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Research article

Phylogenetic relationships of selected Sri Lankan Orchids based on Internal Transcribed Spacer (ITS) sequence analysis

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Abstract: Orchidaceae is a widespread plant family and Sri Lankan orchid flora represents 188 species belonging to 78 genera, including 01 endemic genus (*Adrorhizon*) and 55 endemic species. The main aim of the present research was to characterize selected species of genera *Dendrobium* and *Bulbophyllum* in Sri Lanka with respect to their ITS sequence data and to derive phylogenetic relationships. Modified CTAB protocol was followed for DNA extractions and ITS region was amplified using the primer sets of 17SE and 26SE and phylogenetic trees were constructed based on the available ITS sequences of the Asian species of *Dendrobium* and *Bulbophyllum* by MEGA7 software package. Genetic variation and relationships of six Sri Lankan orchid species; *Dendrobium aphyllum*, *D. crumenatum*, *D. nutantiflorum* endemic species of *Bulbophyllum elliae*, *B. trimenii* and *Eria bicolor* were determined using ITS sequencing. Findings of the analysis conclude, suitability of ITS sequencing as a molecular marker for deriving phylogenetic relationships of genera *Dendrobium* and *Bulbophyllum* with *Eria* as the out group. Further, indiscretions in derived relationships of these taxa with respect to ITS sequences can be interpreted as the effect of geographical isolation occurred during the continental drift.
Keywords: *Dendrobium* - *Bulbophyllum* - Phylogeny - Sri Lankan orchids - ITS sequence.

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INTRODUCTION

The Orchidaceae is one of the most species-rich and cosmopolitan plant families in angiosperms with, approximately 19,500 species (Dressler 1993, Cozzolino & Widmer 2005, Begum *et al.* 2009, Stern 2014). The most recent figure is 26,567 species and 899 genera (http://www.theplantlist.org). They can be found in every biome types except true deserts and polar regions (Jayaweera 1981, Ng & Hew 2000).

Bulbophyllum Thouars is the genus with the highest number of species in the family Orchidaceae (Dressler 1993, Fischer *et al*. 2007, Ribeiro *et al.* 2008) and the number of species comprise in the genus has estimated as about 2400 (Sieder *et al*. 2007). Further, *Dendrobium* Sw. is the third largest genus of orchids representing 1184 species (Begum *et al.* 2009, Leitch *et al*. 2009). Both these genera belong to the subfamily Epidendroideae and tribe Dendrobieae, and genus *Bulbophyllum* is included in subtribe Bulbophyllinae while the genus *Dendrobium* in subtribe Dendrobiinae. These two genera have shown great ornamental and medicinal value. Especially genus *Dendrobium* is broadly categorized as horticultural, agricultural, medicinal or dual-purpose plants considering their utility (Begum *et al*. 2009, Yuan *et al*. 2009). The Asian continent is with the highest orchid diversity while Sri Lanka being one of the tropical islands in the Indian Ocean is rich in orchids having nearly 188 species in 78 genera with fifty-five endemic species and one endemic genus. Eight indigenous *Dendrobium* species; *Dendrobium aphyllum* Roxb**.** (S: Posonmal and Kaputuwesak), *D. panduratum* Lindl., *D. didon* Reichb.f, *D. crumenatum* Swartz (E: Pigeon orchid), *D. nutantiflorum* A**.**D**.** Hawkes & A**.**H**.** Heller, *D. heterocarpum* Wall. ex Lindl. (E: Primrose orchid), *D. bambusifolium* Par. et Reichb.f. and *D. maccarthiae* Thw. (S: Wesak mal) and

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twelve *Bulbophyllum* species; *Bulbophyllum crassifolium* Thwaites ex Trimen, *B. elegans* Gardner ex Thwaites, *B. elliae* Rchb. f., *B. jayaweerae* Fernando et Ormerod, *B. macraei* (Lindl.) Rchb. f., *B. maskeliyense* Livera, *B. petiolare* Thwaites, *B. purpureum* Thwaites, *B. thwaitesii* Rchb. F., *B. tricarinatum* Petch, *B. trimenii* (Hook.f.) J.J. Sm. and *B. wightii* Rchb. f. are found in Sri Lanka. All *Bulbophyllum* species except *B. elegans*, *B. macraei* and *B. maskeliyense* are endemic to Sri Lanka (Fernando & Ormerod 2008). Some of these wild species are very attractive in floriculture, with a high potential of developing as novel cultivars targeting promising features.

