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STUDIES ON MATURASE K SEQUENCES AND SYSTEMATIC CLASSIFICATION OF *BULBOPHYLLUM* IN PENINSULAR MALAYSIA

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Abstract

Bulbophyllum Thou. is a largest genus in *Orchidaceae* family and a well-known plant of tropical area. In this study original nucleotide sequence data of matures K (*mat*K) were collected froms 53 species of *Bulbophyllum* and used to infer the species interrelationship with maximum parsimony and maximum likelihood analysis. Alignment between *mat*K sequences from 53 species of *Bulbophyllum* was performed, and discovered that the average percentage sequence divergence within *Bulbophyllum* species was 2.4%, and maximum in-group divergence was 8.8% between *Bulbophyllum tenuifolium* with *Bulbophyllum ovalifolium* and *Bulbophyllum mutabile*. Furthermore, there are 179 mutation sites and 107 information sites in *mat*K sequences, respectively 20.38% and 12.18%. Molecular systematic analysis of *mat*K was revealed that several of the currently recognised sections are consistent in structure with the viewpoint of traditional classification except of sections *Desmosanthes*, *Hirtula* and *Sestochilus* which was contained misplaced elements. In addition presumably, generic status of section *Cirrhopetalum* cannot longer be supported, as it is deeply embedded within the genus *Bulbophyllum* and section *Desmosanthes* is a sister group to *Cirrhopetalum* with high nodal support.

Introduction

Bulbophyllum is the largest genus of subtribe Bulbophyllinae (Schlechter, 1926). More than 95% of the species in the subtribe belong in the large genus *Bulbophyllum*, with more than 2000 species found mostly in Asia (Seidenfaden & Wood, 1992). Based on historical records of *Bulbophyllum* in Peninsular Malaysia, Ridley (1924) described 93 species without distinct section; Holttum (1953) and Henderson (1954) described 127 and 129 species respectively in 12 sections and 110 species reported in catalogue of the vascular plants of Malaya (Turner, 1995). Latest taxonomy (Seidenfaden & Wood, 1992) described the *Bulbophyllum* in 17 sections with more than 100 species. 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. Nevertheless, areas of earlier collection site were visited for possible sampling. However not all the previous collection sites are forested areas. At least 50% of the recorded collection sites are now palm oil plantation area. Therefore there is possibility to overthrow of several *Bulbophyllum* species 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 (Bartareau, 1994; Borba & Semir, 1998; Tan *et al*., 2002; Nishida *et al*., 2004; Teixeira *et al*., 2004). They are distributed in the most northern parts (Perlis) to the most southern parts (Johor) in peninsular Malaysia and their geography can range from lowland to the highland areas like Cameroon Highlands and Genting Highlands. Orchids of the genus *Bulbophyllum* are one of the important plants in Malaysia in terms of their abundance, but identification of *Bulbophyllum* at species level still remains a problem for practicing taxonomists. In recent years, there have been several attempts to delimit orchid species boundaries based on phylogenetic reconstructions, using chloroplast and nuclear DNA sequences (Dion *et al*., 2008; Fischer *et al*., 2007; Bellstedt *et al*., 2001). Although some of these studies have revealed concordance with species defined by classical taxonomy (Shinwari 1995; Shinwari 2002), others have not. To date, molecular taxonomic work on delimitation of Malaysian *Bulbophyllum* has not been documented. Uncertainty in taxonomic status of the *Bulbophyllum* is a major problem, which requires molecular taxonomic revisions, for example indistinct status of section *Cirrhopetalum*. Botanical treatises by such eminent botanists as Meisner (1842), Endlicher (1837), Bentham and Hooker (1883), Hooker (1890), Pfitzer (1888) and Schlechter (1914) recognize *Cirrhopetalum* as independent genus. Garay (1994) was not concurred with Seidenfaden & Wood (1992) opinion, which had transferred 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.

In this article, chloroplast *mat*K Gene was used to study and discuss the sibship among species of *Bulbophyllum* in order to provide more information for classification of *Bulbophyllum* on molecule level. The *mat*K gene located within the intron of the *trn*K and codes for maturase like protein, which is involved in Group II intron splicing (Turmel *et al*., 2006). The high rate of substitution in this region has resulted in an increased number of parsimony informative sites and strong phylogenetic signal, contributing to its use to discern evolutionary histories at several taxonomic levels (Johnson & Soltis, 1994; Hayashi & Kawano, 2000; Hilu *et al*., 2003; Cameron, 2005; Muller *et al*., 2006). The wealth of phylogenetic information generated from *mat*K has made it an extremely valuable gene for systematic and evolutionary studies. The main objectives of the present

study are to (1) study on *mat*K sequences of *Bulbophyllum*; (2) determine evolutionary relationship of studied taxa based on *mat*K gene; (3) verify the structure of sections.

