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Morphology, Palynology and Molecular Phylogeny of *Barleria cristata* L. (Acanthaceae) Morphotypes from India

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Abstract: *Barleria cristata* L., commonly known as the Philippine violet, is native to South Asia. It is an ornamental plant and is also used for the treatment of a variety of diseases. In India, it is found throughout the country in many forms, varying in its floral attributes (calyx and corolla) and habitat. In order to understand the species limits in *B. cristata*, we studied morphological as well as palynological variation and assessed the phylogenetic relationships among five different morphotypes. The studied morphotypes (populations) came from three phytogeographical regions, namely the Western Ghats, the Deccan Peninsula and the Western Himalaya. The naturally occurring populations from the Deccan Peninsula and the Western Himalaya showed conspicuous differences in their morphology. All the morphotypes had oblate spheroidal, tri-brevicolporate and honey-combed pollen grains which differed only in their quantitative parameters. The distinct-looking morphotypes, namely, Nandi Hills, Uttarakhand and cultivated morphotypes, could not be separated based on pollen characters. Phylogenetic analyses based on chloroplast DNA sequences revealed that our samples formed a clade sister to the *B. cristata* specimen used in the previous study. The genetic variation within morphotypes was not enough for the genomic regions investigated; however, it revealed among morphotype genealogies in detail. Phylogenetic analyses showed that there were three monophyletic groups within the *B. cristata* complex that exhibited some morphological differences. Nevertheless, based on the present sampling, it is not possible to delimit these morphotypes at specific or infraspecific level. To reach such conclusions, further investigations like sampling this species across its distribution range in India and assessment of intraspecific relationships, and their cytogenetical characterization should be done.

Keywords: comparative morphology; ITS; *ndh*F-*trn*L$_{(UAG)}$; Philippine violet; pollen; taxonomy

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1. Introduction

Barleria L. (Barlerieae: Acanthaceae) comprises about 300 species and occurs mainly in the Old World tropics [1]. Species of the genus are usually herbs or shrubs and rarely trees [2]. The major concentrations of diversity and endemism are in Tanzania, Angola, Madagascar and India [1]. The first comprehensive phylogenetic analysis of *Barleria* was carried out by Darbyshire et al. [1] based on *trn*S-G, *ndh*F-*rpl32*-*trn*L$_{(UAG)}$ and the internal transcribed spacer (ITS). A total of 53 species, mostly from Africa, were sampled. They recognised two subgenera, *Barleria* and *Prionitis* (Nees) C.B.Clarke, the latter comprising three sections, *Prionitis* (Nees) Lindau, *Somalia* (Oliv.) Lindau and *Stellatohirta* M.Balkwill. *Chrysothrix* M.Balkwill and *Fissimura* M.Balkwill, the sections belonging to subgenera *Barleria* and *Prionitis*, respectively, in the classification of Balkwill and Balkwill [3] were

subsumed under subgenus *Barleria*. In addition, the section *Cavirostrata* M.Balkwill of the subgenus *Prionitis* was subsumed under section *Somalia* [1]. The species of the subgenus *Barleria* have beak-less capsules that are 4-seeded except in the *Fissimura* clade [1], where they are two-seeded whereas the species of the subgenus *Prionitis* have two- or four-seeded capsules with a prominent beak (solid or partially hollow) [1], except that plants of section *Stellatohirta* have two-seeded fruits with the beak reduced or largely absent [4].

The phylogenetic analysis provides a framework for interdisciplinary investigations in taxonomy, biogeography, evolutionary biology, ecology and conservation [5]. Recently, two phylogenies have been constructed for *Barleria* [1,4]. The phylogenetic results of Darbyshire et al. [1] were not able to clarify relationships in some studied taxa and indicated the need for additional sampling of the species from the Indian landmass and Madagascar as well as the use of restriction site-associated DNA (RAD) sequencing data to resolve the relationships and recent radiation of *Barleria*. Very recently, Comito et al. [4] constructed a RADseq phylogeny wherein they examined 167 species (56% of the total species diversity). Single nucleotide polymorphism (SNP) data were generated using double-digest restriction-site associated DNA sequencing (ddRADseq) and the maximum likelihood phylogeny supported the topology estimated from the chloroplast and nrITS data of Darbyshire et al. [1,4]. Comito et al. [4] also indicated the need for more sampling of Asian species, particularly from the Indian subcontinent, in order to understand the biogeography and diversification of *Barleria*.

The genus *Barleria* can be recognised based on the presence of a calyx with two outer segments large and two inner ones smaller, globose honey-comb pollen and epidermal cells with double cystoliths [6]. *Barleria cristata* L. is the type species of the genus and is commonly known as the Philippine violet. It belongs to the subgenus *Barleria* [1]. The subgenus *Barleria* is further divided into seven monophyletic groups, viz. the *Cristata* clade, the *Ovata* clade, the *Crassa* clade, the *Fissimura* clade, the *Strigosa* clade, the *Monticola* lineage and the *Chrysothrix* lineage [4]. The *Cristata* clade is further split into three subclades, namely, the *Acanthoideae* clade, the core *Cristata* clade and the *Rigida* clade [4]. The core *Cristata* clade includes *B. cristata* and two closely related species, namely, *B. aculeata* Balf.f. (endemic to Socotra) and *B. nitida* Nees (native to India and Sri Lanka) [4].

Barleria cristata is native to South Asia and India; it is found throughout the country [7]. It is cultivated as an ornamental plant for its showy flowers but can also be found as an escape. As the plant is in the horticultural trade, many forms, particularly those varying in corolla colour (such as white, pink, striped and blue), are available. Additionally, the species enjoys varied habitats across the country, and hence, variations may be on account of its plastic response to the existing environmental conditions. *B. cristata* has great importance in different ethnomedical systems for the treatment of a variety of diseases, especially lung disorders and inflammatory conditions [8]. So far, 22 compounds have been reported from the species that fall mainly under the category of flavones and flavonoids, phenolics and phenolic glycosides [9].

Considering the ornamental and medicinal importance, it is necessary to characterise the variation that exists in this species and understand the species limits. Therefore, the aims of this study were to: (i) assess the morphological and palynological variation among the morphotypes, (ii) infer the phylogenetic relationships among the morphotypes using cpDNA (*trn*S-G, *ndh*F-*rpl*32-*trn*$L_{(UAG)}$) and nrDNA (ITS) sequences, and (iii) determine the taxonomic status of the morphotypes, if any.

