Pak. J. Bot., 48(4): 1619-1627, 2016.

INTERGENERIC CLASSIFICATION OF GENUS *BULBOPHYLLUM* FROM PENINSULAR MALAYSIA BASED ON COMBINED MORPHOLOGICAL AND *RBC*L SEQUENCE DATA

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Abstract

Bulbophyllum Thou. is largest genus in Orchidaceae family and a well-known plant of tropical area. The present study provides a comparative morphological study of 38 *Bulbophyllum* spp. as well as molecular sequence analysis of large subunit of rubisco (*rbc*L), to infer the intergeneric classification for studied taxa of genus *Bulbophyllum*. Thirty morphological characters were coded in a data matrix, and used in phenetic analysis. Morphological result was strongly consistent with earlier classification, with exception of *B. auratum*, *B. gracillimum*, *B. mutabile* and *B. limbatum* status. Furthermore Molecular data analysis of *rbc*L was congruent with morphological data in some aspects. Species interrelationships specified using combination of *rbc*L sequence data with morphological data. The results revealed close affiliation in 11 sections of *Bulbophyllum* from Peninsular Malaysia. Consequently, based on this study generic status of sections *Cirrhopetalum* and *Epicrianthes* cannot longer be supported, as they are deeply embedded within the genus *Bulbophyllum*.

Key words: *Bulbophyllum*, Orchidaceae, Peninsular Malaysia, *rbc*L

Introduction

The largest collection of subtribe Bulbophyllinae (Orchidaceae) have been defined as *Bulbophyllum*, a genus which form a large, pantropical, and poorly studied group of orchids in Peninsular Malaysia. *Bulbophyllum* species are mostly epiphytic and they are found in different habitats ranging from (sub) tropical dry forests to wet montane cloud forests and most of them are fly pollinated (Tan *et al*., 2002; Nishida *et al*., 2004; Teixeira *et al*., 2004). Latest traditional taxonomy (Seidenfaden & Wood, 1992) was described more than 100 *Bulbophyllum* species of Peninsular Malaysia into 17 sections. However, based on this study, the members of this large genus have undergone extreme reduction in number of species in the natural habitat and have acquired an efficient adaptation to the canopy environment.

Botanical treatises by such eminent botanists as Meisner (1842), Endlicher (1837), Bentham & Hooker (1883), Hooker (1890), Pfitzer (1888) and Schlechter (1914) recognize *Cirrhopetalum* as a genus. Garay (1994) was not concurred with Seidenfaden & Wood (1992) opinion which had transfered all *Cirrhopetalum* to *Bulbophyllum*. He was believed that section *Cirrhopetalum* is a unique genus in the orchid family because it can be defined unmistakeably through a single character, i.e., the way the lateral sepals are formed at the base of the column foot.

Garay & Kittredge (1985) have proposed a return to separate generic status for section *Epicrianthes* but Vermeulen (1991) was pointed at affinities with section *Leptopus*. Systematic study of combined molecular and morphological data on this genus will gain us a better insight. Most systematists agree that morphological data can be potentially useful in phylogenetic analyses, especially in combination with molecular data (Kron *et al*., 2002; Shinwari *et al*., 1994; Nadeem *et al*., 2013; Jamil *et al*., 2014). The structural portion of this study was performed using qualitative characters of rhizome, pseudobulb, leaf and flower for 38 different species. DNA sequence data from the large subunit of rubisco (*rbc*L) which produces the carbon dioxide fixing enzyme of chloroplast and subsequently combination of morphological binary characters with *rbc*L sequence data were analyzed to infer the intergeneric classification of *Bulbophyllum*. The main objectives of the present study was; 1) to determine and tracing the close relationships among various section belonging to the genus *Bulbophyllum* using *rbc*L sequence data and structural data and 2) to verify the status of sections *Cirrhopetalum* and *Epicrianthes*.

Materials and Methods

Sample collection: For this study, 38 species were collected from a variety of locations in Peninsular Malaysia (Table 1). Species are representing 11 sections as described by Holttum (1953), Seidenfaden & Wood (1992). Morphological characters for both vegetative (included the size of the plant, the characters of rhizome, leaves and pseudobulb) and flower structures (included the size and structure of the petals and sepals, shape of the lip, column, structure of the inflorescence and flowers and so on) were studied for identification and description of *Bulbophyllum* species. Voucher specimens for all accessions have been deposited in herbarium of biology department, Universiti Putra Malaysia (UPM).

Morphological study: To construct the species interrelationship by numerical taxonomy, thirty qualitative characters were selected based on those reported by Fischer *et al*., 2007 and our own field observations (Table 2). Qualitative characters were coded as binary/multistate characters (Table 3).

Table 1. Plant materials used in this study.

