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Taxonomical and molecular authentication of the *Ceropegia candelabrum* L. var. *candelabrum* (Apocynaceae): A new record from Eastern India

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Abstract

Ceropegia candelabrum L. is the lectotype of the genus *Ceropegia* that is native to the Western Ghats of India. This species is reporting for the first time from West Bengal, which is the eastern bottleneck of India. This species has very attractive, eye-catching flower characteristics viz., corolla-tubular; corolla tube long abruptly dilated at the base, claw is fused forming tube-like structure, corolla limb connate at the tip forming a globular cage. We apply both traditional and advanced methodologies to properly authenticate the newly collected species. However, the taxonomy, as well as the molecular (*rbcL, ITS,* and *rbcL+ITS*) data, confirm the identity of that species. Detailed morphology, a new distributional geographic area, and molecular identification are presented here.

Keywords

Ceropegia candelabrum; new distribution; biodiversity; morphology; molecular taxonomy

Introduction

The old-world tropical genus *Ceropegia* L. is the largest in the tribe Ceropegieae and belongs to the family Apocynaceae. It comprises 244 species distributed across Africa, expanding from the east to Arabia, India, China, and the northern part of Australia (1, 2). South Africa, Kenya, Madagascar, and India exhibit the greatest diversity of this genus (3). In India, Ansari (4) is the pioneer worker who worked on Indian *Ceropegia* and recorded 44 species, of which 28 were reported to be endemic. Botanists are constantly fascinated by this genus due to its diverse characteristics such as habit, habitat, flower structure, and ecological adaptations (5). The flower design, corolla size, shape, coloring pattern, and eye-catching flytrap flowers (6) differentiate it from other members of this family. That's why many workers (7-11) worked on this genus and discovered new species or reported the species diversity at various times. Finally, Kambale and Yadav (12) revised the genus *Ceropegia* and recorded 61 taxa from India, of which 44 (72%) are endemic. This study shows the highest diversity of this genus in Maharashtra, with about 25 species, of which 17 are endemic. Besides that, *C. candelabrum* has been reported from Andhra Pradesh, Karnataka, Kerala, and Tamil Nadu. From Sri Lanka, it was reported by Ansari (4), Kambale, and Yadav (12). The Indian *Ceropegia* mainly occurs along steep hill slopes; others grow in rock crevices at low to high-elevation lateritic plateaus along with bushes; others along forest margins, grasslands of dry deciduous forests, shola forest margins, and still others prefer to grow in drier habitats.

We found *Ceropegia candelabrum* for the first time in eastern India on lateritic soil in the *Shorea robusta* forest. Therefore, it is an addition to the flora of Bengal, and it looks similar to *C. intermedia*. The extended distribution, detailed morphology, and molecular evidence are presented here for proper identification.

Materials and Methods

During a field survey (September 2021) in the Tapoban forest (N22°07.454' E87°02.014') of the Raghunathpur area of Jhargram district, West Bengal, we spotted *C. candelabrum* in a single patch under a densely vegetated area of Nayagram Block. The GPS coordinate was recorded by an e-trex10 device, and using this coordinate, a collection map was constructed (Fig. 1) by Esri's ArcGIS software (v. 10.5). This area has an annual normal rainfall of 1400 millimeters per year. The annual maximum temperature varies between 39-42°C, while the minimum temperature varies between 8-9°C (www.wikipwdia.org). No other population of this species was observed in the immediate vicinity. The photographs were taken using a DSLR camera (Canon 550D with an EOS 18-55mm lens). The herbarium was prepared following standard methods (13), and the voucher specimen was deposited in the herbarium (VU/AYAN/017) of Vidyasagar University, Paschim Medinipur (WB). Due to the rarity of the taxon, we collected a single twig and a few flowers. For molecular identification, leaf samples were dried on silica gel. A sampling of the other *Ceropegia* has followed the sectional treatment of Bruyns et al. (14) for phylogeny analysis was downloaded from the Gene Bank (Table 1).

Morphological study: Besides field observation, a detailed study was done in the laboratory using a Leica Stereo Zoom (Model S8APO) microscope. The photographs of dissected floral parts are presented on a photo plate. A freehand drawing has been done here. For the SEM study of the pollinarium, the collected pollinarium was mounted on the aluminum stub with both side carbon taps. After drying, the sample was gold coated under 10µamp, 10-20KV for 120 seconds. A Zeiss Supra-40 microscope (SEM) was used here for this study with 5.00KV extra high tension (EHT) and 5-6 mm working distance (WD). The specimen was properly identified by comparing all the characters with available references like Hooker (15), Ansari (4), Kambale and Yadav (12).

DNA isolation, amplification, and sequencing: Silica dried sample was used for genomic DNA isolation by the NucleoSpin® Plant II Kit (Macherey-Nagel) according to the user manual. After isolation, the quality of the DNA was checked by 0.8% agarose gel electrophoresis, and the DNA profile was visualized on a UV transilluminator. The universally accepted *rbcL* and *ITS* genes were selected for this study. PCR amplification reactions were carried out in a 20 µl reaction volume that contained 1X Phire PCR buffer (containing 1.5 mM $MgCl_2$), 0.2 mM each dNTPs (dATP, dGTP, dCTP, and dTTP), 1µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5 pM of forward and reverse primers. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). The optimal PCR amplification conditions are provided in Table 2. ExoSAP-IT (GE Healthcare) purified the amplified