2. Materials and Methods

2.1. Taxon Sampling and Morphological Analysis

Five morphotypes of *Barleria cristata* were collected during the period 2017–2021. All of them are maintained in the Botanical Garden of Shivaji University, Kolhapur. A total of 20 accessions (4 individuals for each morphotype) were sampled (Figure 1 and Table 1). The morphological analysis was based on observations made on live specimens maintained in the Botanic Garden. Voucher specimens were deposited in SUK (Table 1). Descriptions were

made following the terminology of Hickey and King [10]. Corolla color was determined following the nomenclature of Ridgway [11].

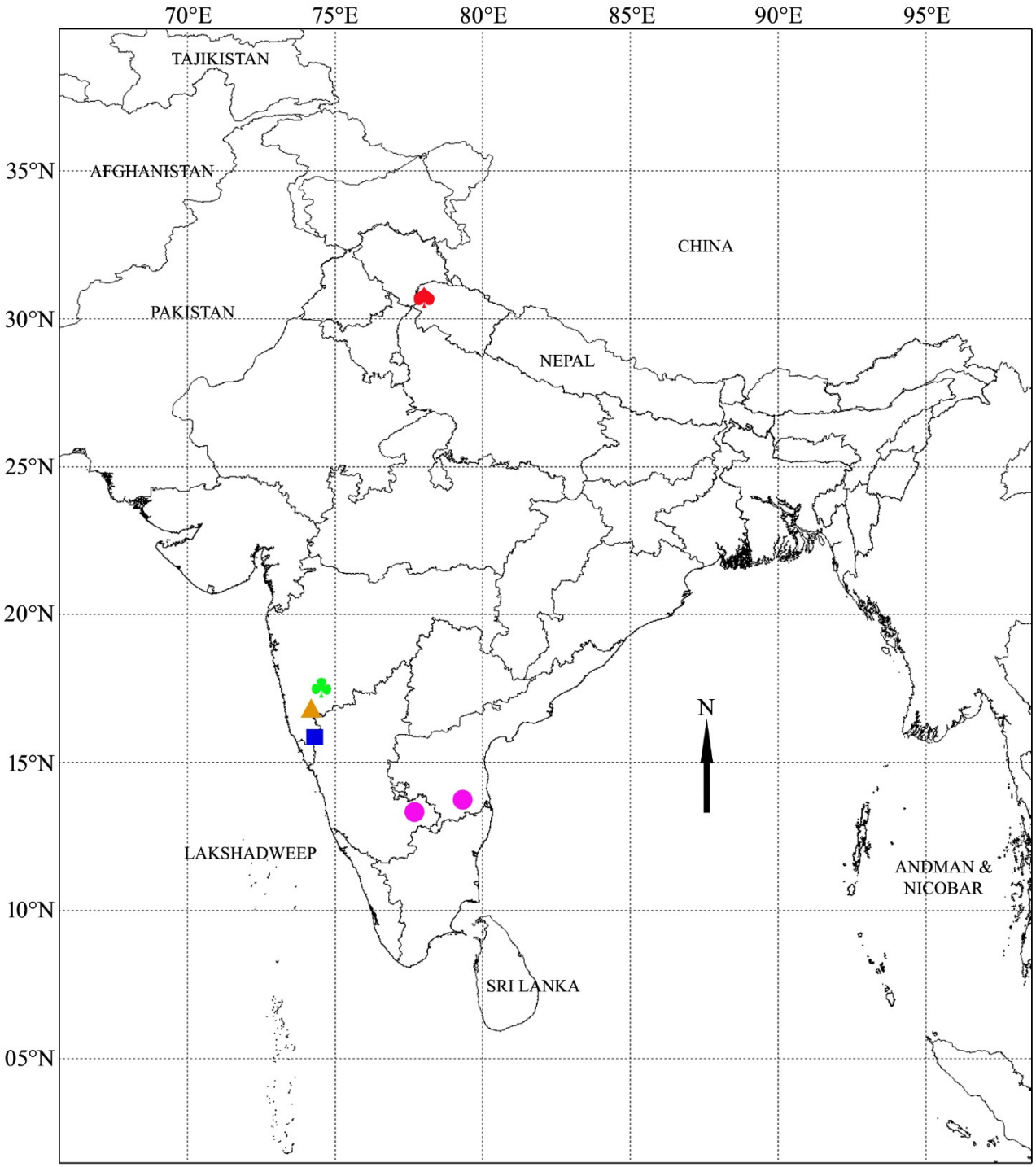

Figure 1. Distribution of *Barleria cristata* morphotypes. ■ *B. cristata* (cultivated form 1); ♣ *B. cristata* (cultivated form 2); ▲ *B. cristata* (cultivated form 3); ● *B. cristata* (Nandi Hills); ♠ *B. cristata* (Uttarakhand).

Table 1. Details of *Barleria cristata* morphotypes (collection locality, GPS coordinates, vouchers and GenBank accession numbers for *trn*S-G, *ndh*F-*trn*L$_{(UAG)}$ and ITS region).

Morphotype	Collection Locality	GPS Coordinates	Voucher ID	GenBank Accession Numbers		
				*trn*S-G	*ndh*F-*trn*L$_{(UAG)}$	ITS
Nandi Hills						
B. cristata	Nandi Hills, Chikkaballapur district, Karnataka	N 13°21.400′ E 77°40.570′	SSP 004	ON667972	ON693951	ON706922
B. cristata	Tirupati, Chittor district, Andhra Pradesh	N 13°41.157′ E 79°20.114′	SSP 051	ON667973	ON693952	ON706923
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 069	ON667974	ON693953	ON706924
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 131	ON667975	ON693954	ON706925
Uttarakhand						
B. cristata	Chakrata, Dehradun district, Uttarakhand	N 30°41.283′ E 77°52.215′	SSP 023	ON667976	ON693955	ON706926
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 055	ON667977	ON693956	ON706927
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 065	ON667978	ON693957	ON706928
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 134	ON667979	ON693958	ON706929
Cultivated form 1						
B. cristata	Kankumbi, Belgaum district, Karnataka	N 15°42.120′ E 74°13.130′	SSP 024	ON667980	ON693959	-
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 058	ON667981	ON693960	-
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 079	ON667982	ON693961	-
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 132	ON667983	ON693962	-
Cultivated form 2						
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 042	ON667984	ON693963	ON706930
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 062	ON667985	ON693964	ON706931
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 071	ON667986	ON693965	ON706932
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 135	ON667987	ON693966	ON706933

