

An overview of floral and vegetative evolution in the Asian clade of *Bulbophyllum* (Orchidaceae)

Nicha Thawara^{1,2}, Panida Kongsawadworakul¹, Piyakaset Suksathan³, Santi Watthana⁴,
Thitiporn Pingyot³, Vincent S.F.T. Merckx^{5,6}, Saroj Ruchisansakun¹

- 1 Department of Plant Science, Faculty of Science, Mahidol University, Bangkok, Thailand
- 2 Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand
- 3 Queen Sirikit Botanic Garden, The Botanical Garden Organization, Chiang Mai, Thailand
- 4 School of Biology, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand
- 5 Understanding Evolution Group, Naturalis Biodiversity Center, Leiden, the Netherlands
- 6 Institute for Biodiversity and Ecosystem Dynamics (IBED), University of Amsterdam, Amsterdam, the Netherlands

Corresponding author: Saroj Ruchisansakun (s.ruchisansakun@gmail.com)

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Abstract

Background and aims – *Bulbophyllum*, the largest genus in Orchidaceae, exhibits a diverse morphology in both reproductive and vegetative characters. While trait diversity and evolution has been extensively studied in Malagasy species and within the *Cirrhopetalum* alliance clade, the evolution of reproductive and vegetative characters at the whole level of the Asian clade remains largely unexplored.

Material and methods – We reconstructed the phylogeny of approximately 11% of all Asian *Bulbophyllum* species using Bayesian inference and maximum likelihood estimation based on nuclear (ITS) and chloroplast (*matK*, *psbA-trnH*) DNA sequence data. This phylogenetic framework allowed us to examine the evolution of two vegetative and four floral characters through ancestral state reconstruction.

Key results and conclusion – The ancestral character states of the Asian clade of *Bulbophyllum* include a single leaf, distinct pseudobulbs, multiple-flowered inflorescences, and lateral and dorsal sepals similar in length. One-leaved pseudobulbs evolved into two-leaved pseudobulbs multiple times. Distinct pseudobulbs gave rise to indistinct pseudobulbs twice. Multiple-flowered inflorescences shifted to solitary flowers and 2–3-flowered inflorescences multiple times, with some instances of evolutionary reversal. Lateral sepal elongation also presents a convergent evolutionary scenario.

Keywords

biodiversity, character evolution, Epidendroideae, epiphytic orchids, ITS, *matK*, phylogeny, *psbA-trnH*, Tropical Asia

INTRODUCTION

Bulbophyllum Thouars is a megadiverse and widely distributed orchid genus with approximately 2,200 accepted species (Gravendeel et al. 2014a; Ya et al. 2021; Nguyen et al. 2022; Moonlight et al. 2024; POWO 2024). Its classification has undergone substantial revisions based on morphological and molecular approaches (Gravendeel et al. 2014b; Hu et al. 2020), with more than 50 generic names being synonymised within *Bulbophyllum* (Gravendeel

et al. 2014a; Vermeulen et al. 2014; POWO 2024). *Bulbophyllum* is pantropical with four geographically-structured clades centred in Madagascar (Fischer et al. 2007), Africa (Vermeulen 1987), Tropical America (Smidt et al. 2011), and Asia/Australasia (Gravendeel et al. 2014a). The last clade comprehends two not fully resolved main subclades, one being predominantly Western Malesian and the other Eastern Malesian (Gravendeel et al. 2014a). The Asian-Pacific region was inferred as the ancestral area of *Bulbophyllum* (Gamish

Table 1. Summary of character evolution in *Bulbophyllum* according to previous studies.

Madagascar: Malagasy <i>Bulbophyllum</i> (Fischer et al. 2007)	
Character	Evolutionary trend
Number of leaves per pseudobulb	2-leaved to 1-leaved
Leaf emergence	Hysteranthous to synanthous
Setaceous peduncle	Absent to present
Number of flowers per inflorescence	Many-flowered to single-flowered
Length of pedicel	Moderate to long to very short
Lip mobility	Movable lip to non-movable lip (enclosed by lateral sepals)
Asia: <i>Cirrhopetalum</i> alliance clade (CAC) (Hu et al. 2020)	
Character	Evolutionary trend
Inflorescence type	Sub-umbellate to racemose
Number of flowers per inflorescence	Many-flowered to 1–3-flowered
Lateral sepal shape	Basally twisted to not basally twisted
Lateral sepal margin connation	Upper margins connate to free
Sepal and petal colour	From other colour to white or yellowish
Floral scent	Imperceptible or decaying organic matter to fruity
Dorsal sepal margin indument	Glabrous to hairy
Petal margin indument	Glabrous to hairy
Spots/markings on sepals and petals	Present to absent
Spots/markings on lip	Present to absent

and Comes 2019). Accordingly, the genus is especially diverse in Asia, where approximately 1,700 species have been recognised across 67 sections (Dressler 1993; Sieder et al. 2009; Vermeulen et al. 2014; Gamish and Comes 2019), occurring in various habitat types and ranging from sea level up to 3,550 m in elevation (Chayamarit et al. 2014; Gravendeel et al. 2014a). Recent analyses of 70 plastid coding regions and nuclear ribosomal DNA cistron data indicate that *Bulbophyllum* diverged into four distinct clades: *Bulbophyllum* sect. *Minutissima* s.s., *Bulbophyllum* sect. *Adelopetalum*, the Afro-Neotropical clade, and the Asian clade (Simpson et al. 2024). Although nuclear ribosomal DNA cistron data reveal a discrepancy regarding the placement of the *Minutissima* s.s. clade and some subclades within the *Adelopetalum* clade, both datasets confirm the recognition of an Asian clade (Simpson et al. 2024). To date, phylogenetic studies of Asian *Bulbophyllum* have been focused on Peninsular Malaysia (Hosseini et al. 2012, 2016; Wonnapijit and Sriboonlert 2015) and the *Cirrhopetalum* alliance (Hu et al. 2020), thus being limited by significant geographic and taxonomic sampling gaps.

The floral morphology of *Bulbophyllum* exhibits remarkable diversity in terms of shape, size, and colour, reflecting adaptations linked to cross-pollination mediated by flies and occasionally auto-pollination (Dressler 1993; Gamisch et al. 2014). Previous research examining trait evolution within *Bulbophyllum* is summarised in Table 1. Synapomorphic combinations of diagnostic characters have been employed to support the Malagasy *Bulbophyllum* clade (Fischer et al. 2007) and the *Cirrhopetalum* alliance clade (Hu et al. 2020). These

character-based analyses have refined the classification of ambiguous groups and enhanced our understanding of clade-specific evolutionary transitions (Hu et al. 2020). While most studies have focused on floral characters, vegetative traits can also provide valuable information for inferring phylogenetic relationships among species (Smidt et al. 2011). Despite the considerable interest in taxonomically complex *Bulbophyllum*, there is currently limited knowledge regarding the evolutionary history of key characters within the Asian clade of *Bulbophyllum*.

This study focuses on the evolution of a selection of morphological characters (Table 2), in the Asian clade of *Bulbophyllum*, which were identified to have played an important role in the evolution of *Bulbophyllum* in general (e.g. Fischer et al. 2007; Hu et al. 2020).

MATERIAL AND METHODS

Taxon sampling

A total of 196 species of the Asian clade of *Bulbophyllum* (covering approximately 11% of the species) were included in phylogenetic analysis. The outgroup consists of nine species, namely three *Bulbophyllum* of the Afro-Neotropical clade, three *Bulbophyllum* sect. *Adelopetalum*, and three *Dendrobium* Sw. species. Forty-three plants from 41 species were sampled from the living collections at Queen Sirikit Botanic Garden (QSBG) in Chiang Mai, Thailand, from which 125 new DNA accessions were obtained (see Suppl. material 1). The voucher specimens were deposited at Queen Sirikit Botanic Garden

Table 2. The six morphological characters used in this study to examine character evolution in the Asian clade of *Bulbophyllum*.