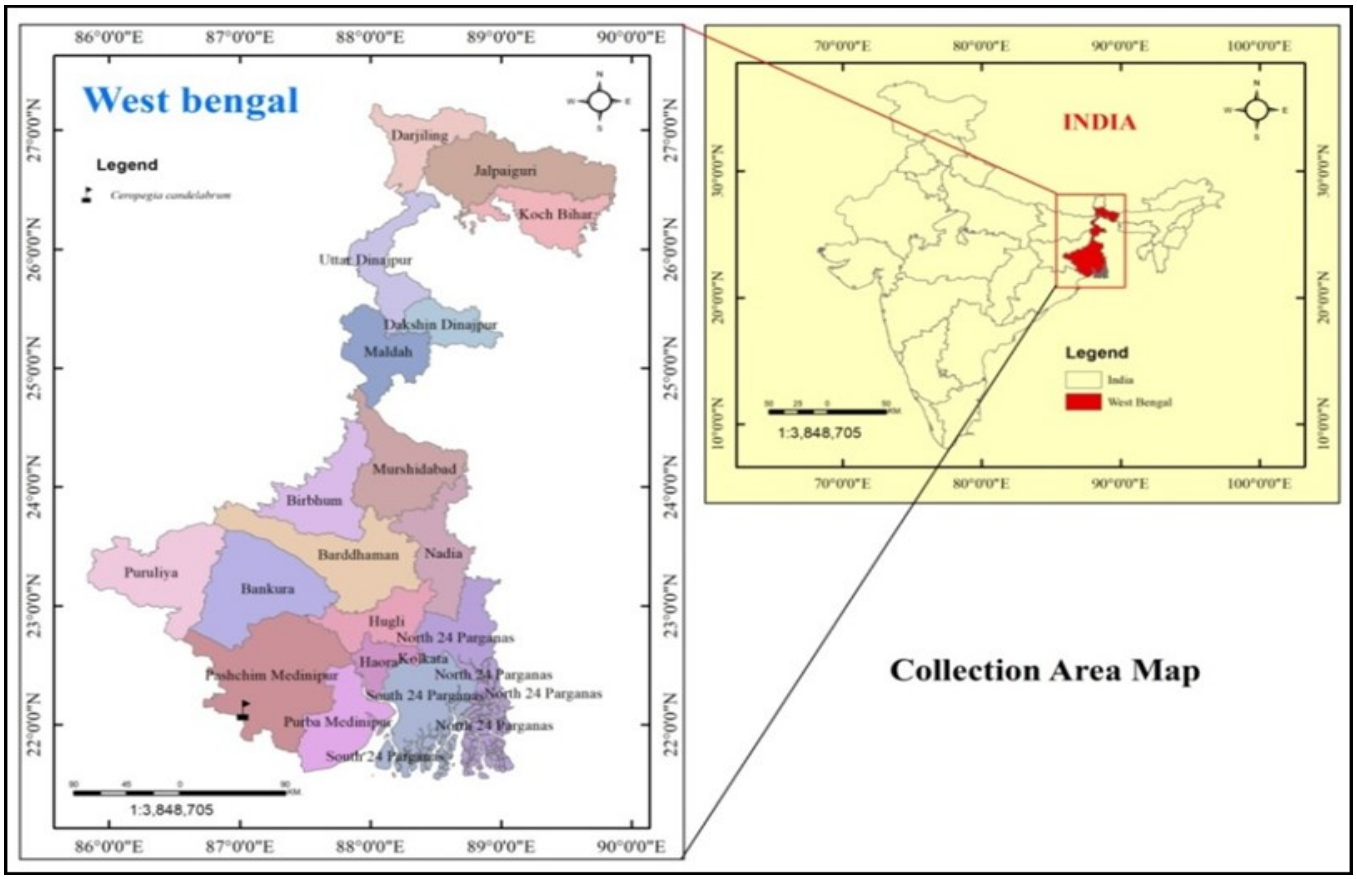

Fig. 1. Map showing location of the taxa

Table 1. Sampling of the Cerpegia species and sequences accessed for this study.

Ceropegia Section (By Bruyns et al. 2017)	**Taxa**	***rbcL***	***ITS***
Aristolochioides (H.Huber) Bruyns	*Ceropegia aristolochioides*Decne.	KR737476 KR737426 KR737337 KR736905	
Chinopegia H.Huber	*Ceropegia pubescens* Wall.	KX910831	AM493280 KP244972
	Ceropegia longfolia Wall.		AM493283
Indopegia H.Huber	*Ceropegia sahyadrica* Ansari & B.G. Kulk.	EU196267	KP244981
	Ceropegia media-(H. Huber) Ansari	EU196266	KP244962
	Ceropegia lawii Hook. f.	EU196265	
	Ceropegia rollae Hemadri	MT219518	
	Ceropegia huberi Ansari		KP244953
	Ceropegia hirsute Wight & Arn.		MT056074
Phalaena H.Huber	*Ceropegia juncea* Roxb.	EU196264 MG254904	
Janthina H.Huber	*Ceropegia intermedia* Wight	EU196263	KP244954 EU106678 AM493285
	Ceropegia elegans Wall.	EU196262	
	Ceropegia thwaitesii Hook.		KP244990
	Ceropegia decaisneana Wight		KP244941
Sarcodactylus	*Ceropegia dichotoma* Haw.	EU196261 MN783772	AM493290 KP244942
	Ceropegia fusca Bolle	MN783773	KT795413
Esculentae Bruyns	*Ceropegia bulbosa* Roxb. var. *bulbosa*	EU196260	
Ceropegia	*Ceropegia candelabrum*L.	**MT066114**	**PP058387** MT056115 MT056127 KP244935
	Ceropegia ravikumariana Kambale & Gnanasek.		MT056117
Carnosae Bruyns	*Ceropegia racemosa* N.E.Br.	KR736405	
Ceropegiella H.Huber	*Ceropegia woodii* Schltr.	X91775	
Tiloris H.Huber	*Ceropegia attenuata* Hook.		KP244928
	Ceropegia pusilla Wight & Arn.		KP244974
	Ceropegia fimbriifera Bedd.		KP244949
	Ceropegia noorjahaniae Ansari		KP244968
	Ceropegia spiralis Wight		KP244984
	Out Group		
	Hemidesmus indicus (L.) R.Br.	MN228499	MW090337 KR259530 KT338797

Table 2. Optimal PCR conditions for the studied genes.

