonal cells of nearly equal size. Cortical parenchyma consists of 8–10 layers of polygonal cells with cellulose walls and uniform size; scattered cells contain cubic calcium oxalate crystals. Sclerenchyma clusters appear as discontinuous rings within the cortical region (**Figure 4**).

The ***stele*** contains a continuous ring of secondary vascular bundles. Primary phloem comprises 2–4 layers of polygonal cells with cellulose walls, arranged in small groups. Secondary phloem consists of 1–3 layers of rectangular cells with undulated cellulose walls, arranged radially. Secondary xylem includes polygonal to circular vessels with lignified walls, arranged in radial rows, and associated parenchyma cells that are

rectangular, lignified, and evenly spaced. Primary xylem contains polygonal or rounded vessels with centrifugal differentiation and lignified walls, along with irregularly arranged parenchyma with cellulose walls. Medullary rays are composed of 1–2 layers of rectangular lignified cells. Internal phloem appears as scattered clusters beneath the primary xylem. The pith consists of irregularly arranged polygonal parenchyma cells (**Figure 4**).

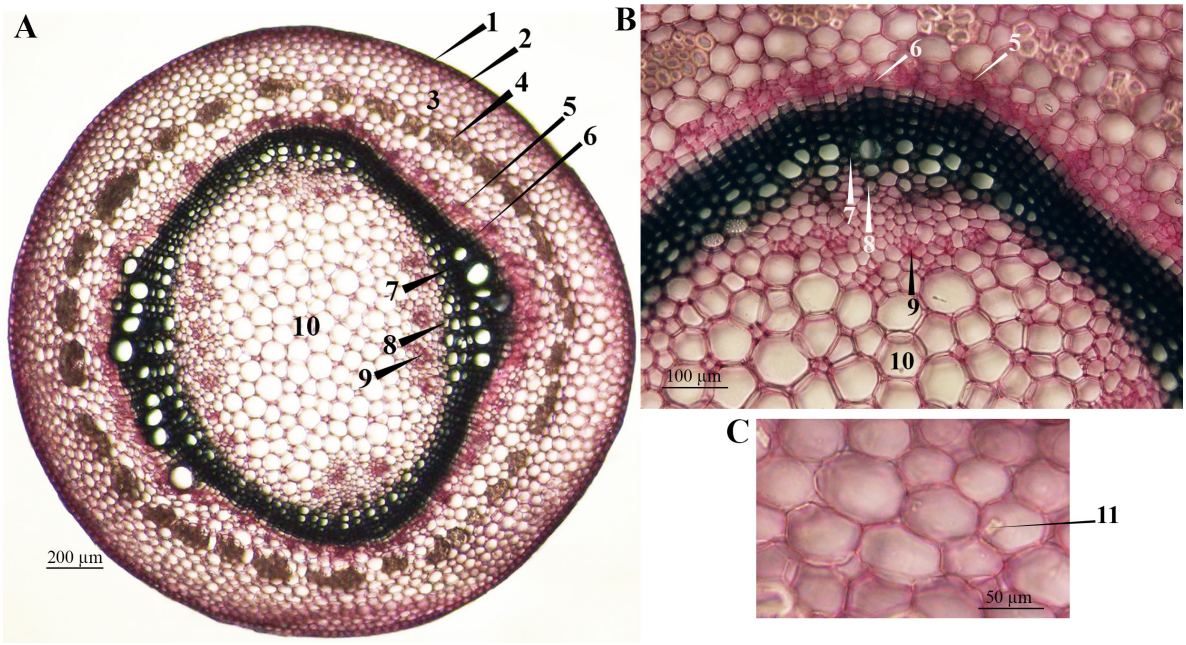

Figure 4. Transverse section of the stem of *C. vietnamensis*. **A:** Whole view. **B:** Stele. **C:** Cubic calcium oxalate crystals. **1:** epidermis, **2:** collenchyma, **3:** cortical parenchyma, **4:** sclerenchyma, **5:** primary phloem, **6:** secondary phloem, **7:** secondary xylem, **8:** primary xylem, **9:** internal phloem, **10:** pith parenchyma, **11:** calcium oxalate crystals.

Root

Root cross-section is nearly circular, divided into a cortical region (occupying three-fourths of the radius) and a stele (occupying one-fourth). ***Cortex:*** the piliferous layer consists of a single layer of polygonal cells with suberized walls, irregular in size, with sparse root hairs. The spongy parenchyma includes 10–12 layers of nearly round to polygonal cells with cellulose walls, irregular in size and arrangement. The endodermis with a conspicuous Casparian strip consists of a single layer of rectangular, regularly shaped cells (**Figure 5**).