Morphological character	States
Number of leaves per pseudobulb	(0) = 1-leaved pseudobulb (1) = 2-leaved pseudobulb
Pseudobulb size	(0) = distinct pseudobulb: diameter of pseudobulb more than 1.5 times the diameter of petiole or leaf base (1) = indistinct pseudobulb: diameter of pseudobulb equal or less than 1.5 times the diameter of petiole or leaf base
Swollen apical sterile flower	(0) = absent (1) = present
Number of flowers per inflorescence	(0) = 1-flowered (1) = 2–3-flowered (2) = multiple-flowered (> 3-flowered)
Connation of lower margin of lateral sepals	(0) = free (1) = connate
Ratio of sepal lengths (lateral to dorsal)	(0) = similar: lateral sepals 1.0–1.5 times as long as the dorsal sepal (1) = different: lateral sepals > 1.5 times as long as the dorsal sepal

Herbarium (QBG). In addition, DNA sequence data for 161 additional *Bulbophyllum* species were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>), comprising 144, 131, and 88 accessions for ITS, *matK*, and *psbA-trnH* markers, respectively (see Suppl. material 2).

The 202 species of *Bulbophyllum* included in this study represent the following sections or alliances: *Adelopetalum* (Fitzg.) J.J.Verm. (3 spp.), *Acrochaene* (Lindl.) J.J.Verm. et al. (1 sp.), *Altisceptrum* J.J.Sm. (1 sp.), *Beccariana* Pfitz. (6 spp.), *Biflorae* Garay et al. (2 spp.), *Biseta* J.J.Verm. ex N.Pearce et al. (1 sp.), *Blepharistes* J.J.Verm. et al. (1 sp.), *Brachyantha* Rchb.f. (10 spp.), *Brachystachyae* Benth. & Hook.f. (5 spp.), *Cirrhopetaloides* Garay et al. (12 spp.), *Cirrhopetalum* Rchb.f. (22 spp.), *Codonosiphon* Schltr. (1 sp.), *Desmosanthes* (Blume) J.J.Sm. (19 spp.), *Drymoda* (Lindl.) J.J.Verm. et al. (1 sp.), *Emarginatae* Garay et al. (4 spp.), *Ephippium* Schltr. (15 spp.), *Epicrianthes* (Blume) Hook.f. (3 spp.), *Eublepharon* J.J.Verm. et al. (3 spp.), *Hirtula* Ridl. (6 spp.), *Hyalosema* Schltr. (1 sp.), *Ione* (Lindl.) J.J.Verm. et al. (3 spp.), *Lemniscata* Pfitz. (10 spp.), *Leopardinae* Benth. & Hook.f. (5 spp.), *Lepidorhiza* Schltr. (3 spp.), *Lupulina* G.A.Fischer (1 sp. from Madagascar), *Macrocaulia* (Blume) Aver. (3 spp.), *Macrosylida* Garay et al. (2 spp.), *Micranthae* Barb. Rodr. (1 sp. from the Neotropics), *Monanthaparva* Ridl. (2 spp.), *Monanthes* (Blume) Aver. (1 sp.), *Monomeria* (Lindl.) J.J.Verm. et al. (1 sp.), *Oxysepala* (Wight) Benth. & Hook.f. (2 spp.), *Physometra* J.J.Verm. et al. (1 sp.), *Ploiarium* Schltr. (1 sp. from Africa), *Plumata* J.J.Verm. et al. (2 spp.), *Polymeres* J.J.Verm. & P.O'Byrne (2 spp.), *Racemosae* Benth. & Hook.f. (6 spp.), *Repantia* J.J.Verm. (1 sp.), *Rhytionanthos* (Garay et al.) J.J.Verm. et al. (3 spp.), *Sestochilus* (Breda) Benth. & Hook.f. (18 spp.), *Stachysanthes* (Blume) Aver. (7 spp.), *Saurocephalum* J.J.Verm. (1 sp.), and *Trias* (Lindl.) J.J.Verm. et al. (9 spp.). Thus, a total of 39 out of 67

sections (Vermeulen 2014) found in Asia and Australasia (58% coverage) were examined.

DNA extraction, PCR amplification, and sequencing

Aligned with the DNA sampling protocol of previous studies (Hosseini et al. 2012, Hosseini and Dadkhah 2015; Wonnapijit and Sriboonlert 2015; Hu et al. 2020), we used three genetic markers, the nuclear ribosomal internal transcribed spacer region (nrITS), and two plastid regions (*matK* and *psbA-trnH*), to produce a phylogenetic tree onto which we mapped these selected traits.

Genomic DNA was extracted from frozen fresh leaves using a modified CTAB method (Doyle and Doyle 1987). Three DNA regions were selected for sequencing: ITS, *matK*, and *psbA-trnH*, based on previous phylogenetic studies (Hu et al. 2020). Information on the primers and PCR conditions is provided in Suppl. material 3. PCR amplification of all fragments was performed in 50- μ l reactions containing 10X Standard Taq Reaction Buffer, 8 μ mol dNTP, 20 μ M of each primer, and Taq DNA Polymerase (New England BioLabs). For ITS mixtures, DMSO was added. PCR products or gel slices were purified using a GEL/PCR Purification Mini Kit (Favogen) following the manufacturer's protocol and were sequenced using the Sanger method by U2Bio (Thailand) Co., Ltd., Bangkok, Thailand.

Phylogenetic analyses

DNA sequences were aligned using the online portal CIPRES Science Gateway (Miller et al. 2010) with MAFFT v.7.471 (Katoh and Toh 2010), and manually adjusted in BioEdit v.7.2.5 (Hall 1999). The MAFFT alignment was performed using the following parameters: automatic selection of an appropriate strategy, a PartTree algorithm for tree building of 6-mer, a 200PAM/kappa of

2, a gap open penalty of 1.53 and an offset value of 0.123. Five datasets (ITS only, *matK* only, *psbA-trnH* only, the combined dataset using all existing taxa from GenBank and our samples, and the pruned dataset using only taxa for which we had data from all three DNA regions) were used for phylogenetic reconstruction under both Bayesian inference (BI) and maximum likelihood (ML).

The model-fit of nucleotide substitution models for each single-marker dataset was assessed using IQ-tree's ModelFinder (Kalyanamorthy et al. 2017), with model selection based on Akaike Information Criterion (AIC). The BI analysis was conducted using MrBayes on XSEDE v.3.2.7a (Ronquist et al. 2012) in the CIPRES portal. In each analysis, four simultaneous Markov Chain Monte Carlo (MCMC) algorithms were run for 10 million generations, with sampling every 1000 generations. The temperature value of the MCMC heated chain was set to 0.2. The initial 25% of generations of the sampled trees were discarded as burn-in. The 50% majority rule consensus tree was used to calculate posterior probabilities (PP). Convergence was assessed by checking that the average standard deviations of split frequency values were < 0.01, estimated sample size (ESS) values were > 200 and potential scale reduction factor (PSRF) values were approaching 1.0.