Materials and Methods

Sample collection: For this study, original *mat*K gene sequences were determined for 53 species of *Bulbophyllum*, including 3 outgroup taxa (*Dendrobium rosellum*, *Dendrobium pahangensis* and *Dendrochilum pallideflavens*). Species are representing 13 sections of *Bulbophyllum* from Peninsular Malaysia as described by Holttum (1953), Seidenfaden and Wood (1992). Most specimens used in this study were collected from a variety of locations in Peninsular Malaysia (Table 1). Voucher specimens for all accessions have been deposited in herbarium of biology department, University Putra Malaysia (UPM).

DNA extraction, PCR amplification and sequencing: DNA was extracted from fresh material using Wizard® Genomic DNA Purification Kit (Promega). The *mat*K gene was amplified from total DNA extracts using the polymerase chain reaction (PCR). Primers 3F_KIM f and 1R_KIM r (CBOL, 2009) were proposed by Ki-Joong Kim have been used for amplification of *mat*K. Reaction mixtures contained approximately 2-8 ng of DNA template, 5 µL of 10× reactions buffer, 2µL dNTPs (each 2.5mM), 2.0 U Taq polymerase and 1µL of each oligonucleotide primer, each at 10 µM concentration, in a final volume 50µL. 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, 1 min 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 *mat*K 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). Measures of nucleotide composition were obtained using the program PAUP* 4.0b10 (Swofford, 2002). Base composition was calculated across all taxa, for 1st, 2nd, and 3rd codon positions and all codon positions combined (Fig. 1). A chi-square (χ^2) test of base heterogeneity was calculated for each codon position and for all codon positions, as implemented in PAUP* 4.0b10. As a heuristic tool to explore the degree of saturation present in the datasets, we plotted raw sequence divergence (uncorrected p distance) vs. number of transition and transversion substitutions for all pairwise comparisons among taxa, for each codon positions. If the codon position sites were saturated, we would expect to see a plateau in such a plot, where little or no additional substitution is detectable with increased p distance. Because no such plateaus are seen (Figs. 2 and 3), we conclude that saturation has not yet occurred in 1st, 2nd, and 3rd position sites. Therefore, we did not exclude characters or employ a weighting scheme in our parsimony analyses.

Molecular data analysis: parsimony and likelihood: One thousand replicates of random stepwise taxon addition were performed to find islands of equally most parsimonious trees, holding ten trees at each step. This was followed by tree bisection-reconnection (TBR) branch-swapping. Clade support was assessed with 1000 bootstrap replicates, with random stepwise taxon addition and TBR branch swapping, but permitting only 1 tree per replicate to be held. For maximum likelihood (ML) analysis, Modeltest 3.7 (Posada & Crandall, 1998) was used to determine the optimal model of nucleotide evolution. The TVM+I+G substitution model (Base = 0.3948, 0.1286, 0.1550) Rmat = (1.0000, 1.1248, 0.2364, 0.2364, 1.1248) with invariable sites (pinvar = 0) and among-site rate heterogeneity (α = 0.3087) was selected using a set of hierarchical likelihood ratio tests (LRTs) implemented in Modeltest. The ML method was then performed to find the optimal ML tree with a heuristic search as implemented in PAUP* 4.0b10, with TBR branch-swapping and 10 random sequence additions.

Result and Discussion

The matK gene sequences and variations: The *mat*K gene sequences obtained from the 53 *Bulbophyllum* species and *Dendrobium rosellum*, *Dendrobium pahangensis* and *Dendrochilum pallideflavens* as outgroup. The aligned sequences consisted of 787 nucleotide sites, which 602 characters were identical among all taxa, 179 sites were variable, and 107 sites were parsimony informative. Average percentage sequence divergence (uncorrected p distance) within *Bulbophyllum* species was 2.4%, and maximum in-group *mat*K divergence was 8.8% (between *B. tenuifolium* with *B. ovalifolium*, and *B. mutabile*). Mean base composition (Fig. 1) was found to be fairly uniform among all taxa analyzed (38.74% A, 15.01% C, 16.2% G and 30.4% T), with a slightly higher proportion of Adenine (43.66%) and lower proportion of guanine (11.24%) at 1st codon positions. Nucleotide composition among all taxa exhibited no significant heterogeneity at all three codon positions: first position, χ^2 = 15.37 (df =162, P =1.00); second position, χ^2 = 4.69 (df =162, P =1.00); and third position, χ^2 = 7.64 (df =162, P =1.00).

The overall transition to transversion (Ti/Tv) ratio was 0.963. Saturation analyses (Griffiths, 1997) were conducted to search for saturated data partitions as a result of multiple substitutions at single sites. Pairwise sequence divergence was compared to pairwise transition and pairwise transversion divergences at first, second and third codon positions separately for the *mat*K gene. The relationships between uncorrected p distance and number of transversion (Fig. 2) and transition (Fig. 3) substitutions of the *mat*K sequences were plotted for all pairwise species comparisons (including outgroup taxa). Plots indicated that, even at 3rd codon positions, no saturation was found in the *mat*K genes and the slope of transversions (G↔T; C↔A) always lies below that of transitions (A↔G; C↔T). This indicates that transversions occur less frequently than transitions and are showing that there are more constraints on the occurrence of transversions than on transitions.