The molecular phylogenetic analysis provides a strong tool in species characterization, identification and resolving taxonomic ambiguities. Currently, many authors have been engaged in using DNA sequence data to resolve phylogenetic and identification ambiguities of plants at different taxonomic levels (Bytebier *et al.* 2007). Further, many studies have shown that multilocus sequence analysis using rDNA-ITS region and m*atk* genes are useful in delineating *Dendrobium* species. However, ITS region has demonstrated relatively high efficiency in discriminating interspecific relationships within various groups of orchids (Wonnapinij & Sriboonlert 2015). Further, nrITS sequence data are sufficient for inferring phylogenetic relationships among *Dendrobium* species and *Bulbophyllum* species (Wonnapinij & Sriboonlert 2015, Moudi & Go 2015). Therefore, the present study aimed to characterize the selected species of genera *Dendrobium* and *Bulbophyllum* in Sri Lanka with respect to their ITS sequence data and to derive their phylogenetic relationships with the species in mainland India and associated islands.

MATERIAL AND METHODS

Plant material

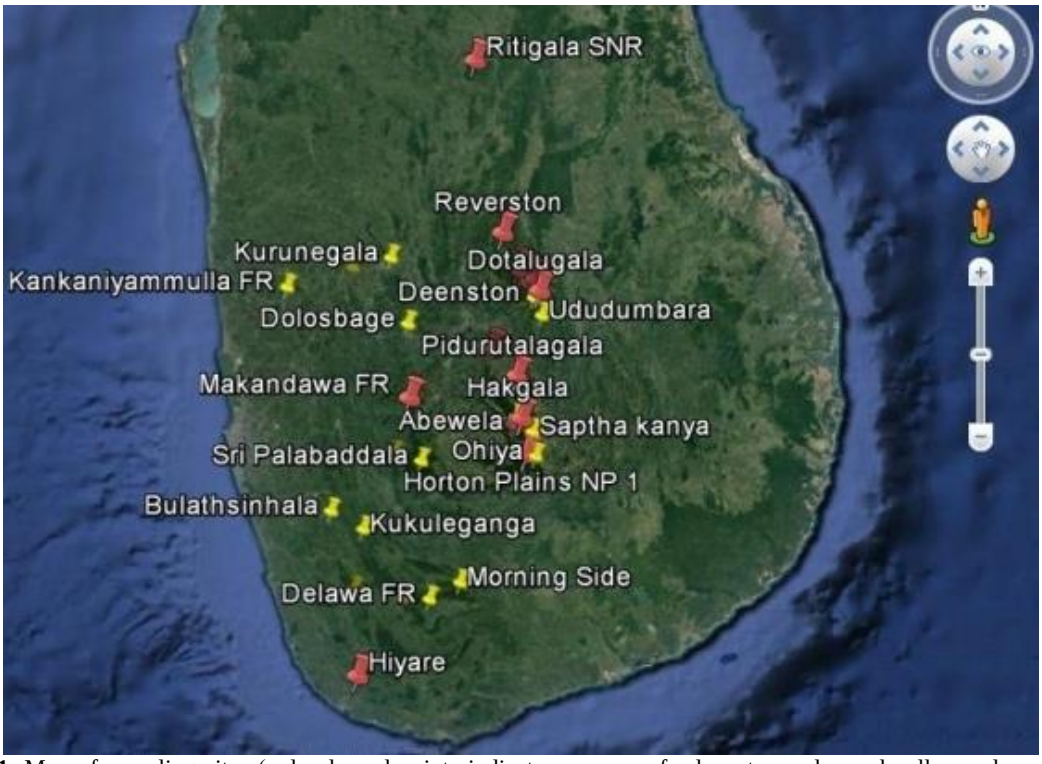

Figure 1. Map of sampling sites (red coloured points indicate presence of relevant samples and yellow coloured points indicate absence of relevant samples).

Forests in wet zone of Sri Lanka were explored for the specimen collection of the two genera *Dendrobium* and *Bulbophyllum* (Fig. 1). Plant material of three species of the genus *Dendrobium*: *D. aphyllum*, *D. crumenatum*, *D. nutantiflorum*, two species of the genus *Bulbophyllum*: *B. elliae* & *B. trimenii* and *Eria bicolor* Lindl. were sampled (Fig. 2). Individuals of the collected taxa were maintained in a greenhouse in the Royal Botanical garden, Peradeniya, Sri Lanka. The identity of the specimens was authenticated against the herbarium specimens in the National Herbarium, Peradeniya, Sri Lanka and voucher specimens were deposited. Nomenclatural priority was given to Fernando & Ormerod (2008) over taxonomic literature on Jayaweera (1981) and world checklist of Orchidaceae (Govaerts *et al.* 2006). *E. bicolor* was selected as the out-group considering its suitability.