The ML analyses were conducted in IQ-TREE v.1.6.12 (Nguyen et al. 2015; Chernomor et al. 2016) with 1,000 replicates of ultrafast bootstraps to obtain nodal support (ultrafast bootstrap support, UFBS) (Hoang et al. 2018). The following settings were used: the “-spp” parameter to specify partitions (each partition has a separate evolution rate), and the “-bb” parameter to define the number of bootstrap replicates. Clades with UFBS > 95% and PP > 0.95 were considered to receive strong support (Alfaro and Holder 2006; Minh et al. 2013). Phylogenetic trees were visualised using Figtree v.1.4 (Rambaut et al. 2021).

Ancestral character state reconstruction

Six morphological characters were studied (Table 2). The characters were coded based on living specimens, photographs, virtual herbarium specimens, and published literature (Seidenfaden and Wood 1992; Seidenfaden 1979; Chen and Vermeulen 2009; Hu et al. 2020). Ancestral state reconstruction was performed using Mesquite v.3.70 (Maddison and Maddison 2021). The reconstruction was plotted on the ML tree derived from the combined dataset. We used Fitch parsimony (Fitch 1971) as the criterion for character optimisation. To account for phylogenetic uncertainty, we traced character histories on 7,500 post burn-in trees from the Bayesian analysis using the ‘Trace Character Over Trees’ command.

Use of AI

The authors utilised ChatGPT-3.5 (Open AI 2024) for grammar checking before submitting the text to a language editing service.

RESULTS

Phylogenetic analyses

The tree topologies obtained from Bayesian inference (BI) and maximum likelihood (ML) analyses from all datasets are consistent. Table 3 summarises the DNA dataset's features, including each individual marker and the combined three-marker dataset, along with the best-fit models for each.

Our results indicate that there is no strong evidence of incongruence among individual gene trees (see Suppl. material 6), although, the tree based on *psbA-trnH* (Suppl. material 6.3) is poorly resolved. The analysis of the combined dataset demonstrated a significant improvement in tree resolution compared to the individual datasets. As a result, we concatenated all markers into a single dataset.

The phylogenetic tree based on the combined three-marker dataset provides support for the monophyly of the Asian clade sensu Simpson et al. (2024) (PP = 1.00, UFBS = 98), within which 37 clades match the sectional classification by Vermeulen (2014) and Hu et al. (2020) (Fig. 1 and Suppl. material 4). Most sections for which we included more than one species are monophyletic with robust support (PP = 1.00, UFBS > 95). However, some sections in the *Cirrhopetalum* alliance clade (CAC) are non-monophyletic because of a small number of outlier species, as well as *B.* sect. *Monanthaparva*, *B.* sect. *Monanthes*, and *B.* sect. *Oxysepala*.

Overall, the results obtained with the pruned dataset (Suppl. material 5) align with those of the combined dataset. Notable differences include: (1) *Bulbophyllum* being separated into 26 lineages due to the absence of representatives from *B.* sect. *Drymoda*, *B.* sect. *Lepidorhiza*, *B.* sect. *Altisceptrum*, *B.* sect. *Hyalosema*, *B.* sect. *Saurocephalum*, *B.* sect. *Codonosiphon*, *B.* sect. *Monanthes*, *B.* sect. *Macrocaulia*, *B.* sect. *Monomeria*, *B.* sect. *Trias*, and *B.* sect. *Polymeres*; (2) the *Biflorae* clade being sister to the *Oxysepala*, *Monanthaparva*, and *Epicrianthes* clades with low support values (PP = 0.56, UFBS = 61). However, this pruned dataset does not falsify the overall phylogenetic relationships.

Character evolution within the Asian clade of *Bulbophyllum*

All morphological characters in *Bulbophyllum* in our study exhibit homoplasy, except for the presence of a swollen apical sterile flower (Fig. 2). Supplementary material 8 provides a summary of the number of state changes. We determined that the ancestral states of the Asian clade of *Bulbophyllum* include having 1-leaved pseudobulbs, distinct pseudobulbs, absence of a swollen apical sterile flower, many-flowered inflorescences, and lateral and dorsal sepals of similar size.

Regarding the number of leaves per pseudobulb, one-leaved pseudobulbs independently shifted into

Table 3. Features of the five datasets used in molecular phylogenetic analysis. The “combined” dataset used all available taxa and the “pruned” dataset used only taxa with data from all three DNA regions (ITS, *matK*, and *psbA-trnH*).

	ITS	<i>matK</i>	<i>psbA-trnH</i>	Combined	Pruned
Number of taxa	190	175	132	207	121
Number of characters (base pairs)	813	1174	912	2899	2899
Number of constant characters	317	808	726	1851	1997
Number of parsimony-uninformative characters	105	139	92	336	301
Number of parsimony-informative characters	391	227	94	712	601
Evolutionary model	GTR+F+I+G4	GTR+F+I+G4	GTR+F+I+G4	-	-

two-leaved pseudobulbs at least four times in *B. sect. Blepharistes*, *B. sect. Drymoda*, *B. sect. Lemniscata*, and *B. sect. Physometra*. Most two-leaved species belong to *B. sect. Lemniscata* (Fig. 2 and Suppl. material 7.1).

Concerning pseudobulb size, indistinct pseudobulbs evolved twice: once in *B. sect. Brachystachyae* and once in *B. sect. Stachysanthes* (Fig. 2 and Suppl. material 7.2).

Floral dimorphism with a swollen apical sterile flower was found only in *B. physometrum*, the only species of *B. sect. Physometra* (Fig. 2 and Suppl. material 7.3).

Inflorescences with multiple flowers evolved into solitary flowers at least eight times, twice within the CAC, and six times outside the CAC: in *B. sect. Leopardinae*; *B. ayuthayense* J.J.Verm., Schuit. & de Vogel (*B. sect. Drymoda*); the clade comprising of *B. sect. Trias*, and *B. sect. Biflorae*; the clade comprising of *B. sect. Sestochilus*, *B. sect. Lepidorhiza*, and *B. sect. Beccariana*; the clade comprising of *B. sect. Epicrianthes*, *B. burfordiense* Garay, Hamer & Siegerist, *B. sect. Polymeres*, *B. saurocephalum* Rchb.f., *B. nitidum* Schltr., *B. sect. Oxysepala*, *B. macphersonii* Rupp, *B. sect. Monanthaparva*, and *B. sect. Macrocaulia*; and in *B. lopolith* J.J.Verm., Schuit. & de Vogel (*B. sect. Ione*). Inflorescences with 2–3 flowers evolved at least three times; once in *B. dayanum* Rchb.f. (*B. sect. Acrochaene*), and at least twice within CAC. Moreover, the solitary-flowered state also reversed back to the 2–3-flowered state at least once in the lineage of *B. sect. Biflorae*, and to the multiple-flowered state at least three times in *B. singaporeanum* Schltr., *B. lasianthum* Lindl., and *B. saurocephalum* (Fig. 2 and Suppl. material 7.4).

For the connation of lower margin of the lateral sepals, the ancestral state of this character is uncertain. The transition from lateral sepals with connate lower margins to free lower margins occurred at least nine times, namely seven times within CAC, and twice in certain species of *B. sect. Lemniscata* (previously known as *B. sect. Pleiophyllum* J.J.Sm.). The reverse transition occurred multiple times throughout the phylogeny, at least 14 times in the CAC and 9 times outside the CAC (Fig. 2 and Suppl. material 7.5).

Flowers with the dorsal and lateral sepals of nearly the same length are ancestral in the Asian clade of

Bulbophyllum. There have been at least five transitions to sepals of different lengths (lateral sepals that are more than 1.5 times as long as the dorsal sepal) within the CAC, with at least four reverse transitions, and three additional transitions in certain species of *B. sect. Lemniscata* (previously known as *B. sect. Tripudianthes* Seidenf.) and *B. sect. Biflorae* (Fig. 2 and Suppl. material 7.6).