Target	**Primer Name**	**Sequence (5' à 3')**	**References**	**PCR Conditions**					
				Intitial denaturation	**Denaturation**	**Annealing temperature**	**Elongation temperature**	**Final extension**	**No. of Cycles**
rbcL	RBCL-AF	ATGTCACCACAAACAGAGACTAAAGC	Levin, 2003	98˚C, 30sec	98˚C, 5sec	58˚C, 10sec	72˚C, 15sec	72˚C, 60sec	40
	RBCL-724R	TCGCATGTACCTGCAGTAGC							
ITS	ITS-5F	GGAAGTAAAAGTCGTAACAAGG	White et al., 1990	98˚C, 30sec	98˚C, 5sec	58˚C, 10sec	72˚C, 15sec	72˚C, 60sec	40
	ITS-4R	TCCTCCGCTTATTGATATGC							

product after amplification to remove unwanted primers and dNTPs. Sequencing was done using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA), following the user manual. After cleaning up, the air-dried product was sequenced in an ABI 3500 DNA Analyzer (Applied Biosystems). The sequence data were checked using Sequence Scanner Software v1 (Applied Biosystems) and the sequence was submitted to the gene bank.

Sequence analysis: Forward and reverse sequence histograms were inspected by Bioedit sequence alignment editor v7.2.5 (16) for trimming the poor quality of 5' & 3' sequence ends and the existing primer sequences. Post-trimming sequences maintained at least 60% of the original read data and this was subjected to the minimum average quality score of Q20 by Finch TV ver. 1.4.0. Those failing to fulfill these criteria were rejected and re-sequenced.

Phylogenetic analysis: The edited sequences were applied for BLAST analysis and identified the sample up to the species level. The correct identified sequences were taken for further analysis, and closely related species were downloaded. Multiple sequence alignment of both the genes (*rbcL* and *ITS*) was performed separately by the MUSCLE algorithm (17) in MEGA XI (18) and aligned data were saved in a FASTA format. Then concatenate the nuclear (*ITS*) and plastid (*rbcL*) genes into one alignment (*rbcL+ITS*) for better resolution of the phylogeny. The alignment characteristics of both the single gene and concatenated genes were calculated in MEGA and presented in Table 3. The data set was further subjected to a selection of best-fit substitution models by jmodel test v2.1.10 (19), which had the lowest AIC scores. The phylogenetic tree was reconstructed by using the Maximum Likelihood (ML) method in MEGA with rapid bootstrapping (500 replicates) and a best-fitted substitution model.

Results

Taxonomic treatment

Ceropegia candelabrum L. Sp. Pl. 1: 211. 1753. Lectotype (designated by Huber, 1957): Plate of Rheed, Hortus Malabaricus 9: 27, t. 16. 1673.

Ceropegia candelabrum L. var. *candelabrum,* H. Huber in Dassan. & Fosberg, Revised Handb. Fl. Ceylon 4: 120. 1983; Ansari, Fasc. Fl. India 16: 11. 1984; Karthik. et al., Fl. Pl. India 1: 161. 2009; Kambale & S.R. Yadav, Asklepios 115: 29. 2013; Kambale & S.R. Yadav, Rheedea 29: 1-115. 2019.

Specimens examined:

India, ***Karnataka***, way from Coorg to Talcauvery, 29.11.2010, S.S. Kambale & S.R. Yadav SUK 2556; Ibid. (grown in the garden), 26.01.2012, S.S. Kambale 5276 (SUK); Madapur, near Karekad Estate, 09.10.1961, A.S. Rao 75018 (BSI); South Canara, 26.10.1900, C.A. Barber 2110 (MH). ***Kerala***, Idukki district, Meenmutty, 02.10.1983, C.N. Mohanan 79904 (MH); Kannur district, Ezhimala, 26.10.1988, Nambiar & Sasidharan 2568 (KFRI); Kollam district, Koni, 27.07.1978, C.N. Mohanan 58318 (CAL); Palakkad district, near Mekaran, 06.09.1913, C.C. Calder & M.S. Ramaswami 601 (CAL); Parambikulam, 11.1910, A. Meebold 712401 (CAL); Thiruvananthapuram district, Bonacaud Tea estate, s.d., J. Joseph 44535 (MH); Ibid., 23.10.2013, S.S. Kambale & S.R. Yadav SSK 96 (SUK). Thrissur district, Mukkali, 28.11.1973, E. Vajravelu 44871; Peechi, KFRI Campus, 29.09.1978, N. Sasidharan 83 (KFRI); SVNP, 02.10.1965, E. Vajravelu 26184 (MH), ***Tamil Nadu***, Chennai district, s.loc., 09.1884, J.S. Gamble 14837 (CAL). ***West Bengal***, Jhargram district, Raghunathpur, Tapoban forest (N22°07.454' E87°02.014'), September 2021, S. Dwari, A.K.Naskar & A.K.Mondal, VU/AYAN/017 (VUH).

