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Strobilanthes glandulata (Acanthaceae), a new species from Sri Lanka based on the morphological and molecular evidences

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Abstract

Strobilanthes glandulata, a new species of Acanthaceae is described from Sri Lanka. It has been previously misidentified as *Strobilanthes lupulina*. It is similar to *S. lupulina* having an inflorescence of heads, funnel-shaped gradually widened corolla, hispids on the stem, and both surfaces of leaves and petioles, but can be easily distinguished from the latter by having different pollens, bracts arranged in well-spaced, margin crenate, and densely covered with glandular-hairy. The phylogenetic analyses indicate that it is a member of the native species of the genus *Strobilanthes* in Sri Lanka. A full description, detailed illustrations, pollen morphology, and complete chloroplast genome are provided.

Keywords: phylogeny, Rambukoluwa, *Strobilanthes lupulina*, taxonomy

Introduction

Strobilanthes Blume (1826: 781) is one of the largest genera in the family Acanthaceae widely distributed in the evergreen forests of tropical Asia to the Pacific and comprising about 450 species (Carine & Scotland 2002, Mabberley 2017, Thomas *et al.* 2020, Wood *et al.* 2021). A tentative list of *Strobilanthes* for Sri Lanka was prepared by Wood (1995, 1998) who recorded 30 species. Later, three additional species were described, i.e., *S. willisii* Carine in Carine *et al.* (2000: 971), *S. cordifolia* (Vahl 1794: 84) Wood (2014: 390), and *S. medahinnensis* Nilanthi in Nilanthi *et al.* (2021: 026).

During the plant explorations on 22nd April 2016, the first and third authors found an interesting *Strobilanthes* population with about 300 individuals growing in the shady places along the stream at Rambukoluwa in the Knuckles Mountain Range. The specimens were collected for the preparation of herbarium and for both morphological and molecular studies. In searching the specimens in various herbaria, one specimen (*Alston 1966*) at PDA collected from Madugoda jungle, which was misidentified as *S. lupulina* Nees (1832: 85) in herbarium, is the same as our material. It is somewhat similar to *S. lupulina* widespread in lowland rain forests and sub montane forests, 100–1800 m, but it is clearly different from the latter and all other known species of the genus by different morphological characters. After carefully compared with all known species (Wood 1994, 1995, 1998, Venu 2006, Wood & Scotland 2009), it is concluded that it represents a new species described below as *S. glandulata* Nilanthi.

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Material and methods

Morphological observation

Morphological traits of new species were compared with *Strobilanthes* descriptions (Wood 1994, 1995, 1998, Venu 2006, Wood & Scotland 2009), as well as herbarium specimens at the national herbarium, Royal botanic gardens, Peradeniya (PDA), online herbaria such as Kew (K), Digital flora of peninsular India by Indian Institute of Science herbarium (JCB), Rijksherbarium, Leiden (L) and online databases of digitized herbarium specimens (JSTOR) with special attention was given to *S. lupulina.* Morphological observations were taken according to Nilanthi *et al*. (2021). The GPS coordination of the collection sites was recorded using GARMIN GPSmap 78s and mapped on floristic map of Sri Lanka using ARC GIS version 10.8.1 ESRI, 2020 (Figure 1). Photographs of important morphological features of fresh specimens and line drawings were prepared.