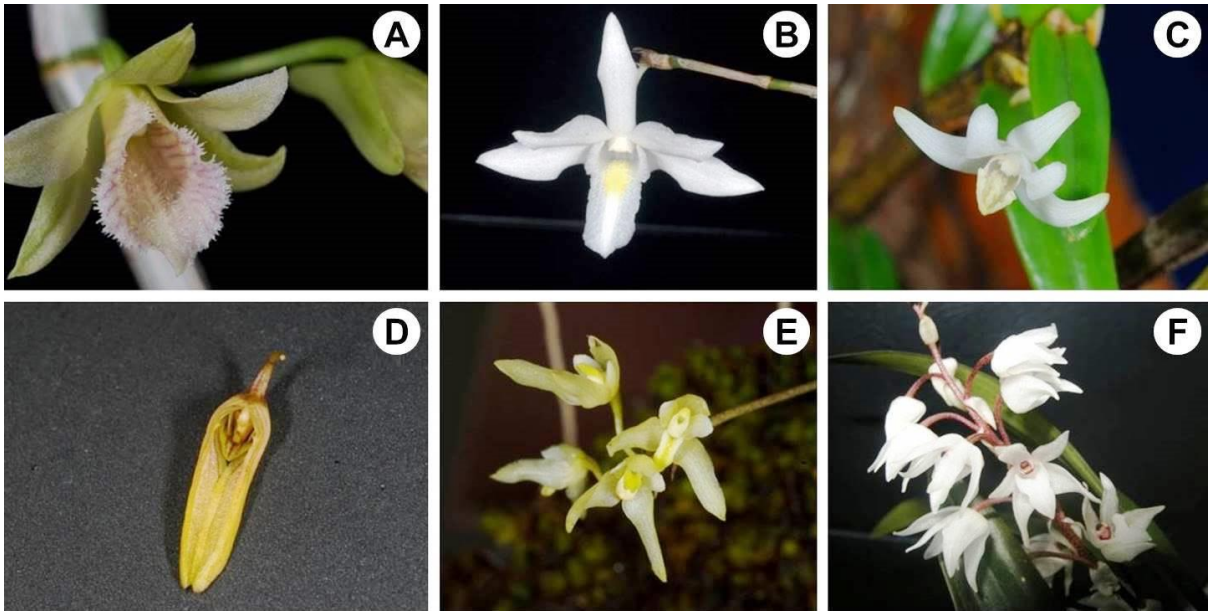

Figure 2. Selected species of *Dendrobium* and *Bulbophyllum* species in Sri Lanka: **A**, *Dendrobium aphyllum* Roxb.; **B**, *Dendrobium crumenatum* Swartz; **C**, *Dendrobium nutantiflorum* A.D. Hawkes & A.H. Heller; **D**, *Bulbophyllum elliae* Rchb. f.; **E**, *Bulbophyllum trimenii* (Hook.f.) J.J. Sm.; **F**, *Eria bicolor* Lindl.

DNA extraction

Plant genomic DNA was extracted at the laboratory, Department of Molecular Biology and Biotechnology, University of Peradeniya, Sri Lanka following the modified CTAB protocol by Russell *et al.* (2009).

Fresh material or silica gel-dried materials were subjected to DNA extractions. Approximately 0.5–1.0 g of fresh and frozen clean plant leaf pieces were grounded in 1 ml of cold sorbitol buffer with a small amount of quartz powder using a mortar and pestle. Further, approximately 50–120 mg of silica gel-dried and clean plant tissue was ground in a 2 ml micro-centrifuge tubes with 4 glass beads using the Mini Beadbeater (BioSpec, UK).

Grounded plant material was transferred into 2 ml micro-centrifuge tubes and cold sorbitol buffer was added up to 2 ml. The plant material was completely dissolved by incubating in the tube on ice for 20 minutes after vortexing. Then the mixture was centrifuged at 10,000 rpm for 10 minutes and the supernatant was decanted carefully. The washing step was carried out repeatedly until the supernatant turns into colourless. After washing 700 µl of 3x CTAB extraction buffer and 30 µl of 30% sarkosyl were added and incubated in a thermo block for one hour at 60ºC. Then 700 µl of chloroform : isoamyl alcohol was added, mixed well by inverting the tube and kept at room temperature for 20 minutes, then centrifuged at 10,000 rpm for 10 minutes at room temperature. The clear upper aqueous layer (~700–800 µl) was transferred to a new micro-centrifuge tube and DNA was precipitated by adding 1/10 volume of 3 M sodium acetate solution (pH 5.2) and 2/3 volume of cold absolute isopropanol, incubating the tubes at -20ºC overnight. Then DNA was pelleted by centrifugation at 14,000 rpm for 20 minutes. The solution was decanted and the pellet was washed twice with 0.5 ml of 75% ethanol (centrifuged at 10,000 rpm for 10 minutes). Ethanol was decanted and the pellet was washed once with 0.5 ml of 100% ethanol, then ethanol was decanted and the pellet was dried at room temperature. Finally, the pellet was dissolved in 50 µl of TE buffer and stored at -20ºC. Purity of the extracted DNA was confirmed by spectrophotometry. DNA quality and quantity were checked on 1% agarose gels with ethidium bromide.

