

Revised morphology and barcoding of *Strobilanthes andersonii* Bedd. a critically endangered plant

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ABSTRACT

Strobilanthes is a genus of perennial flowering shrubs with about 400 species. Due to the infrequent flowering period and monocarpic nature the availability of reproductive parts – the primary requisites for species identification – is comparatively a rare chance. Hence, other methods, such as molecular techniques, are vital to differentiate *Strobilanthes* species in the vegetative stages. The establishment of DNA barcode database is a suitable method. The DNA barcode region ITS nuclear ribosomal DNA of *Strobilanthes andersonii* Bedd was sequenced. A revised morphology of *S. andersonii* Bedd is also prepared. A ML phylogram was constructed to evaluate the phylogenetic perspective of *S. andersonii* Bedd.

1. INTRODUCTION

Strobilanthes (Family: *Acanthaceae*) is a South-East Asian wet tropical evergreen genus that exists in moist forests. This genus with 400 species is the second largest in the family *Acanthaceae* [1], and distributed along the South and Southeast Asia. It reached India after the Indian subcontinent merged Asian mainland through the continental drift. The original genetic stock of *Strobilanthes* was probably a single species, reached from Malaysian region to the Western Ghats [2]. In India, there are 146 species of *Strobilanthes* distributed in the wet non-deciduous forests of the Western Ghats and the Himalayas [3]. Fifty-nine species are reported from South India with maximum endemism in Peninsular India [4]. Beddome incompletely described the species *Strobilanthes andersonii* Bedd in 1864 [5]. Due to the long flowering periodicity, no further collections of this plant were made making the description of this plant incomplete and no further attempt was made on revising the morphology till now. Furthermore, since then, this species was considered extinct [4]. The massive flowering of this species was noticed in the shola forests of Eravikulam National Park in 2008 (2). Pendent flowering spikes with large green bracts were the distinguishing feature of this species. In the present study, the gregarious flowering of *S. andersonii* Bedd is noticed in 2018 in the same locality. Hence, the flowering periodicity of this endemic shrub is ascertained as 10 years.

The recent collection of this species in flowering stage from Eravikulam National Park became a rediscovery of this species. Even though there was previous plant exploration in the same area (Eravikulam NP), there were no report of its existence. It shows that even though the plant flowered according to its own reproductive cycle the botanists who explored this area could not collect it in flowering stage. Furthermore, there was a note on previous reports indicating the huge collection of *Strobilanthes* in vegetative parts [6,7]. This urges a new attempt to identify the species of *Strobilanthes* using the foliar characters. Augustine [2] has proposed a key based on the foliar characters of *Strobilanthes*. The revised morphology of this species based on the recent data is given in this paper. However, a more precise mechanism is the utilization of molecular techniques for identifying the vegetative stages.

DNA Barcoding uses short DNA sequences from nuclear and organelle genomes for the identification of biological specimens [8]. The difference between inter and intra-specific genetic distances within a group of organisms is DNA Barcoding gap [9]. DNA Barcoding can resolve taxonomic and evolutionary problems related to identification [10-12]. In DNA Barcoding, the basic concept is to discriminate the species by the variation in the DNA sequences. In species like *Strobilanthes*, where long flowering periodicity exists, DNA Barcoding is a suitable method for identifying already described species and describing new species.

Nuclear ribosomal DNA (nr DNA) contains 18S, 5.8S, and 26S ribosomal RNA subunit and different spacer DNA regions in between [13]. In the present study, molecular phylogenetic elucidation of *S. andersonii* Bedd and related species of *Strobilanthes* was done using ITS nr DNA.

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Hemigraphis confinis T. Anderson as selected the out-group.

2. MATERIALS AND METHODS

2.1. Taxonomy

First description *S. andersonii* Bedd. Madras J.Lit. and Sci. 1:55, 1864 [5].

2.2. Habitat and Distribution

Evergreen shola forests, 2000 m above the sea level, Kerala, India. The present collection is from Eravikulam National Park, Idukki District, Southern Western Ghats, India.