Code	Section	Taxon	Location	Herbarium/Voucher	Gen bank accession No.
1.	*Hirtula*	*B. dayanum*	Gunung Jerai, Malaysia	UPM/ B0014	JF428000
2.	*Hirtula*	*B. limbatum*	Johor, Malaysia	UPM / B0054	JF428036
3.	*Hirtula*	*B. hirtulum*	Fraser s Hill, Malaysia	UPM / FAN. FH- 314	JF428030
4.	*Cirrhopetalum*	*B. flabellum*	Genting highland, Malaysia	UPM / RG 1945	JF427996
5.	*Cirrhopetalum*	*B. purpurascens*	Cameron Highland, Malaysia	UPM/ B0027	JF428010
6.	*Cirrhopetalum*	*B. vaginatum*	Melacca, Malaysia	UPM/ FAN. FH- 503	JF428005
7.	*Cirrhopetalum*	*B. corolliferum*	Gunung Belumut, Malaysia	UPM / B0026	JF428009
8.	*Cirrhopetalum*	*B. acuminatum*	Gunung Belumut, Malaysia	UPM / RG 2291	JF428021
9.	*Cirrhopetalum*	*B. auratum*	Cameron Highland, Malaysia	UPM / B0060	JF428040
10.	*Cirrhopetalum*	*B. gracillimum*	Genting Highland, Malaysia	UPM / B0053	JF428037
11.	*Aphanobulbon*	*B. flavescens*	Fraser s Hill, Malaysia	UPM / FAN. FH- 062	JF427998
12.	*Aphanobulbon*	*B. mutabile*	Fraser s Hill, Malaysia	UPM / FAN. FH- 105	JF427997
13.	*Aphanobulbon*	*B. linearifolium*	Fraser s Hill, Malaysia	UPM / FAN. FH- 258	JF427999
14.	*Aphanobulbon*	*B. apodum*	Cameron Highlands, Malaysia	UPM / FAN. FH- 276	JF428039
15.	*Aphanobulbon*	*B. odoratum*	Pahang, Malaysia	UPM / B0056	JF428034
16.	*Aphanobulbon*	*B. armeniacum*	Fraser s Hill, Malaysia	UPM / SH.K-105	JF428015
17.	*Desmosanthes*	*B. concinnum*	Genting highland, Malaysia	UPM / RG 2207	JF428006
18.	*Desmosanthes*	*B. sulcatum*	Gunung Jerai, Malaysia	UPM / FAN. FH- 304	JF427995
19.	*Desmosanthes*	*B. angustifolium*	Fraser s Hill, Malaysia	UPM / RG 2313	JF427993
20.	*Desmosanthes*	*B. medusae*	Johor, Malaysia	UPM / B0052	JF428038
21.	*Desmosanthes*	*B. bakhuizenii*	Gunung Jerai, Malaysia	UPM / SH.K-107	JF428019
22.	*Desmosanthes*	*B. obtusum*	Fraser s Hill, Malaysia	UPM / FAN. FH- 172	JF427994
23.	*Sestochilus*	*B. macranthum*	Cameron Highland, Malaysia	UPM / FAN. FH- 153	JF427988
24.	*Sestochilus*	*B. inunctum*	Gunung Jerai, Malaysia	UPM / SH.K-109	JF427989
25.	*Sestochilus*	*B. lobbii*	Cameron Highland, Malaysia	UPM / FAN. FH- 426	JF427991
26.	*Sestochilus*	*B. uniflorum*	Fraser s Hill, Malaysia	UPM / FAN. FH- 107	JF427990
27.	*Sestochilus*	*B. patens*	Gunung Jerai, Malaysia	UPM / B005	JF427992
28.	*Sestochilus*	*B. pileatum*	Gunung Belumut, Malaysia	UPM / RG 2281	JF428007
29.	*Sestochilus*	*B. lasianthum*	Fraser s Hill, Malaysia	UPM / RG 1922	JF428026
30.	*Careyana*	*B. lilacinum*	Gunung Jerai, Malaysia	UPM / B0029	JF428012
31.	*Careyana*	*B. sichyobulbon*	Gunung Jerai, Malaysia	UPM / SH.K-111	JF428002
32.	*Monilibulbus*	*B. stormii*	Cameron Highlands, Malaysia	UPM / B0058	JF428042
33.	*Monilibulbus*	*B. ovalifolium*	Cameron Highland, Malaysia	UPM / RG 2167	JF428003
34.	*Globiceps*	*B. coniferum*	Cameron Highland, Malaysia	UPM / RG1757	JF428041
35.	*Leptopus*	*B. tenuifolium*	Cameron Highland, Malaysia	UPM / B0061	JF428043
36.	*Polyblepharon*	*B. membranaceum*	Fraser s Hill, Malaysia	UPM / B0024	JF428028
37.	*Epicrianthes*	*B. cheiropetalum*	Gunung Jerai, Malaysia Gunung Jerai, Malaysia Gunung Jerai, Malaysia	UPM / B0018	JF428004
38.	*Epicrianthes*	*B. haniffii*	Penang, Malaysia	UPM / B0031	JF428013
39.	*Distichorchis*	*D. pahangensis*	Fraser□ s Hill, Malaysia	UPM / FAN. FH- 180	JF428045

Table 2. List of characters scored for cluster analysis of studied taxa.