Stele: the pericycle is a single layer of polygonal cells with cellulose walls, arranged tightly, and interspersed among endodermal cells. The vascular system contains 2–4 primary xylem bundles alternating with 2–4 primary phloem bundles, separated by medullary rays. Each xylem bundle comprises 2–4 polygonal vessels with lignified walls and radial differentiation. Primary phloem bundles consist of 1–3 layers of polygonal cells with cellulose walls. Medullary rays are made up of 1–2 layers of polygonal cells with cellulose walls. Pith parenchyma consists of polygonal cells with cellulose or lignified walls in older tissues, irregular in size and tightly packed (**Figure 5**).

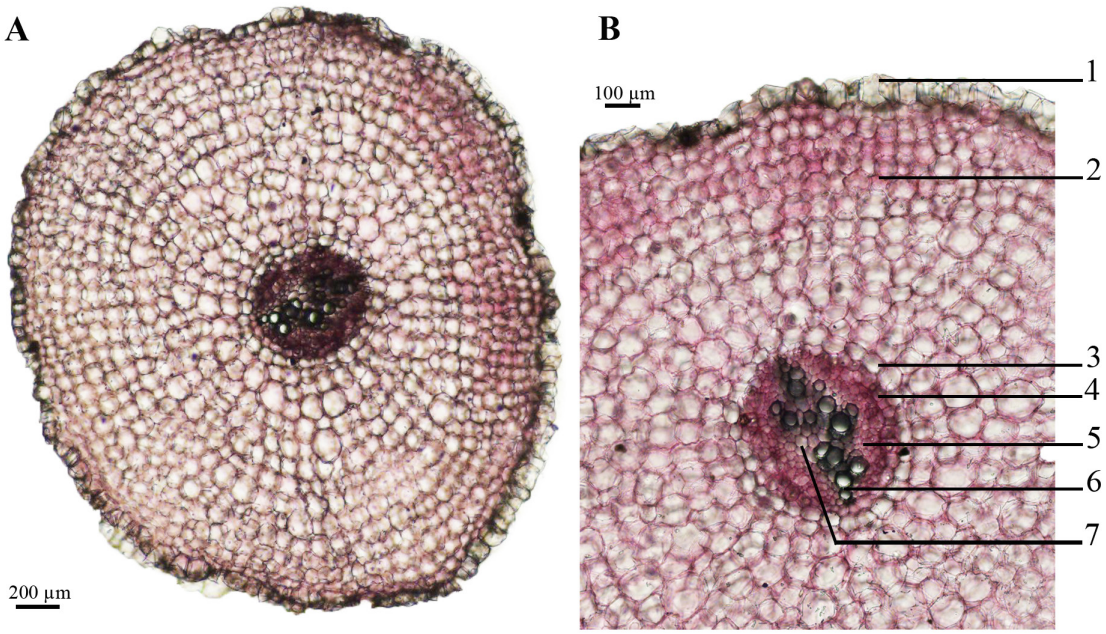

Figure 5. Transverse section of the root of *C. vietnamensis*. **1:** piliferous layer, **2:** cortical parenchyma, **3:** endodermis with Casparian strip, **4:** pericycle, **5:** primary phloem, **6:** primary xylem, **7:** pith parenchyma.

Molecular data

In this study, the *ITS* (Internal Transcribed Spacer) and *trn*L intron regions of *Ceropegia vietnamensis* were successfully amplified and sequenced, providing, to our knowledge, the first published molecular data for this species. The lengths of the *ITS* and *trn*L intron sequences were 557 bp and 467 bp, respectively, and were deposited in the NCBI GenBank database under accession numbers PP975072.1 (*ITS*) and PP975070.1 (*trn*L intron).

For preliminary comparative analyses, the obtained sequences were aligned with those of two morphologically similar species: *C. bulbosa* (*ITS*: KP244993.1; *trn*L: KP245494.1) and *C. murlensis* (*ITS*: MH428808.1), based on previously published data [12-13]. Pairwise alignment between *C. vietnamensis* and *C. bulbosa* revealed 32 nucleotide substitutions and 7 gaps in the *ITS* region, corresponding to 93.0% sequence identity (**Figure 6, Table 1**). In the *trn*L intron, 7 polymorphic sites were detected, resulting in a high sequence identity of 99.0% (**Figure 6, Table 1**). The *ITS* comparison between *C. vietnamensis* and *C. murlensis* showed 21 substitutions and 3 gaps, with 96.0% sequence identity (**Figure 7, Table 1**).