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	JF305780
2.	*Hirtula*	*B. dayanum*	Gunung Jerai, Malaysia	UPM / SH.K-101	JF305799
3.	*Hirtula*	*B. limbatum*	Johor, Malaysia	UPM / B0054	JF305814
4.	*Hirtula*	*B. hirtulum*	Fraser s Hill, Malaysia	UPM / FAN. FH- 314	JF305797
5.	*Hirtula*	*B. hirtulum*	Gunung Belumut, Malaysia	UPM / SH.K-100	JF305810
6.	*Altisceptrum*	*B. farinulentum*	Gunung Jerai, Malaysia	UPM / FAN. FH- 200	JF305803
7.	*Cirrhopetalum*	*B. flabellum*	Genting highland, Malaysia	UPM / RG 1945	JF305776
8.	*Cirrhopetalum*	*B. purpurascens*	Cameron Highland, Malaysia	UPM/ B0027	JF305795
9.	*Cirrhopetalum*	*B. vaginatum*	Melacca, Malaysia	UPM/ FAN. FH- 503	JF305785
10.	*Cirrhopetalum*	*B. corolliferum*	Gunung Belumut, Malaysia	UPM / B0026	JF305789
11.	*Cirrhopetalum*	*B. acuminatum*	Gunung Belumut, Malaysia	UPM / RG 2291	JF305802
12.	*Cirrhopetalum*	*B. auratum*	Cameron Highland, Malaysia	UPM / B0060	JF305817
13.	*Cirrhopetalum*	*B. gracillimum*	Genting Highland, Malaysia	UPM / B0053	JF305813
14.	*Cirrhopetalum*	*B.* sp1	Genting Highland, Malaysia	UPM / SH.K-102	JF305798
15.	*Cirrhopetalum*	*B.* sp2	Gunung Belumut, Malaysia	UPM / SH.K-103	JF305805
16.	*Cirrhopetalum*	*B. dentiferum*	Gua Musang, Kelantan, Malaysia	UPM / B0048	JF305806
17.	*Cirrhopetalum*	*B.* sp3	Gunung Panti, Malaysia	UPM / SH.K-104	JF305808
18.	*Aphanobulbon*	*B. flavescens*	Fraser s Hill, Malaysia	UPM / FAN. FH- 062	JF305778
19.	*Aphanobulbon*	*B. mutabile*	Fraser s Hill, Malaysia	UPM / FAN. FH- 105	JF305777
20.	*Aphanobulbon*	*B. linearifolium*	Fraser s Hill, Malaysia	UPM / FAN. FH- 258	JF305779
21.	*Aphanobulbon*	*B. odoratum*	Pahang, Malaysia	UPM / B0056	JF305793
22.	*Aphanobulbon*	*B. armeniacum*	Fraser s Hill, Malaysia	UPM / SH.K-105	JF305794
23.	*Desmosanthes*	*B. concinnum*	Genting highland, Malaysia	UPM / RG 2207	JF305790
24.	*Desmosanthes*	*B. sulcatum*	Gunung Jerai, Malaysia	UPM / FAN. FH- 304	JF305775
25.	*Desmosanthes*	*B. angustifolium*	Fraser s Hill, Malaysia	UPM / RG 2313	JF305773
26.	*Desmosanthes*	*B. medusa*	Johor, Malaysia	UPM / B0052	JF305812
27.	*Desmosanthes*	*B. bakhuizenii*	Gunung Jerai, Malaysia	UPM / SH.K-107	JF305800
28.	*Desmosanthes*	*B. planibulbe*	Gunung Jerai, Malaysia	UPM / SH.K-108	JF305791
29.	*Desmosanthes*	*B. obtusum*	Fraser s Hill, Malaysia	UPM / FAN. FH- 172	JF305774
30.	*Desmosanthes*	*B.* sp5	Genting Highland, Malaysia	UPM / B0010	JF305786
31.	*Desmosanthes*	*B.* sp6	Gunung Panti, Malaysia	UPM / B0057	JF305816
32.	*Sestochilus*	*B. macranthum*	Cameron Highland, Malaysia	UPM / FAN. FH- 153	JF305768
33.	*Sestochilus*	*B. inunctum*	Gunung Jerai, Malaysia	UPM / SH.K-109	JF305769
34.	*Sestochilus*	*B. lobbii*	Cameron Highland, Malaysia	UPM / FAN. FH- 426	JF305771
35.	*Sestochilus*	*B. uniflorum*	Fraser s Hill, Malaysia	UPM / FAN. FH- 107	JF305770
36.	*Sestochilus*	*B. patens*	Gunung Jerai, Malaysia	UPM / B005	JF305772
37.	*Sestochilus*	*B. pileatum*	Gunung Belumut, Malaysia	UPM / RG 2281	JF305787
38.	*Sestochilus*	*B. lasianthum*	Fraser s Hill, Malaysia	UPM / RG 1922	JF305818
39.	*Sestochilus*	*B. singaporeanum*	Johor, Malaysia	UPM / B0050	JF305811
40.	*Sestochilus*	*B.* sp7	Gunung Belumut, Malaysia	UPM / SH.K-110	JF305807
41.	*Careyana*	*B. lilacinum*	Gunung Jerai, Malaysia	UPM / B0029	JF305796
42.	*Careyana*	*B. sichyobulbon*	Gunung Jerai, Malaysia	UPM / SH.K-111	JF305782
43.	*Monilibulbus*	*B. ovalifolium*	Cameron Highland, Malaysia	UPM / RG 2167	JF305783
44.	*Globiceps*	*B. coniferum*	Cameron Highland, Malaysia	UPM / RG1757	JF305819
45.	*Leptopus*	*B. tenuifolium*	Cameron Highland, Malaysia	UPM / B0061	JF305820
46.	*Polyblepharon*	*B. membranaceum*	Fraser s Hill, Malaysia	UPM / B0024	JF305788
47.	*Polyblepharon*	*B. membranaceum*	Cameron Highlands, Malaysia	UPM / B0045	JF305809
48.	*Epicrianthes*	*B. cheiropetalum*	Gunung Jerai, Malaysia	UPM / B0018	JF305784
49.	*Epicrianthes*	*B. haniffii*	Penang, Malaysia	UPM / B0031	JF305792
50.	*Epicrianthes*	*B. mobilifilum*	Fraser's Hill, Malaysia	UPM / B0015	JF305781
51.	*Ephippium*	*B. restrepia*	Johor, Malaysia	UPM / B0055	JF305815
52.	?	*B.* sp8	Penang, Malaysia	UPM / SH.K-112	JF305801
53.	?	*B.* sp9	Penang, Malaysia	UPM / SH.K-113	JF305804
54.	*Aporum*	*D. rosellum*	Fraser's Hill, Malaysia	UPM / D001	JF305822
55.	*Distichorchis*	*D. pahangensis*	Fraser's Hill, Malaysia	UPM / FAN. FH- 180	JF305823
56.	*Dendrochilum*	*D. pallideflavens*	Genting Highland, Malaysia	UPM / OYS 041	JF305821