No.	Characters description
1.	Size of the plant: 0 = small (≤3 cm) / 1 = intermediate (4-10cm) / 2 = large (≥10cm)
2.	Fresh sheaths or remnants of fresh sheaths covering the rhizome and part of the pseudobulbs: 0 = present/1 = absent
3.	Pseudobulbs: 0 = crowded (distance between bulbs is less than the diameter of a bulb)/ 1 = moderately spaced (distance between bulbs is 1–10 times the diameter of a bulb
4.	Pseudobulb shape: 0= angled/1= ovoid or oblong
5.	Pseudobulb colour: 0 = green& yellow/ 1= brown
6.	Pseudobulbs: 0 =flattened or somewhat flattened /1= no flattened
7.	Pseudobulb: 0= present the hair around the pseudobulb/1= absent the hair
8.	Stem or pseudobulb length: 0= stem/pseudobulb absent or short, 1= elongated
9.	Number of leafs per pseudobulbs: 0 = 1/1 = 2
10.	Leaf apex: 0= blunt/ 1= cleft/ 2= acute or bilobed acute
11.	Leaf thickness: 0 = thick/ 1= thin
12.	Leaf size: 0= small (less than 3 cm long)/ 1= intermediate (between 3-12 cm)/ 2= large (more than 12cm)
13.	Leaf color: 0= green/ 1= otherwise
14.	Peduncle setaceous (bristle like): 0 = yes/1 = no
15.	Inflorescence: 0 = single-flowered/1 = multi-flowered
16.	Flower Size: 0= ≤0.5 cm across, 1= 0.5 - 2 cm across, 2= 2 - 5 cm across
17.	Length of pedicel: 0 = very short (flowers sit on the rachis)/ 1 = moderate to long
18.	Dorsal sepal margin ornamentation: 0 = with hairs/ 1 = glabrous
19.	Surface of dorsal sepal: 0 = with hairs/1 = glabrous
20.	Lateral sepal margin ornamentation: 0 = with hairs/ 1 = glabrous
21.	Surface of lateral sepal: 0 = glabrous/1 = papillose
22.	Sepals length: 0= equal size/1=different size
23.	Sepal markings: 0= without distinct spots or stripes, 1= with distinct spots or stripes
24.	Petal apex margin ornamentation: 0 = with hairs or ciliate/1 = glabrous
25.	Petal apex surface ornamentation: 0 = with hairs, papillose or ciliate/1 = glabrous
26.	Lateral sepals fused (from the column-foot to the middle): 0 = yes/ 1 = no
27.	Lip moveable: 0 = yes/1 = no (lip enclosed by lateral sepals)
28.	Surface of the lip: 0 = papillose or hair/1 = glabrous
29.	Lip apex: 0 = recurved/1 = straight
30.	Column-foot with basal tooth: 0 = present/1 = absent

DNA extraction, PCR amplification and sequencing: DNA was extracted from fresh material using Wizard ® Genomic DNA Purification Kit (Promega). The *rbc*L region was amplified from total DNA extracts using the polymerase chain reaction (PCR). Primers rbcLa_f and rbcLa_ rev were proposed by Consortium for the Barcode of Life (CBOL, 2009) have been used for amplification of *rbc*L. Reaction mixtures contained approximately 2-8 ng of DNA template, 2 µL of 10× reaction buffer, 0.8 µL dNTPs (each 2.5mM), 2.0U Taq polymerase, and 1 µL of each oligonucleotide primer, each at 4 µM concentration, in a final volume 50 µL. The PCR amplification profile included an initial denaturation of 95°C for 4 minutes, 4 cycle of 30 sec denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C then followed by 29 cycle of 30 sec denaturation at 94°C, 1min annealing at 54°C and 1 min extension at 72°C and 5 min final extension at 72°C. Amplified DNA was fractionated by electrophoresis through 3% low-melting agarose gels, recovered from the gels, and purified using Wizard ® PCR Preps DNA Purification System (Promega) according to manufacturer's instructions. Nucleotide sequences of *rbc*L were determined using purified PCR product.

Sequence alignment: Multiple alignments of sequences were performed using CLUSTAL W (Thompson *et al*., 1994). All sequences have been deposited in Genbank (Table 1).

Data analysis: In order to determine the species interrelationships based on morphological characters the species were clustered by unweighted group average (UPGMA) strategy using the simple matching coefficient with NTSYSpc (version 2.1) (Rohlf, 1992). DNA sequence data of *rbc*L and combination of them with morphological data were analyzed with Maximum Parsimony conducted by PAUP* 4.0b10 (Swofford, 2002) and *Dendrobium pahangensis* was designated as outgoup. The MP (Maximum Par-simony) tree was obtained by Heuristic search and boot strap analyses (1000 replications) were performed to test the confidence of every branch in phylogenetic tree.

Table 3. Data matrix of *Bulbophyllum* scored for 30 characters presented in Table 2