Description of the Specimen:

Habit: Terrestrial; Herbaceous; Twiner (Fig. 3A); Size approximately 16–18ft. Root: Root fleshy with globose tuber (Fig. 3&4C). Stem: Aerial; Cauline; Weak Twining; Cylindrical; Terete; Pubescent; Hair, Simple (Fig. 3M&N); Soft, herbaceous with watery latex. Leaves: Exstipulate; Cauline; Simple; Petiolate, petiole 1.2–1.4 cm long, petiole vasculature 1–traced, interpetiolar region smooth, reddish in color, collators present in intrapetiolar region; Leaf base simple; Lamina shape ovate to elliptical, base truncate to rounded, margin entire with few scattered simple hairs, apex acuminate; Texture herbaceous; Phyllotaxy- opposite decussate; Venation reticulate pinnate; Size 6.5×3.2 cm. Inflorescence: Cymose umbellate c.7–10 flowers, peduncle 1–3 cm long, glabrous and longer than pedicle, pedicle length 7–9 mm, glabrous. Flower: Bracteate; Pentamerous; Pentacyclic; Hypogynous; Complete; Perfect; Bisexual; Dichlamydeous; Zygomorphic; Colour greenish yellow at the top and white greenish at the bottom; Size 3–4 cm. Calyx: Sepals–5; Gamosepalous; Aestivation -imbricate; Irregular; Hypogynous; Glabrous; Shape- subulate; Persistent-Marcescent; Color green; Size 4–5mm. Corolla: Petals–5; Gamopetalous; Irregular- Zygomorphic; Hypogynous; Aestivation-valvate; Shape- Tubular; Corolla tube long abruptly dilated at the base, claw are fused forming tube-like structure (Fig. 3&4B). The tube color is greenish yellow at the top and white greenish at the bottom, grayish line is

Table 3. Alignment characteristics of the single and combined genes.

Alignment Gene	Outgroup/ Ceropegia accessions	Length (bp)	Best-fit nucleotide substitution model	Conserved site (%)	Variable site (%)	Parsimony Informative site (%)
rbcL	1/20	1425	JC	1257 (88.21)	51 (3.57)	12 (0.84)
ITS	3/26	730	JC	393 (5.38)	296 (40.54)	196 (26.84)
rbcL+ITS	4/45	2155	T92+G	1650 (76.56)	347 (16.10)	207 (9.60)

present from base to upward on the outer side of the tube. The inner side of the tube is glabrous at the base and hairy from the middle to the top. Corolla limb deep brown to purple at the tip, hairy at the apex, base ovate, connate at the tip forming globular cage, lobes reflex on their back (Fig. 3C& 4E); Deciduous; Size 13–14 mm. Androecium: Fused 5 stamens; Adhesion-Gynostemium; Position-inserted, filament absent; Staminal corona present, corona bi-seriate (Fig.3I& 4G), outer corona–5, entire, hair present at the margin, margin purple in color otherwise yellow, bowl-shaped (Fig. 3H). Inner corona–5, erect, 2.3 mm long, spathulate, convergent at the top, opposite to outer corona, yellow in color, waxy in texture (Fig. 3H&I). Anther cell two, round shaped, without any appendage. Pollinia is present at alternate to staminal corona and its position is the adjacent line of two neighbor anthers.

Pollen masses are solitary in each chamber. Position of the pollinarium horizontal (Fig. 4g), shape- elliptic but base pointed, apex round (Fig. 2B), size 310×121 µm, yellow; Translator size 70–71 µm, attachment to the corpusculum - marginal, abaxial side of the translator with distinct grove but adaxial side smooth (Fig. 2C), apex rounded; Corpusculam size 136×88 µm, apex-bifid, lengthwise a groove present at the middle of the corpusculum, adaxial side smooth (Fig. 2D) but abaxial side with a groove along the axis (Fig. 2E). Gynoecium: Carpels–2; Apocarpous; Sessile; Ovary superior; Surface-glabrous; Unilocular; Placentation: Marginal; Style absent; Stigma short Capitate. Fruit: Follicle size 6–10cm, dry dehiscent, linear, tapering to the apex (Fig. 3F & 4I), brown. Seed: Brown, ovoid, comose, coma long silky white (Fig. 4K). Phenology: August –January

The collected species have glabrous, twiner with linear to orbicular, petiolate leaves, cymes with many flowers having peduncles longer than the pedicles, and a corolla inflated at the base with long-beaked, hairy. The outer 5 corona are entire or truncated with hairy lobes, while the inner corona is erect linear sub-spathulate. These characters were previously noted by Ansari (4), Kambale, and Yadav (12) for *Ceropegia candelabrum* var. *candelabrum*. Therefore, the putative plant is morphologically very likely to be *Ceropegia candelabrum* var. *candelabrum*.

Phylogenetic inference: The aligned *rbcL* and *ITS* gene data sets contain 20 and 26 taxa, respectively, which consist of our sequence and closely related sequences from Gene Bank (Table 1). The *rbcL* data set is 1425bp long and consists of 1257 conserved sites, 51 variable sites, and 12 parsimony informative sites whereas the *ITS* data set is 730bp long, consists of 393 conserved sites, 296 variable sites, and 196 parsimony informative sites. The combined matrix of *rbcL* and *ITS* regions consisted of 2155 characters including the gap. There was a total of 207 parsimony informative sites (Table 3). The collected species belong to the section Ceropegia and its nearest section is Janthina (14). Our *rbcL*-based phylogeny (Fig. 5) has little support for Bruyn's (14) classification and shows these two sections are in a sister clade. The collected species may come with the other reference of C. *candelabrum* but the other *rbcL* reference gene of this species is unavailable in the gene bank. So, it is forming monophyly with section Janthina which is the nearest section of the section Ceropegia.