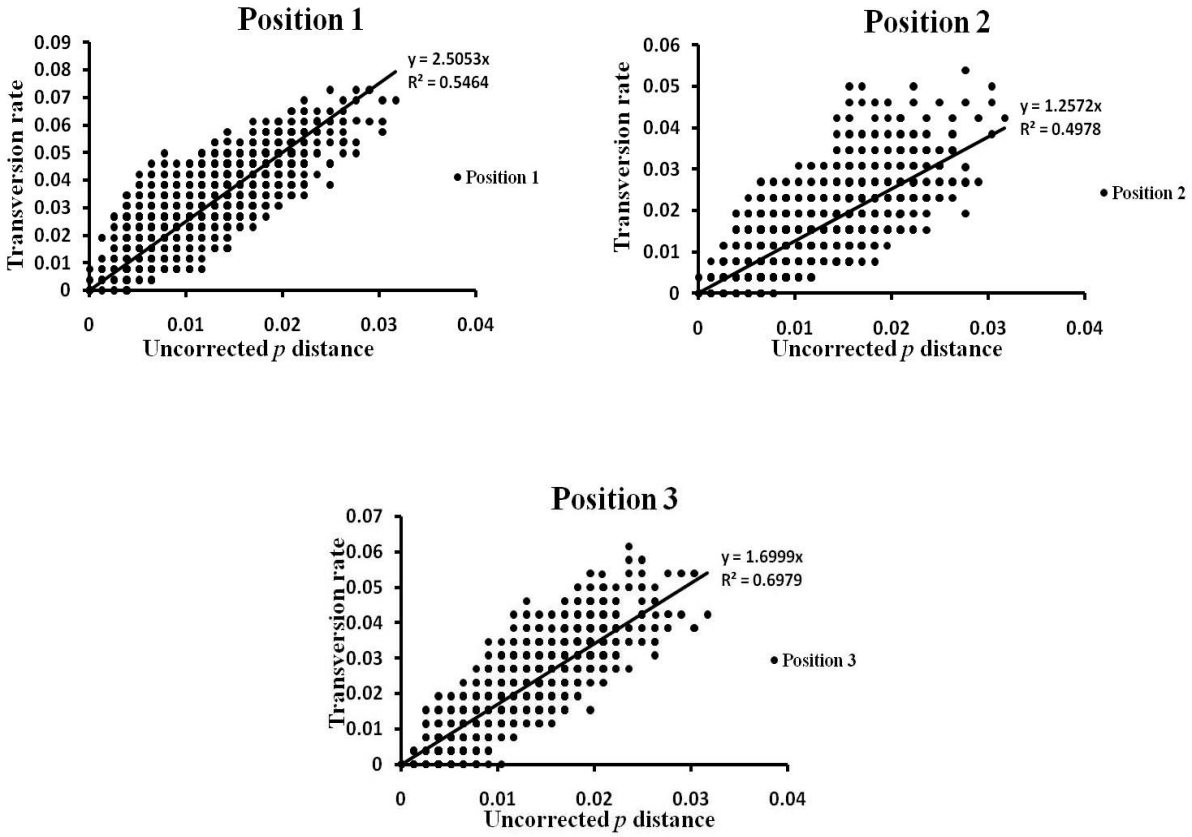

Fig. 2. Saturation plots of transversion rates against uncorrected p-distance at each codon position of matK region.