PCR amplification

DNA from all the collected species were subjected to a polymerase chain reaction (PCR). The internal transcribed spacer (ITS) region was amplified as described by Hidayat *et al.* (2007) and Takamiya *et al.* (2011) using the primer sets of 17SE (ACGAATTCATGGTCCGGTGAAGTGTTCG) and 26SE (TAGAATTCCCCGGTTCGTTCGTCGCCGTTAC). Each PCR reactions was 50 µl, containing 40 ng of genomic DNA as a template, 25 µl of Gotaq PCR master mix, 2.5 µl of each primer at 10 µM and 15.0 µl of Nuclease free water. Reaction conditions were an initial denaturation at 94ºC for 3 min, followed by 30 cycles of 94ºC for 30 S, annealing temperature 55ºC for 30 S and 72ºC for 1 min and 40 S and final extension of 72ºC for 7 min in TAKARA PCR machine. The 5 µl of PCR products were subjected to gel electrophoresis in 1.5% agarose containing ethidium bromide. PCR products were observed using gel documentation system (Vilberlomart).

Sequencing

Sequencing was carried out by ABI 3500 genetic analyzer (Applied Biosystems®) at the Department of Molecular Biology and Biotechnology, University of Peradeniya, Sri Lanka following Sanger method. Thirty micro litters of amplified ITS regions were purified using a Gene Clean kit and subjected to sequencing. The ITS region of each individual PCR product was sequenced in both 5" and 3" directions. Resulted sequences were submitted to the genbank.

Taxa identification

BLAST search was performed for all the obtained ITS sequences and to verify the sequences of other recorded species in the website of the NCBI (http://www.ncbi.nlm.nih.gov/blast/blast.cgi).

Phylogenetic analysis

Phylogenetic analysis was performed to infer the evolutionary relationships of the species compared to mainland India and associated islands in the Indian Ocean using available ITS sequences in the NCBI database (Table 1).

Table 1. List of taxa used for phylogenetic analysis and their country, genbank accession numbers and reference.

Taxon	Country	Gen bank Accession Number	Reference
Dendrobium crumenatum Swartz	Malaysia	KC507780	Moudi & Go (2014)
Dendrobium aloifolium (Blume) Rchb.f.		KC507775	
Bulbophyllum inunctum J.J. Sm.		KC507773	
Bulbophyllum macranthum Lindl.		KC507772	
Dendrobium tosaense Makino	Taiwan	HM590367	Wu *et al.* (2012)
Dendrobium moniliforme (L.) Sw.		HM590369	
Dendrobium somae Hayata		EU840692	
Dendrobium somae Hayata		AF521616	
Dendrobium aloifolium (Blume) Rchb.f.	Thailand	AY239951	Clements (2003);
Dendrobium formosum Roxb. ex Lindl.		AY239967	Clements (2003)
Bulbophyllum siamense Rchb.f.		EF195942	Fischer *et al.* (2007);
Bulbophyllum smitinandii Seidenf. & Thorut		EF195943	Fischer *et al.* (2007)
Dendrobium moniliforme (L.) Sw.	Japan	AY239981	Clements (2003)
Dendrobium parthenium Rchb.f.		AB847668	
Dendrobium papilio Loher		AB847667.	
Dendrobium panduriferum Hook. f.		AB847666	
Dendrobium oligophyllum Gagnep.		AB847665	
Bulbophyllum japonicum Makino		AB786894	
Dendrobium inflatum Rolfe	Indonesia	AY239973	Clements (2003);
Dendrobium lancifolium A. Rich.		AY239976	Clements (2003)
Bulbophyllum hamatipes J.J. Sm.		EF195929	Fischer *et al.* (2007)
Dendrobium papilio Loher	Philippines	AY239987	Clements (2003);
Dendrobium yeageri Ames & Quisumb.		AY240006	Clements (2003)
Bulbophyllum alsiosum Ames		EF195917	Fischer *et al.* (2007);
Bulbophyllum cumingii (Lindl.) Rchb.f.		EF195923	Fischer *et al.* (2007)
Dendrobium aqueum Lindl.	India	KM983096	Gen bank
Dendrobium aphyllum Roxb.		EU840691	
Dendrobium nobile Lindl.		EF618732.1	
Dendrobium chrysanthum Wallich ex Lindl.		AF355572	
Bulbophyllum reptans (Lindl.) Lindl.		JN114443	
Bulbophyllum cariniflorum Rchb. f.		KF866243	
Bulbophyllum careyanum (Hook.) Spreng.		KF866244	
Bulbophyllum nodosum (Rolfe) J.J. Sm.		KF866241	
Bulbophyllum odoratissimum (Sm.) Lindl. ex wall		KF866242	
Dendrobium jenkinsii Wall. ex Lindl.	China	EF629321	Yuan *et al*. (2009);
Dendrobium aphyllum Roxb.		AF355573	Yuan *et al*. (2009);
Bulbophyllum ambrosia (Hance) Schltr.		KC568306	Gen bank
Bulbophyllum odoratissimum (Sm.) Lindley ex wall		FJ428223	
Bulbophyllum affine Lindl.		KC568305	
Dendrobium aphyllum Roxb.	Sri Lanka	MH763848	Present study
Dendrobium crumenatum Swartz		MH763846	
Dendrobium nutantiflorum Hawkes & Heller		MH763847	