2.3. Materials and Methods

S. andersonii used in the study was collected from the evergreen shola forests, Eravikulam National Park, Idukki District, and Kerala in November 2018. The plant species were collected and brought to the laboratory for further molecular analyses. The collected plant specimen, the voucher samples were deposited in the Calicut University Herbarium (CALI) Kerala (Voucher Specimen Number – 7068), (Collection Number – 2101).

2.4. DNA Extraction

The total genomic extraction was done with Nucleospin Plant II kit (Mechery-Nagel) using 100 mg of fresh leaves homogenized using liquid nitrogen. The quality of the isolated DNA was tested by agarose gel electrophoresis followed by gel staining with ethidium bromide.

2.5. Polymerase Chain Reaction (PCR) Amplification and Sequencing

PCR amplifications of the ITS region were carried out in a PCR thermal cycler [14] using the primer pairs [15] and thermo cycling conditions given in Table 1.

Sequencing reaction was performed with the Big Dye Terminator v3.1 cycle sequencing kit [14]. Sequence Scanner Software v1 was used for checking the quality of the sequence (Applied Bio system). Analysis and comparison of the locus were done with the help of BLAST routines [16].

2.6. Phylogenetic Analysis

An ITS phylogram was constructed using ML analysis performed by RAxML with default parameters and 1000 bootstrap replications [17,18]. The criterion used to assess BS support percentages (BP) was as follows: low 50–70%, moderate 71–84%, and strong 95–100. The in group taxa consisted of *S. andersonii* Bedd and 40 other species of *Strobilanthes* retrieved from NCBI Gen Bank [Table 2]. *H. confinis* T. Anderson was the out-group.

3. RESULTS AND DISCUSSION

S. andersonii Bedd is considered to be an extinct species (4), but it is rediscovered recently (2). The flowering periodicity of this plant is 10 years. Hence, identification, discrimination, and morphological

characterization for the purpose of taxonomic studies were difficult. The *S. andersonii* Bedd with long flowering periodicity and monocarpic nature is also a possible cause for their rarity. It is difficult to get the specimens of *Strobilanthes* in flowers. Hence, identification of these species is very challenging in many flora works. Hence, it results in the poor documentation of the diversity of *Strobilanthes*, even though the species is there in its vegetative condition. The characteristics of leaves and other vegetative parts have less importance in species wise diagnostic characteristics. Using ITS nrDNA barcoding, we can expose the genetic relationships among accessions more effectively and accurately [8].

3.1. Revised Morphology

The previous description of this species [5] was incomplete with respect to the floral characteristics which were redressed in the present revised morphology which is elaborated here [Figure 1].

S. andersonii Bedd is a large shrub, growing to 4 m height; young stems angled, hirsute, terete when mature. Leaves simple, opposite; lamina 15–22 × 8–12 cm, ovate, round at base, acuminate at apex, and hirsute-hairy on upper and lower surface; acumen 2–3 cm long, pointed at apex; margin regularly crenate-serrate, ciliate; lateral nerves 6–7 pairs, alternate, regular; intercostae parallel, nervules reticulate, all prominently projected below, and impressed above; and petioles 1.5–4.5 cm long, hirsute-hairy, and hairs light pink. Inflorescences spikes, terminal and upper axillary, usually pendent, 4–7 cm long, 2–2.5 cm broad, cylindrical to obtusely sub-4-angled; bracts many, arranged in four rows, 1.5–2.5 cm across, ovate-orbicular, concave, pale green to pale purple with green-purple nerves, glabrous, margin finely serrulate; serrulations produced in to small spinules; and bracteoles 2, oblanceolate, acute at apex, 1.5 × 0.7 cm, glabrous, greenish white with green nerves, ciliate along the margins. Flowers 15–30 in each spike, densely packed, usually a pair of two opposite flowers or rarely two pairs bloom together; sepals 1.6 cm long, linear-lanceolate, acute, glabrous, ciliate along the margin, pale green; corolla 2.1 cm long, 2.5 cm across, sub-campanulate; tubular below,



Figure 1: *Strobilanthes andersonii* Bedd. (a) Habit, (b) One spike inflorescence (c) One flower longitudinally sectioned.