Table 1. *Cont.*

Morphotype	Collection Locality	GPS Coordinates	Voucher ID	GenBank Accession Numbers		
Cultivated form 3						
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 040	ON667988	ON693967	ON706934
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 060	ON667989	ON693968	ON706935
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 070	ON667990	ON693969	ON706936
B. cristata	Lead Botanical Garden, Shivaji University, Kolhapur, Maharashtra, India	N 16°40.310′ E 74°15.190′	SSP 133	ON667991	ON693970	ON706937

2.2. Palynology

Flowering specimens were collected from various localities as well as from the Botanical Garden, Shivaji University, Kolhapur. Pollen grains were stored in glacial acetic acid and then acetolysed in freshly prepared mixture of acetic anhydride ($C_4H_6O_3$): concentrated sulphuric acid (H_2SO_4) (9:1) following the technique of Erdtman [12] and Yadav et al. [13]. Measurements of at least 20 pollen grains for each morphotype were taken by making semi-permanent preparation of acetolysed pollen grains mounted in glycerine jelly. Pollen grains were examined under light microscope (LM) and scanning electron microscope (SEM). Mean values and standard deviation were calculated. Values of Polar axis length (P) and Equatorial diameter (E) were calculated to find out P/E ratio. For SEM, acetolysed samples were dehydrated in ethanol series and mounted on the stub using double-sided sticky tape. Air-dried samples were then coated with gold using Emscope SC 500 sputter coater and observed under JEOL JSM 6360 scanning electron microscope at 5 to 15 kV. The terminology used for pollen grain description follows Erdtman [14,15] and Punt et al. [16]. All the values are represented as mean ± SD. The data were subjected to one-way analysis of variance (ANOVA) and significant differences between mean values were determined by Duncan's multiple range test ($p < 0.05$) using SPSS Statistics for Windows, version 16.0 (SPSS Inc., Chicago, IL, USA).

2.3. DNA Extraction, PCR and DNA Sequencing

Total Genomic DNA was extracted from young and fresh leaves of collected plant species using the modified CTAB method [17] with some modifications mentioned in Tamboli et al. [18] or with Qiagen DNeasy kits (Qiagen, Germantown, MD, USA). Intergenic spacer region *trn*S-G was amplified using primers 5′*trn*S + 3′*trn*G [19] and the spacers *ndh*F-*rpl*32-*trn*L$_{(UAG)}$ were amplified using primers 5′*ndh*F + 3′*rpl*32R and 5′*rpl*32F + 3′*trn*L$_{(UAG)}$ [20]. To amplify ITS region we tried two different combinations of primer such as ITSLeu1 [21]-ITS4 [22] and AB101-AB102 [23]. PCR reactions were performed in a 30 µL medium containing 15 µL nuclease-free water, 10 µL EmeraldAmp GT PCR Master Mix (TaKaRa, Kusatsu, Shiga, Japan) and 1 µL each forward and reverse primer (10 µM). PCR reactions were carried out in a Thermal cycler (TaKaRa, Kusatsu, Shiga, Japan). The amplified PCR product was further purified and sequenced at Macrogen Corporation (Seoul, Korea). PCR protocol for amplification of *trn*S-G and *ndh*F-*rpl*32-*trn*L$_{(UAG)}$ regions was same; 94 °C 3 min, 40 cycles (94 °C for 40 s, 55 °C for 45 s and 72 °C for 1 min) and final extension at 72 °C for 3 min. For nrITS it was; 94 °C for 4 min, 35 cycles (94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min) and final extension at 72 °C for 10 min. Sequences of *trn*S-G, *ndh*F-*rpl*32-*trn*L$_{(UAG)}$ and ITS region were submitted to the GenBank NCBI database. GenBank accession numbers are provided in Table 1.

2.4. Sequence Alignment and Phylogenetic Analyses

We have amplified and sequenced the plastid intergenic spacers *trn*S-G and *ndh*F-*rpl*32-*trn*L$_{(UAG)}$, and nrITS as these have been previously used to resolve phylogenetic relationships in *Barleria* [1]. We retrieved DNA sequences of all the 53 *Barleria* species and 9 outgroup species used in the study of Darbyshire et al. [1] from NCBI GenBank database. Details of sequence taken from NCBI GenBank database were shown in Table S1. A total of 56 sequences generated in this study and were incorporated into the phylogeny of Darbyshire et al. [1] to resolve this *B. cristata* complex. To root the phylogenetic hypothesis, we used the same 9 outgroup species which were used by Darbyshire et al. [1] as these outgroup taxa are from Barlerieae as recognised by McDade et al. [24]. DNA sequences were checked and edited using Sequencher v 5.1 (Gene Codes Corporation, Ann Arbor, MI, USA) [25]. Multiple sequence alignment was carried out using MUSCLE [26] implemented in MEGA X [27]. Alignments of each gene were then further refined using GBlocks v. 0.91b [28]. Two datasets were prepared for phylogenetic analyses; (a) cpDNA (*ndh*F-*trn*L$_{(UAG)}$+*trn*S-G) and (b) ITS. *ndh*F-*trn*L$_{(UAG)}$ and *trn*S-G sequence datasets were concatenated into a single matrix using SequenceMatrix [29]. Incongruence among cpDNA and nrITS datasets was determined by comparing topologies of cpDNA and nrITS phylogeny. Phylogenetic analyses of cpDNA and nrITS dataset were carried out separately using maximum likelihood (ML) and Bayesian inference (BI). jModelTest 2 [30] on XSEDE on the CIPRES science gateway (http://www.phylo.org/ (accessed on 23 May 2022)) [31] was used to find out the fittest nucleotide substitution model for the datasets under the Akaike Information Criterion (AIC). The best-fit nucleotide model for nrITS dataset was GTR+I+G, and for cpDNA it was GTR+G. ML analyses were conducted using RAxML-HPC v.8 [32] on XSEDE via CIPRES portal [31] with rapid bootstrap algorithm using GTR+G as default model for all the datasets and by employing 1000 bootstrap replicates. BI was performed using MrBayes v.3.2.6 [33] on XSEDE via CIPRES portal [31]. Markov Chain Monte Carlo (MCMC) was conducted using the best-fit nucleotide model and consisted of four independent runs of 50,000,000 generations with a tree sampled every 1000 generations. The first 25% trees were discarded as burn-in and the remaining were used to generate 50% Majority-rule consensus Bayesian tree. Average standard deviation of split frequencies at the end of run was below 0.01. In Tracer 1.7.1 [34], the effective sample size (ESS) presented values well above 200 for all statistics, which ensured that the selected settings were sufficient for the sampling. Estimation of Average pairwise genetic distance within morphotypes and among morphotypes based on each gene was done using MEGA X [27].