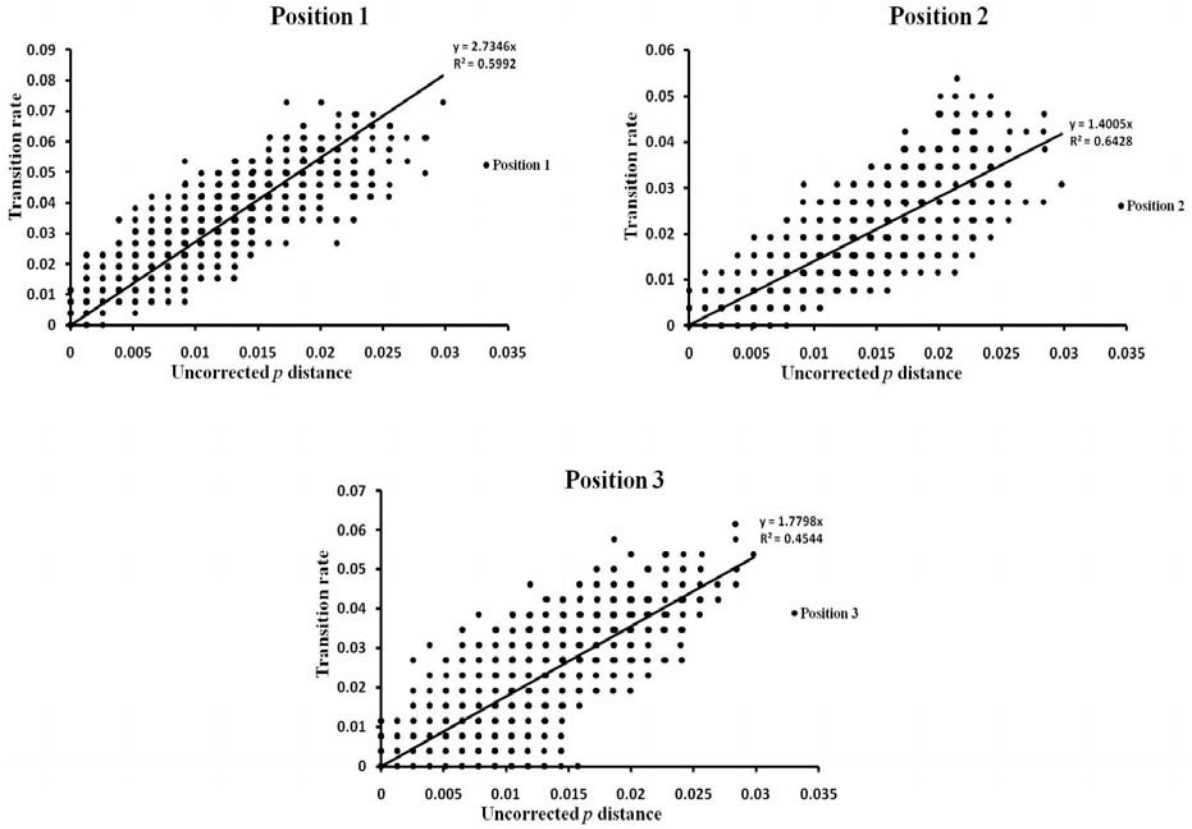

Fig. 3. Saturation plots of transition rates against uncorrected p-distance at each codon position of *mat*K region.

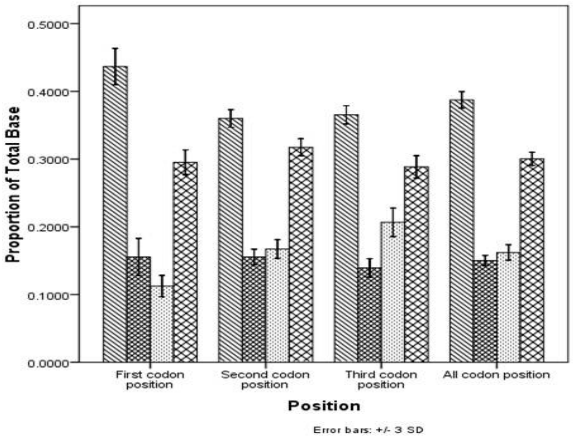

Fig. 1. Base composition for each codon position of the 787-bp aligned *mat*K region, averaged over all samples. Error bars depict minimum to maximum range. A: adenine, C: cytosine, G: guanine, T: thymine.