Bulbophyllum elliae Rchb.f.	Sri Lanka	MH763845	Present study
Bulbophyllum trimenii (Hook.f.) J.J. Sm.		MH763849	
Eria bicolor Lindl.		MH763844	

The phylogenetic tree was constructed referring to Feng *et al.* (2015). ITS sequences were aligned initially with Clustal W in MEGA7 (Kumar *et al.* 2016). Then the phylogeny was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The bootstrap consensus tree inferred from 500 replicates was used to represent the evolutionary history of the analyzed taxa and branches corresponding to partitions reproduced in less than 50% bootstrap replicates were not considered (Felsenstein 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and were in the units of the number of base substitutions per site. All the missing data were eliminated, by the software package MEGA7, to minimize the errors that could have occurred in the analysis process.

RESULTS

Phylogeny of Dendrobium species

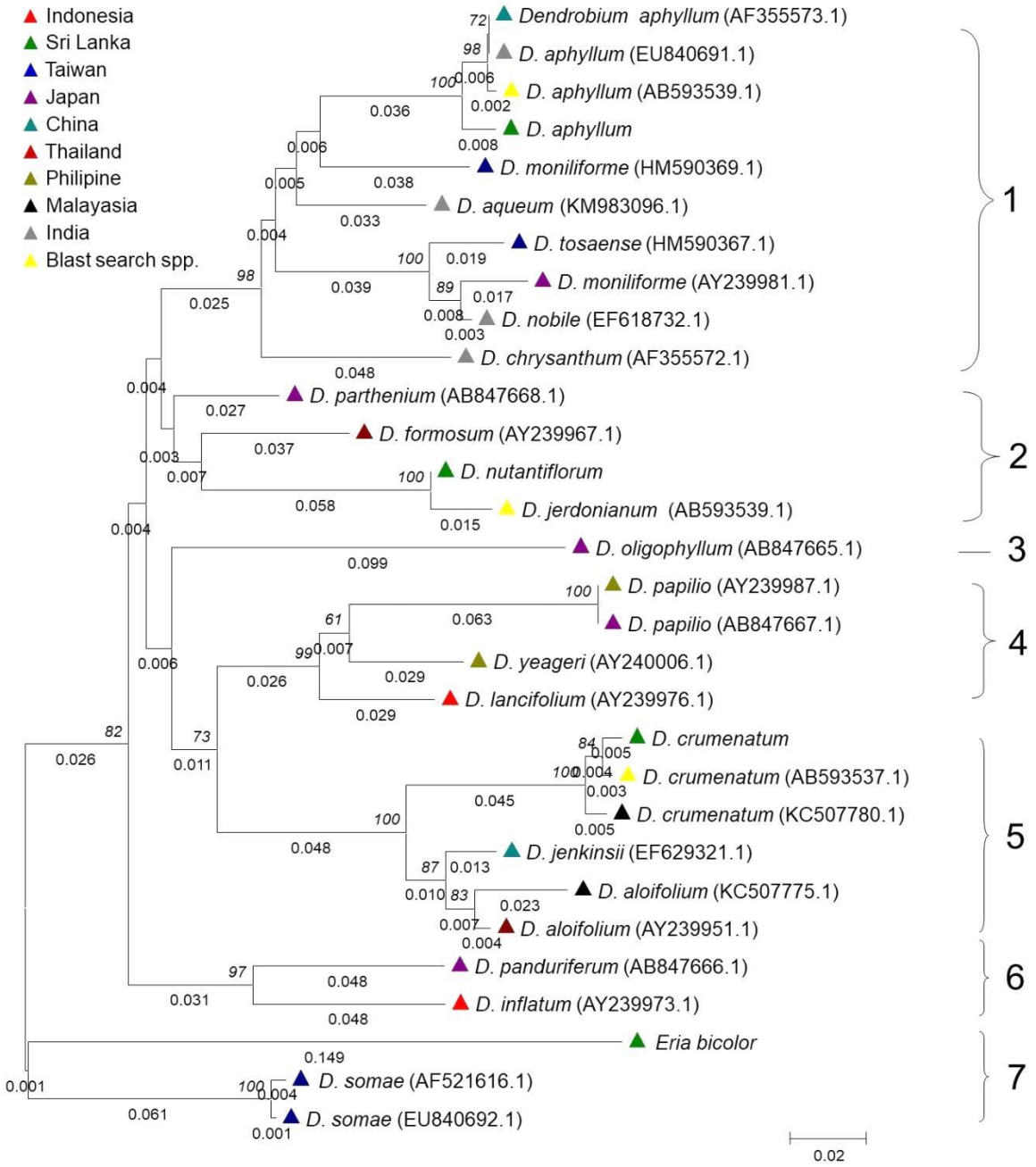

Figure 3. ITS sequencing data based phylogenetic relationships of *Dendrobium* taxa from mainland India and associated islands were inferred by the Neighbor-Joining method in MEGA7. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Bootstrap values above 50% calculated are stated. The scale bar represents five base substitutions for 100 nucleotide positions.