3. Results

3.1. Morphological Analyses

All the morphotypes maintained in the Botanical Garden exhibited differences in their morphology (Table 2). The inflorescence was a unilateral cyme in the case of *B. cristata* (Nandi Hills), whereas the rest of the morphotypes had cymes combining dichasial and monochasial elements. Bracts shapes ranged from linear to linear-lanceolate. The most conspicuous differences were observed in the features of the calyx and corolla (Figure 2). The shape of the inner calyx lobes was linear in all the morphotypes. The outer calyx lobes were elliptic in *B. cristata* (Nandi Hills), whereas the rest of the morphotypes had lanceovate outer lobes. The number of teeth on the margin of the outer lobes was maximum in *B. cristata* (Nandi Hills) (7–22). The corolla colour and lobe (shape and apex) varied in all the morphotypes (Table 2 and Figure 2). In *B. cristata* (cultivated form 1) and *B. cristata* (Nandi Hills), the apex of the lower lobe was retuse, while in others it was either rounded or acute.

Table 2. Comparative morphology of *Barleria cristata* morphotypes. Variable characters indicated in bold.

Attributes	*Barleria cristata* (Cultivated form 1)	*Barleria cristata* (Cultivated form 2)	*Barleria cristata* (Cultivated form 3)	*Barleria cristata* (Nandi Hills)	*Barleria cristata* (Uttarakhand)
Leaves	Elliptic, 4.5–13.7 × 1.4–4.9 cm; venation eucamptodromous, one main vein from base, 5–8 pairs of principal laterals	Elliptic, 4–14.3 × 2–5.7 cm; venation eucamptodromous, one main vein from base, 5–8 pairs of principal laterals	Elliptic, 7.4–20.3 × 2.3–7.2 cm; venation eucamptodromous, one main vein from base, 5–7 pairs of principal laterals	Elliptic-lanceolate, 3.6–12.2 × 1.1–5.2 cm; venation eucamptodromous, one main vein from **base, 6–10 pairs of principal laterals**	Elliptic, 2–8.1 × 1–2.9 cm; venation eucamptodromous, one main vein from base, 4–5 pairs of principal laterals
Inflorescence	Cymes combining dichasial and monochasial elements with 1–6 flowers maturing sequentially	Cymes combining dichasial and monochasial elements with 1–7 flowers maturing sequentially	Cymes combining dichasial and monochasial elements with 1–8 flowers maturing sequentially	**Unilateral cyme with 1–2 flowers maturing sequentially**	Cymes combining dichasial and monochasial elements with 1–5 flowers maturing sequentially
Bracts	**Linear**, 0.8–1 × 0.1–0.15 cm, surface ciliate throughout, **eglandular**	Linear-lanceolate, 1.4–1.8 × 0.2–0.3 cm, surface ciliate throughout with stalked cupular glands near margins	**Linear**, 0.4–0.7 × 0.05–0.1 cm, surface with very few scattered stalked glandular trichomes	Linear-lanceolate, 1.4–1.8 × 0.2–0.3 cm, surface ciliate throughout with stalked cupular glandular trichomes near margins	**Linear**, 0.4–0.7 × 0.05–0.1 cm, surface ciliate throughout with very few scattered stalked glandular trichomes
Calyx	Outer lobes lance-ovate, 1.7–2.2 × 0.7–0.8 cm; apex narrowly acute, spinose; margins dentate; teeth 0.1–0.2 cm long, 7–12	Outer lobes lance-ovate, 1.3–2.4 × 0.5–0.9 cm; apex narrowly acute, spinose; margins dentate; teeth 0.1–0.3 cm long, 8–15	Outer lobes lance-ovate, 1.6–2.4 × 0.6–0.8 cm; apex narrowly acute, spinose; margins dentate; teeth 0.1–0.3 cm long, 8–15	**Outer lobes elliptic**, 1.8–2.4 × 0.8–0.9 cm; apex acute, spinose; margins dentate; teeth 0.1–0.2 cm long, **16–22**	Outer lobes lance-ovate, 1.1–2.1 × 0.4–0.6 cm, apex narrowly acute, spinose; margins dentate; teeth 0.05–0.1 cm long, 9–11
	Inner lobes linear, 0.6–0.8 × 0.15–0.4 cm	Inner lobes linear, 0.6–0.8 × 0.15–0.2 cm	Inner lobes linear, 0.7–0.8 × 0.15–0.3 cm	Inner lobes linear, 0.6–0.9 × 0.1–0.2 cm	Inner lobes linear, 0.7–0.8 × 0.1 cm
Corolla	**Deep Rose Pink**; upper lobes elliptic to broadly elliptic, apex acute or rounded; lower lobes broadly elliptic or suborbicular, apex retuse	**White**; upper lobes elliptic to broadly elliptic, apex acute or rounded; lower lobes broadly elliptic or suborbicular, apex rounded or acute	**Lavender-violet**; upper lobes lobes elliptic to broadly elliptic, apex acute or rounded; lower lobes very broadly elliptic or suborbicular, apex rounded or acute	**Rose Pink; upper lobes obovate or oblanceovate**, apex acute; **lower lobes suborbicular or widely obovate**, apex retuse	**Chinese violet; upper lobes oblanceovate or elliptic**, apex rounded or slightly retuse; **lower lobes broadly elliptic or suborbicular, apex rounded**
Stamens	**3–3.2 cm long; rose pink**	1.4–2.8 cm long; anthers lavender grey	2.1–2.5 cm long; anthers lavender grey	1.6–2.0 cm long; **anthers wood browny**	1.5–2.6 cm long; anthers lavender grey
Gynoecium	Style 3.9–4.2 cm long, **glabrous except near the base (few eglandular hairs)**	Style 3.4–4.7 cm long, glabrous throughout	Style 3.2–4.6 cm long, glabrous throughout	Style 3.4–4.7 cm long, **hirsute at base**	Style 2.7–3.8 cm long, glabrous throughout