Table 1: Primers and reaction conditions.

DNA region	Primer pairs	Primer sequence (5-3)	Thermo cycling conditions
ITS	ITS-5F ITS-4R	GGAAGTAAAAGTCGTAACAAGG TCCTCCGCTTATTGATATGC	98°C 30 s, 98°C 5 s, 58°C 10 s, 72°C 15 s, 72°C 60 s, 4°C-40 cycles

Table 2: List of Plant names, accession number, and collection place of *Strobilanthes* species, retrieved from NCBI Gen Bank.

S. No.	Species Name and Nucleotide No.	GenBank No. ITS	Native Range
1	<i>Strobilanthes andersonii</i> Bedd. 608	MT571642.1	Eravikulam National Park, Southern Western Ghats, India
2	<i>Strobilanthes kunthiana</i> (Nees) T.Anderson 674	AY489377.1	Southern Western Ghats, India
3	<i>Strobilanthes ciliate</i> Nees 674	AY489381.1	Western Peninsular India
4	<i>Strobilanthes barbata</i> Nees 673	AY489382.1	Southern Western Ghats, India
5	<i>Strobilanthes neilgherrensis</i> Bedd 676	AY489373.1	Southern Western Ghats, India
6	<i>Strobilanthes pulneyensis</i> Clarke 628	AY489374.1	Southern Western Ghats, India
7	<i>Strobilanthes lawsonii</i> Gamble 675	AY489375.1	Southern Western Ghats, India
8	<i>Strobilanthes stenodon</i> C.B.Clarke 673	AY489372.1	India
9	<i>Strobilanthes andamanensis</i> Bor 675	AY489386.1	Andaman
10	<i>Strobilanthes lupulina</i> Nees 675	AY489397.1	Southern Western Ghats, India
11	<i>Strobilanthes decurrens</i> Nees 675	AY489390.1	Southern Western Ghats, India (TN)
12	<i>Strobilanthes micrantha</i> Wight 675	AY489388.1	Southern Western Ghats, India
13	<i>Strobilanthes asper</i> Venu& P.Danie 1675	AY489399.1	SW.India
14	<i>Strobilanthes anceps</i> Nees 677	AY489395.1	India
15	<i>Strobilanthes punctata</i> Nees 678	AY489396.1	India
16	<i>Strobilanthes rubicunda</i> (Nees) T Anderson 674	AY489370.1	Southern Western Ghats, India
17	<i>Strobilanthes involucrate</i> (Blume) Bremek 676	AY489358.1	Java
18	<i>Strobilanthes anceps</i> Nees 664	JX443807.1	S. India, Sri. Lanka
19	<i>Strobilanthes steenisiana</i> J.R.Benn 676	AY489357.1	Java
20	<i>Strobilanthes</i> sp. "Hongkong Island" 675	AY489355.1	Hongkong
21	<i>Strobilanthes walker</i> Arn.ex Nees 674	AY489391.1	Southern Western Ghats, India
22	<i>Strobilanthes filiformis</i> Blume 673	AY489353.1	Java
23	<i>Strobilanthes imbricate</i> Nees 672	AY489362.1	Indo-China
24	<i>Strobilanthes bibracteata</i> Blume 675	AY489359.1	Java
25	<i>Strobilanthes isophylla</i> (Nees) T.Anderson 674	AY489352.1	Bhutan to Bangladesh
26	<i>Strobilanthes cernua</i> Blume 662	AY489361.1	Java
27	<i>Strobilanthes japonica</i> (Thunb) Miq. 675	AY489356.1	Japan, East Asia
28	<i>Strobilanthes alata</i> Nees 678	AY489360.1	Java
29	<i>Strobilanthes repanda</i> (Blume) J.R.Benn 679	AY489366.1	Java
30	<i>Strobilanthes pluriformis</i> C.B. Clarke 639	AY489346.1	Philippines
31	<i>Strobilanthes aprica</i> var. <i>pedunculata</i> Craib 673	AY489345.1	South China to Indo-China
32	<i>Strobilanthes habracanthoides</i> J.R.I Wood 663	AY489369.1	India
33	<i>Strobilanthes capitata</i> (Nees) T.Anderson 650	AY489349.1	India
34	<i>Strobilanthes versicolor</i> Diels 699	MT914278.1	Tibet to China
35	<i>Strobilanthes speciosa</i> Blume 643	AY489348.1	Java
36	<i>Strobilanthes oligocephala</i> T. Anderson ex C.B. Clark 626	AY489351.1	Tibet and Assam
37	<i>Strobilanthes dyeriana</i> (Mast.) J.R.I.Wood 644	AY489367.1	Java
38	<i>Strobilanthes attenuata</i> Nees 643	AY489344.1	India
39	<i>Strobilanthes multidentata</i> C.B.Clarke 633	AY489350.1	India
40	<i>Strobilanthes galeopsis</i> Stapf 626	AY489354.1	Borneo
41	<i>Strobilanthes pulcherrima</i> T.Anderson 663	AY489368	Sri Lanka
42	<i>Hemigraphis confinis</i> T.Anderson 680	AY489400.1	Java