OTUs	Character																												
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	20	1	2	3	4	5	6	7	8	9	30
B. dayanum	1	0	1	1	1	1	0	0	0	0	1	0	1	1	1	0	1	0	0	0	0	1	1	0	0	1	1	1	1	0
B. limbatum	1	0	1	1	0	0	1	0	0	1	1	0	1	1	0	0	1	0	0	0	0	1	1	0	0	1	0	1	1	0
B. hirtulum	1	0	0	1	1	1	1	0	0	0	1	0	1	1	0	1	1	1	0	0	0	1	1	0	0	1	1	1	1	1
B. flabellum	1	1	1	0	0	1	1	0	0	0	1	0	1	1	1	0	1	1	0	1	0	1	0	0	1	0	1	1	0	0
B. purpurascens	1	1	1	1	1	1	1	0	0	0	1	0	1	1	0	1	1	1	0	1	1	1	1	0	1	0	1	1	0	1
B. vaginatum	1	1	1	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	0	1	0	0	1	0	1	0	1	1	0	0
B. corolliferum	1	1	1	0	0	1	1	0	0	0	1	0	1	1	0	0	1	1	0	1	0	0	0	0	1	0	1	1	0	0
B. acuminatum	1	1	1	0	0	1	1	0	2	0	1	0	1	1	0	0	1	1	0	1	0	0	0	0	1	0	1	1	0	0
B. auratum	1	1	1	0	0	1	1	0	0	0	1	0	1	1	0	0	1	1	1	0	0	0	0	0	1	0	0	1	0	1
B. gracillimum	1	1	1	0	0	1	1	0	0	1	1	0	1	1	0	0	1	1	1	0	0	1	0	0	1	0	0	1	0	1
B. flavescens	2	1	1	1	0	1	1	0	0	0	2	0	1	1	0	1	1	1	0	0	1	0	1	0	0	0	1	1	0	1
B. mutabile	0	0	1	1	0	1	1	0	0	0	0	0	1	1	0	1	1	1	0	0	1	1	1	0	1	0	1	1	0	1
B. linearifolium	1	1	1	1	0	1	1	0	0	0	2	0	1	1	0	1	1	1	0	0	1	1	1	0	1	0	1	1	0	1
B. apodum	2	1	1	1	0	1	1	0	0	0	2	0	1	1	0	1	1	1	0	0	1	1	1	0	1	0	1	1	0	1
B. odoratum	2	1	1	1	0	1	1	0	0	0	2	0	1	1	0	1	1	1	0	0	1	1	1	0	0	0	1	1	0	1
B. armeniacum	1	1	1	1	0	1	1	0	0	0	1	0	1	1	0	1	1	1	0	0	1	1	1	1	0	0	1	1	0	1
B. concinnum	1	1	1	1	0	1	1	0	2	1	1	0	1	1	0	1	1	1	0	0	1	1	1	0	1	1	1	1	1	1
B. sulcatum	0	1	1	1	0	1	1	0	0	1	1	0	1	1	0	1	1	1	0	0	1	1	1	0	1	0	1	1	0	1
B. angustifolium	1	1	1	1	0	1	1	0	2	1	1	0	1	1	0	1	1	1	0	1	1	1	1	0	1	0	1	1	0	1
B. medusae	1	1	1	0	0	1	1	0	1	0	1	0	1	1	0	1	1	1	0	1	1	1	1	0	1	0	1	1	0	1
B. bakhuizenii	1	1	1	1	0	0	1	0	0	1	1	0	1	1	0	1	1	1	0	0	1	1	1	0	1	0	1	1	0	1
B. obtusum	1	1	1	1	0	1	1	0	0	1	1	0	1	1	0	1	1	1	0	0	1	1	1	0	1	0	1	1	0	1
B. macranthum	2	0	1	1	0	1	0	0	0	0	2	0	1	0	1	1	1	1	0	0	1	1	1	0	1	0	1	1	0	1
B. inunctum	2	0	1	1	0	1	0	0	2	1	2	0	1	0	1	1	1	1	0	0	1	1	1	0	0	0	1	1	0	1
B. lobbii	2	0	1	1	0	1	0	0	0	0	2	0	1	0	1	1	1	1	0	0	1	1	1	0	1	0	1	1	0	1
B. uniflorum	2	0	1	1	0	0	0	0	2	0	2	0	1	0	1	1	1	1	0	0	1	1	1	0	1	0	1	1	0	1
B. patens	2	0	1	1	0	1	0	0	1	0	2	0	1	0	1	1	1	1	0	0	1	1	1	0	1	1	1	1	1	1
B. pileatum	2	0	1	1	0	1	0	0	0	1	2	0	1	0	1	1	1	1	0	0	1	1	1	0	1	1	1	1	1	1
B. lasianthum	2	0	1	1	0	0	0	0	0	0	2	0	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	1	1
B. lilacinum	2	1	1	0	0	1	1	0	0	0	2	0	1	1	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1
B. sichyobulbon	2	1	1	0	0	1	1	0	0	0	2	0	1	1	0	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1
B. stormii	1	1	0	1	0	0	1	0	0	1	0	0	1	0	1	1	1	1	1	0	1	1	1	0	1	0	1	1	0	1
B. ovalifolium	0	1	0	1	0	0	1	0	0	1	0	0	1	0	1	1	1	1	0	1	1	1	1	0	0	0	1	1	0	1
B. coniferum	1	0	0	1	0	1	1	0	0	0	1	0	1	1	0	2	2	2	0	1	2	0	0	0	0	0	1	1	0	0
B. tenuifolium	0	1	1	0	1	1	1	0	0	1	0	0	1	0	1	1	1	1	0	0	1	1	1	0	0	1	1	1	1	0
B. membranaceum	0	1	1	1	0	1	1	0	2	1	0	0	1	0	0	1	1	1	0	0	1	1	1	0	0	0	1	1	0	1
B. cheiropetalum	1	0	1	0	1	1	1	0	2	0	1	1	1	0	0	1	1	1	0	0	0	0	1	0	0	1	1	1	1	1
B. haniffii	1	0	1	0	0	1	1	0	2	0	1	1	1	0	0	1	1	1	0	0	0	0	1	0	0	1	1	1	1	1

Result and Discussion

Species interrelationship based on morphological characters: Two major clusters are evident in Fig. 1 and consisted of 11 sections of *Bulbophyllum* which have been described by Seidenfaden & Wood (1992). The first cluster contains species of section *Hirtula*. Despite the fact that *Bulbophyllum limbatum* was described inside section *Hirtula* (Seidenfaden & Wood, 1992), in this analysis it was close to section *Cirrhopetalum* and *Careyana*. Meanwhile, Holttum (1953) described *B. lilacinum* (section *Careyana*) and *B. limbatum* (section *Hirtula*) in a same section. So status of *B. limbatum* in this analysis was fairly corresponded with Holttum's suggestion.

