



Molecular phylogenetics of species of *Bulbophyllum* sect. *Trias* (Orchidaceae; Epidendroideae; Malaxidae) based on nrITS and plastid *rbcL* and *matK*

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Abstract

Phylogenetic relationships among species of *Bulbophyllum* sect. *Trias* and related taxa in *Bulbophyllum* has not been previously studied due to scarcity of appropriate samples. In this study, we aimed to assess the relationship of these taxa and investigate interspecific relationships of these orchids using DNA sequences from two plastid genes, *rbcL* and *matK*, and one nuclear region, nuclear ribosomal internal transcribed spacer, nrITS. Our results showed that all *Bulbophyllum* sect. *Trias* species were embedded among other groups of *Bulbophyllum*, supporting the already published transfer of *Trias* to synonymy of *Bulbophyllum*. All trees revealed that the Indochinese sect. *Trias* form a monophyletic group that could be divided into three groups coincident with their vegetative characters. This study further shows that nrITS sequences can be sufficient for inferring phylogenetic relationship among *Trias* species, although this marker and the combination of this plus the plastid genes are not able to distinguish the differences between some closely related species.

Introduction

Trias Lindley (1830: 60) is a genus of miniature epiphytic orchids consisting of at least 15 species (Dressler 1993, Cameron *et al.* 1999, Chase *et al.* 2003, van den Berg *et al.* 2005, Smidt *et al.* 2011). These epiphytic orchids are indigenous to tropical and subtropical rain forests in Southeast Asia, India, China and Australia (Liu *et al.* 2007), where they grow primarily on shady rocks along streams and in open woodlands (Liu *et al.* 2007, Schuiteman *et al.* 2008). The unique features of *Trias* are not just restricted to their floral morphology but are also found in their compact pseudobulbs and leaf shapes. Their characteristics were defined by Seidenfaden (1986) as having horn-like prolonged operculum with uniform broad triangular-ovate sepals and petals that are smaller than the sepals and mostly linear, rising vertically on each side of their column. Dressler (1993) included 15 orchid genera within this subtribe. However, recent evidence from molecular analyses has demonstrated that all former genera of Bulbophyllinae including *Trias* should be subsumed in *Bulbophyllum* Thouars (1822: t. 3; Pridgeon *et al.* 2014, Vermeulen *et al.* 2014). *Trias* was placed in *Bulbophyllum* in tribe Malaxideae, subtribe Bulbophyllinae (Chase *et al.* 2015).

The recent combination of molecular data with morphometric data has resulted in enhanced classification techniques over traditional approaches to taxonomy, with molecular analysis enabling a new classification of Orchidaceae (Chase *et al.* 2003, 2015). Sequences of both plastid DNA regions and nrITS have been used for phylogenetic analysis in Orchidaceae (Neubig *et al.* 2009, Li *et al.* 2011a, Batista *et al.* 2013, Xiang *et al.* 2013, Luo *et al.* 2014). Among the plastid DNA regions, the plastid *rbcL* and *matK* genes are the most commonly used regions as core barcodes (Hollingsworth *et al.* 2011). Plastid *rbcL* is the most commonly used gene and has been proven to aid in identification at a variety of taxonomic levels as it contains no length variation and has sufficient sequence divergence particularly at the genus level (Cameron *et al.* 1999). Similarly, the plastid gene *matK* has also been commonly used for assessing the phylogenetic relationships in Orchidaceae (Kores *et al.* 2000, Bateman *et al.* 2003, Ponsie *et al.* 2007, Hosseini *et al.* 2012, Batista *et al.* 2013) as it is one of the most rapidly evolving genes of the plastid genome (Hilu & Liang 1997, Hollingsworth *et al.* 2011). In addition to plastid genes, several studies have applied nuclear DNA regions, such as internal transcribed spacer of ribosomal DNA (nrITS), to phylogenetic analysis in order to identify relationship among species of orchids (Hollingsworth 2011). Many recent studies combined sequences of this region with plastid regions, such as *matK*, *rbcL*, *trnL-F* spacer, *trnL* intron, *psbA-trnH* spacer and *trnS-trnG* spacer (Kocyan *et al.* 2004, Smidt *et al.* 2011, Xiang *et al.* 2013) to improve resolution of the phylogenetic trees of various groups of orchids. ITS not only

contains enough variable sites for separation of taxa but also flanks conserved rDNA regions that facilitate amplification of this region, thereby enhancing assessment of phylogenetic relationships within Orchidaceae, particularly at species level (Chen *et al.* 2010, Yao *et al.* 2010).

To date, by employing a combination of molecular markers, numerous genera of orchids have been reassessed e.g. *Ophrys* Linnaeus (1753: 945; Soliva *et al.* 2001), *Coelogyne* Lindley (1821: t. 33; Gravendeel *et al.* 2001), *Galeandra* Lindley (1830: t. 8; Monteiro *et al.* 2010), *Dendrobium* Swartz (1799: 82; Xiang *et al.* 2013), *Habenaria* Willdenow (1805: 5; Batista *et al.* 2013), *Cypripedium* Linnaeus (1753: 951; Li *et al.* 2011b) and *Bulbophyllum* (Fischer *et al.* 2007, Smidt *et al.* 2011, Hosseini *et al.* 2012). *Trias* has not fully been investigated previously, and only a few studies have assessed its relationship with *Bulbophyllum* (Gravendeel *et al.* 2004, Pridgeon *et al.* 2014). The research presented here aims to examine the relationship between *Trias* and related taxa in *Bulbophyllum* and investigate relationships among the Indochinese *Trias* species. In order to achieve these goals, DNA sequences of three regions, one nuclear DNA region (nrITS) and two plastid genes (*matK* and *rbcL*) were amplified, sequenced and analysed.

Materials and methods

Taxon sampling

Eight Indochinese species of *Bulbophyllum* sect. *Trias* (Lindl.) Vermeulen, Schuiteman & de Vogel (2014: 110) (1–4 samples per species) were available for this study. These eight Indochinese *Trias* species were obtained from the Wiruch Nursery in Chiangmai and maintained in the greenhouse at the Department of Genetics, Kasetsart University, Bangkok: *Bulbophyllum cambodianum* (Christenson 2003: 542) Vermeulen, Schuiteman & de Vogel (2014: 110), *B. disciflorum* Rolfe (1895: 7), *B. leion* Vermeulen, Schuiteman & de Vogel (2014: 111), *B. meson* Vermeulen, Schuiteman & de Vogel (2014: 111), *B. nasutum* Reichenbach (1871: 1482), *B. pictum* Parish & Reichenbach, (1874: 150), *B. roseum* Ridley (1896: 276), *B. oblongum* Reichenbach (1861: 249). One species from the closely related sect. *Drymoda* (Lindley 1838: t. 8) Vermeulen, Schuiteman & de Vogel (2014: 102), *B. ayuthayense* Vermeulen, Schuiteman & de Vogel (2014: 102), was also collected from the same nursery. In order to examine the relationship between sect. *Trias* and other taxa of *Bulbophyllum*, GenBank database sequences of 32 species of *Bulbophyllum* were downloaded. Sequence data of five species of *Dendrobium* were further retrieved from GenBank to be used as the outgroup. Voucher information and GenBank accession numbers of all sequences included in this study are listed in Table 1.