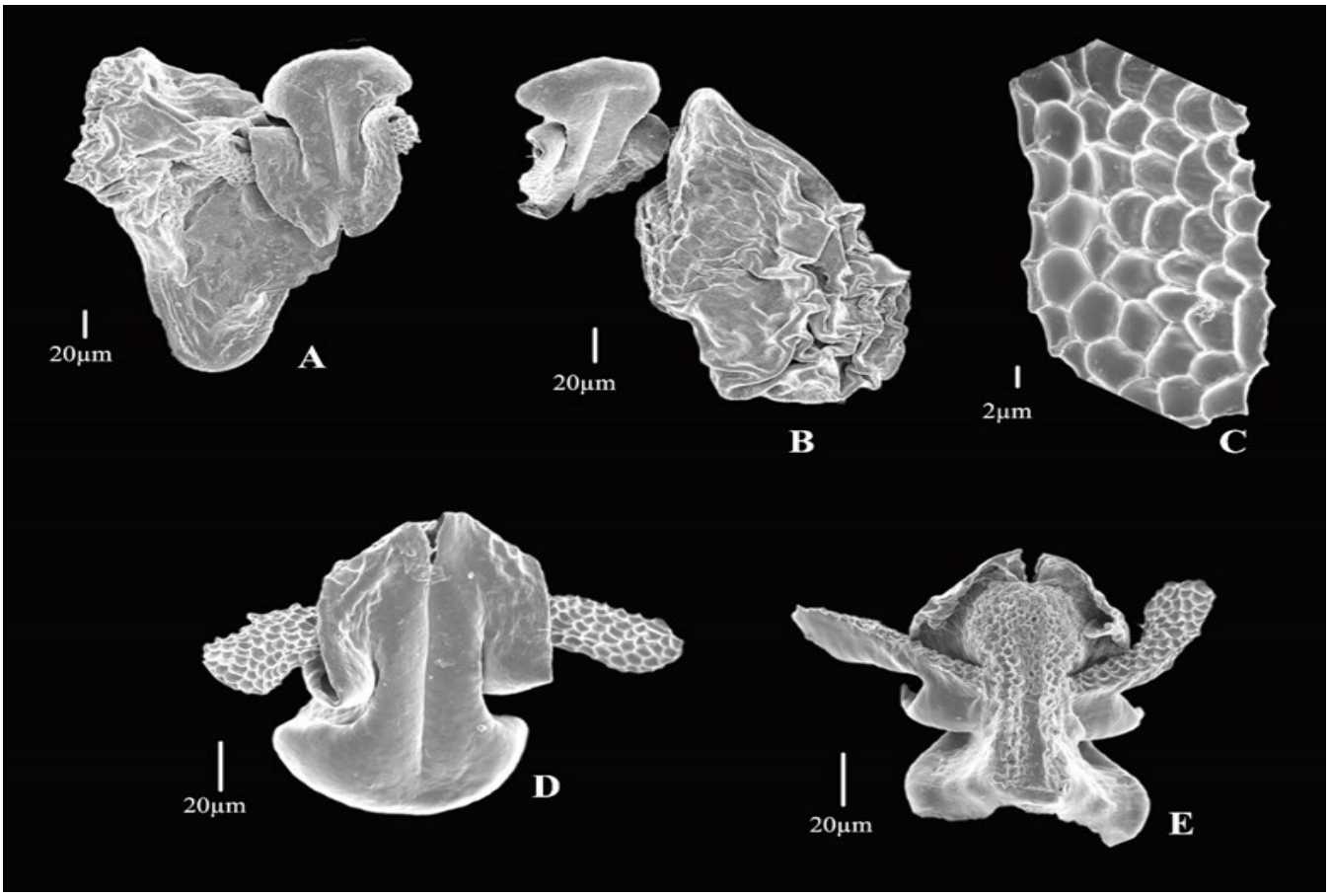

Fig. 2. SEM image of Pollinarium; A & B. Pollinarium with single pollinia; C. Translator grove; D. Adaxial side of corpusculum; E. Abaxial side of corpusculum.