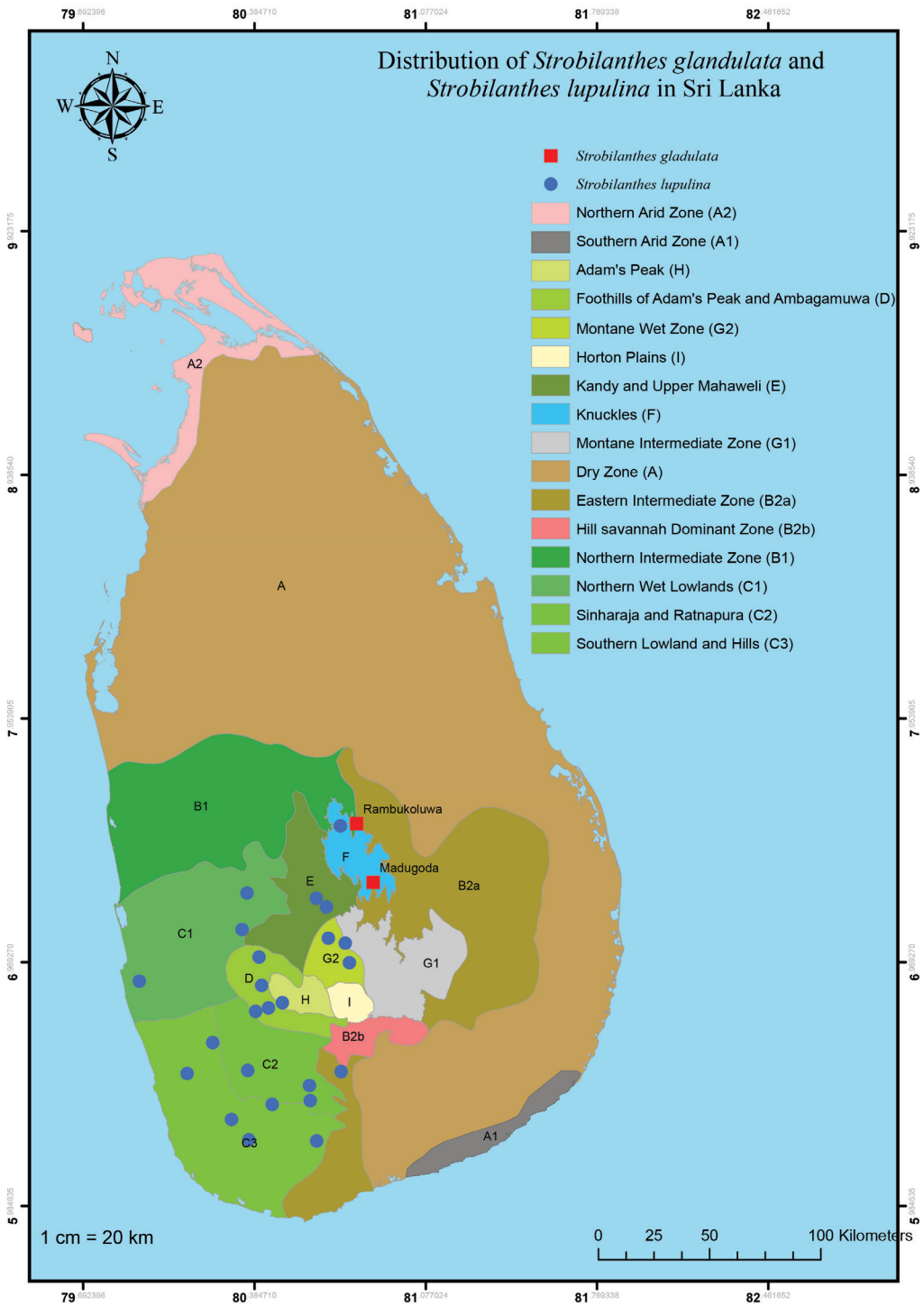

FIGURE 1. Distribution of *Strobilanthes glandulata* and *S. lupulina* in Sri Lanka.

Immature leaves of *S. glandulata* and *S. lupulina* were harvested and stored in -80 °C for DNA extraction and chloroplast genome (cpDNA) analysis. Voucher specimens; *S. glandulata* of Rambukoluwa, E 202704 N 258834, 282 m, *Nilanthi RMRN_123* and *S. lupulina* of Bogawanthalawa, E 195839 N 148042, 1300 m, *Nilanthi RMRN_112* are deposited at PDA.

The pollen grains were obtained from the dried specimen *Alston 1966* housed at PDA and studied using scanning electronic microscope (SEM). The measurements were obtained with the Hitachi SU6600 Analytical Variable Pressure FE-SEM software and ImageJ 1.45 software (Abramoff *et al*. 2004). The readings are given based on five measured pollen grains. The classes of pollen were determined following the classification proposed by Walker & Doyle (1975).

Molecular authentication

DNA extraction and sequencing

Genomic DNA from leaf samples of *S. glandulata* and *S. lupulina* was extracted using the plant DNAeasy mini kit (Qiagen 69106) following the manufacturer's guidelines. The quality and quantity of extracted DNA were processed with Nanodrop 2000 spectrophotometer (Thermo Scientific) and Agarose gel electrophoresis. About 1 µg of genomic DNA was sent to Admera, USA for high throughput sequencing. Genomic DNA sequencing was done using Illumina technology as previously described (Shinozaki *et al.* 1986).

Chloroplast genome assembly and analysis

The Chloroplast genome assembly was done as previously reported (Nilanthi *et al.* 2021). Approximately 90 million reads were available in the sequencing dataset for each species. To create contigs three chloroplast genome assemblers were used. The assemblers were GetOrganelle v.1.7.1 (Jin *et al*. 2020), NOVOPlasty v3.7 (Dierckxsens *et al.* 2017), and Fast-Plast v.1.2.8 (McKain *et al.* 2017). If the contigs were not complete genomes, a reads mapping strategy was followed to fill the gaps between contigs. To annotate the genomes Geseq v1.84 (Tillich *et al.* 2017) was used. The genomes were visualized using OrganellarGenomeDRAW (OGDRAW) v1.3.1 (Greiner *et al.* 2019). MAFFT v7.450 (algorithm FFT-NS-2) (Katoh & Standley 2013) was used to align the two sequences on the Geneious platform (Kearse *et al.* 2012). The DNA polymorphism of the species was analyzed by loading the alignment to DnaSP v6.12.03 (Rozas *et al.* 2017). The nucleotide variability (Pi) parameter was calculated using a sliding window (window length - 600, step size - 200). Further, Simple Sequence Repeats of the two sequences were detected using Krait v1.3.3 (Du *et al.* 2018). The threshold values for the number of repeats were set to 8,4,3,3, and 3 for mono-, di, -tri, tetra, and pentanucleotide SSRs respectively. The threshold value for nucleotide variability - Pi, was set to 0.05. The assembled chloroplast sequences of *S. lupulina* (MW802211) and *S. glandulata* (MW802212) were submitted to GenBank.

Results

Morphological evidence

New species with inflorescences of heads, funnel-shaped gradually widened corollas, hispids on the stem, both surfaces of leaves, and petioles are closely related to *S. lupulina*. However, it differs by much-branched habit with rounded slender stems, unequal ovate leaves with margin dentate, flowers in oval elongate heads longer than 4.5 cm, well-spaced bracts with crenate margin and dense glandular-hairs, corolla longer than 2.1 cm and pale purple (Table 1).

Pollen morphology

Moreover, *S. glandulata* having quantitatively and qualitatively different pollens compared to *S. lupulina* (Figure 2). Following the images of SEM were calculated the polar axis and equatorial axis diameter. In *S. lupulina* the polar length ranges from 48.10–54.90 µm, and equatorial length ranges from 32.16–36.10 µm, while the polar length of *S. glandulata* ranges from 45.73–49.08 µm and equatorial length ranges from 34.28–38.54 µm. In accordance with the dimensions, the polar length/equatorial length ratio, the pollen grains of *S. lupulina* and *S. glandulata* have an ellipsoidal shape with 3 ditches (tricolpate type). The size (P/E ratio) of *S. lupulina* (1.5) is larger than *S. glandulata* (1.3).

TABLE 1. Comparison of morphological characters of *Strobilanthes glandulata* and *S. lupulina*.