TABLE 1. Voucher information and GenBank accession numbers.

Taxon	Voucher/Isolate*	GenBank accession number		
		<i>rbcL</i>	<i>matK</i>	ITS
<i>Bulbophyllum</i> sect. <i>Trias</i>				
<i>B. cambodianum</i>	AS2013Tc01	KM924492	KM924446	KM924469
<i>B. cambodianum</i>	AS2013Tc02	KM924493	KM924447	KM924470
<i>B. cambodianum</i>	AS2013Tc03	KM924494	KM924448	KM924471
<i>B. cambodianum</i>	AS2013Tc04	KM924495	KM924449	KM924472
<i>B. disciflorum</i>	AS2013Td01	KM924499	KM924453	KM924476
<i>B. disciflorum</i>	AS2013Td02	KM924500	KM924454	KM924477
<i>B. disciflorum</i>	AS2013Td03	KM924501	KM924455	KM924478
<i>B. disciflorum</i>	AS2013Td04	KM924502	KM924456	KM924479
<i>B. meson</i>	AS2013Ti01	KM924512	KM924466	KM924489
<i>B. meson</i>	AS2013Ti02	KM924513	KM924467	KM924490
<i>B. leion</i>	AS2013Tm01	KM924506	KM924460	KM924483
<i>B. leion</i>	AS2013Tm02	KM924507	KM924461	KM924484
<i>B. nasutum</i>	AS2013Tn01	KM924509	KM924463	KM924486
<i>B. nasutum</i>	AS2013Tn02	KM924510	KM924464	KM924487
<i>B. nasutum</i>	AS2013Tn03	KM924511	KM924465	KM924488
<i>B. oblongum</i>	AS2013To01	KM924496	KM924450	KM924473
<i>B. oblongum</i>	AS2013To02	KM924497	KM924451	KM924474

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TABLE 1. (Continued)

Taxon	Voucher/Isolate*	GenBank accession number		
		<i>rbcL</i>	<i>matK</i>	ITS
<i>B. oblongum</i>	AS2013To03	KM924498	KM924452	KM924475
<i>B. pictum</i>	AS2013Tp01	KM924503	KM924457	KM924480
<i>B. pictum</i>	AS2013Tp02	KM924504	KM924458	KM924481
<i>B. pictum</i>	AS2013Tp03	KM924505	KM924459	KM924482
<i>B. roseum</i>	AS2013Tr01	KM924508	KM924462	KM924485
Sect. <i>Drymoda</i>				
<i>B. ayuthayense</i>	AS2013Drys01	KM924514	KM924468	KM924491
Sect. <i>Sestochilus</i>				
<i>B. inunctum</i>	B002	JF427989.1	JF305769.1	JF428110.1
<i>B. lobbii</i>	B004	JF427991.1	JF305771.1	JF428099.1
<i>B. lobbii</i>	-	-	-	AF521074
<i>B. pileatum</i>	B0022	JF428007.1	JF305787.1	JF428105.1
Sect. <i>Stenochilus</i>				
<i>B. patens</i>	B005	JF427992.1	JF305772.1	JF428100.1
<i>B. macranthum</i>	B001	JF427988.1	JF305768.1	JF428097.1
Sect. <i>Beccariana</i>				
<i>B. lasianthum</i>	B0041	JF428026.1	JF305818.1	JF428116.1
<i>B. uniflorum</i>	B003	JF427990.1	JF305770.1	JF428098.1
Sect. <i>Epicrianthes</i>				
<i>B. cheiropetalum</i>	B0018	JF428004.1	JF305784.1	JF428109.1
Sect. <i>Monanthaparva</i>				
<i>B. membranaceum</i>	B0024	JF428028.1	JF305788.1	JF428107.1
Sect. <i>Cirrhopetalum</i>				
<i>B. acuminatum</i>	B0025	JF428021.1	JF305802.1	JF428117.1
Sect. <i>Acrochaene</i>				
<i>B. dayanum</i>	B0014	JF428000.1	JF305780.1	JF428119.1
<i>B. dayanum</i>	B0038	JF428018.1	JF305799.1	JF428120.1
Sect. <i>Hirtula</i>				
<i>B. hirtulum</i>	B0033	JF428030.1	JF305797.1	JF428126.1
<i>B. hirtulum</i>	B0049	JF428031.1	JF305810.1	JF428127.1
<i>B. limbatum</i>	B0054	JF428036.1	JF305814.1	JF428122.1
Sect. <i>Racemosae</i>				
<i>B. lilacinum</i>	B0029	JF428012.1	JF305796.1	JF428111.1
Sect. <i>Stachysanthes</i>				
<i>B. armeniacum</i>	B0030	JF428015.1	JF305794.1	JF428112.1
<i>B. flavescens</i>	B0012	JF427998.1	JF305778.1	JF428102.1
<i>B. linearifolium</i>	B0013	JF427999.1	JF305779.1	JF428103.1
Sect. <i>Hoplandra</i>				
<i>B. restrepia</i>	B0055	JF428035.1	JF305815.1	JF428123.1
Sect. <i>Napelli</i>				
<i>B. napelli</i>	ECS-ITS29	-	-	GQ339711
<i>B. cantagallense</i>	ECS-ITS40	-	-	GQ339722
<i>B. atropurpureum</i>	ECS-ITS24	-	-	GQ339706
Sect. <i>Dydactyle</i>				
<i>B. meridense</i>	ECS-ITS30	-	-	GQ339712
<i>B. exaltatum</i>	ECS-ITS33	-	-	GQ339715

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TABLE 1. (Continued)

Taxon	Voucher/Isolate*	GenBank accession number		
		<i>rbcL</i>	<i>matK</i>	ITS
Sect. <i>Xiphizusa</i>				
<i>B. ciluliae</i>	ECS-ITS16	-	-	GQ339698
<i>B. gladiatum</i>	ECS-ITS36	-	-	GQ339718
<i>B. melloi</i>	ECS-ITS37	-	-	GQ339719
Sect. <i>Bulbophyllaria</i>				
<i>B. cirrhosum</i>	ECS-ITS2	-	-	GQ339684
<i>B. bracteolatum</i>	ECS-ITS5	-	-	GQ339687
Sect. <i>Micranthae</i>				
<i>B. epiphytum</i>	ECS-ITS11	-	-	GQ339693
<i>B. micranthum</i>	ECS-ITS15	-	-	GQ339697
Sect. <i>Furvescens</i>				
<i>B. nagelii</i>	ECS-ITS38	-	-	GQ339720
Sect. <i>Megaclinium</i>				
<i>B. falcatum</i>	ECS-ITS1	-	-	GQ339683
Outgroup				
<i>Dendrobium (Epigeneium) rotundatum</i>	Jin X-H 13049			KF143740.1
<i>D. mariae (E. clemensiae)</i>	Jin X-H 10120	KF177670.1	KF143739.1	KF143530.1
<i>D. rosellum</i>	D001	JF428046.1	JF305822.1	JF428129.1
<i>D. parciflorum</i>	Jin X-H 10113	KF177607.1	KF143676.1	KF143466.1
<i>D. pulchellum</i>	Jin X-H 11878	KF177644.1	KF143712.1	KF143503.1

* Specimens of *Trias* and *Drymoda* were deposited at Bangkok Herbarium (BK).