DISCUSSION

In this study, we focus on the Asian clade of *Bulbophyllum* redefined by Simpson et al. (2024), which is a subclade of the Asian-Pacific clade (e.g. Gamisch and Comes 2019) excluding *B. sect. Adelopetalum* and *B. sect. Minutissima*. Our phylogenetic hypothesis aligns with traditional sectional delimitation. Most morphological characters in this study are homoplastic, while the presence of a swollen apical sterile flower is here confirmed as an autapomorphy of *B. physometrum* (Vermeulen et al. 2017). Identifying specific synapomorphies to distinguish sections within *Bulbophyllum* remains a challenging task. The combination of multiple characters is typically required for defining clades (Hosseini et al. 2016).

Phylogenetic relationships within the Asian clade of *Bulbophyllum*

This study is based on the most comprehensive molecular sampling of Asian *Bulbophyllum*, including the first sequences for 24 species (Suppl. material 1). It provides valuable insights into sectional delimitation within the Asian clade of *Bulbophyllum*.

Within the *Cirrhopetalum* alliance clade (CAC), we included previously unstudied species in our analysis (*B. ovatum* Seidenf., *B. trigonopus* (Rchb.f.) P.T.Ong, *B. cf. scabratum* Rchb.f., *B. bakhuizenii* Steenis). Despite our smaller combined dataset (excluding the *Xdh* marker), our findings align with those of Hu et al. (2020), reaffirming the relationships within the CAC. Our results confirm that the CAC comprises *B. sect. Brachyantha*, *B. sect. Cirrhopetaloides*, *B. sect. Cirrhopetalum*, *B. sect. Desmosanthes*, *B. sect. Emarginatae*, *B. sect. Ehippium*, *B.*

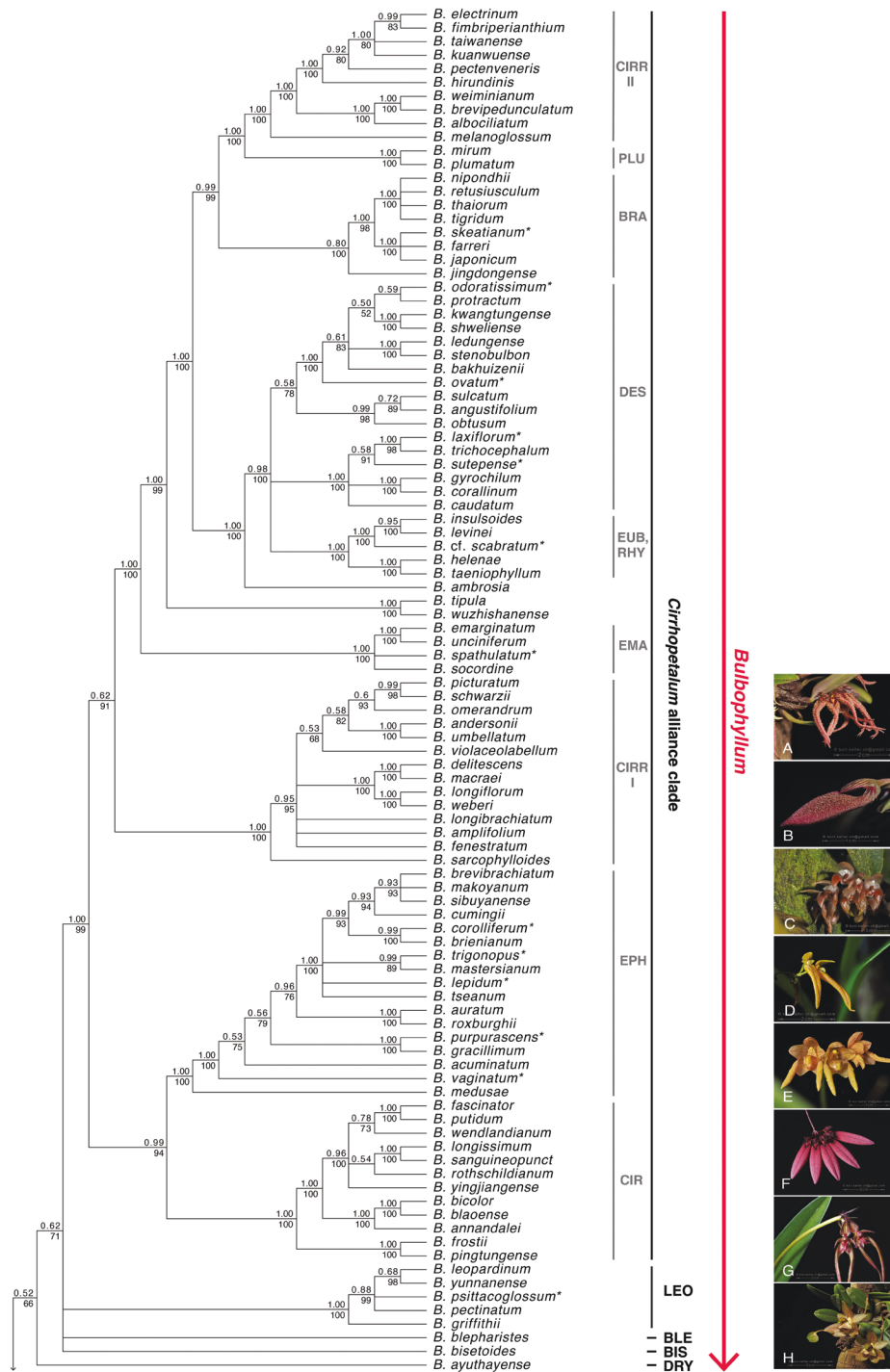


Figure 1. Majority-rule consensus tree resulting from Bayesian inference analysis based on the combined nuclear and chloroplast dataset. Values above and below branches indicate posterior probabilities (PP) and ultrafast bootstrap support (UFBS). An asterisk (*) indicates sequences that were newly generated in this study. Photos show the morphological diversity of Asian *Bulbophyllum*. A. *B. thaiorum* (BRA). B. *B. mirum* (PLU). C. *B. corallinum* (DES). D. *B. helenae* (RHY). E. *B. umbellatum* (CIRR). F. *B. acuminatum* (EPH). G. *B. wendlandianum* (CIR). H. *B. pectinatum* (LEO). I. *B. affine* (SES). J. *B. ecornutum* (BEC). K. *B. apodum* (STA). L. *B. lindleyanum* (HIR). M. *B. repens* (BSC). N. *B. physometrum* (PHY). O. *B. epicranthes* (EPI). P. *B. hirtum* (LEM). Q. *B. careyanum* (RAC). R. *B. nasutum* (TRI). Photo credit: Kurt Keller. Sectional placement of taxa is indicated by abbreviations: ACR (Acrochaene), ADL (Adelopetalum), ALT (Altisceptrum), BEC (Beccariana), BIF (Biflorae), BIS (Biseta), BLE (Blepharistes), BRA (Brachyantha), BSC (Brachystachyae), CIR (Cirrhopetaloides), CIRR (Cirrhopetalum), COD (Codonosiphon), DES (Desmosanthes), DRY (Drymoda), EMA (Emarginatae), EPH (Ephippium), EPI (Epicrianthes), EUB (Eublepharon), HIR (Hirtula), HYA (Hyalosema), ION (Ione), LEM (Lemniscata), LEO (Leopardinae), LEP (Lepidorhiza), MAC (Macrocaulia), MNO (Monomeria), MNP (Monanthaparva), MNT (Monanthes), OXY (Oxysepala), PHY (Physometra), PLU (Plumata), POL (Polymeres), RAC (Racemosae), REP (Repantia), RHY (Rhytionanthos), SES (Sestochilus), SAU (Saurocephalum), STA (Stachysanthes), and TRI (Trias).