Second major cluster consisted of following sections: 1) section *Cirrhopetalum* with some analogous characters, such as umbellate inflorescence or unequal size of sepals. In this analysis *B. gracillimum* and *B. auratum* parted from the *Cirrhopetalum*, and this was different with previous taxonomy (Seidenfaden &Wood, 1992). 2) Section *Aphanobulbon* with identical characters such as inconspicuous pseudobulb, raceme inflorescence and short stelids on column redound to the close species relationship. *Bulbophyllum mutabile* was described in section *Aphanobulbon*, but in this analysis it was far away. Plant size of *B. mutabile* is much smaller and the inflorescence is bearing 2-3 flowers in opposition of other species (30-60 flowers). However, inconspicuous pseudobulb was an ordinary character for all species of *Aphanobulbon*. In this

case, study on anatomical characters could be useful. 3) Next sub-cluster; contain 6 species of section *Desmosanthes*, shown close affiliation with section *Aphanobulbon*. All species in section *Desmosanthes* were small in size (1-6 cm) with raceme or subumbellate inflorescence and creeping or hanging rhizome. *Bulbophyllum medusae* with long lateral sepals same as species of section *Cirrhopetalum*, were placed inside section *Desmosanthes* as well, and this was corresponded with latest description. 4) Next sub-cluster consisted of seven species of section *Sestochilus* which was in agreement with Seidenfaden & Wood (1992) suggestion. Their rather large flowers with large and non-ciliate petals were recognized these species in the same group. 5) *Bulbophyllum lilacinum* and *B. sichyobulbon* in the next sub-cluster were described under section *Careyana* and this was corresponded with Seidenfaden & Wood (1992). 6) Section *Monilibulbus* in the next sub-cluster contains 2 species. Distinguished characters for section *Monilibulbus* are flattened and very close pseudobulb with erect top. Seidenfaden & Wood (1992) with Holttum (1964) had same suggestion for status of these species. *Bulbophyllum tenuifolium* and *B. membranaceum* have very tiny flower and papillose lip, like *B. ovalifolium*, so they were placed in the same cluster. 7) *Bulbophyllum cheiropetalum* and *B. haniffii* were placed in section *Epicrianthes* and this is in agreement with earlier classification (Seidenfaden & Wood, 1992). Papillose lip as a key character used to place *B. coniferum* (Section *Globiceps*) close to section *Epicrianthes*. Holttum (1964) placed this species in section 12 and Seidenfaden & Wood (1992) proposed section *Globiceps*.

The *rbc*L Data Analysis: *rbc*L gene sequences were obtained from the 38 *Bulbophyllum* species and *Dendrobium pahangensis*. The aligned sequences consisted of 493 nucleotide sites. 469 characters were identical among all taxa, 32 sites were variable, and 14 were parsimony informative. Average percentage sequence divergence (uncorrected p distance) within *Bulbophyllum* species was 0.8 %, and maximum in-group *rbc*L divergence was 2.4% (between *B. sulcatum* with *B. ovalifolium*).

The consensus tree (Fig. 2) inferred from 370 most parsimonious trees is shown. Branches corresponding to partitions reproduced in less than 50 % trees are collapsed. [Tree length= 103, consistency index (CI) = 0.72, retention index (RI) = 0.85, homoplasy index (HI) = 0.70]. The percentages of parsimonious trees in which the associated taxa clustered together are shown next to the branches.