PCR amplification and sequencing

ITS, *rbcL* and *matK* genes were amplified from DNA using the Phire Plant Direct PCR Kit (Thermo Scientific, USA). Primers used for *rbcL*, *matK*, and ITS amplification were as follows: *rbcL* (Hasebe *et al.* 1994, Asahina *et al.* 2010); *matK* (Topik *et al.* 2005, Asahina *et al.* 2010; ITSF-orc (5'-AGAAGTCGTAACAAGGTTTCCG-3') and ITSR-orc (5'-TCCGCTTATGATATGCTTAAA-3') for ITS (developed for this study). Fresh orchid leaves were punctured with Harris Uni-Core™ 0.50 mm and released into the PCR reaction. PCR was performed in a Veriti® Thermal Cycler (Applied Biosystems, US) in a 50 µl reaction containing 1x Phire Reaction Buffer, 5 pmol of each primer, 1U of Phire Hot Start II DNA Polymerase (Thermo Scientific, USA). PCR conditions used to amplify all three DNA regions were as follows: initial denaturation at 98°C for 5 min, followed by 40 cycles of denaturation at 98°C for 40 sec, annealing at 50°C for 30 sec, extension at 72°C for 40 sec, and a final extension step at 72°C for 5 min. Purified PCR products were sequenced with an ABI 3730XL DNA Analyzer (Applied Biosystems, USA) at Macrogen (South Korea). All sequences were blasted against the GenBank database using blastn (<http://blast.ncbi.nlm.nih.gov>) with default parameters to verify an appropriate sequence.

Sequence alignment

DNA sequences of each region were aligned using MAFFT (Kato & Standley 2013). All aligned sequences were visually inspected and manually adjusted using Aliview (Larsson 2014). In the case of aligned ITS1–5.8S–ITS2 sequences, the primary aligned sequences were manually adjusted based on the 3' end of the 5.8s *rDNA* sequence annotated by ITS2 database (Schultz *et al.* 2006, Selig *et al.* 2008, Schultz & Wolf 2009, Koetschan *et al.* 2010, Koetschan *et al.* 2012, Merget *et al.* 2012) and the annotation of ITS1 region of the database sequences. ITS sequences were submitted to ITS2 database (Schultz *et al.* 2006, Selig *et al.* 2008, Schultz & Wolf 2009, Koetschan *et al.* 2010, Koetschan *et al.* 2012, Merget *et al.* 2012) to locate ITS2 region using annotate function (Schultz *et al.* 2006, Selig *et al.* 2008, Schultz & Wolf 2009, Koetschan *et al.* 2010, Koetschan *et al.* 2012, Merget *et al.* 2012). Secondary structures of the annotated ITS2 sequences were then predicted using predict function of the ITS2 database that attempts to fold sequences directly or uses homology modeling method (Wolf *et al.* 2005, Keller *et al.* 2009, Koetschan *et al.* 2010). Both sequences and secondary structure information of the ITS2 region were aligned and manually adjusted using

4SALE 1.7 (Seibel *et al.* 2006, Seibel *et al.* 2008). ITS2 aligned sequences obtained from MAFFT were replaced by aligned ITS2 sequences provided by 4SALE 1.7. The *matK* aligned sequences were manually adjusted based on the annotation of the complete *matK* sequences including sequences of *Dendrobium mariae* (*Epigeneium clemensiae*) Schuiteman & Adams (2011: 66), *D. parciflorum* Reichenbach ex Lindley (1859: 4) and *D. pulchellum* Roxburgh (1832: 486). In the case of *rbcL* aligned sequences, the primary aligned sequences were manually adjusted based on the reading frame information defined in the annotation of the database sequences. The combined *matK*–*rbcL* aligned matrix was assembled using SequenceMatrix1.7.8 (Vaidya *et al.* 2011).

Phylogenetic analyses

The adjusted aligned ITS and combined *matK*–*rbcL* sequences were submitted to PartitionFinder (Lanfear *et al.* 2012) to select the best partitioning scheme and model of sequence evolution. To analyze each aligned matrix, the branch lengths parameter was set to linked. The list of evolutionary models to be searched by this program was restricted to the models used in MrBayes RAXml and BEAST. All possible schemes were searched. The best evolutionary models for each partition and the partitioning scheme were selected based on Bayesian Information Criterion (BIC; Posada & Buckley 2004). The selected best model of sequence evolution was then used with MrBayes v3.2.2 (Ronquist *et al.* 2012) and maximum likelihood method in raxmlGUI 1.3.1 (Silvestro & Michalak 2012). *Dendrobium pulchellum* was set as the outgroup for every analysis.

The partitioning scheme and model of sequence evolution were set as suggested by the program PartitionFinder (Lanfear *et al.* 2012). Four parameters: substitution rates, frequencies of each nucleotide, gamma shape parameter, and proportion of invariable sites, were adjusted for each partition. Four Markov chain Monte Carlo (MCMC) analyzes were used for each run and two independent runs for each dataset. The MCMC analyzes were run for 500,000 generations; however, it was set to stop when the average deviation of split frequencies was less than 0.01. The parameter values and trees were sampled every 1,000 generations. The first 25% of the samples were discarded as burn-in.

The partitioning scheme was set as suggested by PartitionFinder (Lanfear *et al.* 2012). The GTR substitution model was used for every analysis. The gamma distribution of rate variation across sites and proportion of invariable sites were set as suggested by the PartitionFinder (Lanfear *et al.* 2012). Rapid bootstrap analysis with 1000 replicates was used.

A tree was constructed by StarBEAST v.2.1.3 (Heled & Drummond 2010, Bouckaert *et al.* 2014). Multiple specimens of the same species were mapped to a single taxon, except for four specimens of *B. cambodianum* that were mapped to two terminals: *B. cambodianum* I and *B. cambodianum* II. ITS, *matK*, and *rbcL* with additional partitioning based on codon position (1+2,3) were treated as separated partitions. The *matK* gene partition was linked to the third base of *rbcL* codon partition and the generalized time-reversible model with gamma distribution (GTR+G) was applied to these partitions. The Tamura-Nei model with gamma distribution (TN93+G) was applied to ITS1–ITS2 partition. The HKY model with gamma distribution (HKY+G) was applied to 5.8s rDNA partition. The HKY model with invariable proportion of sites (HKY+I) was applied to *rbcL* partition that contains only the first and the second base of its codon. For every partition, a strict molecular clock model was applied. A piecewise linear and constant root was set as a population size model. Speciation was set as Yule model. For MCMC process, 50 million steps were run without pre-burnin, and the parameter values were stored to a log file every 5,000 steps. TreeAnnotator (Sieder *et al.* 2007) was applied to summarize a set of trees provided by StarBEAST (Heled & Drummond 2010, Bouckaert *et al.* 2014). Burn-in percentage and posterior probability limit were set as 25% and 0.5, respectively. A maximum clade credibility tree with median node heights was set as a targeted tree.