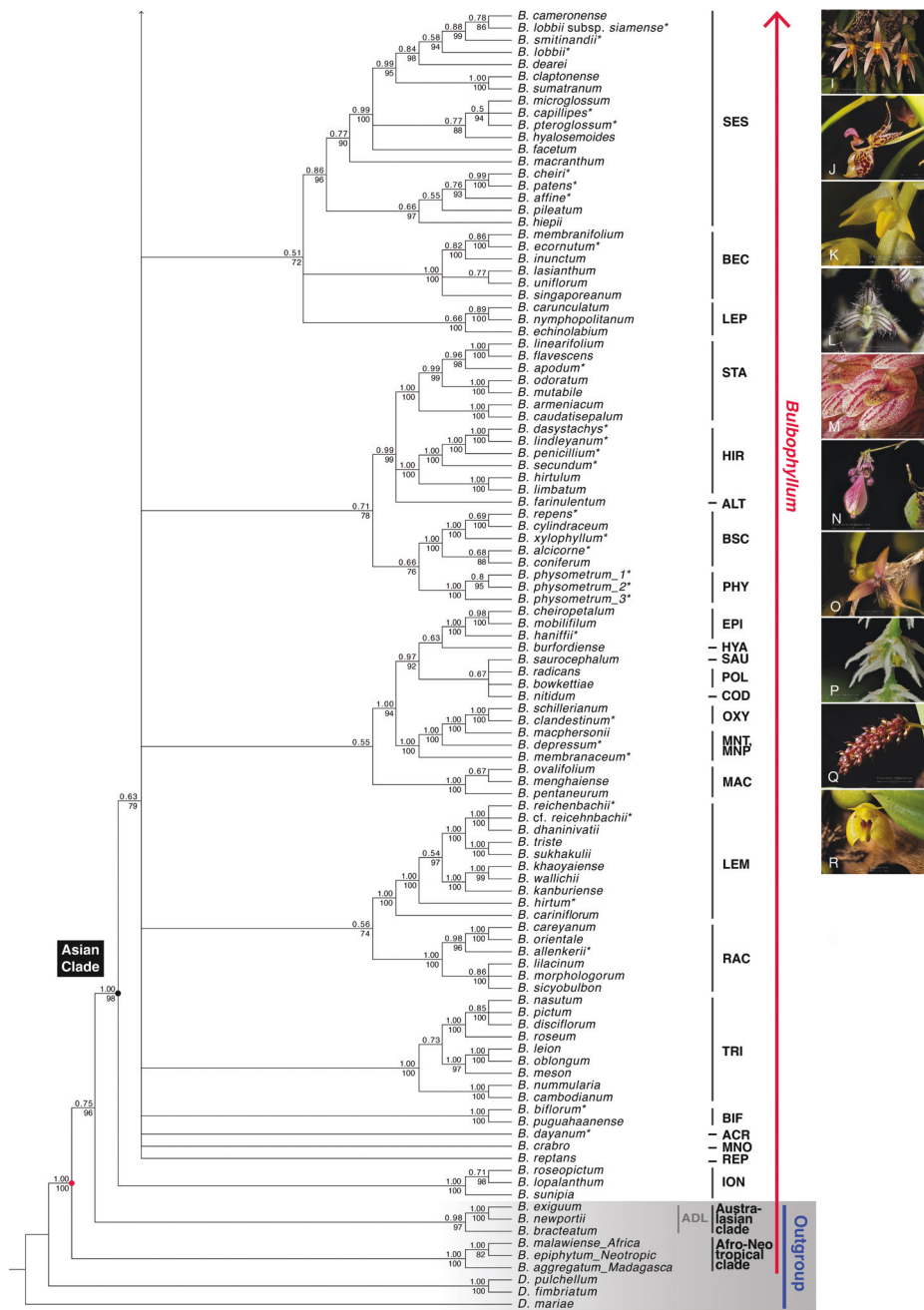


Figure 1 (continued). Majority-rule consensus tree resulting from Bayesian inference analysis based on the combined nuclear and chloroplast dataset. Values above and below branches indicate posterior probabilities (PP) and ultrafast bootstrap support (UFBS). An asterisk (*) indicates sequences that were newly generated in this study. Photos show the morphological diversity of Asian *Bulbophyllum*. A. *B. thaiorum* (BRA). B. *B. mirum* (PLU). C. *B. corallinum* (DES). D. *B. helenae* (RHY). E. *B. umbellatum* (CIRR). F. *B. acuminatum* (EPH). G. *B. wendlandianum* (CIR). H. *B. pectinatum* (LEO). I. *B. affine* (SES). J. *B. ecornutum* (BEC). K. *B. apodum* (STA). L. *B. lindleyanum* (HIR). M. *B. repens* (BSC). N. *B. physometrum* (PHY). O. *B. epicranthes* (EPI). P. *B. hirtum* (LEM). Q. *B. careyanum* (RAC). R. *B. nasutum* (TRI). Photo credit: Kurt Keller. Sectional placement of taxa is indicated by abbreviations: ACR (Acrochaene), ADL (Adelopetalum), ALT (Altisceptrum), BEC (Beccariana), BIF (Biflorae), BIS (Biseta), BLE (Blepharistes), BRA (Brachyantha), BSC (Brachystachyae), CIR (Cirrhopetaloides), CIRR (Cirrhopetalum), COD (Codonosiphon), DES (Desmosanthes), DRY (Drymoda), EMA (Emarginatae), EPH (Ephippium), EPI (Epicranthes), EUB (Eublepharon), HIR (Hirtula), HYA (Hyalosema), ION (Ione), LEM (Lemniscata), LEO (Leopardinae), LEP (Lepidorhiza), MAC (Macrocaulia), MNO (Monomeria), MNP (Monanthaparva), MNT (Monanthes), OXY (Oxysepala), PHY (Physometra), PLU (Plumata), POL (Polymeres), RAC (Racemosae), REP (Repantia), RHY (Rhytionanthos), SES (Sestochilus), SAU (Saurocephalum), STA (Stachysanthes), and TRI (Trias).