broadened above; tubular lower part cylindrical, 0.7 cm long, deep violet inside, glabrous; upper portion sub-campanulate, 1.4 cm broad, white-pale blue with deep violet prominent nerves, glabrous; lobes equal, orbicular, round to sub-cordate at apex, entire or shallowly crenate along the margins, glabrous, pale blue with thin blue nerves, spreading, twisted; stamen 4, free; filaments unequal, in 2 pairs, attached together into a small sheath just above the tubular part of

the corolla tube; longer filaments 18 mm long, 1.5 mm thick, violet below, white above, glabrous; shorter filaments 12 mm long, inner, similar to the outer long filaments; anthers similar, just at the mouth of the corolla, oblong, shallowly cleft to sagittate at base, 5 mm long, pale pink, anthers of long stamens open first; ovary on a disc, 4 mm long, ovoid, glabrous; ovules 4, style 2.1 cm long, slender, glabrous, pink; stigma pointed into a white spot; and disk present below the

ovary, 2 mm thick, yellow. Fruit 2 × 0.7 cm, ovoid, acute, glabrous; seeds four, orbicular, and compressed.

3.2. Phylogenetic Analysis

Presently, the phylogram based on ITS nr DNA gene sequences grouped all available 40 species into two clades [Figure 2]. The *Strobilanthes* species of Peninsular India are distributed in both. These clades were further divided into sub clades. Clade I consisted of 9 taxa divided into two groups (BS 100%). *S. andersonii* Bedd is coming under Group A. Group B contains 8 taxa with BSS of 51%. The species coming under group B are *Strobilanthes pulneyensis* Clark, *Strobilanthes barbata* Nees, *Strobilanthes lawsonii* Gamble, *Strobilanthes neilgherrensis* Bedd, *Strobilanthes ciliata* Nees, *Strobilanthes kunthiana* (Nees) T. Anderson, *Strobilanthes stenodon* C. B. Clark, and *Strobilanthes andamanensis* Bor. Among these, all species except *S. andamanensis* Bor is native to Southern Western Ghats, India. *S. andamanensis* Boris is from Andaman Islands. The Indian *Strobilanthes* species *S. andersonii*

Bedd, *S. pulneyensis* Clark, *S. barbata* Nees, *S. lawsonii* Gamble, *S. neilgherrensis* Bedd, *Strobilanthes ciliata* Nees, *S. kunthiana* (Nees) T. Anderson, *S. stenodon* C. B. Clark, and *S. andamanensis* Bor formed the most recent clade I with high boot strap support (BSS of 100%). The members of these clade can be considered as recent radiations and with Clade II as a sister clade.

Clade II consisted of 32 species and was divided into five sub-groups. Group A contains *Strobilanthes japonica*, the native of Japan, East Asia with BSS 39%. Group B contains *Strobilanthes galeopsis* Stapf is from S.E. Asia with BSS 20%. Group C contains 22 species with BSS 58%.