The phylogenetic trees obtained from MrBayes v3.2.2 (Ronquist *et al.* 2012), raxmlGUI (Silvestro & Michalak 2012) and the tree constructed using StarBEAST (Heled & Drummond 2010, Bouckaert *et al.* 2014) were viewed and adjusted using FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Results

Sequence alignment and partition analysis:—All sequences obtained in this study were compared to GenBank database sequences using NCBI nucleotide BLAST (blastn) (<http://blast.ncbi.nlm.nih.gov>), and the results confirmed that these sequences are from orchids. In the case of plastid genes, *rbcL* and *matK*, the aligned sequences without gaps of all sequences including sequences obtained from this analysis and selected database sequences were 481 bp and 780 bp, respectively. The aligned ITS matrix with gaps was 765 bp long.

Due to different evolutionary constraints on different codon positions and different functions between the two ITS and 5.8S rDNA regions, partition analysis was applied to examine these differences. As shown in Table 2, the best scheme for partitioning ITS region was the separation of the two spacers from the 5.8S rDNA. In the case of *matK*–*rbcL* sequences, the best scheme was composed of two partitions: one was the combination of all codon positions in *matK* gene and the third codon position in the *rbcL* gene and another was the combination of the first and the second codon position in the *rbcL* gene. The model of sequence evolution for each partition is shown in Table 2.

TABLE 2. The best scheme of partitioning and model of sequence evolution for each partition.

DNA region	Number of partitions	Subset partitions	Evolutionary model
ITS	2	ITS1 and ITS2	GTR+G ^{a,b}
		5.8s rDNA	GTR+G ^a ,K80+G ^b
<i>matK</i> – <i>rbcL</i>	2	<i>matK</i> and the third base of codon of <i>rbcL</i>	GTR+I+G ^a , GTR+G ^b
		The first and the second base of codon of <i>rbcL</i> gene	GTR+I+G ^a ,JC+I ^b

a: The evolutionary model applied to construct phylogenetic tree based on Maximum likelihood method using program RAxML (Silvestro & Michalak 2012).

b: The evolutionary model applied to construct phylogenetic tree based on Bayesian method using program Mr.Bayes (Ronquist *et al.* 2012).

Phylogenetic analysis of ITS:—We performed phylogenetic analysis on aligned sequences of ITS from the orchid species listed in Table 1. The Bayesian analysis produced a tree topology similar to that based on ML. Here we only present the Bayesian tree (Fig. 1) with the posterior probabilities (PP) and ML bootstrap percentages (BP).

A group of former *Trias* species, *Drymoda* and all *Bulbophyllum* species were clustered together with high support (100 BP, 1.00 PP). The topology of this cluster showed that the *Bulbophyllum* species included in this study could be divided into two groups corresponding well with their provenance: Asia (87 BP, 1.00 PP) and South America/Africa (97 BP, 1.00 PP). All *Trias* and *Drymoda* species were included in the group of Asian *Bulbophyllum*. This cluster of Asian *Bulbophyllum* could be divided into six clades: T, B1, B2, B3, B4 and B5. Clade T (100 BP, 1.00 PP) comprised all *Trias* species. Within the T clade, *Trias* species could be further separated into three groups. Group T1 (100 BP, 1.00 PP) comprised *B. cambodianum*. Group T2 (99 BP, 1.00 PP) comprised *B. disciflorum*, *B. nasutum*, *B. pictum* and *B. roseum* and group T3 (66 BP, 0.72 PP) comprised *B. meson*, *B. leion* and *B. oblongum*. In group T1 accessions 2, 3, and 4 of *B. cambodianum* were sister to accession 1. Clade B1 (99 BP, 1.00 PP) was comprised of seven species of sect. *Sestochilus* (Breda 1827: t. 3) Bentham & Hooker (1883: 502), *Stenochilus* Smith (1914: 33) and *Beccariana* Pfitzer in Engler and Prantl (1888: 179). Clade B2 (98 BP, 1.00 PP) comprised *B. membranaceum* Teijsmann & Binnendijk (1854: 488), sect. *Monanthaparva* Ridley (1896: 269) and *B. cheiropetalum* Ridley (1926: 477; sect. *Epicrianthes* (Blume 1825: 306) Hooker (1890: 753). Clade B3 (69 BP, 0.99 PP) included three members from sect. *Stachysanthes* (Blume 1825: t. 64) Vermeulen & O’Byrne (2008: 152). Clade B4 (100% BS and 1.00 PP) comprised *B. restrepia* Ridley (1893: 365; sect. *Hoplandra* Vermeulen (2008: 147) and two species from sect. *Hirtula* Ridley (1908: 68): *B. hirtulum* Ridley (1900: 71) and *B. limbatum* Lindley (1840: 171). *Bulbophyllum ayuthayense* was sister to these clades (T and B1–B4), and clade B5 (51 BP, 0.95 PP) included *B. dayanum* Reichenbach (1865: 434; sect. *Acrochaena* Lindley (1853: 1), *B. acuminatum* Ridley (1907: 81; sect. *Cirrhopetalum* (Lindley 1830: 45) Reichenbach 1861: 259) and *B. lilacinum* Ridley (1896: 276; sect. *Racemosae* Bentham & Hooker (1883: 502).

Bulbophyllum species in the cluster of South American *Bulbophyllum* could be separated into five clades: B6, B7, B8, B9 and B10. Clade B6 (100 BP, 1.00 PP) comprised three *Bulbophyllum* species of sect. *Napelli* Reichenbach (1861: 249). Clade B7 (100 BP, 1.00 PP) comprised three members of sect. *Xiphizusa* Reichenbach (1861: 919). Clade B8 (100 BP, 1.00 PP) included two species from sect. *Dydactyle* Lindley (1902: 595). Clade B9 (100 BP, 1.00 PP) comprised two members of sect. *Micranthae* Barbosa Rodrigues (1882: 117), and clade B10 (85 BP, 0.92 PP) comprised two species from sect. *Bulbophyllaria* Reichenbach (1864: 613). *Bulbophyllum nagelii* Williams, (1939: 144; sect. *Furvescens* Smidt, Borba and van den Berg [2011: 1061]) was sister to the other South American species of *Bulbophyllum*. The African *Bulbophyllum*, *B. falcatum* Reichenbach (1861: 258; sect. *Megaclinium* Fischer & Vermeulen 2014: 15) was sister to these South American clades.