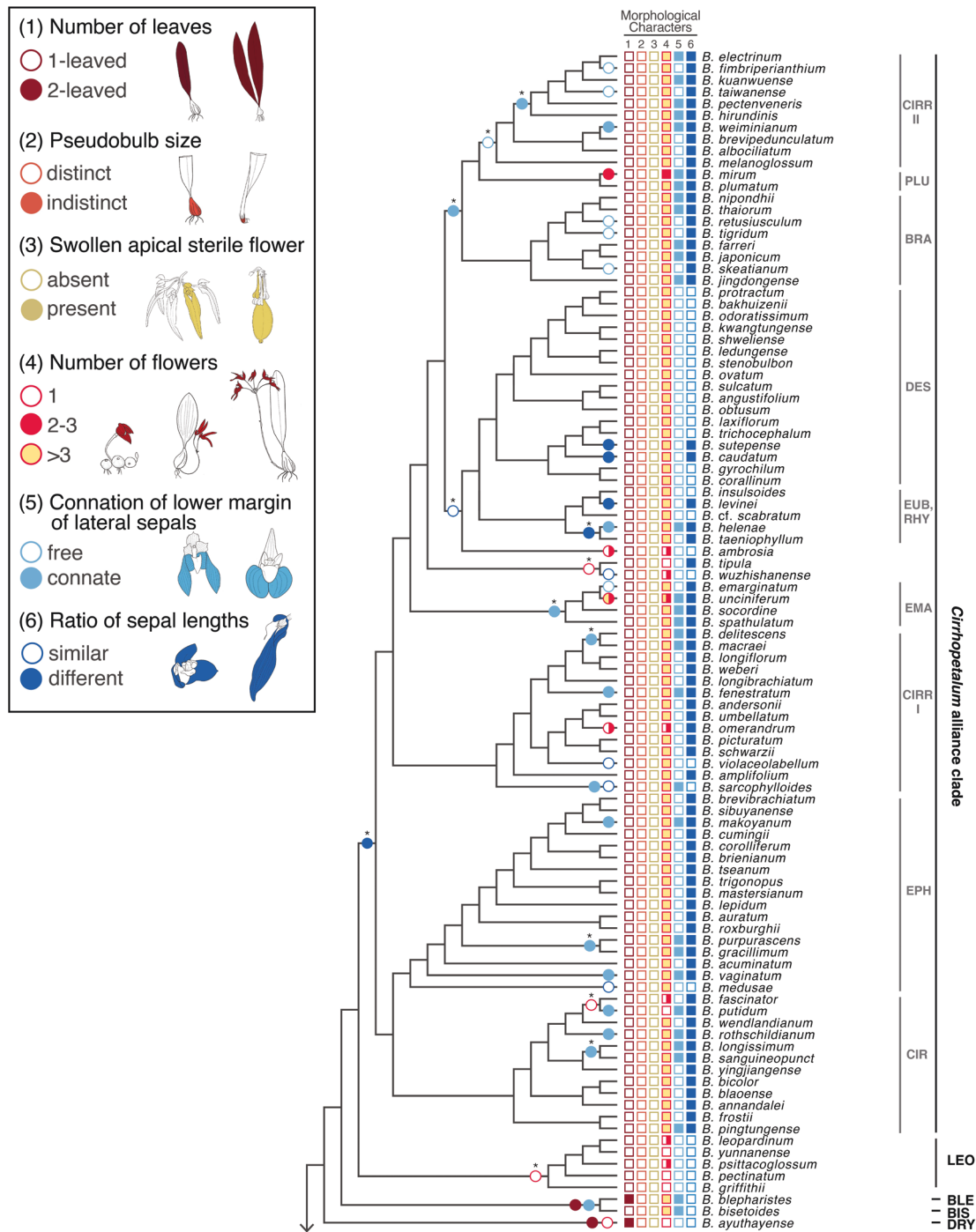


Figure 2. Ancestral character state reconstruction in the Asian clade of *Bulbophyllum* summarised on the maximum-likelihood (ML) tree derived from the combined dataset. Boxes at the tree tips show the character states of the six morphological characters examined for each taxon, while circles found along tree branches indicate character state transitions. An asterisk (*) indicates the transitions at the nodes with PP \geq 0.95 and UFBS \geq 95. Sectional placement of taxa is indicated by abbreviations: ACR (*Acrochaena*), ALT (*Altisceptrum*), BEC (*Beccariana*), BIF (*Biflorae*), BIS (*Biseta*), BLE (*Blepharistes*), BRA (*Brachyantha*), BSC (*Brachystachyae*), CIR (*Cirrhopedaloides*), CIRR (*Cirrhopedalum*), COD (*Codonosiphon*), DES (*Desmosanthes*), DRY (*Drymoda*), EMA (*Emarginatae*), EPH (*Ephippium*), EPI (*Epicriantes*), EUB (*Eublepharon*), HIR (*Hirtula*), HYA (*Hyalosema*), ION (*Ione*), LEM (*Lemniscata*), LEO (*Leopardinae*), LEP (*Lepidorrhiza*), MAC (*Macrocaulia*), MNO (*Monomeria*), MNP (*Monanthaparva*), MNT (*Monanthes*), OXY (*Oxysepala*), PHY (*Physometra*), PLU (*Plumata*), POL (*Polymeres*), RAC (*Racemosae*), REP (*Repantia*), RHY (*Rhytionanthos*), SES (*Sestochilus*), SAU (*Saurocephalum*), STA (*Stachysanthes*), and TRI (*Trias*).