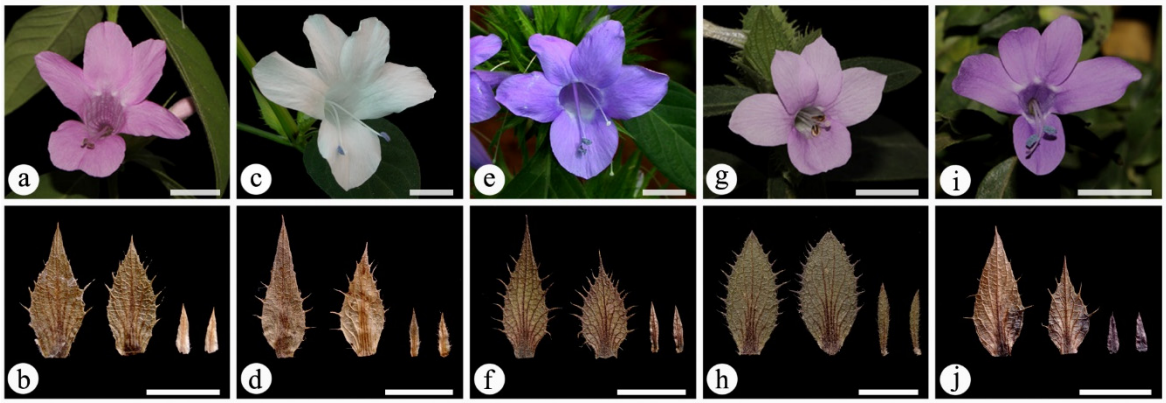

Figure 2. *Barleria cristata* morphotypes; (**a**,**c**,**e**,**g**,**i**) corolla lobes, front view; (**b**,**d**,**f**,**h**,**j**) two larger outer calyx lobes (adaxial and abaxial), two smaller inner calyx lobes. (**a**,**b**) *B. cristata* (cultivated form 1); (**c**,**d**) *B. cristata* (cultivated form 2); (**e**,**f**) *B. cristata* (cultivated form 3); (**g**,**h**) *B. cristata* (Nandi Hills); (**i**,**j**) *B. cristata* (Uttarakhand). Scale bars = 1 cm.

3.2. Palynology

Pollen grains were oblate spheroidal, isopolar and radially symmetrical. They were tri-brevicolporate and honey-combed. Colpi had tapering ends. Columellae number varied in each lumen. Morus was sharply winding and simplicolumellate. Nexine formed a layer with small and large granules (Figures 3 and 4). The maximum values for polar (P) (100.73 ± 4.38 µm) and equatorial (E) (104.13 ± 4.25 µm) lengths were recorded for the pollen grains of *B. cristata* (cultivated form 3). Colpus width (16.50 ± 1.78 µm) and pore diameter (11.93 ± 1.63 µm) were maximum in the case of *B. cristata* (Nandi Hills). The other quantitative features are listed in Table 3. Analysis of variance showed a statistically significant difference ($p < 0.05$) between *B. cristata* morphotypes from Nandi Hills and Uttarakhand.

Table 3. Pollen grain features of *Barleria cristata* morphotypes. E = Equatorial length, P = Polar length.

Morphotypes	P (µm) ± SD	E (µm) ± SD	P/E	Lumina Diameter (µm) ± SD	Colpus Length (µm) ± SD	Colpus Width (µm) ± SD	Pore Diameter (µm) ± SD
B. cristata (Cultivated form 1)	79.79 ± 2.80 ^{d}	83.51 ± 3.91 ^{d}	0.96 ^{a}	11.91 ± 2.32 ^{b}	40.92 ± 3.46 ^{b}	9.86 ± 1.24 ^{d}	9.78 ± 1.13 ^{c}
B. cristata (Cultivated form 2)	99.98 ± 4.16 ^{a}	104.13 ± 4.02 ^{a}	0.96 ^{a}	17.39 ± 3.57 ^{a}	38.23 ± 4.90 ^{c}	11.50 ± 1.69 ^{c}	8.51 ± 1.40 ^{d}
B. cristata (Cultivated form 3)	100.73 ± 4.38 ^{a}	104.13 ± 4.25 ^{a}	0.97 ^{a}	16.32 ± 4.25 ^{a}	35.28 ± 4.61 ^{d}	9.34 ± 1.60 ^{d}	9.12 ± 1.14 ^{bc}
B. cristata (Nandi Hills)	87.98 ± 4.49 ^{c}	91.06 ± 4.69 ^{c}	0.97 ^{a}	13.15 ± 2.95 ^{b}	47.28 ± 3.11 ^{a}	16.50 ± 1.78 ^{a}	11.93 ± 1.63 ^{a}
B. cristata (Uttarakhand)	93.39 ± 4.16 ^{b}	97.75 ± 4.19 ^{b}	0.96 ^{a}	15.42 ± 4.31 ^{a}	39.63 ± 3.69 ^{bc}	12.47 ± 1.29 ^{b}	9.47 ± 1.21 ^{b}

Mean values with different alphabets in column showed statistically significant differences ($p < 0.05$) according to Duncan's multiple range test.