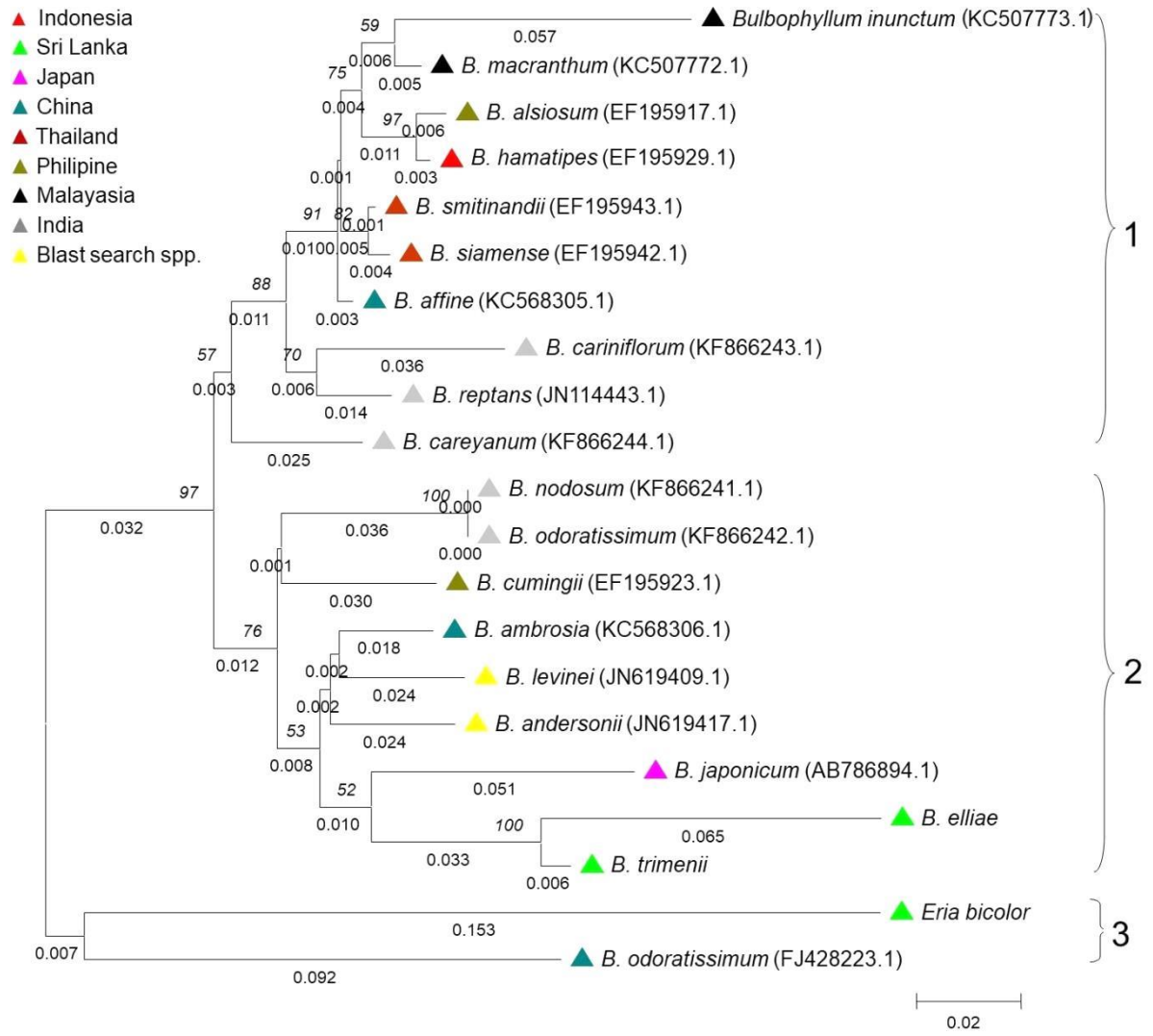

Figure 4. ITS sequencing data based phylogenetic relationships of *Bulbophyllum* taxa from mainland India and associated islands were inferred by the Neighbor-Joining method in MEGA7. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Bootstrap values above 50% calculated are stated. The scale bar represents five base substitutions for 100 nucleotide positions.

Seven clusters were derived in the phylogenetic tree for *Dendrobium* taxa from mainland India and associated islands (Fig. 3). The tree was rooted on the selected out-group; *Eria bicolor* and *Dendrobium somae* (AF521616.1 and EU840692.1) from Taiwan. The other *Dendrobium* species were separated into six clusters with bootstrap value of 82. Based on the clustering pattern *D. panduriferum* (AB847666.1) from Japan and *D. inflatum* (AY239973.1) from Indonesia in cluster 6 can be considered as basal members of the analysed taxa.

Dendrobium species from India; *D. aphyllum* (EU840691.1), *D. aqueum* (KM983096.1), *D. nobile* (EF618732.1) and *D. chrysanthum* (AF355572.1), China; *D. aphyllum* (AF355573.1), Taiwan; *D. moniliforme* (HM590369.1), *D. tosaense* (HM590367.1), Japan; *D. moniliforme* (AY239981.1), and *D. aphyllum* (AB593539.1) formed into cluster 1 together with *D. aphyllum* from Sri Lanka with bootstrap value of 98. However, *D. aphyllum* was distinct within the cluster 1 with bootstrap value of 100. Further, *D. aphyllum* from India, China and other blast searched species have formed a separate clade with bootstrap value of 98, without indicating any evolutionary distance while *D. aphyllum* of Sri Lanka has separated into a line expressing evolutionary distance.