Characters	***S. glandulata***	***S. lupulina***†
Habit	much-branched shrub	erect shrub
Stem	slender, rounded	very stout, quadrangular
Leaf dimension	3–14 × 1.5–8 cm	2.5–16 × 1.5–6 cm
Leaf shape	ovate	broadly eliptic
Leaf apex	acuminate	shortly acuminate
Leaf margin	dentate	serrate
Leaf veins	5–7 pairs	9–11 pairs
Petiole length	1.3–7.5 cm long	0.3–7.5 cm long
Inflorescence	oval-elongated head	head
Head length	4.5–6.1 cm long	2–4.5 cm long
Bract shape	broadly ovate,concave	ovate, strongly concave
Bract colour	green	whitish, pink or green
Bract margin	crenate	entire
Bract indumentum	densely glandular hairy	glabrous
Bract length	10–15 mm long	13–18 mm long
Calyx length	5–6 mm	5–6 mm long
Bract:calyx ratio	2:1	3:1
Corolla colour	pale purple	white or purple
Length of corolla tube	21–25 mm long	18–25 mm long
Flower diameter	8–12 mm	6–10 mm

†Wood, 1998

In respect to the exine adornment of the pollen grain of *S. lupulina,* the ribs equal in length and all ribs joined at poles. The width of the ribs ranges from 3.23–3.94 µm. While *S. glandulata* has 3–4 ribs which are completely encircle the poles of the pollen and those ribs have solid aggregates of raised supratectal elements. The width of each ribs ranges from 4.44–5.02 µm. However, the number of ribs are same for both the species. The size and the shape of the endoaperture are different in the two species. In *S. lupulina* the length of the aperture ranges between 12.81–15.84 µm and the width of the aperture ranges in between 5.21–5.81 µm. The shape of the endoaperture in *S.lupulina* is circular/lalongate. In respect to *S. lupulina* the enopaerture of *S. glandulata* is larger in size. The length of the aperture ranges in between 16.10–17.25 µm, while the width ranges from 6.24–8.55 µm and the shape of the endoaperture is lalongate.

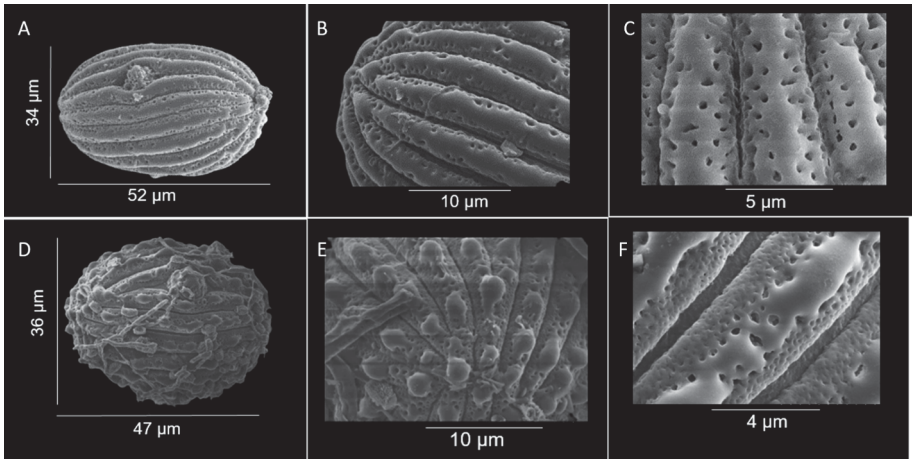

FIGURE 2. Pollen morphology of *S. glandulata* and *S. lupulina* observed under SEM. A–C. *Strobilanthes lupulina.* A: Equatorial view, B, C: Exine ornamentation. D–F. *Strobilanthes glandulata*. D: Equatorial view, E, F: Exine ornamentation.

Molecular evidence

Chloroplast genome assembly and analysis

GetOrganelle could assembled a single complete contig to cover the complete chloroplast genome of *S. glandulata.* For *S. lupulina,* the contigs were assembled using all three assemblers (GetOrganelle, NOVOPlasty, and Fast-Plast). The contigs produced a complete genome for *S. lupulina* after using *S. glandulata* as a reference. The difference in length of the cp genome of two species was 31 bases (Table 2). The pairwise alignment of the two sequences revealed a 259 base-pair variations. This difference was caused by 104 SNPs and 155 indels. The maximum indel length was 17, and the minimum was 1. Both genomes possessed a quadripartite structure - a single sequence copy (SSC) region, a large sequence copy (LSC) region, and two inverted repeats (IRA and IRB) regions. The GC contents, the number of protein-coding, tRNA, and rRNA genes of the two genomes were very similar. DnaSP analysis revealed four highly polymorphic regions in the genomes.Three regions were intergenic spacers (IGS) (rpI20-rps12, ccsA-ndhD, and ndhD-psaC), and the fourth region was the second half of the ycf2 gene. Interestingly, universally accepted barcoding regions such as matK, rbcL, and psbA-trnH had very low variability (matK - 1SNP, rbcL, and psbA-trnH - no SNPs). The highest variability was in the ycf2 region (Pi>0.02). This information is vital when designing more accurate DNA barcodes. The SSR distribution of the two sequences was also very similar (Table 3). A/T mononucleotide SSR type had the highest frequency. It confirms the previous findings of the low GC content (high AT content) in the chloroplast genomes (Eguiluz *et al.* 2017). The second highest frequent SSR type was trinucleotide microsatellites. Based on the SSR location, the number of SSR located outside coding regions (~63.3%) was higher than the SSRs within coding regions (~36.7%). The SSR information is useful when developing unique molecular markers to identify the two species (Figure 3 & 4, Table 2 & 3).