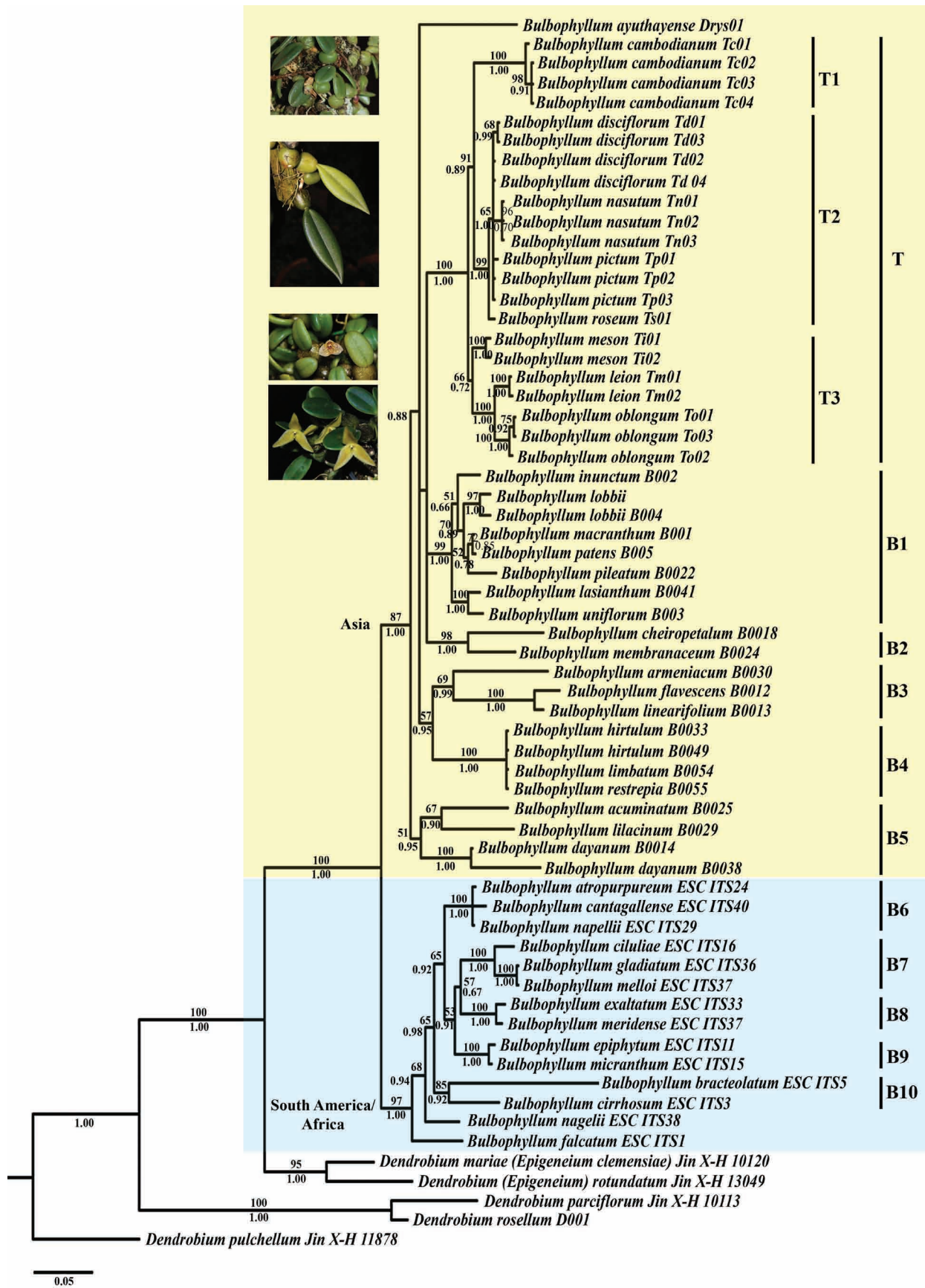


FIGURE 1. Phylogenetic tree of *Trias* and *Bulbophyllum* based on nuclear ITS sequence. The Bayesian tree of *Trias* species, *Drymoda* and 32 *Bulbophyllum* species with five outgroup species. Numbers above branches are maximum likelihood bootstrap percentage (BP), numbers below branches are Bayesian posterior probabilities (PP). A dash (-) above branches are values below 50 BP. Clades of *Trias* and *Bulbophyllum* are indicated. *Trias* photographs represent vegetative morphological characters unique to each *Trias* clade.

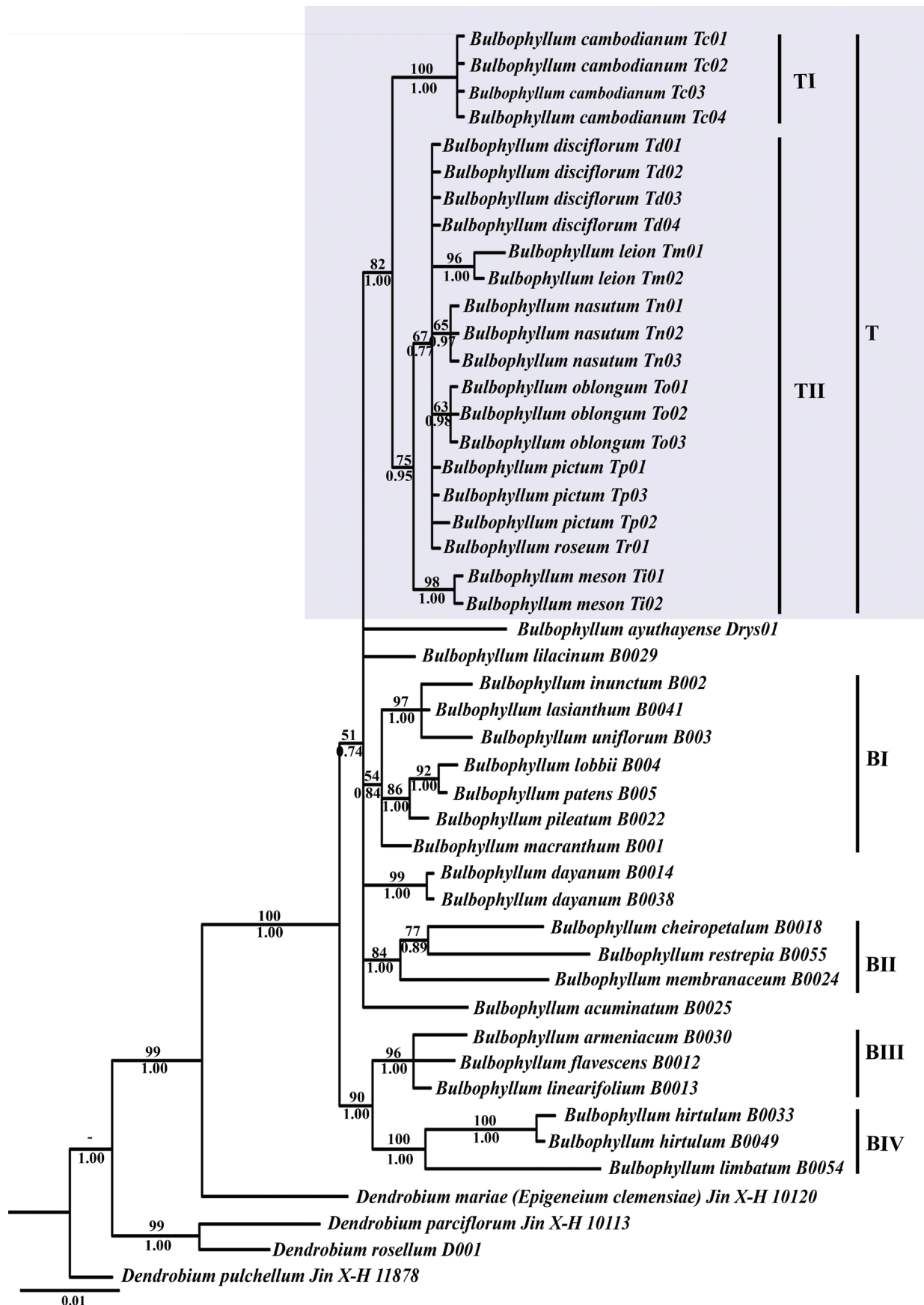


FIGURE 2. The phylogenetic tree of *Trias* and *Bulbophyllum* based on plastid *matK-rbcL* sequence. The Bayesian tree of *Trias* species, *Drymoda* and 18 Asian *Bulbophyllum* species with four outgroup species: *Dendrobium pulchellum*, *D. parciflorum*, *D. rosellum* and *D. mariae*. Numbers above branches are maximum likelihood bootstrap percentage (BP), numbers below branches are Bayesian posterior probabilities (PP). Clades of *Trias* and *Bulbophyllum* are indicated.