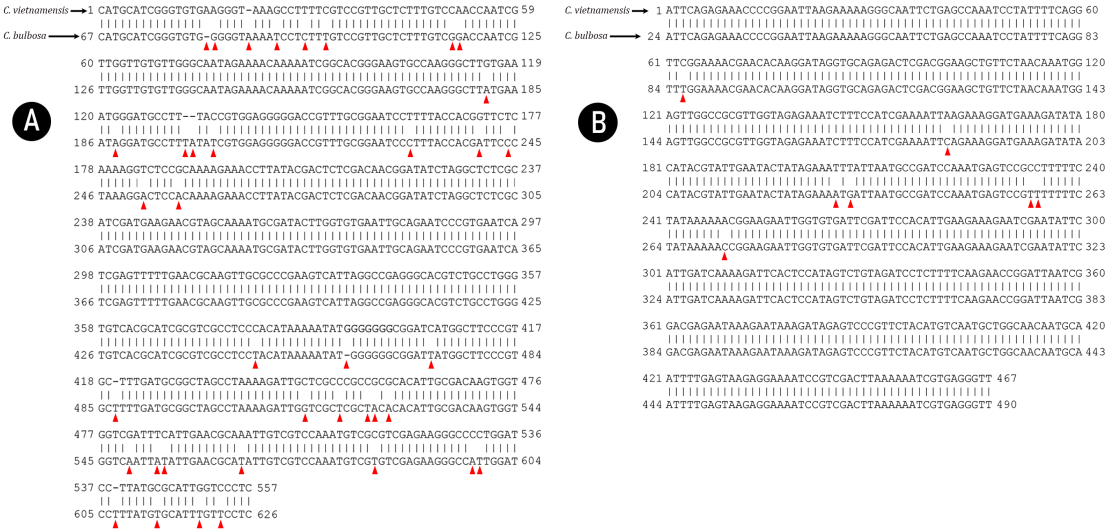

Figure 6. Pairwise alignments of (**A**) the *ITS* region and (**B**) the *trn*L intron region between *C. vietnamensis* (top row) and *C. bulbosa* (bottom row). The red triangles indicate nucleotide differences between the two species.

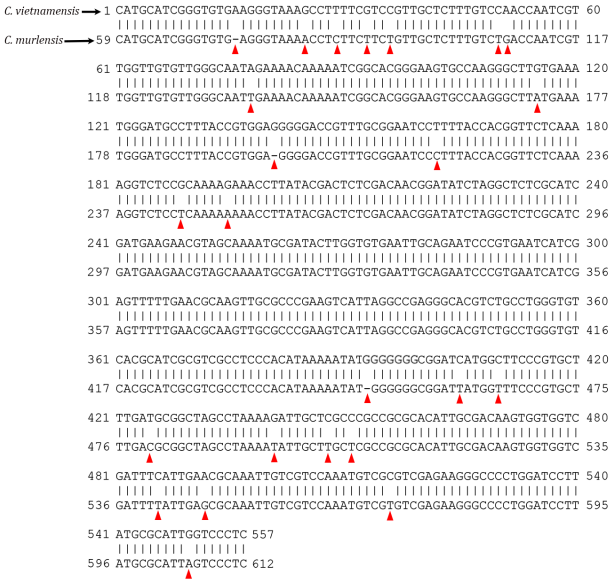

Figure 7. Pairwise alignments of the *ITS* region between *C. vietnamensis* (top row) and *C. murlensis* **(bottom row).** The red triangles indicate nucleotide differences between the two species.

Table 1. Comparative Analysis of ITS and trnL intron regions in Ceropegia vietnamensis

Comparison	Region	Aligned length (bp)	SNPs	Gaps	Identity (%)
C. vietnamensis vs C. bulbosa	*ITS*	557	32	7	93.0
C. vietnamensis vs C. bulbosa	*trn*L intron	467	7	0	99.0
C. vietnamensis vs C. murlensis	*ITS*	557	21	3	96.0

DISCUSSION

Ceropegia vietnamensis was first described by Luu et al., who noted its morphological resemblance to *C. laotica*, *C. bulbosa*, and *C. murlensis*, particularly in general habit and floral coloration. However, clear morphological differences among these closely related species were documented in the taxonomic treatment by Luu et al., [9]. The microanatomical results presented in this study offer additional characters for interspecific comparison within *Ceropegia*, a genus currently divided into 63 sections according to Bruyns et al., [3], with particular relevance to section Esculentae Bruyns.