TABLE 2. Base pair difference between cp genome of *Strobilanthes lupulina* and *S. glandulata.*

Species	***S. lupulina***	***S. glandulata***
Genome size (bp)	144647	144616
LSC length (bp)	93191	94174
SSC length (bp)	17767	17774
IR length (bp)	16340	16334
GC content (%)	38.3	38.3
GC content in LSC (%)	36.6	36.6
GC content in SSC (%)	32.7	32.7
GC content in IR (%)	46.1	46.1
tRNA genes	37	37
rRNA genes	8	8

TABLE 3. SSR type, location and the count in the cp genome of *Strobilanthes lupulina* and *S. glandulata.*

Species	SSR type					Location		
	Mono	Di	Tri	Tetra	Penta	Non coding	Coding	Total
S. lupulina	87	39	72	12	2	131	81	212
S. glandulata	87	40	71	11	1	136	74	210

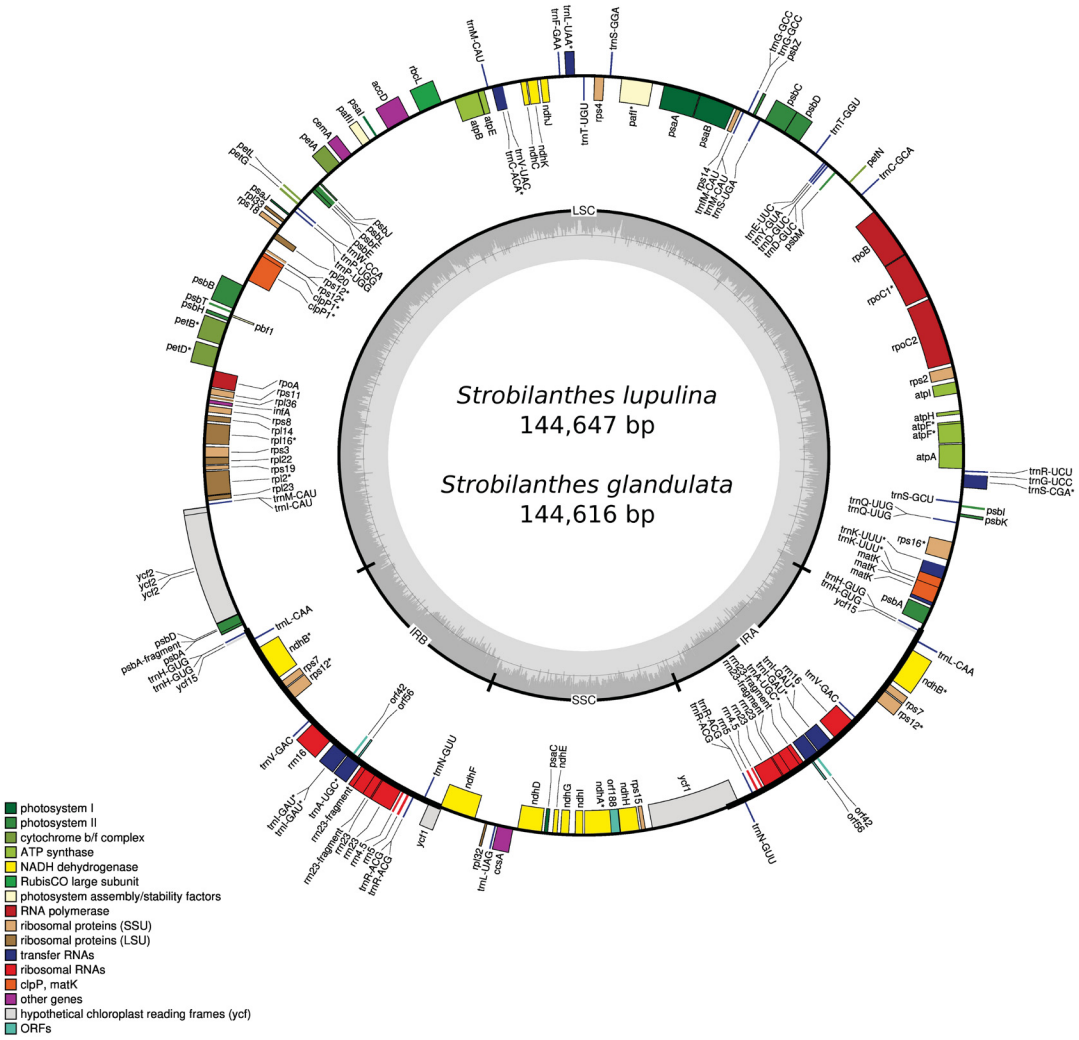

FIGURE 3. Chloroplast genome maps of *Strobilanthes lupulina and S. glandulata.*