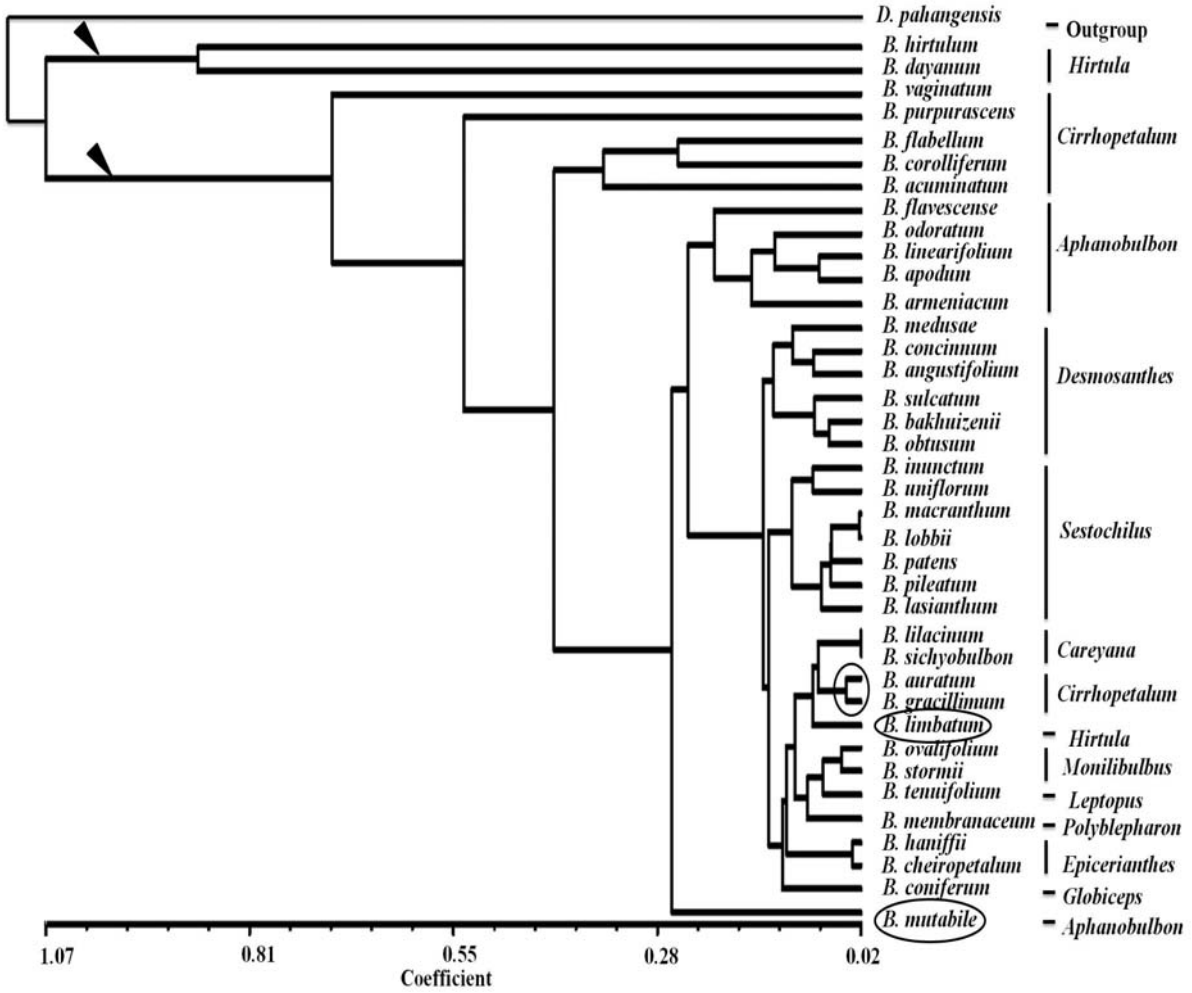

Fig. 1. Relationships of *Bulbophyllum* species using qualitative morphological characters.

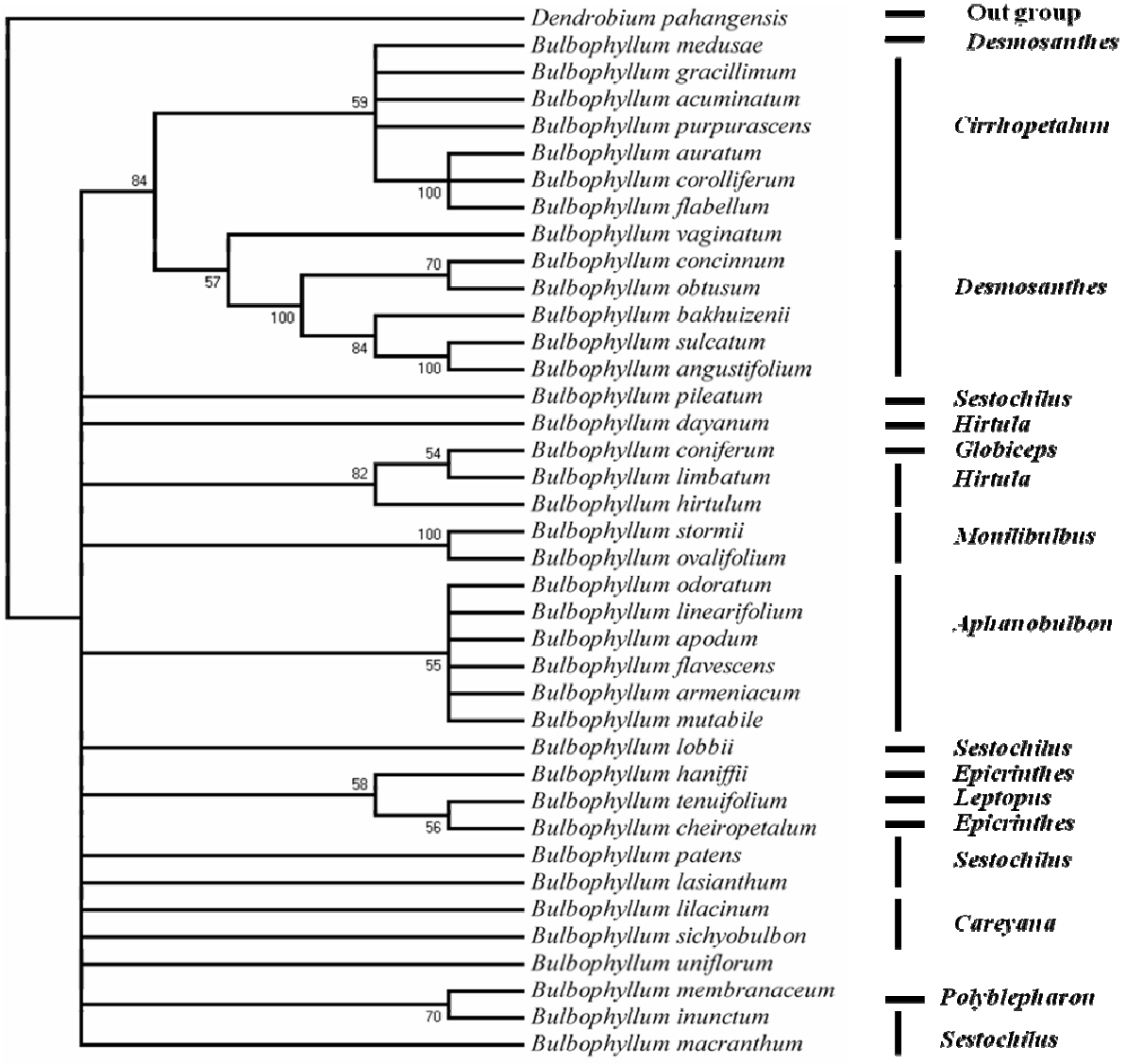

Fig. 2. The consensus tree inferred from 370 most parsimonious trees is shown for *rbc*L region. Bootstrap percentage ≥ 50 are indicated above the nodes.