Analysis of *mat*K sequence data: Unweighted parsimony analysis resulted in 3058 equally parsimonious trees [Tree length= 324, consistency index (CI) =0.64, retention index (RI) =0.69, homoplasy index (HI) = 0.35]; the 50% majority-rule consensus tree is shown in Fig. 4. In the MP tree, three (A–C) main clades were present. Clade A (BP76) divided to six sub-clades. The first and second sub-clades consisted of taxa assigned to section *Cirrhopetalum* (BP100) and two unidentified species (*B.* sp8 and *B.* sp9), the third sub-clade of section *Desmosanthes* (BP100), the forth sub-clade of section *Careyana* (BP100), the fifth sub-clade of *B. dayanum* and *B. macranthum* (BP100) the sixth sub-clade of section *Sestochilus* (BP52). Clad B was made up of sections *Hirtula*, *Altisceptrum* and *Aphanobulbon* (BP100). In clade C, *B. ovalifolium* and *B. coniferum* placed close together (BP100) connect with species of sections *Ephippium*, *Leptopus*, *Polyblepharon* and *Epicrianthes* (BP68). The ML tree (Fig. 5) shows the same tree topology as MP. It is a good bet that if different methods give the same tree, then a robust estimation will be acquire.

Sectional relationships of studied taxa: From *mat*K analysis, the most significant result regarding the taxonomy of *Bulbophyllum* is demonstration of monophyly of studied taxa. All of the sections with the exception of section *Hirtula* and *Sestochilus* described by Holttum (1953), form strongly supported monophyletic groups. Unique morphological synapomorphies characterizing clades are scarce, but supporting combinations of characters are abundant. Vermeulen (1991) characterized section *Desmosanthes* (BP100/BP100, clade A) by small plants, distinct pseudobulb, inflorescence with two or more very tiny flowers except of *B. medusae* with long lateral sepals, rachis very short and flowers arranged on subumbellate inflorescence. Combination of characters with homoplasious status formed section *Desmosanthes*. Based on this study this section was sister to section *Cirrhopetalum* with strong support (BP100/BP100) and *B. medusae* was misplaced inside section *Desmosanthes*. Unique synapomorphy character of section *Cirrhopetalum* (longer length of lateral sepals than dorsal sepal) and angled pseudobulb are prominent character and the same features can be observed in *B. medusae*. Leaf shape and scape, which covered by several sheaths, are similar with *B. vaginatum.* However non fringed edges of petals, dorsal sepal and free base lateral sepals differentiated this species from *Cirrhopetalum* but based on *mat*K data analysis we propose *B. medusae* inside section *Cirrhopetalum*. *Cirrhopetalum* with 80 species around the world is a section, which was always, be considering as a separate genus. Umbellate inflorescence, longer length of lateral sepals than dorsal sepal, petals and dorsal sepal with fringed edges and angled pseudobulb characterizes the predominantly section *Cirrhopetalum*. The *mat*K analysis revealed common ancestor between the *Bulbophyllum* and *Cirrhopetalum* and there was no evidence to accept generic status of *Cirrhopetalum.*

In second sub-clade, based on morphological evidence of leaf and pseudobulb (thick leaf with blunt tip and angled pseudobulb) two unidentified species are highly similar with section *Cirrhopetalum* even they are in the same clade with *B. dentiferum* (BP100/ BP95). Reproductive characters were not available otherwise we could say these species are intermediate between sections *Desmosanthes* and *Cirrhopetalum*. *Bulbophyllum lilacinum* and *B. sichyobulbon* (BP100/BP100) described in section *Careyana* (Seidenfaden & wood, 1992) meanwhile Holttum (1953) assigned *B. lilacinum* in section 12 along with few species of *Sestochilus*. Based on this study Section *Careyana* was independent from *Sestochilus.*

Section *Hirtula* was paraphyletic. *Hirtula* is consisting of 3 species (*B. hirtulum*, *B. dayanum* and *B. limbatum*) (Seidenfaden & Wood, 1992). Holttum (1953) describe *B. hirtulum* in section 9 and *B. hespidum* (synonym of *B. dayanum*) in section 12 along with *B. limbatum*. For *B. hirtulum* and *B. limbatum* Ridley (1924) proposed *Hirtula* as a sectional name. Following combination of characters are common in section *Hirtula*: Inflorescence with more than one flowers, equal length and similar structure of sepals and ciliate petals. Based on result section *Hirtula* has been divided into two separated clades; *B. Hirtula* and *B. limbatum* sister group to section *Aphanobulbon* (clade B) and *B. dayanum* in clade A.