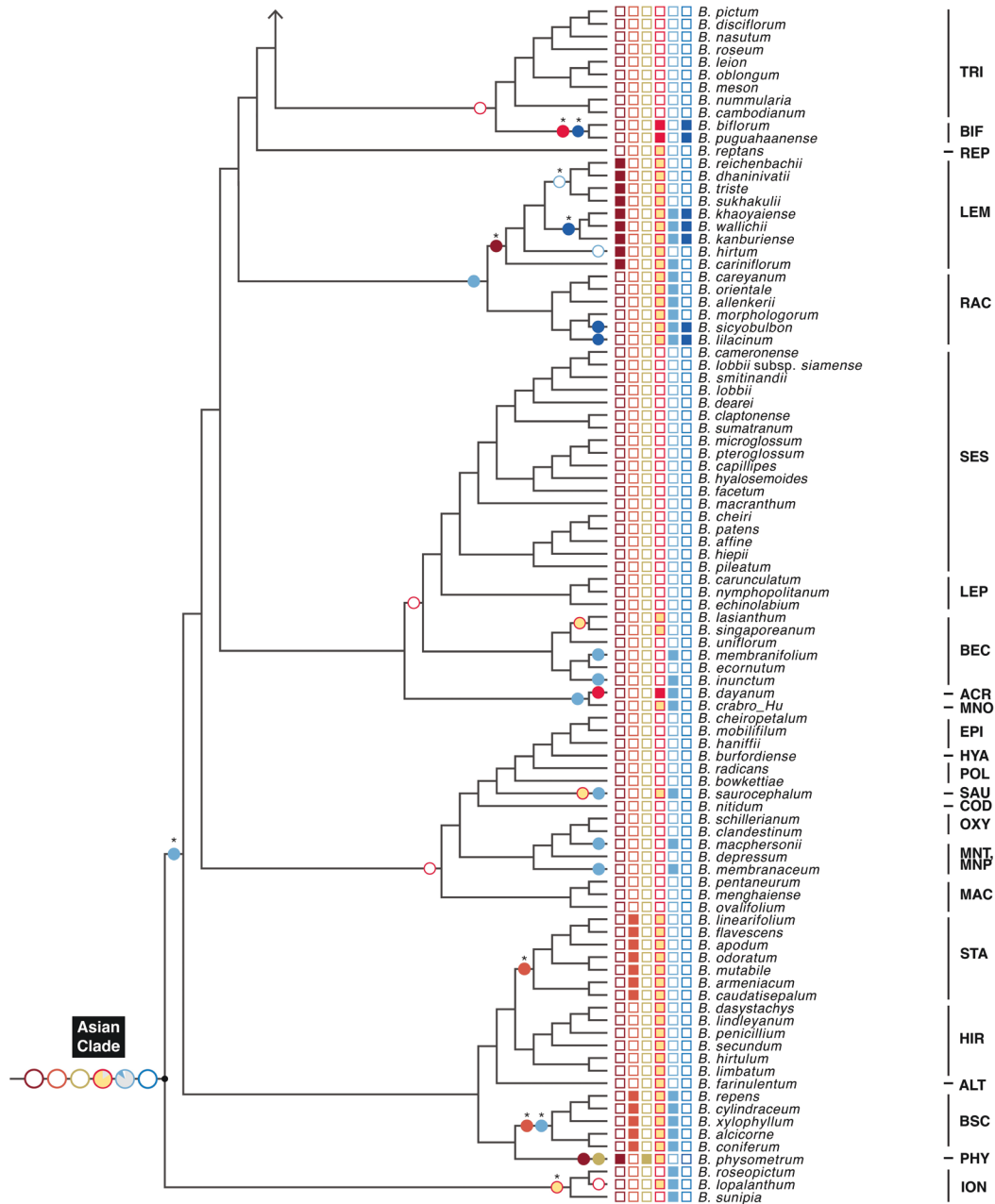


Figure 2 (continued). Ancestral character state reconstruction in the Asian clade of *Bulbophyllum* summarised on the maximum-likelihood (ML) tree derived from the combined dataset. Boxes at the tree tips show the character states of the six morphological characters examined for each taxon, while circles found along tree branches indicate character state transitions. An asterisk (*) indicates the transitions at the nodes with PP ≥ 0.95 and UFBS ≥ 95. Sectional placement of taxa is indicated by abbreviations: ACR (*Acrochaene*), ALT (*Altisceptrum*), BEC (*Beccariana*), BIF (*Biflorae*), BIS (*Biseta*), BLE (*Blepharistes*), BRA (*Brachyantha*), BSC (*Brachystachyae*), CIR (*Cirrhopetaloides*), CIRR (*Cirrhopetalum*), COD (*Codonosiphon*), DES (*Desmosanthes*), DRY (*Drymoda*), EMA (*Emarginatae*), EPH (*Ephippium*), EPI (*Epicrianthes*), EUB (*Eublepharon*), HIR (*Hirtula*), HYA (*Hyalosema*), ION (*Ione*), LEM (*Lemniscata*), LEO (*Leopardinae*), LEP (*Lepidorhiza*), MAC (*Macrocaulia*), MNO (*Monomeria*), MNP (*Monanthaparva*), MNT (*Monanthes*), OXY (*Oxysepala*), PHY (*Physometra*), PLU (*Plumata*), POL (*Polymeres*), RAC (*Racemosae*), REP (*Repantia*), RHY (*Rhytionanthos*), SES (*Sestochilus*), SAU (*Saurocephalum*), STA (*Stachysanthes*), and TRI (*Trias*).

sect. *Eublepharon*, *B. sect. Macrosylida*, *B. sect. Plumata*, and *B. sect. Rhytionanthos*. However, most sections within the CAC are non-monophyletic as the existing sections include a small number of outliers. Thus, a major revision of this group is needed (Hu et al. 2020).

In non-CAC taxa, our results suggest that *B. sect. Beccariana* (PP = 1.00, UFBS = 100), and *B. sect. Sestochilus* (PP = 0.86, UFBS = 96) are monophyletic, contrasting with the results of Simpson et al. (2024). However, some species examined by Simpson et al. (2024) were not included in this analysis. The morphology of these two sections is very similar, therefore their definition may need to be refined (Vermeulen et al. 2015).

Our analysis of non-CAC taxa, using the three-marker dataset (ITS, *matK*, and *psbA-trnH*), revealed that *B. sect. Physometra* and *B. sect. Hirtula* are further apart than previously believed. This differs from a study by Nowak et al. (2023), which suggested a connection between *B. physometrum* and representatives of *B. sect. Hirtula*. However, their analysis did not include some key taxa, such as *B. sect. Altisceptrum*, *B. sect. Brachystachyae*, and *B. sect. Stachysanthes*. By incorporating these sections, *B. sect. Physometra* appears closely related to *B. sect. Brachystachyae* with low support value (PP = 0.66, UFBS = 76), and has affinities with *B. sect. Hirtula*, *B. sect. Altisceptrum* and *B. sect. Stachysanthes* according to ML analysis.

ML analysis hints for a close relationship between *B. sect. Racemosae* and *B. sect. Lemniscata* (PP = 0.56, UFBS = 74), supported by morphological traits such as the multiple-flowered racemes and the connation of the lower margin of the lateral sepals. In contrast, Hosseini et al. (2016), proposed a close relationship between *B. sect. Racemosae* and *B. sect. Cirrhopetalum* based on a four-marker dataset. However, their analysis did not include phylogenetically critical taxa such as *B. sect. Lemniscata* and *B. sect. Leopardinae*.

Consistent with Hosseini et al. (2012, 2016), *Bulbophyllum sect. Stachysanthes* is closely related to *B. sect. Altisceptrum*, *B. sect. Brachystachyae*, and *B. sect. Hirtula*. Additionally, our findings support the merging of *Bulbophyllum sect. Sestochilus* with *B. sect. Stenochilus* (including *B. macranthum* Lindl., *B. affine* Lindl.) proposed by Vermeulen et al. (2015).

Uncertainties still exist in the phylogenetic tree of the Asian clade of *Bulbophyllum*, notably the position of *B. sect. Physometra*. A broader sampling, including a minimum of three representatives from each polyspecific section and taxonomically puzzling species (e.g. *B. planibulbe* (Ridl.) Ridl., *B. polliculosum* Seidenf.) and/or sections (*B. sect. Biseta*, *B. sect. Pelma* (Finet) Schltr., *B. sect. Repantia* J.J.Verm. ex N.Pearce, P.J.Cribb & Renz), is key to gain a more comprehensive understanding of this group. Moreover, the inclusion of more DNA markers, especially plastome markers, can offer valuable insight into relationships among closely related species as in Neotropical *Bulbophyllum* (Zavala-Páez et al. 2020), along with the adoption of targeted next-generation sequencing

approaches utilising the Angiosperms353 (Johnson et al. 2019) or Orchidaceae963 (Eserman et al. 2021) probes.

Character evolution in the Asian clade of *Bulbophyllum*

Evolution of two-leaved pseudobulbs

Most *Bulbophyllum* species in the Asian clade possess one-leaved pseudobulbs, making this trait a practical clue for field identification against other orchid genera. Accordingly, the presence of a two-leaved pseudobulb is frequently employed to key together some unique *Bulbophyllum* species (Seidenfaden 1979; Kasetluksamee and Ngerensaengsaruy 2009), which might be assumed as forming a natural group. However, our findings indicate that the two-leaved pseudobulb has independently evolved from a one-leaved pseudobulb ancestor at least four times in Asian clade of *Bulbophyllum*. *Bulbophyllum* species with two-leaved pseudobulbs are typically found in exposed micro-habitats with direct sunlight, while the species with one leaf are usually found in shaded areas (Chayamarit et al. 2014). This transition in the leaf number within the Asian clade of *Bulbophyllum* can be attributed to an adaptation to divergent light availability. The presence of a single large leaf, which appears to be symplesiomorphic in *Bulbophyllum*, maximises light capture for photosynthesis in shaded habitats. Conversely, in more well-lit conditions, the presence of two leaves enables the orchid to optimise light exposure by adjusting leaf orientation and the angle between the leaves and the pseudobulb (Strauss et al. 2020). In contrast, most Malagasy *Bulbophyllum* have two-leaved pseudobulbs, which is considered the ancestral state for Malagasy species. The possible reversals to the one-leaved pseudobulb state in some Malagasy species (Fischer et al. 2007), may relate to the invasion of more shaded habitats in the island.