In maximum parsimony tree of *rbc*L data, separation of species in some clades was not strongly supported but for some others, it was congruent with morphological studies. This result was anticipated, since most of the nucleotides in *rbc*L gene among the studied taxa were identical. Following information was obtained from Fig. 2. Section *Cirrhopetalum* and *Desmosanthes* had very close affiliation with almost strong bootstrap support (BP84); even *B. medusae* were nested inside *Cirrhopetalum* and *B. vaginatum* inside *Desmosanthes*. The MP tree has revealed unresolved status for species of section *Sestochilus*. This result was corresponding with *mat*K sequence analysis (Gravendeel *et al*., 2006; Hosseini *et al*., 2012) Based on the result these species must define as a new different section or sections. *Bulbophyllum dayanum* cannot longer be defined in section *Hirtula*. This result was consistent with *mat*K sequences (Hosseini *et al*., 2012) and combined different regions analysis (Hosseini *et al*., 2016). Similar results were reported by studies using *rbc*L (Shinwari *et al*., 2011, 2014). Furthermore, *B. limbatum* has shown close relationship with *B. coniferum*. Sample developing can help to improve the status of section *Globiceps*. The status of section *Monilibulbus* and *Aphanobulbon* were consistent with morphological result but species of section *Aphanobulbon* were appeared in the polytomous clade. So, status of this section could not be fully ascertained. MP analysis showed close relationship of section *Epicrianthes* and *Leptopus*. Nevertheless, the addition of a new molecular datasets with enhance number of species need to clarifying of the sectional delimitation. Based on the result *Bulbophyllum inunctum* can be transferred into section *Polyblepharon* (BP=70). But since the other results (Hosseini *et al*., 2012; Hosseini *et al*., 2016) were inconsistent with this situation, so the alteration cannot be proposed easily.

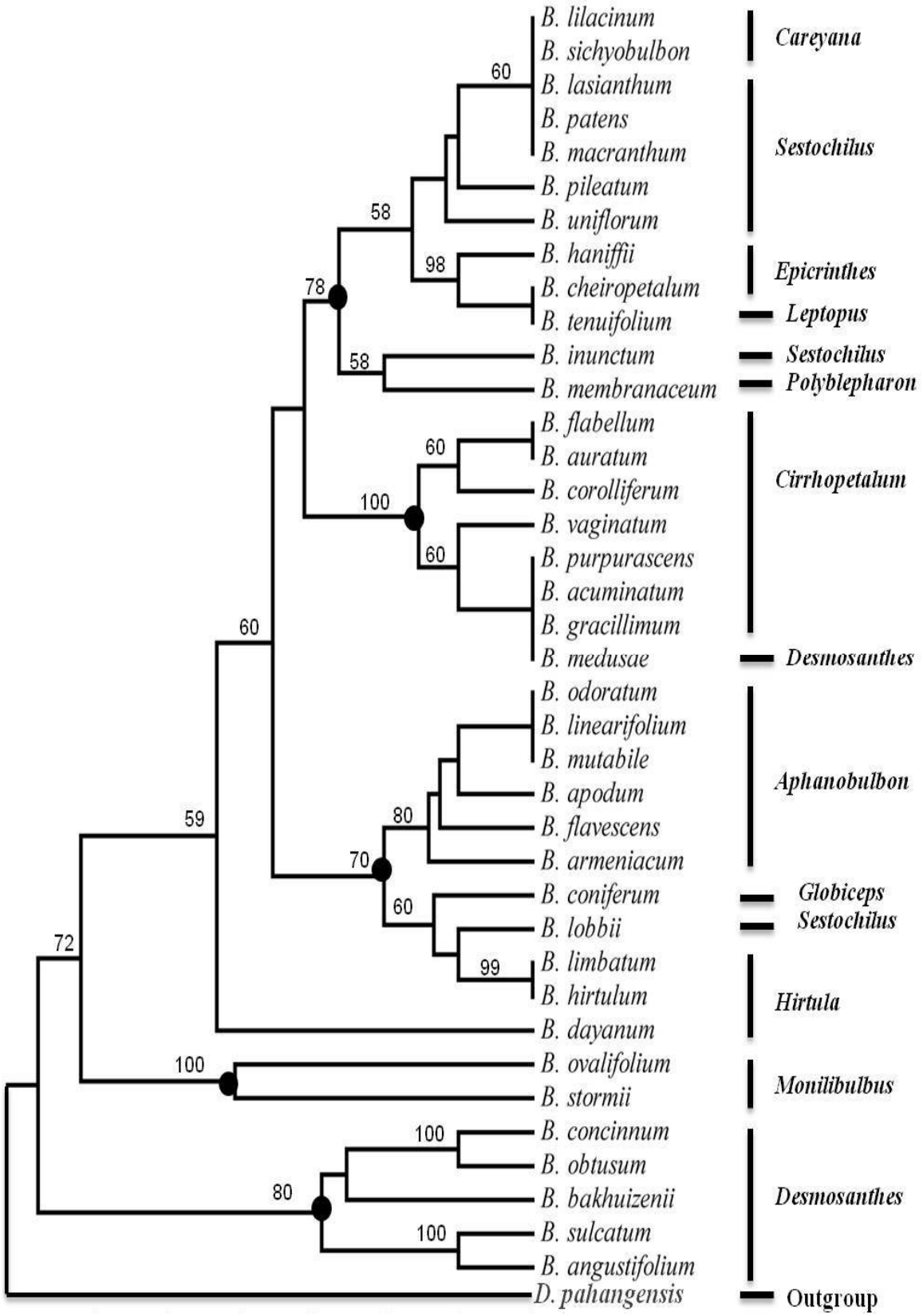

Fig. 3. The consensus tree inferred from 329 most parsimonious trees is shown for combined morphological and molecular data. Bootstrap percentage ≥ 50 are indicated above the nodes. Nodes for the recognize sub-clusters are marked with black dot.