All species of section *Sestochilus* are large plants with distinct pseudobulb and rhizome covered by chaffy sheaths. They have one non-resupinate flower with the exception of *B. singaporeanum* and *B. lasianthum* which have many flowers on racemose inflorescence and glabrous petals are more than half as long as the sepals. Seidenfaden and Wood (1992) used the same features to characterize this section. Holttum (1964) proposed species of section *Sestochilus* into two separate sections; *Stenochilus* with one-flowered inflorescence and non resupinate flowers and *Sestochilus* with one to two or more-flowered inflorescences. Meanwhile Vermeulen (1991) found this as unnatural division and proposed only one section *Sestochilus* but analysis of *mat*K sequence data showed polyphyletic status for section *Sestochilus* and species divided into separate clades that are not very closely related.

The majority of the species in section *Aphanobulbon* (clade B) are; small to medium-sized plants with very small or sometimes undetectable pseudobulbs, multi-flowered raceme inflorescences and hairy lip (except of *B. linearifolium* and *B. mutabile*). Vermeulen (1991) used the majority of the mentioned characters to recognize *Aphanobulbon*. Based on this study section *Aphanobulbon* is a sister group to section *Altisceptrum*, *B. hirtulum* and *B. limbatum*. (BP100, BP96).

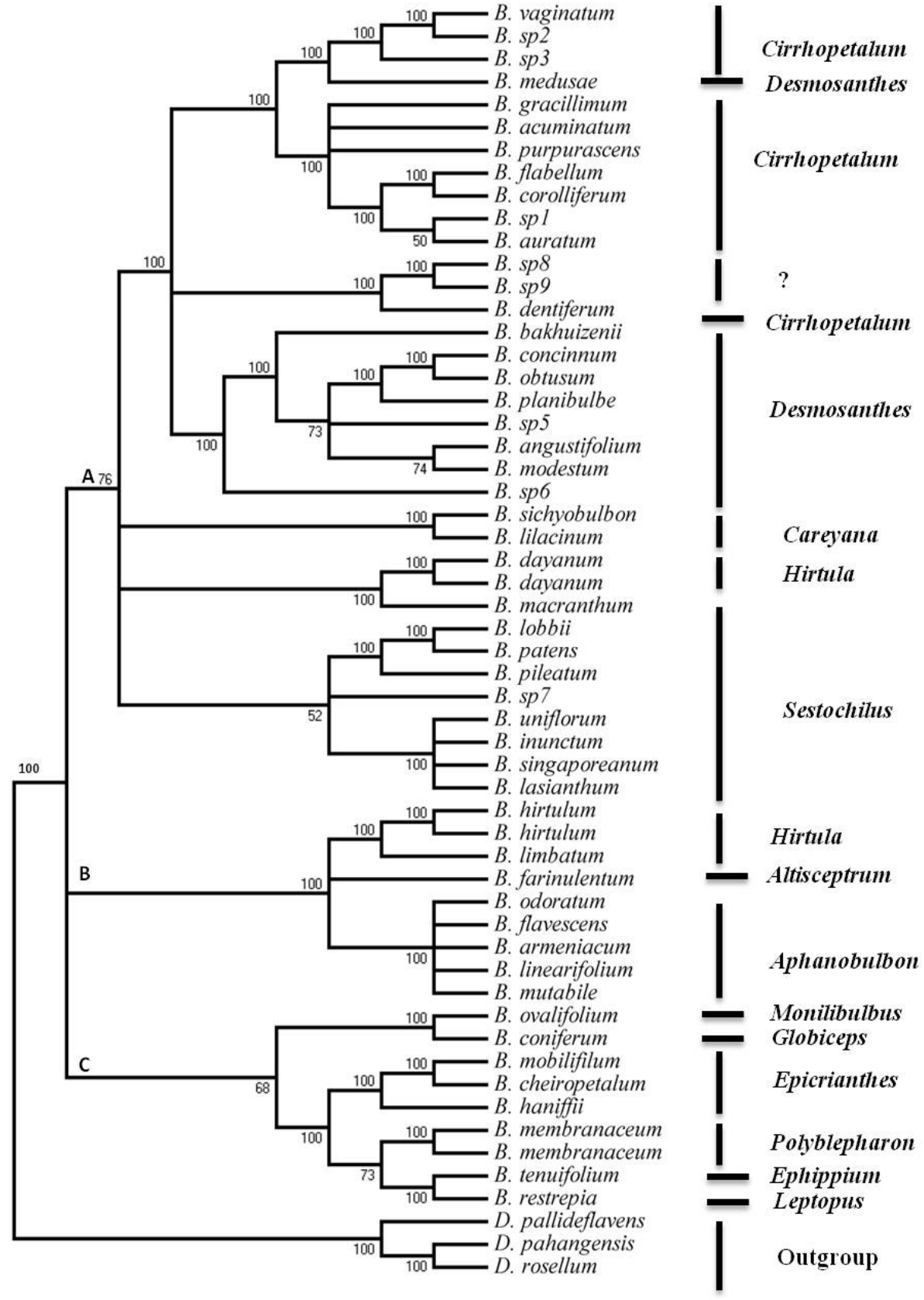

Fig. 4. The strict consensus tree inferred from 3058 most parsimonious trees is shown for matK region. Bootstrap percentage > 50 are indicated above the nodes.

