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Molecular systematics of *Dendrobium* (Orchidaceae, Dendrobieae) from mainland Asia based on plastid and nuclear sequences

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ABSTRACT

Dendrobium is one of the three largest genera and presents some of the most intricate taxonomic problems in the family Orchidaceae. Based on five DNA markers and a broad sampling of *Dendrobium* and its relatives from mainland Asia (109 species), our results indicate that mainland Asia *Dendrobium* is divided into eight clades (with two unplaced species) that form polytomies along the spine of the cladogram. Both *Dendrobium* and *Epigeneium* are well supported as monophyletic, whereas sect. *Dendrobium*, sect. *Densiflora*, sect. *Breviflores*, sect. *Holochrysa*, are paraphyletic/polyphyletic. Many ignored phylogenetic relationships, such as the one of major clades formed by *D. jenkinsii* and *D. lindleyi* (two members of sect. *Densiflora*), the *Aphyllum* group, the *Devonianum* group, the *Catenatum* group, the *Crepidatum* group, and the *Dendrobium moniliforme* complex are well supported by both molecular and morphological evidence. Based on our data, we propose to broaden sect. *Dendrobium* to include sect. *Stuposa*, sect. *Breviflores*, and sect. *Holochrysa* and to establish a new section to accommodate *D. jenkinsii* and *D. lindleyi*. Our results indicated that it is preferable to use a broad generic concept of *Dendrobium* and to pursue an improved infrageneric classification at sectional level, taking into account both morphology and current molecular findings.

1. Introduction

The genus *Dendrobium* belongs to the tribe Dendrobieae (Orchidaceae: Epidendroideae) and is one of the three largest genera in the family Orchidaceae with approximately 800–1500 species (Cribb and Govaerts, 2005; Wood, 2006; Zhu et al., 2009), mainly distributed in tropical Asia, Australasia, and Australia, with a few species extending into the temperate Asian regions and New Zealand. *Dendrobium* is characterized by lateral inflorescences arising from the upper part of the shoot, lateral sepals forming a mentum with the column foot, and four naked pollinia. An autapomorphy of this genus is that the rostellum is hollow and swollen and when touched released an opaque, glue-like liquid. The taxonomy of *Dendrobium* is considered to present one of the most intricate problems in Orchidaceae, due to the morphological diversity, wide distribution range, large number of species, and the intergrading and overlapping morphological variation within and among species (Adams, 2011; Morris et al., 1996; Yukawa and Uehara, 1996). Since the establishment of *Dendrobium* by Swartz (Swartz, 1799), various generic delimitations and infrageneric systems have been proposed based on morphological characters (Adams, 2011; Brieger, 1981; Burke et al., 2008; Clements, 2003, 2006; Clements and Jones, 2002; Kranzlin, 1910; Schlechter, 1912; Schuiteman, 2011; Seidenfaden, 1985; Tang and Wang, 1951; Wood, 2006; Yukawa et al., 2000). Lindley (1829) established the subtribe Dendrobiinae, initially recognizing four sections in *Dendrobium* (Lindley, 1830), and later expanding the number of sections to 10 (Lindley and Paxton, 1851). Schlechter (1912) proposed an infrageneric system of *Dendrobium* with four subgenera and 41 sections, which was followed by most authors with a few modifications recognizing a broad *Dendrobium* with a reasonably natural infrageneric system at section level (not at subgenus level). Brieger (1981) divided the Dendrobiinae into 28 genera, most of which correspond to Schlechter's sections of *Dendrobium*. Recently, results of molecular analyses have shed new light on the taxonomy of *Dendrobium*. Most phylogenetic reconstructions indicate that the subtribe Dendrobiinae can be subdivided into three main

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clades (Burke et al., 2008; Clements, 2003, 2006; Schuiteman, 2011; Yukawa et al., 1993, 2000). At the same time, many morphological characters of the Dendrobinae appear to be homoplasious, and several previously defined infrageneric taxa of *Dendrobium* are not monophyletic (Adams, 2011; Brieger, 1981; Burke et al., 2008; Clements, 2003, 2006; Clements and Jones, 2002; Kranzlin, 1910; Schlechter, 1912; Schuiteman, 2011; Seidenfaden, 1985; Tang and Wang, 1951; Wood, 2006; Yukawa et al., 2000). Some divergent ideas about the taxonomy and classification of *Dendrobium* have been put forward. Based on analyses of nuclear ITS, Clements and Jones (2002) and Clements (2003, 2006) divided the broad *Dendrobium* s.l. into three subtribes with approximately 50 genera. Also based on ITS data, Schuiteman (2011) proposed a broader *Dendrobium*, and subsumed several small genera of the subtribe Dendrobiinae into *Dendrobium*. Dressler (1993) and Wood (2006) presented a traditional, morphology-based consensus view of *Dendrobium* and Dendrobiinae with five or six genera.