Group C contains species such as *Strobilanthes steenisiana* J.R. Benn, *Strobilanthes rubicunda* Nees T. Anderson, *Strobilanthes involucreata* (Blume) Bremik, *Strobilanthes versicolor* Diels, *Strobilanthes pulcherrima* T. Anderson, *Strobilanthes bibracteata* Blume, *Strobilanthes dyeriana* (Mast) J.R.I Wood, *Strobilanthes repanda* Blum J.R. Benn, *Strobilanthes oligocephala* T. Anderson ex C. B. Clark, *Strobilanthes multidens*

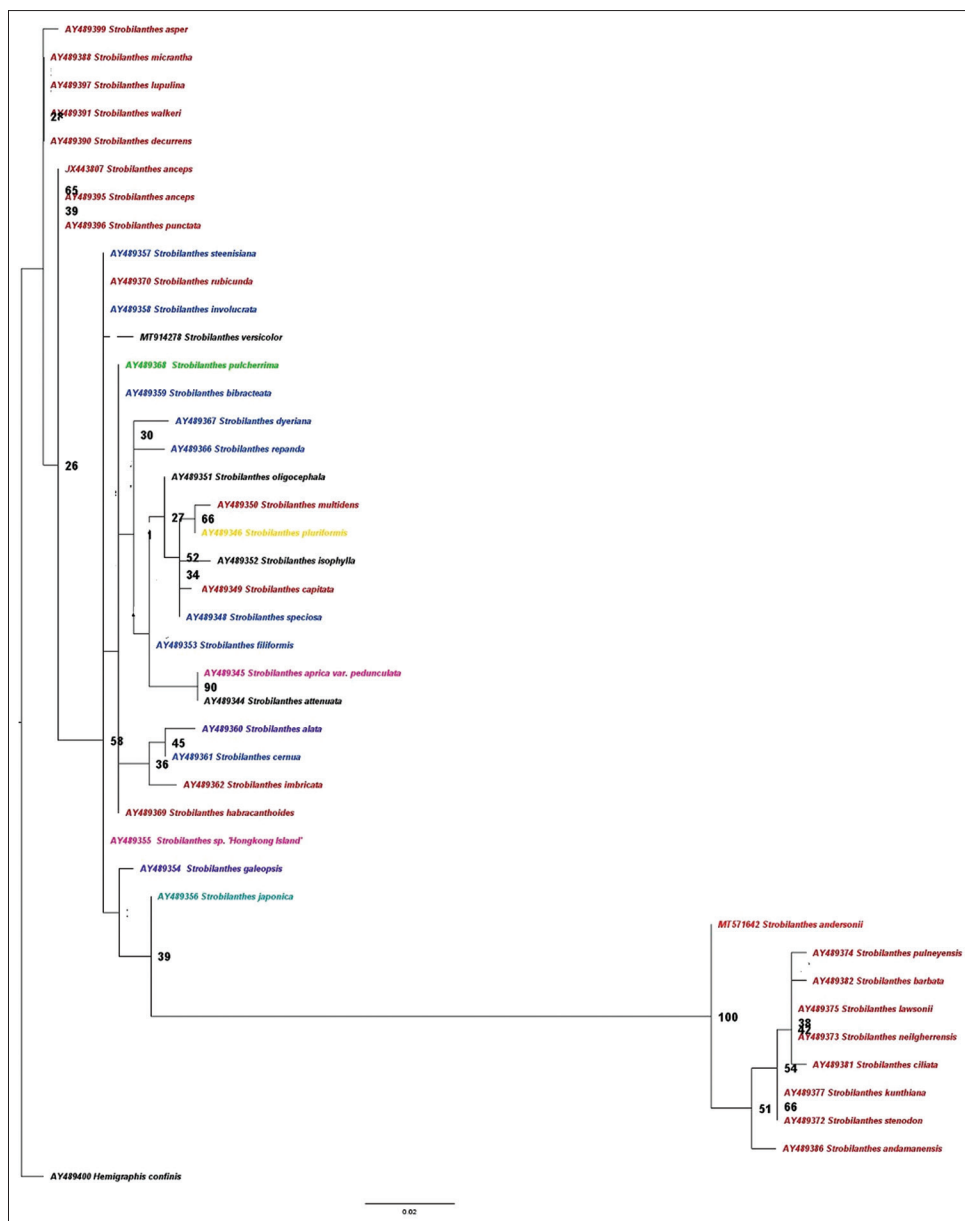


Figure 2: ML tree inferred with RAXML of nr DNA (ITS) of *Strobilanthes andersonii* Bedd compared with 40 species of *Strobilanthes*.