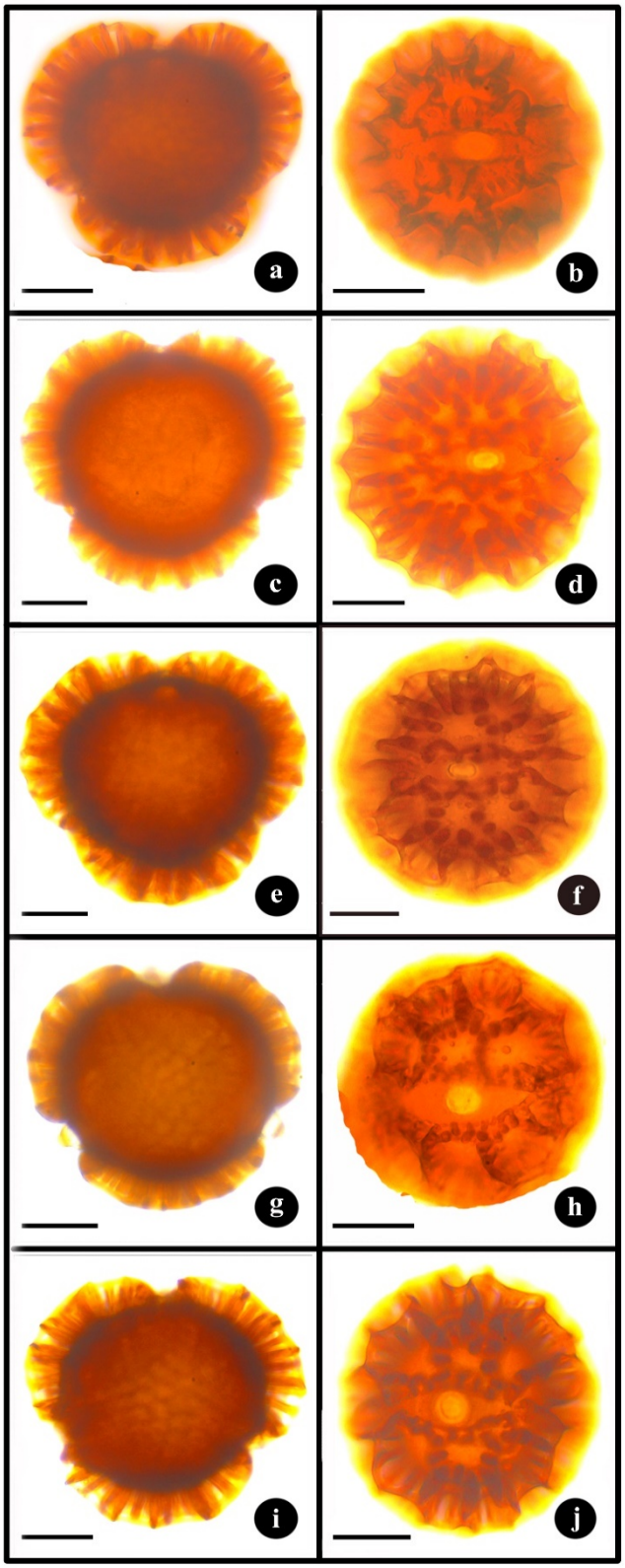

Figure 3. Light photomicrographs of *Barleria cristata* morphotypes (**a**–**j**). polar view (**a**,**c**,**e**,**g**,**i**), aperture view (**b**,**d**,**f**,**h**,**j**). (**a**,**b**) *B. cristata* (cultivated form 1); (**c**,**d**) *B. cristata* (cultivated form 2); (**e**,**f**) *B. cristata* (cultivated form 3); (**g**,**h**) *B. cristata* (Nandi Hills); (**i**,**j**) *B. cristata* (Uttarakhand). Scale bars (**a**–**j**) = 50 μm.

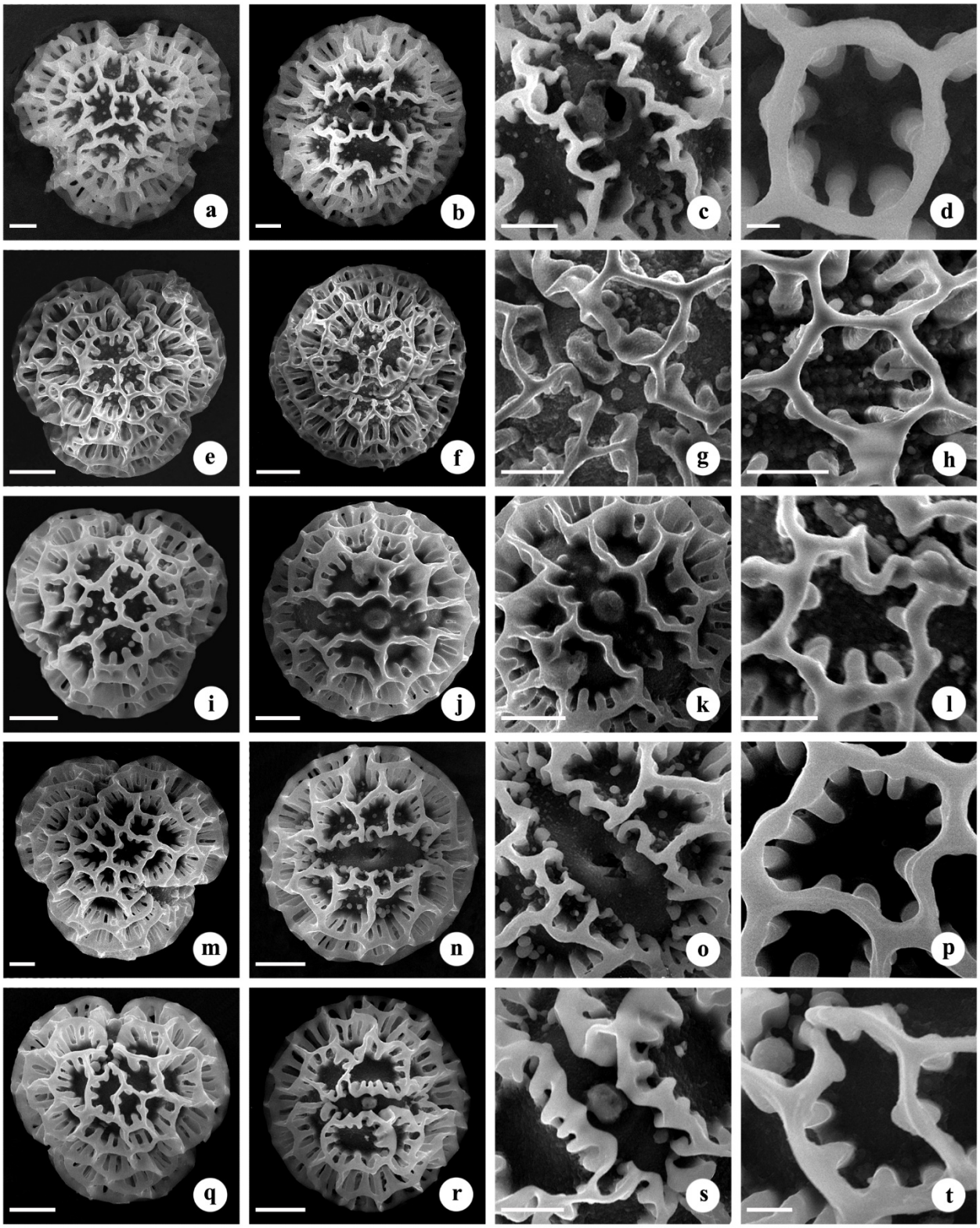

Figure 4. Scanning electron photomicrographs (polar view, equatorial view, enlarged view of aperture, enlarged view of tectum, respectively) of *Barleria cristata* morphotypes. (**a**–**d**) *B. cristata* cultivated form 1); (**e**–**h**) *B. cristata* (cultivated form 2); (**i**–**l**) *B. cristata* (cultivated form 3); (**m**–**p**) *B. cristata* (Nandi Hills); (**q**–**t**) *B. cristata* (Uttarakhand). Scale bars (**e**,**f**,**i**,**j**,**n**,**q**,**r**) = 20 µm; (**a**–**c**,**g**,**h**,**k**–**m**,**o**,**s**) = 10 µm; (**p**,**t**) = 5 µm; (**d**) = 2 µm.