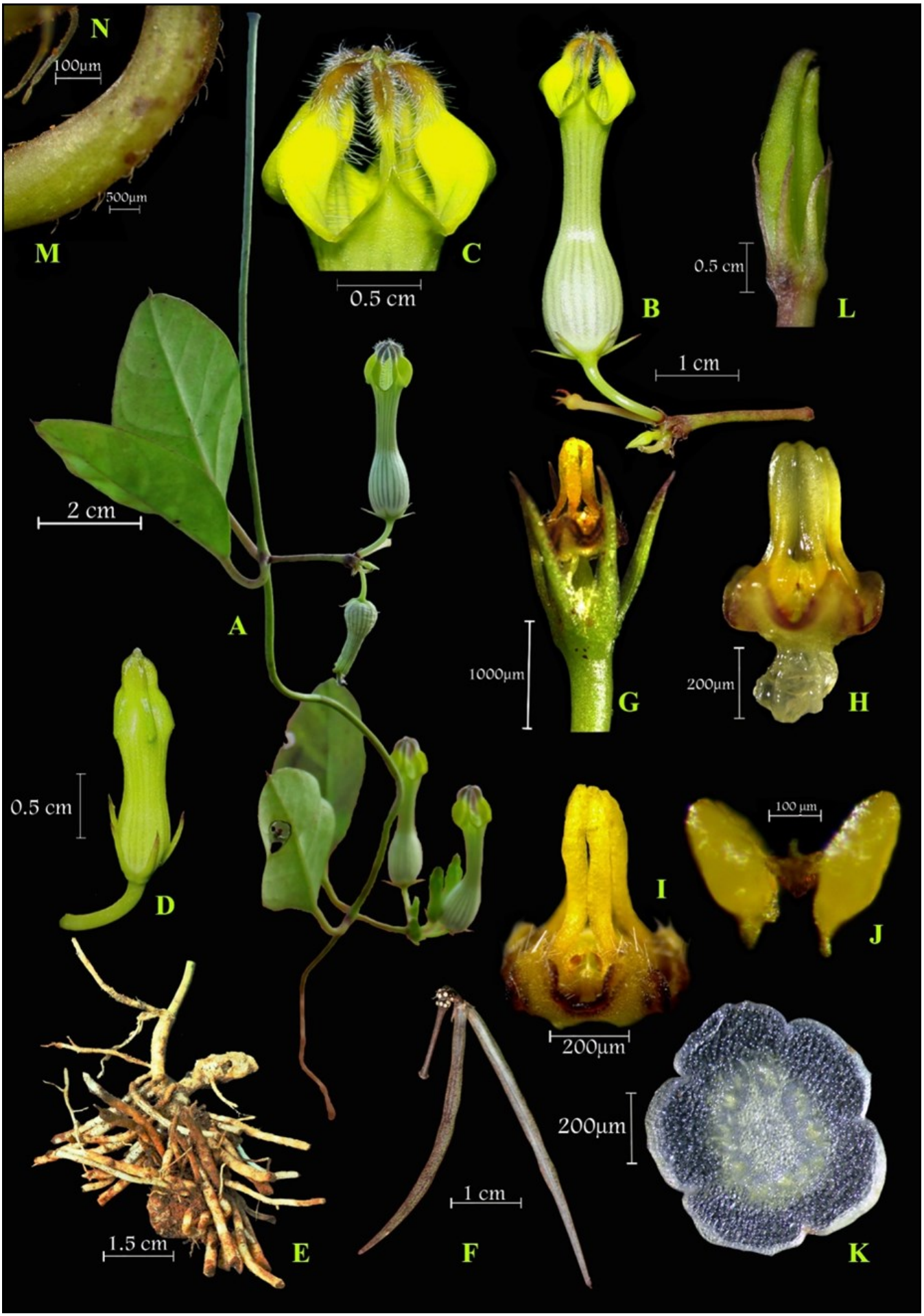

Fig. 3. *Ceropegia candelabrum* : A. flowering twig; B. Side view of the flower; C. Corolla tip showing corolla lobes; D. An immature flower; E. Tuber with roots; F. Fruit (follicle); G. Corona with calyx; H. Close-up view of corona; I. Side view of Corona tip;J.Pollinia; K. T.S of stem; L. Immature fruit; M. Close-up view of stem showing hairs; N. Close-up of hairs.