Although there are some agreements, for example, on the exclusion of *Oxystophyllum* and *Pseuderia* from the Dendrobiinae, such a tremendous divergence of taxonomic views on *Dendrobium* s.l. has led to considerable taxonomic uncertainty and debates. As previous results of molecular systematics were largely based on sparse sampling across *Dendrobium*, or focused on a single section, and mainly utilized either a single DNA marker (especially ITS), or two, some conclusions and results were weakly supported or even without statistical support. Therefore, it is desirable to understand the phylogenetic relationships within *Dendrobium* s.l., as well as the delimitation of the infrageneric taxa, the evolutionary history of morphological characters that characterize the infrageneric taxa, and the interrelationships among major clades within *Dendrobium*, to base the analyses on multiple DNA markers and a denser sampling across *Dendrobium*. However, it is impossible to sample all of the species throughout the range of this large and widespread genus. In the present study, phylogenetic relationships were inferred using five DNA markers (plastid *rbcL*, *matK*, *trnH-psbA* spacer and *trnL* intron and nuclear ITS sequences), with sampling of *Dendrobium* primarily from mainland Asia with the aims of (1) understanding the delimitation of the infrageneric taxa of *Dendrobium* from mainland Asia; (2) reconstructing the phylogenetic relationships within sect. *Dendrobium*; (3) investigating the interrelationships among major infrageneric taxa from mainland Asia; (4) reconsidering the taxonomy of *Dendrobium*.

2. Materials and methods

2.1. Taxon sampling

The taxon sampling in this study included 192 accessions of 109 *Dendrobium* species and of the traditionally recognized genera *Epigeneium* and *Flickingeria* species, in order to represent the geographic and taxonomic diversity in mainland Asia. *Bulbophyllum newportii*, *Oxystophyllumchangjiangense*, *Liparis kumokiri* and *Malaxis spicata* were used as outgroups. The species and voucher specimens used in this study and the GenBank accession numbers are listed in Table A.1.

2.2. Molecular methods

Total genomic DNA from silica-dried material was extracted using a modified CTAB protocol from Doyle and Doyle (1987). For this study, four plastid markers (the coding gene *rbcL*, *matK*, *trnH-psbA* spacer and *trnL* intron) and the internal transcribed spacers from the nuclear ribosomal DNA (ITS) were used. The PCR and sequencing primers for *rbcL*, *matK*, *trnH-psbA* spacer, *trnL* intron and ITS are listed in Table A.2. The selected DNA regions were amplified using a standard polymerase chain reaction (PCR). The sequencing reactions were performed using the ABI Prism Bigdye Terminator Cycle Sequencing Kit (Applied Biosystems, ABI).

2.3. Phylogenetic analyses

Sequences were aligned using the program Clustal X 1.83 (Thompson et al., 1997) and manually adjusted using BioEdit (Hall, 1999). The homogeneity between the nrDNA ITS data and the combined plastid dataset (*rbcL*, *matK*, *trnH-psbA* spacer and *trnL* intron) was tested using the incongruence length difference (ILD) test (Farris et al., 1995), implemented in PAUP v4.0b10 (Swofford, 2002). Following Cunningham (Cunningham, 1997), a significance level of $P = 0.01$ was adopted for this test. In the present study, the congruence between data sets was assessed by a comparing the topologies and support values of the strict consensus trees of data partitions. This "hard incongruence" test was performed directly by visually comparing the support and resolution of each of the clades in the separate analyses that had a higher bootstrap percentage (BP) and posterior probability (PP) than $BP > 75$ and $PP > 90$ (Norup et al., 2006; Wiens, 1998).

The phylogenetic analyses for each matrix were performed using the maximum parsimony (MP) and Bayesian inference (BI) methods in PAUP v4.0b10 (Swofford, 2002) and MrBayes v3.0b4 (Ronquist and Huelsenbeck, 2003), respectively.

For the MP analyses, heuristic searches were conducted with 1000 replicates of random addition, one tree held at each step during the stepwise addition, tree-bisection-reconnection (TBR) branch swapping, MulTrees in effect, and steepest descent off. All of the characters were unordered and equally weighted, and the gaps were coded as missing data. To evaluate the node support, bootstrap analyses (Felsenstein, 1988) were performed using 1000 replicates, with 10 random taxon additions and heuristic search options.

Prior to the Bayesian analysis, the Akaike Information Criterion (AIC) implemented in ModelTest v3.7 (Posada and Crandall, 1998) was used to select the best-fit model of molecular evolution for each dataset. For the BI analyses, four chains of the Markov Chain Monte Carlo (MCMC) chains were run, sampling one tree every 1000 generations for 5,000,000 generations, starting with a random tree. Majority rule (>50%) consensus trees were constructed after removing the "burn-in period" samples (the first 25% of the sampled trees).

2.4. Character reconstruction

The evolution of five diagnostic morphological characters that have been used to define infrageneric taxa of *Dendrobium* from mainland Asia was reconstructed using a maximum likelihood approach with the Markov *k*-state one-parameter (Mk1) model implemented in Mesquite v2.74 (http://mesquiteproject.org/mesquite/mesquite.html). The five reconstructed characters are: (1) distinct elongate rhizome present; (2) leaf sheathing at base; (3) bright yellow flowers; (4) stems and leaves hairy; (5) hairs on stems and leaves blackish. For the purpose of this analysis we followed the infrageneric system of *Dendrobium* as presented by Wood (2006) and Zhu et al. (2009).

3. Results

3.1. Sequences and alignment

In this study, 527 new sequences were obtained. Sequence lengths were as follows: 875 bp for ITS region, 1315 bp for *rbcL*,