Phylogenetic analyses of *matK*–*rbcL* sequences:—Due to a limited number of *matK* and *rbcL* sequences of *Bulbophyllum* species deposited in the GenBank database, South American/African *Bulbophyllum* species could not be included in this analysis. Both *matK* and *rbcL* sequences of *Dendrobium (Epigeneium) rotundatum* Hooker (1890: 712) were excluded from this analysis because its *matK* sequence generated a large gap in the alignment. The same methods as above were used to analyze the *matK*–*rbcL* sequences with similar results for each method, so we only present the Bayesian tree with the PP and BP displayed (Fig. 2).

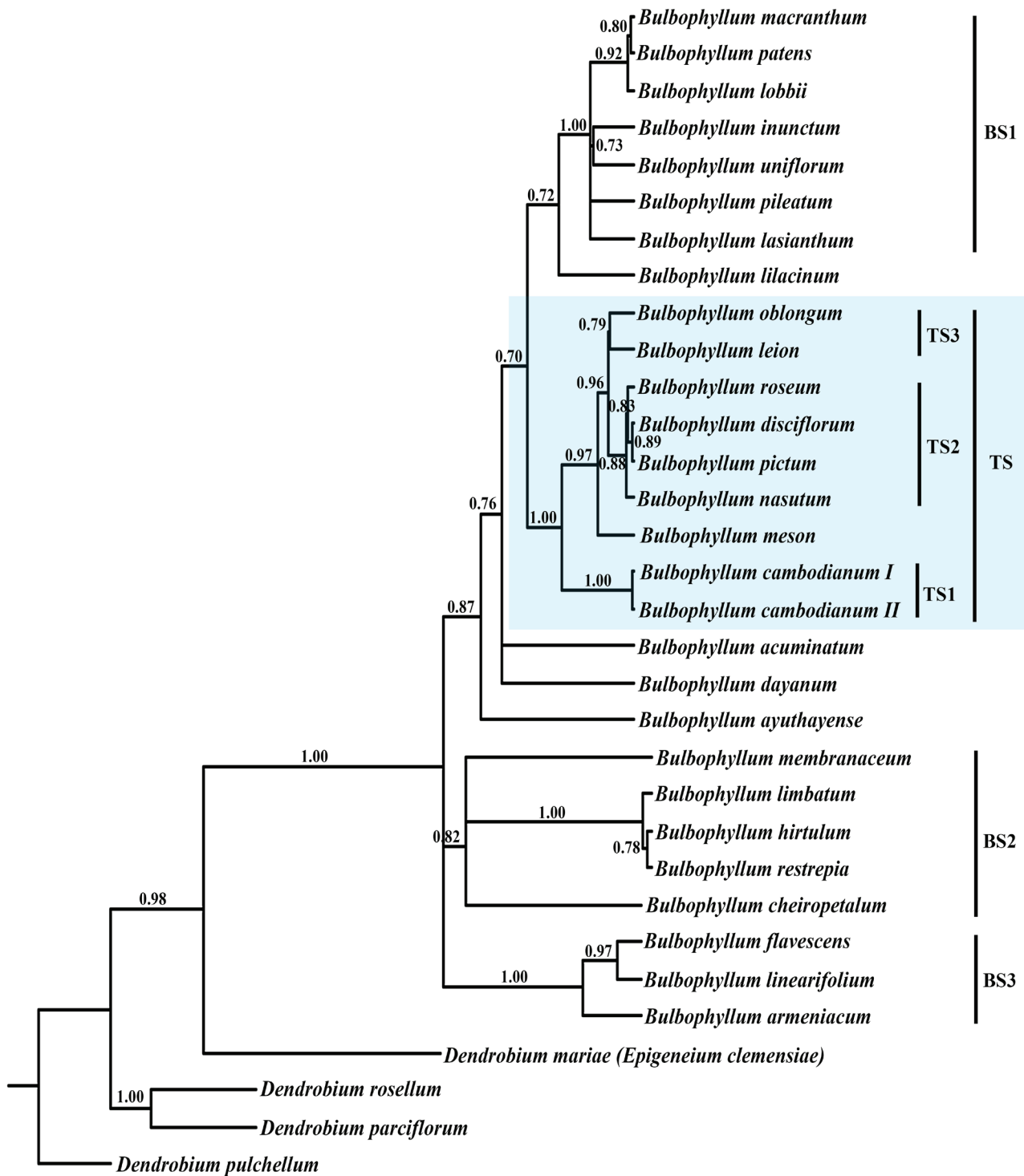


FIGURE 3. The species tree of *Trias* and *Bulbophyllum* based on ITS and plastid *matK*–*rbcL* sequences. The species tree constructed by StatBEAST utilizing ITS, *matK*, and *rbcL* for eight *Trias* species, *Drymoda* and 18 *Bulbophyllum* species with four outgroup species: *Dendrobium pulchellum*, *D. parciflorum*, *D. rosellum* and *D. mariae*. Numbers above branches are posterior probabilities (PP). Clades of *Trias* and *Bulbophyllum* are indicated.

The topology of the tree constructed from the combined *matK-rbcL* sequences suggested that all Asian *Bulbophyllum* (including former *Trias* species) included in this study could be separated into five clades: T, BI, BII, BIII and BIV. Importantly, this tree showed that all *Bulbophyllum* species (including *Trias* and *Drymoda* species) were clustered together, with high support (100 BP, 1.00 PP). This tree also showed that all species of former *Trias* and *Drymoda* species were embedded within groups of Asian *Bulbophyllum*.

Regarding the former *Trias* species, this tree showed them to be monophyletic (82 BP, 1.00 PP). This sect. *Trias* clade could be further divided into two groups. The first group (TI) comprised all *B. cambodianum* samples (100 BP, 1.00 PP). The second group (TII; 75 BP, 0.95 PP) comprised all other *Trias* species. *Bulbophyllum meson* was sister to the rest of group TII.