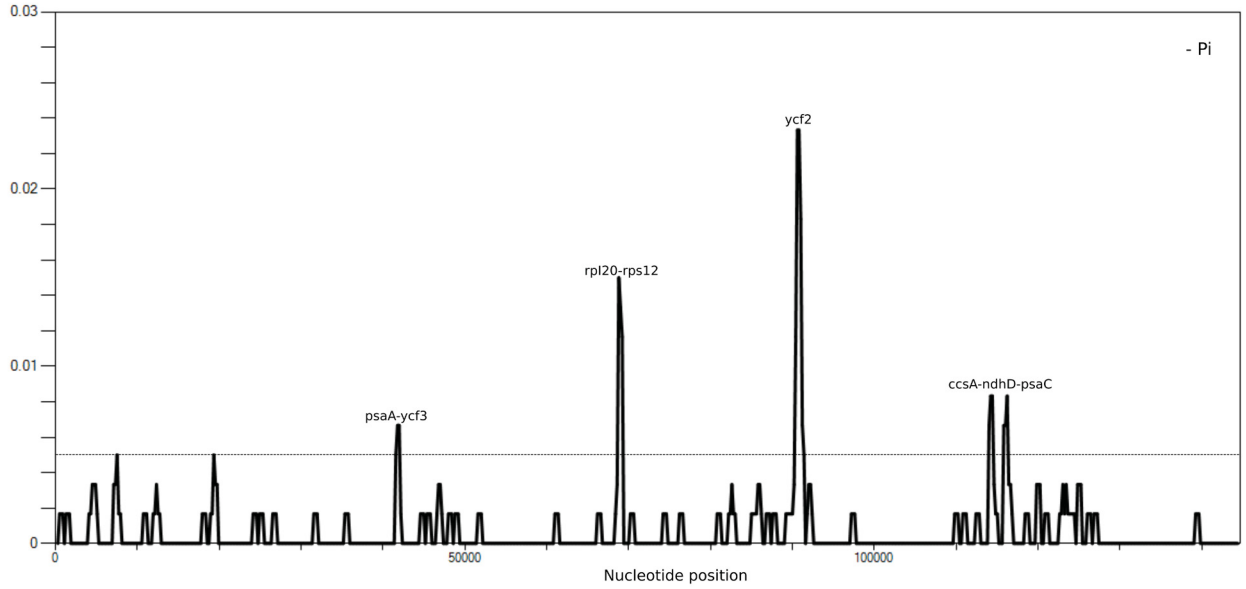

FIGURE 4. DnaSP DNA polymorphism analysis - Nucleotide variability (Pi) comparison between *Strobilanthes lupulina* and *S. glandulata.* The window length and step size were set to 600bp and 200bp respectively. The most varying regions and the commonly used barcoding regions are listed against the base-pair differences found.

Taxonomic treatment

Strobilanthes glandulata Nilanthi, *sp. nov.* (Figures 5 & 7)

Type:—SRI LANKA. Central province: Kandy District, Madugoda, 14 Feb 1928, *Alston 1966* (holotype PDA!).

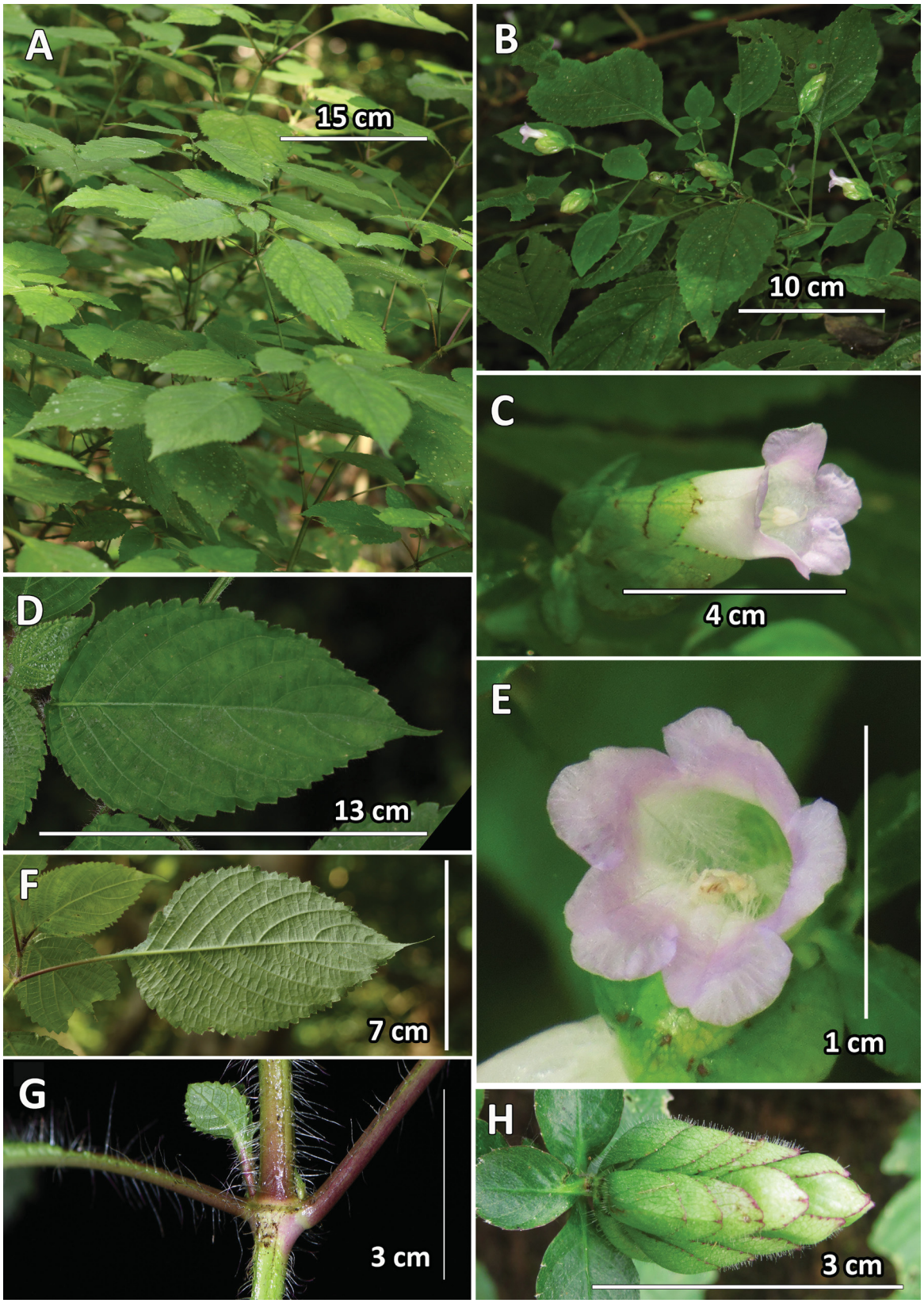

FIGURE 5. *Strobilanthes glandulata.* A. Habit; B. Flowering branch; C. Inflorescence; D & F. Leaves adaxial and abaxial view; E. front view of corolla; G. Internode; H. Bracts.