Blast search produced the cluster 2 representing species of Japan; *D. parthenium* (AB847668.1), Thailand; *D. formosum* (AY239967.1) and Sri Lanka; *D. nutantiflorum* together with *D. jerdonianum* (AB593539.1). However, *D. nutantiflorum* of Sri Lanka and *D. jerdonianum* (AB593539.1) have shown close relatedness by producing an internal cluster with bootstrap value of 100.

Species of Philippine; *D. papilio* (AY239987.1) and *D. yeageri* (AY240006.1), Japan; *D. papilio* (AB847667.1) and Indonesia; *D. lancifolium* (AY239976.1) have formed the cluster 4 by clustering *D. papilio* of Japan and Philippine with bootstrap value of 100 and showing high evolutionary relatedness.

Cluster 5 consists of *Dendrobium* species of Malaysia; *D. crumenatum* (KC507780.1), *D. aloifolium* (KC507775.1), China; *D. jenkinsii* (EF629321.1), Thailand (*D. aloifolium* (AY239951.1) and Sri Lanka; *D. crumenatum*, in which *D. crumenatum* have formed a seperated clade with the bootstrap value of 100. *D. crumenatum* of Sri Lanka and *D. crumenatum* (AB593537.1) resulted by NCBI blast search are together while separating *D. crumenatum* (KC507780.1) of Malaysia by bootstrap value of 100. However, *D. crumenatum* of Sri Lanka and *D. crumenatum* (AB593537.1) have formed internal clades with bootstrap value of 84.

Phylogeny of Bulbophyllum species

The phylogenetic tree of *Bulbophyllum* taxa derived using the information of taxa of mainland India and associated Islands have produced three clusters (Fig. 4). The phylogenetic tree has rooted in the selected outgroup; *Eria bicolor* and *Bulbophyllum odoratissimum* (FJ428223.1) from China.