Combined molecular and morphological data analysis: *rbc*L sequence data contain 493 characters, were combined with 30 morphological characters in a single data matrix. Systematic classification of combined data was analyzed by PAUP* 4.0b10 using Maximum Parsimony. The consensus tree inferred from 329 most parsimonious trees (Fig. 3). The consistency index is 0.66 and the retention index is 0.86. Codon positions were included $1^{st}+2^{nd}+3^{rd}$.

Three groups were obtained from combined data analyses which are consistent with the structure of sections in the recent classification by Seidenfaden & Wood (1992); 1) section *Monilibulbus* comprises *B. ovalifolium* and *B. stormii*. These species are tiny, single flowered and they have very close bilaterally flattened pseudobulb; 2) section *Epicrianthes* has defined by thick leaf, single flower on the short pedicel, lip with elaborate construction (cover with small vesicles and hairs) and diverging petals. The last character is a unique synapomorphy character for this group. Garay & Kittredge (1985) have proposed generic status for the section *Epicrianthes* but the analyses were not supported section *Epicrianthes* as a separated genus; 3) section *Careyana* comprises *B. lilacinum* and *B. sichyobulbon*. Holttum (1964) assigned *B. lilacinum* in section 12 along with a few species of section *Sestochilus*. In this study, cluster analysis of structural characters as well as combined data showed close relationship between sections *Careyana* and *Sestochilus* which corresponded well with Holttum (1964) □s suggestion.

The combined data analysis was revealed structure of following sections inconsistent with prior taxonomy: 1) Section *Sestochilus* was divided into separate clades that are not in close relationship. However, morphological clustering was corresponded with prior classification (Seidenfaden & Wood, 1992). Species of section *Sestochilus* are large plants with distinct pseudobulb. Rhizome in all species is covering by sheaths. They have one non-resupinate flower except for *B. lasianthum* (many flowers on racemose inflorescence). Another explicit character is glabrous petals. They were more than half as long as sepals. In spite of the same characters in species structure, *B. inunctum* and *B. lobbii* have placed in different clades; 2) Section *Cirrhopetalum* with 80 species around the world, always it has been considered as a separate genus (Garay *et al*., 1994). Based on this research section *Cirrhopetalum* cannot be considered at generic level, because the section deeply nested inside genus *Bulbophyllum* (BP100). Umbellate inflorescence, longer length of lateral sepals than dorsal sepal, fringed edges of petals as well as dorsal sepal and angled pseudobulb characterizes the predominantly section *Cirrhopetalum*. Based on morphological clustering (Fig. 1) species of section *Cirrhopetalum* were placed into separate clusters which means combination of morphological characters have not sufficient power to classification of these species in one dependent group. Combination of *rbc*L sequence data with morphological data corroborated opinion of traditional classification for section *Cirrhopetalum*, but combined data analysis were placed *B. medusae* from section *Desmosanthes* inside section *Cirrhopetalum*; 3) Some Homoplasious characters formed section *Desmosanthes*. Vermeulen (1991) characterized section *Desmosanthes* by small plants, distinct pseudobulb, inflorescence with two or more flowers, rachis very short and flowers arranged on subumbellate inflorescence with very tiny flowers except of *B. medusae*. Specific characters of section *Cirrhopetalum* were observed in *B. medusae* and this can be a reason for new status of this species. 4) Third sub-clade consisted of section *Aphanobulbon*. Species in section *Aphanobulbon* were small to medium-sized plants with very small or sometimes undetectable pseudobulbs as unique character and multi-flowered raceme inflorescences. Most of the species recognise by a hairy lip except of *B. linearifolium* and *B. mutabile*. Vermeulen (1991) used majority of above characters to recognize section *Aphanobulbon*. In this analysis section *Aphanobulbon* had a close relationship with species of sections *Globiceps*, *Hirtula* and *B. lobbii* with moderately strong support (BP70). However, analysis of anatomical data along with molecular data is required to support their new status; 5) based on this research we proposed *B. dayanum* in a separate new section and this species must be removed from section *Hirtula*. Moreover, morphological analysis was supported this inference.

In this research, the clustering result based on morphological data was strongly congruent with the viewpoint of traditional taxonomy. Combination of *rbc*L sequence data along with morphological data were provided acceptable information sites and especially were suitable for study on systematic classification among sections (Shinwari *et al*., 2002). However, it was partially different from the outlook of conventional taxonomy but it was supported in other aspects, which indicates that the evolution in plant morphology and molecular level are perhaps not coincident. In view of the fact that there are a few studies in phylogenetic and systematic classification of *Bulbophyllum* on the molecular level, accordingly, the results in this research with differences from the traditional classification need further be validated.

Acknowledgments

This research work is made possible through Research University Grant scheme no. 19148/4 made available by University of Kurdistan.

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(Received for publication 20 July 2015)