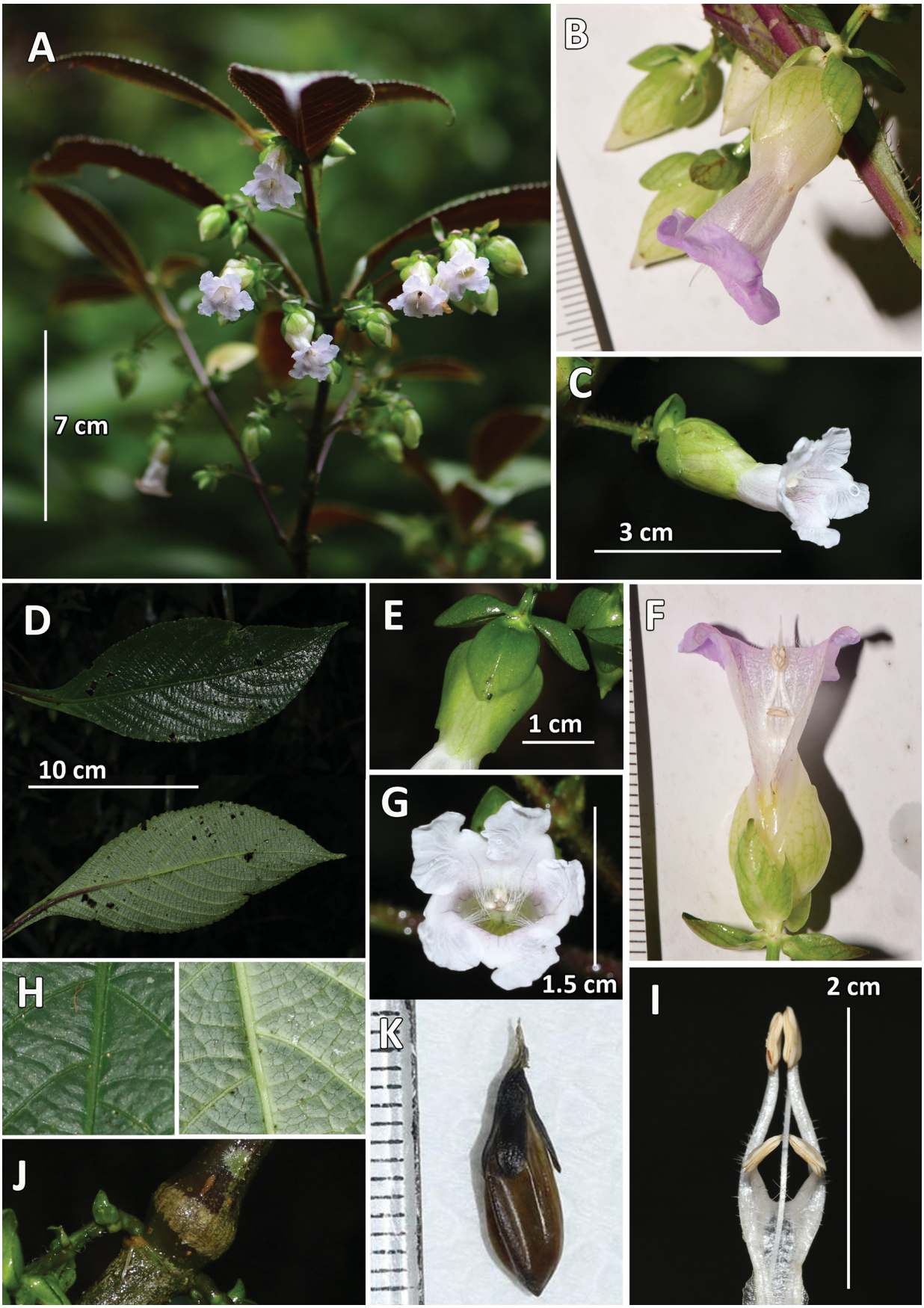

FIGURE 6. *Strobilanthes lupulina.* A. Flowering branch; B–C Inflorescence; D. Leaves adaxial and abaxial view; E. Bracts; F. Corolla split open showing the stamens; G. front view of corolla; H. Close up of the leaf surface; I. Stamens; J. Internode; K. Capsule.