Other Asian *Bulbophyllum* can be separated into four clades. Clade BI (54 BP, 0.84 PP) comprised seven species: *B. inunctum* Smith (1906: 215), *B. lasianthum* Lindley (1855: 53), *B. uniflorum* Griffith (1851: 293), *B. lobbii* Lindley (1847: sub t. 29), *B. patens* King ex Hooker (1890: 187), *B. pileatum* Lindley (1844: 73), and *B. macranthum* Lindley (1844: t. 13), which belong to sections *Sestochilus*, *Stenochilus* and *Beccariana*. Clade BII (84 BP, 1.00 PP) comprised three species from three sections: sect. *Monanthaparva* (*B. membranaceum*), sect. *Hoplandra* (*B. restrepia*) and sect. *Epicrianthes* (*B. cheiropetalum*). Clade BIII (96 BP, 1.00 PP) comprised three members of sect. *Stachysanthes*: *B. linearifolium* King & Pantling (1897: 586), *B. flavescens* Lindley (1830: 54) and *B. armeniacum* Smith (1917: 70). Finally, clade BIV (100 BP, 1.00 PP) comprised two species of sect. *Hirtula*: *B. hirtulum* and *B. limbatum*. Based on the tree topology, other four *Bulbophyllum* species: *B. ayuthayense* (sect. *Drymoda*), *B. lilacinum* (sect. *Racemosae*), *B. dayanum* (sect. *Acrochaene*.) and *B. acuminatum* (sect. *Cirrhopetalum*), are sister to all clades of *Trias* species and clade BI and BII.

Two major differences between the nrITS region and plastid *matK-rbcL* results were observed. The first one was the clustering among *Trias* species and the second one was the grouping within some clades of other Asian *Bulbophyllum*. The tree constructed from *matK-rbcL* sequences indicated two groups within the *Trias* clade, whereas that of ITS had three groups within this clade. Considering members of each group, we observed that TII (75 BP, 0.95 PP) with *matK-rbcL* tree was the combination of T2 (99 BP, 1.00 PP) and T3 (66 BP, 0.72 PP) for ITS. ITS provided greater resolution than the plastid genes.

Species tree analysis:—Different genes could provide different tree topologies that could be embedded within a shared species tree. By using four *Dendrobium* species as outgroups, all *Trias* and some *Bulbophyllum* species formed a clade (1.00 PP) with four subclades: TS, BS1, BS2 and BS3 (Fig. 3). Asian species of *Bulbophyllum* could be divided into three clades: BS1, BS2 and BS3. Considering the relationship between the *Trias* and *Bulbophyllum*, the *Trias* clade was closely related with *Bulbophyllum* group of clade BS1, specifically here *B. lilacinum* (0.70 PP). This group of *Trias*, BS1 clades and *B. lilacinum* was sister to *B. dayanum* and *B. acuminatum*. This group of *Trias* and *Bulbophyllum* were sister to *Bulbophyllum* clades BS2 and BS3.

Discussion

The discriminatory power of nuclear ITS ribosomal DNA sequences and two plastid gene sequences from the group of *Trias* species was evaluated in this study. All trees presented in this study showed that *Trias* and *Bulbophyllum* shared a common ancestor (100 BP, 1.00 PP), within which clade the *Trias* species formed a subclade, showing a close relationship between the *Trias* clade and species of sects. *Sestochilus*, *Stenochilus* and *Beccariana*. The placement of these Indochinese *Trias* species in the clades of other *Bulbophyllum* sections supported the position of these orchid taxa within the *Bulbophyllum* (Pridgeon *et al.* 2014, Vermeulen *et al.* 2014).

By comparing the topology of the trees, both Bayesian and ML trees constructed from ITS region sequences had resolution higher than the ones constructed from combined plastid genes. The topology of the ITS tree also corresponded well with the species tree constructed based on all markers included in this study. In orchids, the ITS region demonstrated relatively high efficiency in discriminating interspecific relationship within various groups of orchids when used in combination with plastid markers, such as *rbcL*, *matK*, *trnS-trnG*, *trnH-psbA* spacers and *trnL* intron (Smidt *et al.* 2011, Batista *et al.* 2013, Xiang *et al.* 2013). An extensive study that aimed to assess the core seed plant barcode (*rbcL+matK*) and the other two regions: nrITS and plastid *trnH-psbA*, showed that nrITS demonstrated the highest discriminatory power among the four markers in almost all 23 angiosperm orders tested (Li *et al.* 2011), a result supported by our results.

For the *Trias* clade, the ITS tree (Fig. 1) demonstrated that it could be separated into three groups: T1 (*B.*

cambodianum), T2 (*B. nasutum*, *B. disciflorum*, *B. pictum*, and *B. roseum*), and T3 (*B. meson*, *B. oblongum* and *B. leion*). The *matK-rbcL* tree (Fig. 2) indicated that the *Trias* clade included two groups: T1 and TII. All samples of *B. cambodianum* were clustered in one group (T1) as in the ITS tree. The other seven *Trias* species were clustered in the TII group with one species, *B. meson*, sister to the other six species. The species tree clustered members in groups T1 and T2 of the ITS tree and in group T1 of the *matK-rbcL* tree. The species tree only partially supported the clustering of members in ITS group T3; it showed a group of *B. oblongum* and *B. leion* (group TS3) but indicated that *B. meson* was sister to TS2 (*B. rosea*, *B. disciflorum*, *B. pictum*, and *B. nasutum*) and TS3. The clustering of *Trias* demonstrated by the ITS tree and the species tree corresponded well with the similarity of vegetative growth characteristics among members of each group. Vegetative characteristics were also useful for distinguishing clades within *Dendrobium* (Adams 2011, Xiang *et al.* 2013).

ITS group T1 (100 BP, 1.00 PP) comprised four specimens of two morphologically divergent accessions of *B. cambodianum*. The vegetative features of these specimens were similar, and all were recognised taxonomically as *B. cambodianum*, but the flowers were different. Specimen 1 (Christenson 2003; Fig. 4A) has pale yellow flowers with purple dots on the sepals, both edges of petals and proximal half of the lip, with the shape of the dorsal sepal and lateral sepals being slightly different and the dorsal slightly more slender than the laterals. The operculum is yellow and terete with a prolongation longer than its body, and the leaves of this specimen are leathery, blue green with an obtuse apex. Specimens 2–4 (Fig. 4B) have similarly shaped sepals with purple dots that are slightly darker and more densely distributed in the basal part of the sepals than in the apex and darker purple dots that cover the petals including the lip densely and with the dorsal sepal bent forward at almost a 90-degree angle. The operculum is conical-cylindrical obtuse. The lip is ovate, obtuse, slightly retuse, and pale yellow with dark purple dots. Their leaves are pale green, 2.0–2.5 × 2.0–2.5 cm long. Our ITS trees demonstrated that the *B. cambodianum* specimens 2–4 were grouped together (98 BP, 0.91 PP), which coincides with the morphology of these specimens. Based on both tree topology and morphological characters, we speculate that these four *B. cambodianum* specimens could either be a natural variation of *B. cambodianum* or be an entirely different species. The lack of geographical information for these accessions of sect. *Trias* prevented us from making further speculation in this study. More samples of known provenance are needed to examine this issue in the future.