Bhandari et al., [11] reported the micromorphological traits of *Ceropegia bulbosa* var. *lushii*, a taxon closely related to *C. vietnamensis* [9]. In general, the anatomical structure of the vegetative organs (roots, stems, and leaves) of *C. vietnamensis* shows substantial similarity to other congeners, including *C. bulbosa* var. *lushii* [11]. Notably, both species share the presence of internal phloem in the stem and leaf vasculature—an uncommon trait among angiosperms.

Nevertheless, *C. vietnamensis* can be distinguished from *C. bulbosa* var. *lushii* by the following microanatomical features: (i) Root: beneath the piliferous layer, *C. vietnamensis* exhibits parenchymatous tissue without a differentiated exodermis, which contrasts with the well-developed exodermis found in *C. bulbosa* var. *lushii* [11]; (ii) Stem: the presence of cubic calcium oxalate crystals is evident in *C. vietnamensis*, whereas these crystals were not observed in *C. bulbosa* var. *lushii*; (iii) Leaf: the palisade parenchyma of *C. vietnamensis* is composed of 3–4 layers of elongated, narrow cells, compared to only 1–2 layers of shorter cells in *C. bulbosa* var. *lushii* [11].

To date, molecular data are available for *C. bulbosa* and *C. murlensis*, but not for *C. laotica* [12-13]. In this study, preliminary comparisons of the *ITS* and *trn*L intron sequences revealed moderate levels of sequence divergence between *C. vietnamensis* and the morphologically similar species *C. bulbosa* and *C. murlensis*. These differences were especially notable in the *ITS* region, where sequence identity ranged from 93.0% to 96.0%.

Although the molecular dataset in this study is limited in both taxon sampling and analytical depth, the findings are consistent with previous research that employed *ITS* and *trn*L markers to distinguish closely related *Ceropegia* species. For example, these regions were instrumental in the identification of new species such as *C. murlensis* and *C. mizoramensis* [13], as well as *C. maharashtrensis* and *C. karulensis* from India [17]. Furthermore, DNA barcoding using plastid markers such as *trn*L, *trn*L-F, *trn*T-L, and *psb*A-*trn*H has been effective in resolving taxonomic ambiguities across the genus, including the identification of *C. citrina* in Thailand [16], *C. lodarensis* in Arabia [14], and rare taxa such as *C. odorata* and *C. hirsuta* [15].

It is important to note, however, that the molecular analysis in the present study did not include phylogenetic reconstruction and was limited to pairwise sequence comparisons with only two related taxa. Consequently, while the observed sequence variation provides preliminary molecular support for the distinctiveness of *C. vietnamensis*, it does not clarify its phylogenetic placement within the genus or among related clades. These findings should therefore be interpreted cautiously and viewed as a complement to the morphological and anatomical evidence, rather than as conclusive taxonomic proof.

Future studies should aim to include a broader sampling of *Ceropegia* species, incorporate additional molecular markers (both nuclear and plastid), and employ phylogenetic inference methods to comprehensively assess the evolutionary relationships and systematic position of *C. vietnamensis*.

CONCLUSIONS

This study presents the first comprehensive characterization of *Ceropegia vietnamensis*, integrating morphological, anatomical, and preliminary molecular evidence. The findings enhance our understanding of this species and provide a foundational framework for future taxonomic and phylogenetic research within the genus *Ceropegia*.

ACKNOWLEDGEMENTS

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ETHICAL CONSIDERATIONS

This study did not require formal ethical approval, as it involved only the collection of non-protected, non-endangered plant material and did not include any research involving human or animal subjects. The collection was conducted in accordance with current Vietnamese legislation, including the Biodiversity Law No. 20/2008/QH12 and the Law on Environmental Protection (2020). Specimens were deposited in the Herbarium of the University of Science, Vietnam National University – Ho Chi Minh City (PHH), ensuring transparency and facilitating future verification.

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DECLARATION OF COMPETING INTEREST

The authors confirm that there are no competing interests to disclose.

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