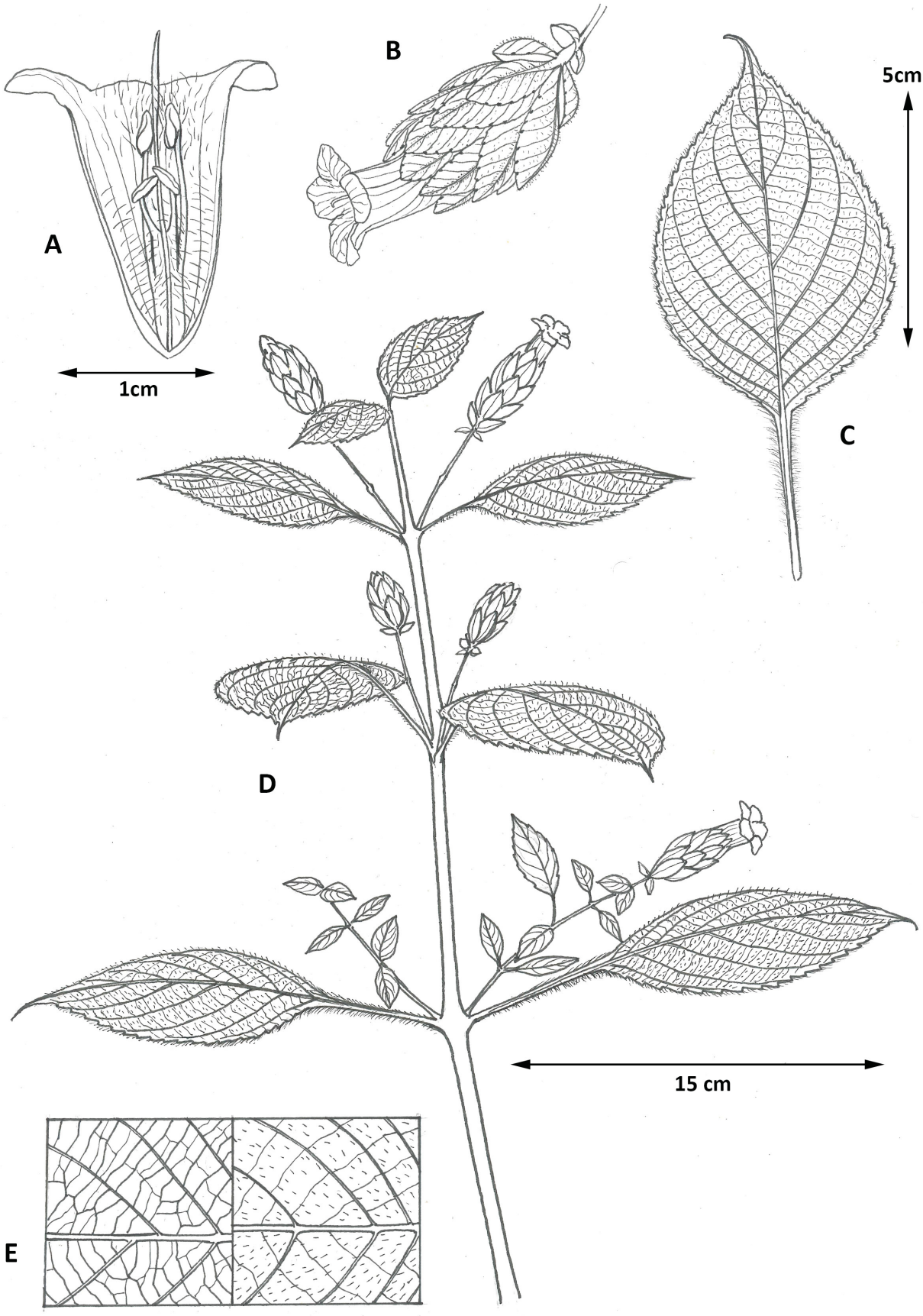

FIGURE 7. *Strobilanthes glandulata*. A. Corolla split open showing the stamens; B. Inflorescence; C. Leaf; D. Flowering branch; E. Close up of the leaf surfaces.

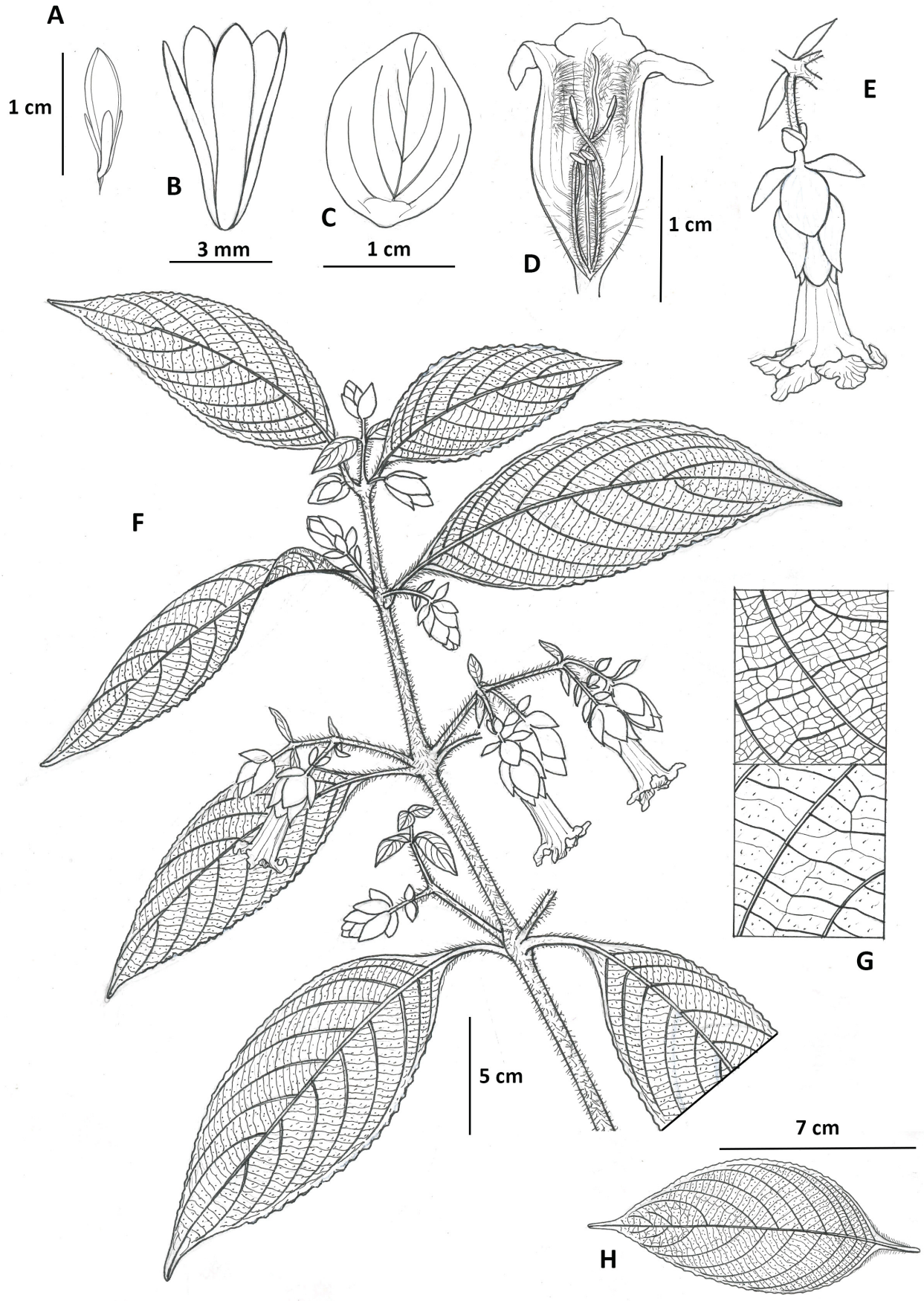

FIGURE 8. *Strobilanthes lupulina*. A. Capsule; B. Calyx; C. Bracts; D. Corolla split open showing the stamens; E. Inflorescences; F. Flowering branch; G. Close up of the leaf surfaces; H. Leaf.

