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Molecular systematics of genus *Bulbophyllum* (Orchidaceae) in Peninsular Malaysia based on combined nuclear and plastid DNA sequences

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ABSTRACT

Phylogenetic relationships were inferred for representative *Bulbophyllum* species of 13 sections from subtribe Bulbophyllinae (Epidendroideae, Orchidaceae) in Peninsular Malaysia. The combined data matrix consists of sequences from ITS nuclear gene region and *trn*L-F, *mat*K, and *rbc*L plastid gene regions with 3114 characters. Molecular data were analysed using parsimony and Bayesian inference. The results show that several recognized sections are monophyletic. Section *Hirtula* with paraphyletic status must split up and section *Desmosanthes* contain misplaced elements. Furthermore, generic status of *Cirrhopetalum* and *Epicrianthes* cannot be supported, because they are deeply embedded within the genus *Bulbophyllum*. Section *Desmosanthes* is recognized as the closest group to section *Cirrhopetalum*; therefore, they can be merged in some aspects.

1. Introduction

Bulbophyllum Thouars is the largest genus of the pantropical subtribe Bulbophyllinae and comprises approximately 2032 species (Frodin, 2004). Sieder (2007) reported 184 species with 48 endemic species from Malaysia. It is mostly an epiphytic species found in different habitats, ranging from (sub) tropical dry forests to wet montane cloud forests and most of them are adapted to fly pollination (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 highland areas like Cameroon and Genting Highlands. In recent years, several molecular phylogenetic studies of orchids have been published (Van Den Berg et al., 2005; Carlsward et al., 2006) placing genus *Bulbophyllum* as sister to the genus *Dendrobium* within tribe Dendrobieae in the higher Epidendroids clade of subfamily Epidendroideae. 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 practising taxonomists. Uncertainty in taxonomic status of the *Bulbophyllum* is a major problem which requires molecular taxonomic revision.

Attempts to split up this gargantuan genus into smaller, more manageable units have generally faltered due to the fact that most of the main groups differ only in rather loose combinations of characters. In every section, there were species that do not

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possess all the diagnostic characters of that section, and yet seem to belong in it; conversely, species that seemingly belong to a certain group based on its diagnostic characters sometimes show other characters that contradict the affinity (Schuiteman et al., 2010). Such genera as *Cirrhopetalum* have been split off, but based on molecular studies of *mat*K sequences analysis (Gravendeel et al., 2004; Hosseini et al., 2012), generic status of *Cirrhopetalum* failed. In this study, analysis of some *Bulbophyllum* species with putative out groups was performed on 4 DNA regions: plastid *trn*L-F spacer, *mat*K gene, *rbc*L gene, and the ITS data. In this paper, the main objectives were to clarify the internal topology within *Bulbophyllum* spp., to test the monophyly of studied taxa, and to explicate the generic status of sections *Cirrhopetalum* and *Epicrianthes* based on reconstructed multi-gene based phylogeny.

2. Materials and methods

2.1. Taxon sampling

Sampling of species required for this study was one of the greatest challenges faced. It was not possible to cover every part of the forested area in Peninsular Malaysia during this study period. 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, it was not possible to obtain some of the species required for this study.

Some of the collected samples were without flower, so the correct name of these species could not be declared. In this case, blooming season needs to be waited for or the plants should be placed in green house. However, some species in the new conditions could not survive and some of them never flower even after three years in the green house. Species representing 13 sections of Peninsular Malaysian *Bulbophyllum*, as described by Seidenfaden and Wood (1992), were sampled (Table 1).

For some sections, it was not possible to identify all the accessions to species or to apply a valid name based on only vegetative characters, so they appeared as B. sp1- B. sp9.

Living samples were collected in Peninsular Malaysia in the wild or taken from plants in the green house of University Putra Malaysia. Herbarium dried samples from the herbarium in Biology Department of University Putra Malaysia were also used for some species. Voucher specimens for all accessions were deposited in herbarium of Biology Department, University Putra Malaysia (UPM). Three taxa (*Dendrobium rosellum*, *Dendrobium pahangensis* and *Dendrochilum pallideflavens*) were selected as the out group.

2.2. DNA extraction, amplification, and sequencing

DNA was extracted from the herbarium or fresh material using Wizard® Genomic DNA Purification Kit (Promega). Polymerase chain reaction (PCR) amplification was performed using Bioline Biolasetaq (5 U/ul), Bioline 10 × biolase buffer, dNTPs (10 mM) following the manufacturer's protocol and 2–8 ng template DNA for a 50 μl reaction mixture.

The Primers 26SE and 17SE of Sun et al. (1994) were used for the amplification of the nrITS regions with the following PCR program: an initial 2 min premelt at 94 °C and 34 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C and 1 min extension at 72 °C followed by a final extension for 7 min. For sequencing, the primers ITS4 and ITS5 (White et al., 1990) were used. The cpDNA *trn*L–*trn*F intron was amplified using forward primer designed by Fischer et al. (2007) and universal reverse primer designed by Taberlet et al. (1991). The *rbc*L and *mat*K chloroplast region were amplified using the designed primers pair by David Erickson and Ki-Joong Kim, respectively, from the Consortium for the Barcode of Life (CBOL, 2009). These primers were amplified partial sequences of *rbc*L and *mat*K with length of 500–600 bp and 1000 bp, respectively.

Amplification of cpDNA was carried out with an initial 3 min premelt at 94 °C and 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C and 1 min 40 s extension at 72 °C followed by a final extension for 7 min at 72 °C. DNA was purified from gel slice or PCR product by Wizard® SV Gel and PCR clean-up system (Promega) following the manufacturer's protocol. All DNA sequences produced for this study were submitted to GenBank. For several species, it was not possible to amplify all the molecular markers.

2.3. DNA sequence alignment

Multiple sequence alignments were performed using Clustal W (Thompson et al., 1994) with default settings by MEGA 4 and BioEdit softwares. Sequences from forward and reverse primers were assembled and compared. Verified sequences were visually inspected and manually adjusted using these softwares. The primer part was trimmed from the first of each sequence, because the character base sequence should not include primer. Then, the end of the sequences was matched together and the unmatched sequence was trimmed. In the *trn*L– *trn*F matrix, an unalignable 64 bp part of the spacer region had to be excluded from the phylogenetic analyses for all the species analyzed.

2.4. Phylogenetic analysis

Maximum parsimony (MP) analyses were undertaken for the individual markers (nrITS, *trn*L–F, *mat*K, and *rbc*L), for the combined cpDNA dataset and for all the molecular markers combined using PAUP* 4.0b10 (Swofford, 2002). To find islands of equally most parsimonious trees, maximum parsimony analyses were run using a heuristic search strategy of branch-