All the other *Bulbophyllum* species were clustered into two with the bootstrap value of 97. Species of India, China and Philippine were positioned in both clusters; cluster 1 and cluster 2. Only the species; *B. alsiosum* (EF195917.1) of Philippine and *B. hamatipes* (EF195929.1) from Indonesia have formed an internal cluster within cluster 1 with the bootstrap value of 97 while all the other species of the same country were not formed any internal clusters with species from different countries. While *B. odoratissimum* (FJ428223.1) in China is placed in the root of the tree, *B. odoratissimum* (KF866242.1) of India has formed a separate cluster together with *B. nodosum* (KF866241.1) of India within the cluster 2 with bootstrap value of 100.

Sri Lankan *Bulbophyllum* species (*B. elliae* and *B. trimenii*) and *B. japonicum* (AB786894.1) of Japan have formed a common cluster with bootstrap value of 52 within the cluster 2. Furthermore, Sri Lankan *Bulbophyllum* taxa have formed a separate cluster together with bootstrap value of 100. However, Chinese *B. odoratissimum* (FJ428223.1) has shown close relatedness with the out-group, *Eria bicolor* while Indian *Bulbophyllum odoratissimum* (KF866242.1) has grouped with other *Bulbophyllum* species.

DISCUSSION AND CONCLUSION

Findings of the research can be considered as the first attempt in using ITS sequencing for determination of genetic variation and inferring phylogenetic relationships of six species of Sri Lankan orchids; *Dendrobium aphyllum*, *D. crumenatum*, *D. nutantiflorum*, endemic species of *Bulbophyllum elliae*, *B. trimenii* and *Eria bicolor* according to the currently available research information. Most of the previous studies on species characterization, identification, phylogeny and genetic variation in orchid genera were based on another molecular sequencing; matK, trnH-psbA, ITS, trnL-F, rbcL and rps16, and psaB (Kores *et al.* 2001, Kocyan *et al.* 2004, Fischer *et al.* 2007, Pansarina *et al.* 2008, Yao *et al.* 2009, Takamiya *et al.* 2011). However, in orchids, it was proven that the ITS region demonstrates relatively high efficiency in deriving interspecific relationships (Wonnapinij & Sriboonlert 2015). Further, Wonnapinij & Sriboonlert (2015) and Moudi & Go (2015) reported that nrITS sequence data are sufficient for inferring phylogenetic relationships of *Dendrobium* species and *Bulbophyllum* species. In agreement with the previous research findings, the results of the present study also have proved the facts in favour with the above conclusions. The authenticity of all sequences of the analysed samples, obtained in the study, was confirmed with Gene Bank database sequences using NCBI nucleotide BLAST (blastn) (http://blast.ncbi.nlm.nih.gov) (Table 2). Based on the findings of the present analysis, it can be concluded that ITS sequencing as are liable molecular marker for deriving phylogenetic relationships of genera *Dendrobium* and *Bulbophyllum* with *Eria* as the out-group.

Table 2. Nucleotide blast of ITS regions of studied Sri Lankan taxa.

Taxa	Primer	Description	Query Cover (%)	E value	Identity (%)
Dendrobium aphyllum Roxb.	17SE	*Dendrobium aphyllum* genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 26S rRNA, partial sequence, bio_material: TBG<JPN>:122508	100	0	98
	26SE	*Dendrobium aphyllum* genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 26S rRNA, partial sequence, bio_material: TBG<JPN>:122508	100	0	99
Dendrobium crumenatum Swartz	17SE	*Dendrobium crumenatum* genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 26S rRNA, partial sequence, bio_material: TBG<JPN>:115833	100	0	99
	26SE	*Dendrobium crumenatum* genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 26S rRNA, partial sequence, bio material: TBG<JPN>:115833	100	0	99

Dendrobium nutantiflorum Hawkes & Heller	17SE	*Dendrobium jerdonianum* genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 26S rRNA, partial sequence	100	0	98
	26SE	*Dendrobium jerdonianum* genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 26S rRNA, partial sequence	100	0	98
Bulbophyllum elliae Rchb.f.	17SE	*Bulbophyllum levinei* isolate CBSDLITS 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	100	0	91
	26SE	*Bulbophyllum levinei* isolate CBSDLITS 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	100	0	92
Bulbophyllum trimenii (Hook.f.) J.J. Sm.	17SE	*Bulbophyllum andersonii* isolate SMSDLITS 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	100	0	88
	26SE	*Bulbophyllum andersonii* isolate SMSDLITS 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	100	0	94
Eria bicolor Lindl.	17SE	*Pinalia spicata* voucher SBB-0241 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	100	0	90
	26SE	*Pinalia spicata* voucher SBB-0241 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	100	0	92

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