Diagnosis:—*Strobilanthes glandulata* is closely related to *S. lupulina* (Figure 6 & 8).but differs by having slender rounded (not stout quadrangular) stem, ovate (not broadly elliptic) leaves, leaf margin dentate (not serrate), flowers in oval-elongated heads (not in short heads), bracts well-spaced (not compact), margin crenate (not entire), and 3-4 ribs which are completely encircle the poles of pollen (all ribs joined at poles)

Description:—Shrubs,1–2 m high. Stem slender, rounded, glabrescent below, hispid-pubescent above, much-branched, young shoots pale green, becoming dark brown when old. Leaves unequal, variable in size; blades ovate, 3–14 × 1.5–8 cm, apex acuminate, base attenuate, margin dentate, hispid on the upper surface, bullate above, cystoliths numerous, decurrent on the petiole; petioles 1.3–7.5 cm long, hispid. Inflorescences terminal and axillary oval-elongated heads; peduncles 3.5–4 cm long, hispid; heads 4.5–6.1 cm long. Bracts distinct, broadly ovate, obtuse, concave, margin crenate with dotted glands, densely covered with glandular-hairs, lower bracts 3–10 × 2–4 mm, smaller than the upper bracts 10–15 × 4–7.5 mm. Calyx 5–6 mm long, oblong, glabrous, lobes equal. Corolla 21–25 mm long, 8–12 mm wide, pale purple, funnel-shaped, glabrous outside, glandular pubescent inside, lobes ovate, obtuse, 3 mm long, 4 mm wide. Stamens 4, included, 2-longer and 2-shorter, longer stamina filament 15–18 mm long, shorter stamina filament 7–9 mm long, stamina filament pubescent. Pollen ellipsoid, tricolporate, P/E = 1.3, ribs 18, ribs have solid aggregates of raised supratectal elements, endoaperture elongate (Figure 2). Ovary ca. 2×1 mm, apex pubescent, style 18–22 mm long, filiform, pubescent on lower part; stigma linear, ca. 2 mm long, simple, glabrous. Capsule not seen.

Etymology:—The epithet "*glandulata*" refers to the glandular-hairs on the bracts.

Phenology:—Flowering from March to May.

Habitat:—The new species grows in the shady places among rocks in the banks of streams and edges of nature trails (Figure 9).

FIGURE 9. Habitats of *Strobilanthes glandulata.*

Distribution:—It is rather restricted in undisturbed habitat in Rambukoluwa, at an elevation of 282 m towards the eastern slope of the Knuckles Mountain and Madugoda in Sri Lanka. Whereas *S. lupulina* shows quite widespread distributions at low and high altitudes of the wet zone at an elevation of 100–1800m.

Conservation Status:—From 2016 to 2021, we visited Madugoda for several times. However we were unable to rediscover *S. glandulata* from Madugoda. Destruction of natural habitats due to agriculture and development projects could be the main reason for the disappearance of this species from the type locality. As a result, the study determined that the only remaining population of *S. glandulata* is in Rambukoluwa. Approximately, 300 individuals have been noted in the Rambukoluwa area, and it seems that the species is well-adapted to the habitat. There is a threat to this population due to road construction for the Kaluganga project. Therefore, *S. glandulata* could be classified as Critically Endangered (B1ab, ii, iii, v; B2ab, ii, iii, v) following the IUCN Status Categories under Criterion

Additional specimens of *Strobilanthes glandulata* examined (paratypes):—Matale District, Eastern slope of Knuckles mountain at Rambukoluwa, E 202704 N 258834, 282 m, 24 April 2019, RMRN_123 (PDA!).

***S*pecimens of *Strobilanthes lupulina* examined:—Matale District:** Lagalla, Sept 1893, *Trimen s.n.*. (PDA); **Kagalle District:** Kalugala, 28 Aug 1927, *Alston 877* (PDA); Across river from Kitulgala, 6 Nov 1967, *Comanor 540* (PDA); Ambarawella, 23 Aug 1968, *Amaratunga 1629* (PDA); **Kalutara District:** Kalugala, 28 Jun 1975, *Waas 1290* (PDA); Kalwana-Agalawatta, 27 mile post, 9 Sep 1975, *Sumithraarachchi et al. 1010* (PDA); Kalugala Forest, Pahala

Hewessa, 6 Nov 1975, *Sohmer & Waas 10276* (PDA); half mile north of Pahala Hewessa, 17 Feb 1977. *B. & K. Bremer 810* (PDA), RATNAPURA DISTRICT: Kokawatte, Sinharaja Forest, Dec 1893, *Trimen S.N.* (PDA); **Nuwara Eliya District:** Gardner in *Thwaites C.P. 254* (PDA); Road from Pundaluoya to highway A5 and Nuwara Eliya, 26 Dec 1970, *Theobald & Krahulik 2818* (PDA).

Discussion

Pollen morphology reported to have considerable taxonomic importance (Erdtman, 1986), therefore the variability of pollen morphology in *Strobilanthes* is potentially a useful character to delimit taxa in this group (Chen *et al.* 2019). The pollen morphology of south Indian and Sri Lankan *Strobilanthes* is well documented and recognized (Carine & Scotland, 1998). The new species, *S. glandulata*, has sub-prolate pollens with straight ribs. According to the recognized ellipsoid pollen diversity by Bremekamp (1944), there is much variation in the ornamentation of the longitudinal ribs between pseudocolpi, which accounted for 6 different Types. According to classification of (Carine & Scotland, 1998), *S. glandulata* belongs to Type 5, which has the pollen grains with discontinuous aggregations of exine along the middle of each rib, while *S. lupulina* corresponds with Type 2, which has prolate grains. *S. glandulata* is distinct from the other species in Type 5. *S. heteromalla* also belongs to the same type, but has higher P/E ratio. Accordingly, *S. lupulina* and *S. glandulata* can be distinguished from each other by the size and shape of the pollen grains, and the exine ornamentation in the longitudinal ribs.

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