C. B. Clark, *Strobilanthes pluriformis* C. B. Clark, *Strobilanthes isophylla* (Nees) T. Anderson, *Strobilanthes capitata* Nees T. Anderson, *Strobilanthes speciosa* Blume, *Strobilanthes filiformis* Blume, *Strobilanthes aprica* var. *pedunculata* Craib, *Strobilanthes attenuate* Nees, *Strobilanthes alata* Nees, *Strobilanthes cernua* Blume, *Strobilanthes imbricate* Nees, *Strobilanthes habracanthoides* J. R. I. Wood, *Strobilanthes* spp. "Hong Kong Island." In these species *S. rubicunda* Nees T. Anderson, *S. multidentis* C.B. Clark, *S. capitata* Nees T. Anderson, *S. attenuate* Nees, *S. imbricate* Nees, and *S. habracanthoides* J.R. I. Wood which are from India. *S. steenisiana* J.R.Benn, *S. involucrate* (Blume) Bremik, *S. bibracteata* Blume, *S. dyeriana* (Mast) J.R.I Wood, *S. repanda* Blum J. R.Benn, *S. speciosa* Blume, *S. filiformis* Blume, *S. alata* Nees, and *S. cernua* Blume are native of Java. The Tibetan species is *S. versicolor* Diels, *S. oligocephala* T. Anderson ex C, B. Clark and *S. aprica* var. *pedunculata* Craib. The Group C represents taxa from geographically diverse regions of India, Java, and Tibet forming a weakly supported group (BSS 58%).

Group D contains three species, two species of *Strobilanthes anceps* Nees and *Strobilanthes punctate* Nees which are from South Asia BS value 26%.

Group E contains five species. They are *Strobilanthes asper* Venu and Daniel, *Strobilanthes micrantha* Wight, *Strobilanthes lupulina* Nees, *Strobilanthes walkeri* Arn. ex Nees, and *Strobilanthes decurrens* Nees. These species are native of Southern Western Ghats, India. *S. japonica* is the sister taxa to this clade. Clade II is the basal clade with taxa showing an early establishment and wide geographical distribution. Members of this clade might have been the earliest radiations in the Southern Western Ghats from an ancestor with South Asian origin.

S. andersonii Bedd and its related species were descended from clade II and recently established in Western Ghats due to adaptive radiation. In evolutionary radiation, a fast escalation in the diversity of a group of organisms occurs by frequent speciation within a particular clade with fast evolutionary and ecological divergence in geographical location [19]. *S. andersonii* Bedd has close affinity with other Indian species. Moreover, the analysis shows that South Indian species belong to recent lineages of *Strobilanthes*.

4. CONCLUSION

By revisiting the taxonomic description of this species hitherto considered as extinct, a proper key for identifying the species can be formulated for field identification of the species. Furthermore, it will accelerate the conservation efforts of this species with high endemism by demarcating the areas of its occurrence a further clarification on the taxonomic ambiguity was attempted by the phylogenetic study. Internal transcribed spacer nuclear DNA sequences were suitable in taxonomic studies of *Strobilanthes* even if the flowers are not accessible. In the present study, sequence analysis of *S. andersonii* Bedd using ITS sequences was performed to clarify phylogenetic relationships and found that this species is recent radiation in the South Western Ghats.

5. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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7. CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

8. ETHICAL APPROVALS

Forest Department of Kerala, India providing permission to collect the plant sample and this study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

Plant sample information and data available in Table:2.

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