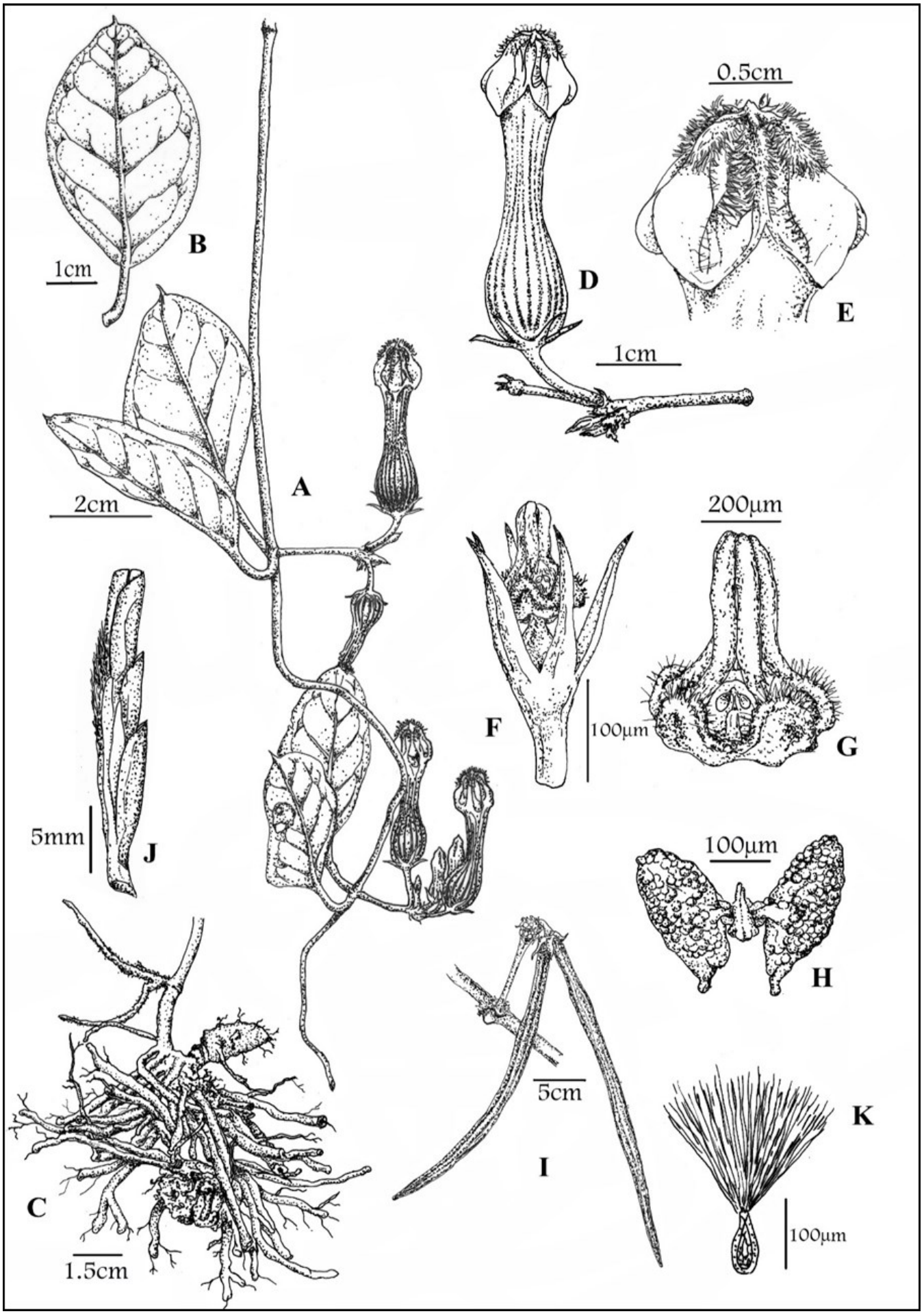

Fig. 4. Illustration of ***Ceropegia candelabrum*: A.**flowering twig; **B.**leaf; **C.** Tuber with roots; **D.** flower; **E.** Corolla tip showing corolla lobes; **F.**Corona; **G.** Tip of corona; **H.** Pollinia;**I.** Fruit (follicle); **J.**Arrangement of seeds; **K.** Seed bearing pappus.

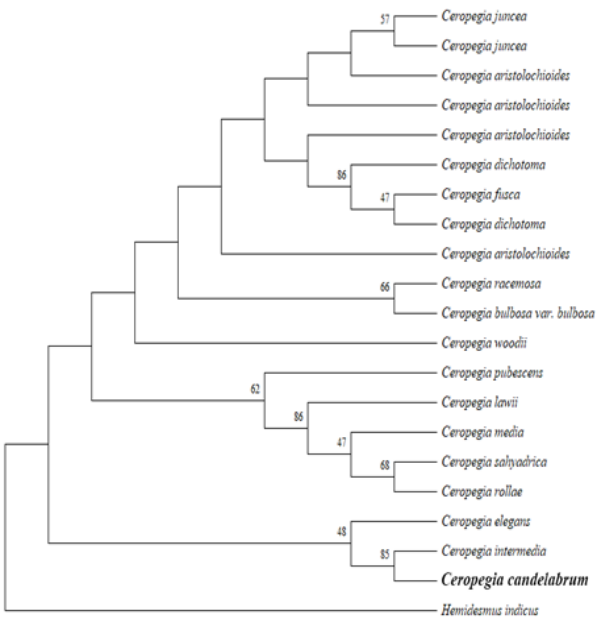

Fig. 5. Maximum Likelihood (ML) tree of *rbcL* data. Bootstrap values above the branches.

However, *ITS*-based phylogeny solves this issue; the collected species are grouped with reference *C. candelabrum* (Fig. 6). All the *C. candelabrum* in this data set are developed in a monophyletic group (Section Ceropegia) with 83 bootstraps supported. Both the individual phylogenies show *C. intermedia* closely related to *C. candelabrum*. However, the tree resolution based on combined gene data is better than that of individual genes. The tree topology of the combined matrix (*rbcL*+*ITS*) distinctly separates these two closely related species. Our combined tree (Fig. 7) suggests that the collected plant is grouped with *C. candelabrum* with strong support (BS=98). Therefore, the putative plant is most likely *C. candelabrum*.

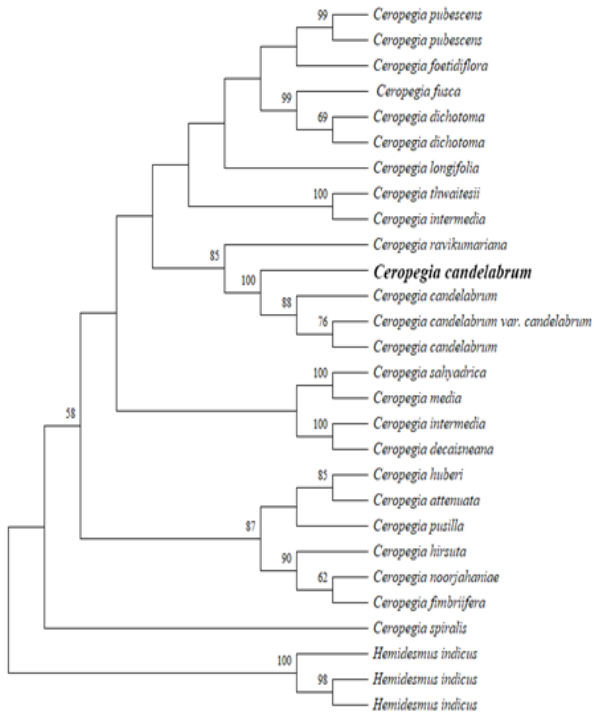

Fig. 6. Maximum Likelihood (ML) tree of *ITS* data. Bootstrap values above the branches.