3.3. Phylogenetic Analyses

The cpDNA (*ndh*F-*trn*L$_{(UAG)}$+*trn*S-G) dataset included 85 accessions (53 *Barleria* species and 9 outgroups) and contained 1930 characters. The ITS dataset contained 501 characters and included 73 accessions (48 *Barleria* species and 7 outgroups). The phylogenetic analyses based on cpDNA (Figure 5) data and ITS data (Figure S1) showed conflict in the relationship. A previous study by Darbyshire et al. [1] also reported the conflict between cpDNA and ITS data, but the conflict was resolved by placing missing ITS sequences for two taxa, namely, *B. aristata* I.Darbysh. and *B. limnogeton* S.Moore, in the ITS dataset used in that study. In the present study, the conflict between cpDNA and ITS data was greater due to the addition of ITS sequences from morphotypes of *B. cristata*. The results of the cpDNA data were congruent with the phylogenetic results of Darbyshire et al. [1], but the ITS results were anomalous. The ITS region has been challenging to sequence following the Sanger method for Barleriae (L.A. McDade and al. unpub.). Hence, we focused on the phylogenetic results obtained from the cpDNA dataset. The cpDNA phylogeny resolved the relationship within subg. *Prionitis* and subg. *Barleria* with a strong Bayesian posterior probability value (BI PP) and maximum likelihood bootstrap support (ML BS). All the studied accessions of five different *B. cristata* morphotypes and the GenBank retrieved accessions of *B. cristata* (Hu, S.Y. and But, P. 23180), which were sampled from China in the previous study [1], were placed in subg. *Barleria* under the clade *Cristata* complex (Figure 5). *B. cristata* (Hu, S.Y. and But, P. 23180) showed sister relationships with five different *B. cristata* morphotypes with BI = 1 and ML BS = 100 (Figure 5). The accessions of cultivated form 1, Nandi Hills and Uttarakhand each formed a single group with strong BI PP and ML BS support. The cpDNA phylogeny was unable to resolve the relationship among accessions of cultivated form 2 and cultivated form 3, as all the accessions of these two morphotypes formed a single group with BI = 0.96 and ML BS = 62 (Figure 5). The average genetic distance within the sequences of five morphotypes was not significant (Table S2). The average genetic distance among the sequences of morphotypes for the cpDNA region was also not significant, but for the ITS region it ranged from 0.00805 to 0.19023 (Table S2).

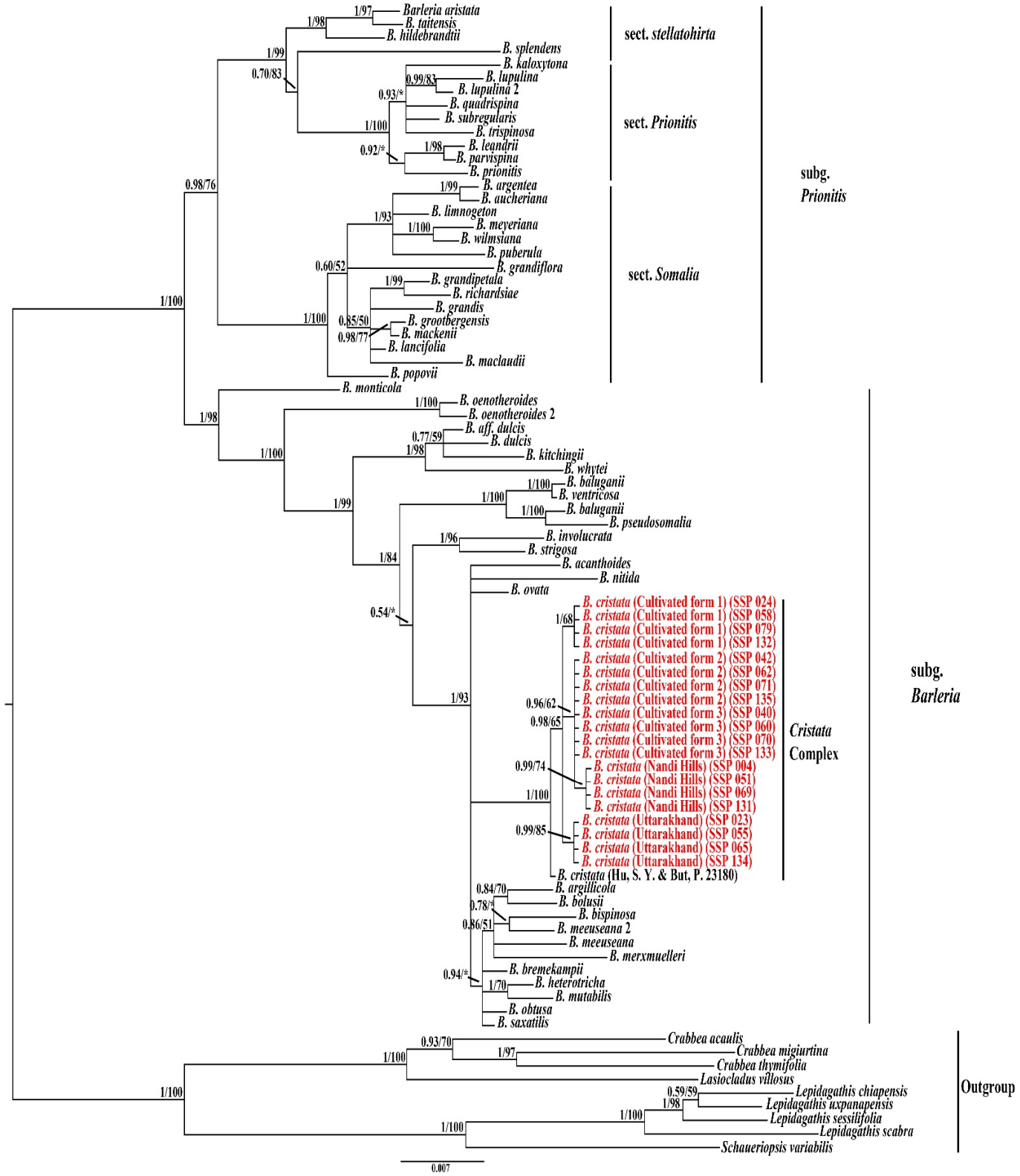

Figure 5. 50% Majority-rule consensus Bayesian phylogenetic tree based on cpDNA data (*ndh*F-*trn*L$_{(UAG)}$+*trn*S-G). Bayesian posterior probability values and maximum likelihood bootstrap values (BPP/ML BS) are provided above branches. Asterisk (*) indicates ML BS values < 50%. The taxa sampled in this study are in red color and their voucher details in parentheses.