Parallel evolution of indistinct pseudobulbs

The shape and size of pseudobulbs among sympodial orchids are highly variable. This organ contributes both to water and nutrient storage (Dressler 1993; Zhang et al. 2018). In the case of the Asian clade of *Bulbophyllum*, the pseudobulb has shifted from being distinct to becoming indistinct in two clades. There is parallel evolution of this trait in *B. sect. Brachystachyae* and *B. sect. Stachysanthes* possibly due to similar ecological constraints, namely water availability. Indeed, these species with indistinct pseudobulbs are commonly found in montane areas in Peninsular Malaysia, Sumatra, Java, Borneo, eastwards to the western Pacific region (Vermeulen 2014; Vermeulen et al. 2015), where water availability is rarely a limiting factor. In areas with dry season, in mainland Southeast Asia, larger pseudobulbs are adaptive in water storage. In these areas there is probably strong selection against smaller pseudobulbs. Additionally, pseudobulbs serve as an important pool of water and nutrients not only for orchids themselves but also for herbivores. Thus, the

pressure from herbivory may favour the evolution of smaller pseudobulbs (Ribeiro et al. 1994; Li et al. 2022), which in turn may be less susceptible to rot in excessively humid conditions (Körner et al. 1989). Pseudobulb size thus probably responds to multiple trade-offs in the allocation of resources to water-storage tissue.

The evolution of a unique autapomorphy in *B. physometrum*

Dimorphic flowers are uncommon in orchids. Monomorphic flowers are ancestral in *Bulbophyllum*, being present in the majority of the species. Monomorphic flowers shifted to dimorphic flowers with a swollen apical sterile flower only once in *B. physometrum*. During our field observations, we noticed that *B. physometrum* grows in open areas on tall trees, and its large sterile flower swings by the gentle draft of the wind, likely acting as a visual cue in attracting pollinators from a distance, as it was previously remarked by Vermeulen et al. (2017).

Multiple-flowered inflorescences are ancestral in the Asian clade of *Bulbophyllum*

Inflorescences with multiple flowers is the ancestral state within the Asian clade of *Bulbophyllum*. There have been several transitions to solitary flowers and occasionally transitions to inflorescences with 2–3 flowers, as it had already been inferred within the CAC (Hu et al. 2020). Pollinators often visit multiple flowers on a multiple flowered inflorescence (Li et al. 2010), which can lead to self-pollination within the same plant. In contrast, pollinators of single flowers are more likely to have pollinators fly away after visiting just one flower, reducing the chance of self-pollination. This means that single-flowered plants might produce seeds with better genetic quality than those with many flowers (Sun et al. 2018). Additionally, there may be a trade-off between present reproduction and future growth. Compared to having inflorescence with multiple flowers, bearing a single flower may require relatively low energy for flowering and fruiting (Sun et al. 2018). Thus, in environments where resources are limited, solitary flowers may be favoured. Moreover, this pattern was also observed in Malagasy *Bulbophyllum* (Fischer et al. 2007).

Additionally, this transition may result from pollinator shifts. Several pollination studies have demonstrated that single-flowered species tend to exhibit larger flowers and rely on large flies as their pollinators. For example, *B. patens* King ex Hook.f. is pollinated by male fruit flies of the genus *Bactrocera* (Tan and Nishida 2000). On the contrary, multiple-flowered species typically have smaller flowers pollinated by small flies or fruit flies. For instance, *B. nipondhii* Seidenf. is pollinated by female scuttle flies of the genus *Megaselia*, while *B. penicillium* is pollinated by minute fruit flies of the genus *Drosophila* (Liu et al. 2010; Pakum et al. 2019). The transition from multiple-flowered inflorescences to solitary flowers may thus be inextricably linked to the pollinators' preferences.

Connate or free-margined lateral sepals

The ancestral state of this character is uncertain in the Asian clade of *Bulbophyllum*. Throughout evolution, there have been numerous instances of transitions from free-margined lateral sepals to connate-margined lateral sepals, along with multiple cases of reversals. The connation of lateral sepals along the lower margin is present in different clades, exhibiting clade-specific patterns. For example, *B. sect. Brachystachyae* is known for having lateral sepals connate along the lower margin, creating a saucer-like structure (Seidenfaden and Wood 1992). Additionally, it is important to highlight that the connation of sepals can also occur on the upper margin, representing a distinct evolutionary pathway independent from the connation observed along the lower margin (Hu et al. 2020). Upper margin connation serves as a distinguishing feature of the CAC, being only absent in *B. sect. Desmosanthes*, *B. sect. Eublepharon*, *B. sect. Rhytionanthos*, and *B. ambrosia*, which have free lateral sepals (Hu et al. 2020).

Convergent patterns of lateral sepal elongation

Flowers that possess lateral and dorsal sepals of similar length are typically observed in non-CAC taxa, but they have independently evolved in *B. sect. Biflorae*, *B. sect. Lemniscata*, *B. sect. Racemosae*. In contrast, CAC taxa commonly exhibit a difference in length between their lateral and dorsal sepals, except for the lineage that includes *B. sect. Desmosanthes*, *B. sect. Eublepharon*, *B. ambrosia* (Hance) Schltr., *B. wuzhishanense* X.H.Jin, *B. violaceolabellum* Seidenf., and *B. sarcophylloides* Garay, Hamer & Siegerist. Hu et al. (2020) identified this subclade, primarily consisting of species from *B. sect. Desmosanthes*, by their racemose inflorescences and the lateral sepals, which are free and equal in size to the dorsal sepals. The connation and size variations of the sepals contribute to diverse floral architectures, possibly involved in pollinator attraction. These adaptations are often associated with specialised pollination strategies. The connation of lateral sepals may create a landing platform for pollinators, directing them towards the column and lip. This guidance increases the probability of successful pollination by facilitating the interaction between pollinators and the pollinaria/stigma (Ruchisansakun et al. 2016).

CONCLUSION

Our study contributes to the understanding of the evolutionary patterns of taxonomically significant traits within the Asian clade of *Bulbophyllum*. The ancestral character analysis, using combined DNA regions (ITS, *matK*, and *psbA-trnH*), uncovered that several traits commonly used in traditional taxonomic classification, including leaf count, pseudobulb size, flower count, the connation of the lower margin of the lateral sepals, and sepal length ratio, have undergone multiple independent changes, resulting in homoplasy. The dimorphic flowers with a swollen ovary are identified as an automorphic

character of *B. physometrum*. While these characters can still be useful when combined with other traits to distinguish different sections, the improvement of molecular phylogenetic frameworks through phylogenomics, will offer more opportunities to study the evolution of additional characters in the Asian clade of *Bulbophyllum*.

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SUPPLEMENTARY MATERIALS

Supplementary material 1

Voucher information and GenBank accession numbers of the newly generated sequences.

<https://doi.org/10.5091/plecevo.114642.suppl1>

Supplementary material 2

Voucher information and GenBank accession numbers of sequences generated in previous studies.

<https://doi.org/10.5091/plecevo.114642.suppl2>

Supplementary material 3

List of primers with thermocycling conditions used for PCR amplification.

<https://doi.org/10.5091/plecevo.114642.suppl3>

Supplementary material 4

Maximum likelihood (ML) tree with branch lengths based on combined nuclear and chloroplast sequences.

<https://doi.org/10.5091/plecevo.114642.suppl4>

Supplementary material 5

The phylogenetic tree based on the pruned dataset containing only taxa having data for all three DNA regions.

<https://doi.org/10.5091/plecevo.114642.suppl5>

Supplementary material 6

Phylogenetic trees based on single-maker datasets.

<https://doi.org/10.5091/plecevo.114642.suppl6>

Supplementary material 7

Ancestral character state reconstruction in the Asian clade of *Bulbophyllum* for each character.

<https://doi.org/10.5091/plecevo.114642.suppl7>

Supplementary material 8

Summary of state changes throughout trees for each character.

<https://doi.org/10.5091/plecevo.114642.suppl8>

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