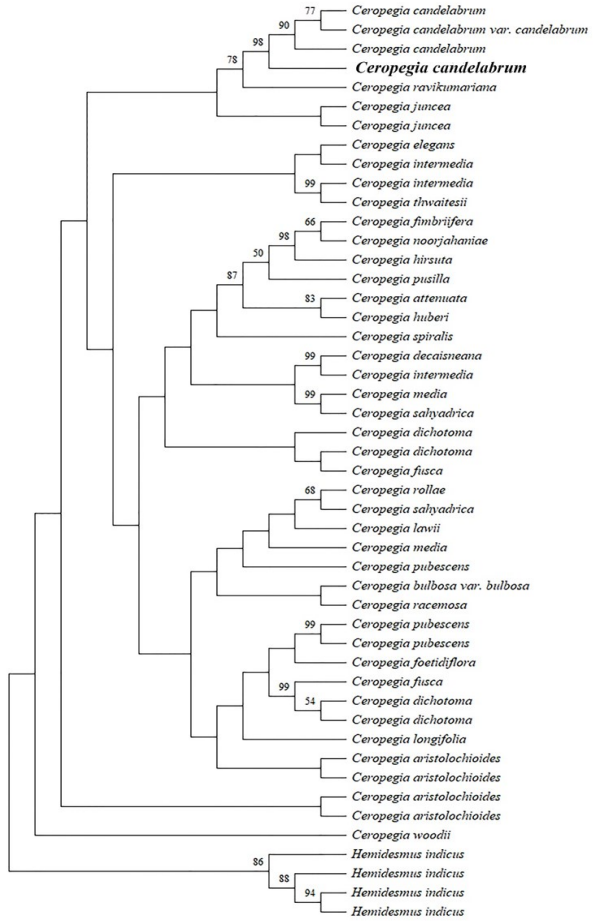

Fig. 7. Maximum Likelihood (ML) tree of *rbcL*+*ITS* data. Bootstrap values above the branches.

Discussion

In summary, the morphological data strongly support that the studied plant is *Ceropegia candelabrum* L. var. *candelabrum*. This result also supports the Kambale and Yadav (2019) work. However, morphologically, it has a close similarity with *C. intermedia*. These two species are related due to their similar morphological characteristics *viz.* slender climbers; perennial, herbaceous stem; cordate to ovate-lanceolate, petiolate, glabrous leaves; corolla with a slender tube inflated at the base and lobes joined at the apex. However, from our observation, there are some differences listed in Table 4. After successful taxonomical treatment, we proceed with molecular authentication. The *rbcL* and *ITS* genes are selected due to their universality, conserve site, and variable site at the inter-intra-specific level. The BLAST nucleotide search of the final *rbcL* sequence shows 100% identities with *C. intermedia* due to the lack of further accession of *C. candelabrum* in the database. *ITS* shows the successful BLAST analysis. All the highest identity percentage accessions of *rbcL* and *ITS* are saved and rearranged according to the latest classification. The Bayesian inference phylogeny based on the *ITS* gene gives a very good result and successfully discriminates the closely related species *C. intermedia* as *rbcL* can't be discriminated well. All the multiple accessions of *C. candelabrum* in the present treatment develop a monophyletic group with the present species and have significant posterior probability values. Although the combined matrix-based phylogeny does not well

separate all the *C. candelabrum* accessions. But still, taxonomy and the ITS gene are good enough for the correct authentication of the taxa. This species is the first time reported from West Bengal, and it is also a new addition to Bengal flora.

Conclusion

The presented morphological details and molecular analysis confirm that the collected species is *Ceropegia candelabrum* L. var. *candelabrum.* This is the first report of this fascinating species from Eastern India. It is a very rare taxon in this part of the country. The detailed morphological characters helped to differentiate the taxon at the intra-specific level, and the single *ITS* gene and combined *rbcL+ITS* genes distinctly identified the species.

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Authors' contributions

The first author designed, worked, and wrote up the manuscript and prepared it for correspondence to the journal. The second author collects the plant specimen, the third author gives the free-hand drawing and the fourth author supervises the whole work. Finally, all the authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None.

Table 4. Morphological similarity and dissimilarity between closely related species.

Similarity			
Sr. No.	**Characters**	***C. candelabrum***	***C. intermedia* Kambale and Yadav (2019)**
1.	Habit	Twiner herb	Twiner herb
2.	Rootstock	Tuberous with small fascicled or fibrous	Globose tuberous
3.	Calyx	Glabrous	Glabrous
4.	Corolla	Corolla 13-14 mm. Tubular; corolla tube long and abruptly dilated at base, claw is fused forming tube-like structure.	Corolla 1.9–2.6 cm long, purple; tube 1.5–2 cm long, curved, abruptly dilated at base, narrow at the middle, funnel-shaped at mouth.
5.	Staminal Corona	Corona bi-seriate	Corona bi-seriate
Dissimilarity			
Sr. No.	**Characters**	***C. candelabrum***	***C. intermedia* Kambale and Yadav (2019)**
1.	Hair on stem	Pubescent	Glabrous
2.	Lamina shape	Ovate to elliptical	Ovate-lanceolate
3.	Corolla	The tube color is greenish yellow at the top and white greenish at the bottom, greyish line is present from base to upward on the outer side of the tube. The inner side of the tube is glabrous at the base and hairy from the middle to the top. Corolla limbs are a deep brown to purple at the tip, hairy at the apex, base ovate, connate at the tip forming a globular cage, lobes reflex on their back	Glabrous, greenish at the dilated base, purple otherwise; lobes 4–8 mm long, ovate-lanceolate, connate at tip forming ellipsoid-ovoid cage, lobes folded on their back, connate at middle, deep purple and hairy throughout at the upper half, faint in the lower half.
4.	Staminal Corona	Corona bi-seriate, outer corona-5, entire, hair present at the margin, margin purple in color otherwise yellow, bowl-shaped. Inner corona-5, erect, 2.3 mm long, spathulate, convergent at the top, opposite to outer corona, yellow in color, waxy in texture	Corona bi-seriate; outer of 5 entire lobes, glabrous; inner of 5 erect, c.1.5 mm long, spathulate - clavate lobes, opposite with outer corona.

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