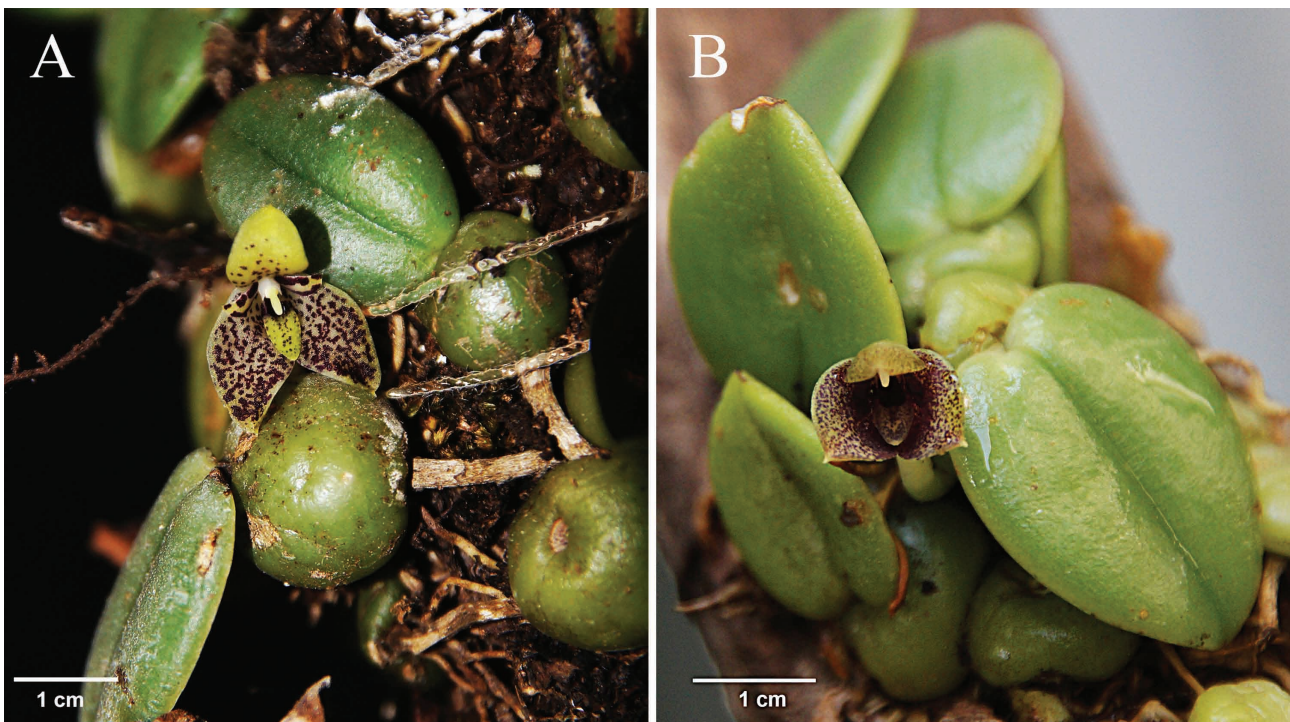


FIGURE 4. *Bulbophyllum cambodianum*. Two *Bulbophyllum cambodianum* specimens with different flower morphology. *B. cambodianum* I (A) has pale yellow flower with purple dots on the sepals, both edges of petals and proximal half of the lip, with the top sepal and the lateral sepals shapes being slightly different and the top being slightly slender than the lateral. *B. cambodianum* II (B), on the other hand, has equally shaped sepals, with purple dots on its flower that are slightly darker and more densely distributed in the proximal end of sepals than in the apex and having darker purple dots that cover the petals including the lip very densely with its dorsal sepal bent forward at almost a 90-degree angle.

The ITS T2 group (99 BP, 1.00 PP) and species tree TS2 (0.88 PP) comprise four species: *B. disciflorum*, *B. nasutum*, *B. pictum* and *B. roseum*. These four species share similar habits with long (5–10 cm) linear acute leaves and distant ovate pseudobulbs. Neither ITS nor *matK-rbcL* could clearly separate samples of *B. disciflorum* from those of *B. pictum*, which corresponds well with the similar flower morphology of these taxa. They have pale yellow flowers with reddish-purple dots densely clustered rather than evenly on the sepals. The lip and petals are covered densely with these purple dots. The sepals and petals of *B. disciflorum* are linear and acute, whereas all three sepals of *B. pictum* are ovate triangular. The operculum of *B. disciflorum* is yellow whereas that of *B. pictum* is pale yellow to white. Although the colour and pattern of *B. disciflorum* and *B. nasutum* are different, the shape of the flowers is similar.

ITS group T3 (66 BP, 0.72 PP) contained the smallest species of sect. *Trias*, compared to the other two groups described previously; it includes *B. meson*, *B. leion*, and *B. oblongum*. In addition, all three *Trias* species in this group share similar habits. Pseudobulbs of this group are globose to slightly ovate, 4–15 mm in diameter. Leaves are glabrous, subobtusate, 0.5–4.0 × 0.5–1.0 cm. Flowers are yellow sometimes with pinkish-red sepalal veins. Their operculum has a similar Y-fork projection. The flower morphology of *B. meson* was slightly different from the other two species. Both *B. leion* and *B. oblongum* flowers are yellow with similarly sized sepals, whereas the flower of *B. meson* is yellowish-white and has ovate sepals with reddish-purple stripes on all sepals. In addition, the lateral sepals of *B. meson* are slightly larger than dorsal sepal. The morphological differences between *B. meson* and the other two *Trias* species in this group correspond well with the topology of group T3 with *B. meson* samples as sister to the samples of *B. leion* and *B. oblongum*. These morphological differences could further explain why *B. meson* was not clustered with *B. leion* and *B. oblongum* in the species tree.

The clusters of *Bulbophyllum* in the ITS tree formed two distinct groups: Asian and South American/African *Bulbophyllum* (87 BP, 1.00 PP for the former and 97 BP, 1.00 PP for the latter). In other studies as here, 15 formerly recognised genera of Bulbophyllinae were suggested to be subsumed into *Bulbophyllum* with some of these genera, i.e. *Trias* and *Drymoda*, treated as sections in *Bulbophyllum*. Other genera, i.e. *Sunipia*, were included in existing sections (Pridgeon *et al.* 2014, Vermeulen *et al.* 2014).

The low resolution of the *matK-rbcL* tree could either be a consequence of using only a small region of *matK* and *rbcL* or related to the limitations of these two markers in species identification of these orchid taxa. Although the *rbcL* and *matK* sequences of all *Trias* species generated in this study were nearly complete with the size of approximately 1,300 bp and 1,500 bp, respectively, all database sequences of *Bulbophyllum* were only 481 bp, and 780 bp long, respectively. The length of aligned *rbcL* and *matK* sequences applied to phylogenetic analyses were only 481 and 780 bp, respectively. It is therefore possible that informative parts of these plastid genes were unintentionally excluded. In addition, the discriminatory power of *rbcL* and *matK* sequence may be limited to genus level (Hosseini *et al.* 2012, Moudi *et al.* 2013). The inadequate resolution of *matK-rbcL* tree called for the use of other DNA markers such as nrITS region in the phylogenetic analysis in order to resolve the relationship among *Trias* species.

Even though this study revealed relationships between *Trias* and other groups of *Bulbophyllum* as well as those between some sect. *Trias* species, a number of questions are still unanswered. For example, what is the placement of other *Trias* species in relation to this Indochinese group; what is a suitable marker for identifying sect. *Trias* species; and how do the complex sections of *Bulbophyllum* related to each other? Even though these questions involve different systematic levels, all of them demand the same requirements: development of new pipeline or markers to identify species and sections.

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