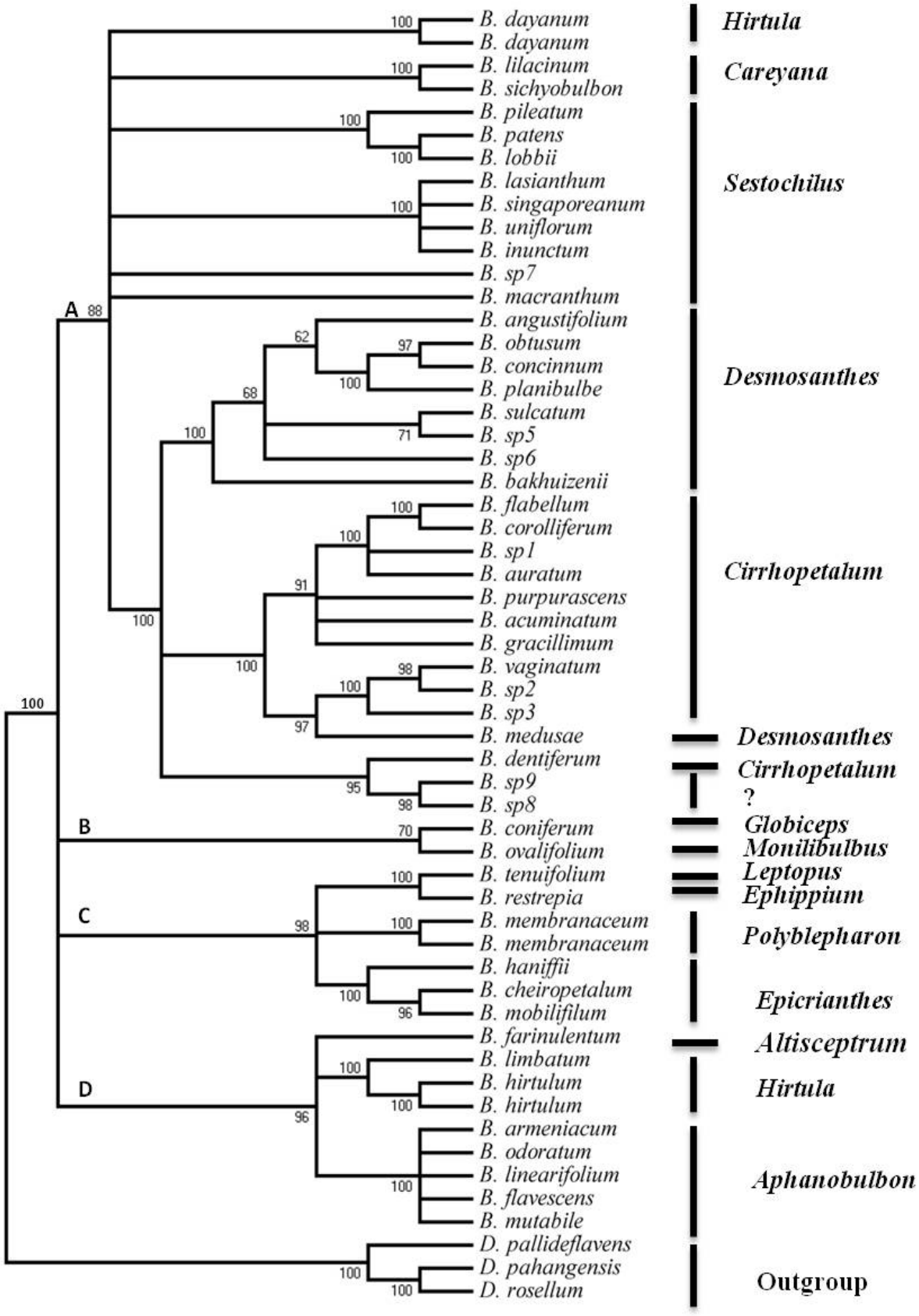

Fig. 5. Tree is resulting from maximum likelihood analysis of the matK gene dataset. Numbers at nodes represent percent recovery in bootstrap analysis (1000 replicates).

MP and ML analyses showed close relationship of sections *Ephippium*, *Leptopus*, *Polyblepharon* and *Epicrianthes* with high bootstrap support (BP100/ BP98). These sections with few numbers of species recognized. Enhance species number for each section is proposed to confirm their status. *Bulbophyllum coniferum* and *B. ovalifolium* placed in the same clade with moderately high support (BP100/BP70). Sample developing can help to improve the status of sections *Globiceps* and *Monilibulbus* as well. In this study as first, considerable evidence has been made in delimiting natural sections and the relationships amongst species could be unequivocally determined. Nevertheless, the addition of a new molecular dataset with enhance number of species will allow significant progress in clarifying the sectional delimitation in *Bulbophyllum*. Present studies will help in finding DNA barcodes beside other conserve genes like rbcL earlier reported by several authors (Shinwari *et al*., 1994, Shinwari 1998 & 2000).

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