4. Discussion

Barleria cristata is a widespread species in India that is cultivated as an ornamental but also grows naturally. Horticultural interest in the species has led to the cytogenetical characterization of some of its morphotypes. The chromosome number in *Barleria* species is usually based on $x = 10$ [35]. The different horticultural varieties show the presence of $2n = 32, 34, 36, 38, 40$ and 42 chromosomes that have been attributed to aneuploid alterations and hybridization resulting in a complex [36]. Nevertheless, there are no corresponding meiotic or pollen viability studies for these varieties that can further ascertain the origin of these unusual chromosome numbers. In the present investigation, the studied morphotypes (populations) came from three phytogeographical regions, namely, the Western Ghats (all the cultivated forms), the Deccan Peninsula (forms from Nandi Hills and Tirupati) and the Western Himalayas (Uttarakhand form). The naturally occurring morphotypes from the Deccan Peninsula and Western Himalaya show conspicuous differences in their morphology. These morphotypes are geographically isolated, and therefore, the variation arising on account of admixture is not possible. The variation in these morphotypes is the outcome of evolution under unique edaphoclimatic regimes or their independent origins. In the case of *Flemingia* subg. *Rhynchosioides* Baker, it has been observed by Gavade et al. [37] that the differences in the morphology of *F. tuberosa* Dalzell and other species of the subg. *Rhychosioides* could be because of their different niches or independent origin. On the other hand, the morphotypes from the Western Ghats all exploit similar niches. They have escaped in the nature, are mainly cultivated as ornamentals, and hence show very similar morphology.

All the morphotypes shared common features of pollen grains and differed only in their quantitative parameters. The distinct-looking morphotypes, namely, Nandi Hills and Uttarakhand and cultivated morphotypes, cannot be separated based on pollen characteristics. Although Al-Hakimi et al. [38], based on their studies on pollen features of nine *Barleria* species from Yemen, concluded that the species within the sections could be differentiated by some characters. They observed that within section *Barleria, B. acanthoides* Vahl and *B. aculeata* Balf.f. can be differentiated by the dimensions of their lumina, columellae, exine thickness and ora. Similarly, *B. parviflora* R.Br. and T.Anderson and *B. orbicularis* Hochst. Ex T.Anderson (section *Somalia*) may be separated utilising equatorial and polar dimensions, columellae, ora and apocolpium sizes. However, Al-Hakimi et al. [38] could not differentiate the pollen features of *B. ventricosa* Hochst. Ex Nees (section *Fissimura*) from species of other sections. Therefore, it is clear that quantitative pollen features are not taxonomically important. In our studies, the differences in qualitative pollen features were not conspicuous. This could be because of their genetic homogeneity, meaning that all the morphotypes constitute a single species. Therefore, pollen variation at the intraspecific level is not significant. The variation in quantitative parameters of pollen grains could have been governed by the edaphoclimatic regimes in which these morphotypes exist. The assessment of infraspecific variations and their subsequent delimitation has not been attempted in any of the Indian species of *Barleria* so far. Although such studies are available for African *Barleria* species. Nyirenda and Balkwill [39] carried out phenetic studies on three morphologically similar species of *Barleria*, namely, *B. bechuanensis* C.B.Clarke, *B. irritans* Nees and *B. jubata* S.Moore. They recorded the morphological characters in the form of matrices and performed Cluster analysis of qualitative and quantitative characters to study the variation within the three species. They observed that *B. bechuanensis* and *B. irritans* are species complexes with two species each, and the variation within these species can be recognised at the species level. Combinations of numerical analysis of the morphological data and phylogenetic analysis based on morphological and molecular data have been used previously to study plant species complexes [40]. In the present case, morphological variation within the morphotypes was not significant enough to perform numerical analysis. Accordingly, we only discussed morphological variation present among the morphotypes. Nevertheless, we carried out a phylogenetic analysis of all the sampled accessions of five morphotypes to study molecular variation present among and within morphotypes. The

placing of all *B. cristata* accessions into the previous comprehensive phylogeny of Darbyshire et al. [1] revealed that *B. cristata* shows considerable variation. Our samples were recovered as a clade sister to the samples (Hu, S.Y. and But, P. 23180) used by Darbyshire et al. [1]. The genetic variation within morphotypes was not enough; however, it revealed among morphotype genealogies in detail. It is clear from the analyses based on cpDNA from mostly cultivated samples that there are three monophyletic groups within the *B. cristata* complex, namely, group 1 comprising (specimens of cultivated form 1), group 2 (specimens of cultivated form 2, cultivated form 3 and Nandi Hills) and group 3 (specimens from Uttarakhand) (Figure 5). It was observed that the sample collected by Hu and But from China fell outside the three clades formed by our samples from India indicating the existence of tremendous variation in the *B. cristata* complex and calling for an integrated taxonomic approach to study and delimit this complex. It is very surprising that even after being a widespread species, limited DNA sequence data are available for this species. The phylogeny given by Darbyshire et al. [1] represents only one sample of it. Comito et al. [4] generated ddRAD sequencing data for multiple specimens of *B. cristata* collected from the USA (CSULB Greenhouse (LOB)), China (Gooligong Shan Biodiversity Survey 15,945 (CAS)) and Nepal (Grey-Wilson, C. Phillips 865B (K)). But in order to compare those specimens with Indian specimens, there is a need to generate ddRAD sequencing data from Indian *B. cristata* morphotypes. It is hoped that the present work will stimulate taxonomic work on widespread polymorphic species in general and on *B. cristata* in particular. The results based on cpDNA from mostly cultivated samples are, in our opinion, conclusive and can serve as a hypothesis of the phylogenetic relationship in the *B. cristata* complex.

5. Conclusions

This study has contributed important details to our understanding of the morphological and phylogenetic relationships within the morphotypes of the *B. cristata* complex. The three monophyletic groups exhibit some morphological differences among them. Nevertheless, it is not possible to delimit these morphotypes at species or variety level as the sampling represents only three geographical regions of this widely distributed species. In fact, we could not ascertain whether these variations were discrete or continuous. It would be worthwhile to study this species across its distribution range in India and assess both inter and intraspecific relationships. Furthermore, cytogenetical characterization can throw some light on the evolution of these morphotypes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14080677/s1, Table S1: Details of species included in phylogenetic analysis; Table S2: Estimate of average pairwise genetic distance among and within five morphotypes of *B. cristata* for *trn*S-G, *ndh*F-*trn*$L_{(UAG)}$ and ITS region. Figure S1: 50% Majority-rule consensus Bayesian phylogenetic tree based on ITS region. Supplementary Material S1: Aligned combined sequence data (*ndh*F-*trn*$L_{(UAG)}$+*trn*S-G) matrix. Supplementary Material S2: Aligned ITS sequence data matrix.

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