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Molecular systematics of subtribe Orchidinae and Asian taxa of Habenariinae (Orchideae, Orchidaceae) based on plastid *matK*, *rbcL* and nuclear ITS

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ABSTRACT

The subtribe Orchidinae, distributed predominantly in Eastern Asia and the Mediterranean, presents some of the most intricate taxonomic problems in the family Orchidaceae with respect to generic delimitation. Based on three DNA markers (plastid *matK*, *rbcL*, and nuclear ITS), morphological characters, and a broad sampling of Orchidinae and selected Habenariinae mainly from Asia (a total of 153 accessions of 145 species in 31 genera), generic delimitation and phylogenetic relationships within the subtribe Orchidinae and Habenariinae from Asia were assessed. Orchidinae and Asian Habenariinae are monophyletic, and Orchidinae is divided into distinct superclades. Many genera, such as *Amitostigma*, *Habenaria*, *Hemipilia*, *Herminium*, *Platanthera*, *Peristylus* and *Ponerorchis*, are not monophyletic. *Habenaria* is subdivided into two distantly related groups, while *Platanthera* is subdivided into three even more disparate groups. Many previously undetected phylogenetic relationships, such as clades formed by the *Amitostigma–Neottianthe–Ponerorchis* complex, *Platanthera latilabris* group, *Ponerorchis chrysea*, *Sirindhornia*, and *Tsaiorchis*, are well supported by both molecular and morphological evidence. We propose to combine *Hemipiliopsis* with *Hemipilia*, *Amitostigma* and *Neottianthe* with *Ponerorchis*, *Smithorchis* with *Platanthera*, and *Aceratorchis* and *Neolindleya* with *Galearis*, and to establish a new genus to accommodate *Ponerorchis chrysea*. *Tsaiorchis* and *Sirindhornia* are two distinctive genera supported by both molecular data and morphological characters. A new genus, *Hsenhsua*, and 41 new combinations are proposed based on these findings.

1. Introduction

The subtribe Orchidinae consist of about 35 genera and 350–400 species, distributed mainly in Eastern Asia, the Mediterranean Region with a few species extending in Northern America, Southern America and Africa (Chen et al., 2009; Dressler, 1993; Kraenzlin, 1901; Lang, 1999; Pearce and Cribb, 2002; Pridgeon et al., 2001). Orchidinae is similar to Habenariinae in habitat preferences and many vegetative and floral characters, but these two subtribes can be distinguished on the basis of the structures of stigma: Orchidinae usually has a concave and sessile stigma often with confluent lobes, whereas Habenariinae usually has stalked, convex and distinct stigma lobes (Dressler, 1993; Pridgeon et al., 2001). This morphological distinction has been supported by molecular evidence (Douzery et al., 1999). However, Inda et al. (2010, 2012) indicated that Habenariinae s.l. is paraphyletic, and several genera from Africa, such as *Stenoglottis*, *Cynorkis*, and *Holothrix*, were resolved as successive sister to Orchidinae + *Habenaria* and its alliance. Batista et al. (2013) showed that the Habenariinae clade (formed by *Habenaria* s.l. + *Cynorkis* + *Stenoglottis*) is sister to the Orchidinae clade formed by (*Orchis* + *Platanthera*). In practice, it can be difficult to distinguish between concave sessile stigma lobes and stalked convex ones in some alpine taxa, such as *Androcorys*, *Herminium*, *Peristylus*, *Ponerorchis*, and *Smithorchis*

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(Dressler, 1993; Lang, 1999; Pridgeon et al., 2001), while some true *Platanthera* species (Orchidinae) clearly have stalked stigma lobes. Kurzweil and Weber (1992), Pridgeon et al. (2001) and Chase et al. (2003) even suggested abandoning the recognition of Orchidinae and Habenariinae as distinct clades.

Orchidinae is a medium-sized subtribe in Orchidaceae and one of many well-studied groups in Orchidaceae, however, the generic delimitation and classification within Orchidinae are still problematic and is complicated by the morphological diversity, wide distribution range, homoplasy of characters considered diagnostic at generic level, and the intergrading and overlapping morphological variation between genera (Aceto et al., 1999; Bateman et al., 2003, 2009; Box et al., 2008; Dressler, 1981, 1993; Hapeman and Inoue, 1997; Jin and Efimov, 2012; Luer, 1975; Pridgeon et al., 2001; Soliva et al., 2001; Tyteca and Klein, 2008). Based on morphological characters and/or analyses of molecular data, various generic delimitations and taxonomies have been proposed, especially on some systematically difficult genera, such as *Dactylorhiza*, *Orchis* s.l., *Platanthera*, *Ponerorchis*, and *Tsaiorchis* (Aceto et al., 1999; Bateman et al., 2003, 2009; Hapeman and Inoue, 1997; Hooker, 1890; King and Pantling, 1896, 1898; Lang, 1998, 1999; Soliva et al., 2001; Luer, 1975; Pridgeon et al., 2001; Tyteca and Klein, 2008).

As previous molecular systematics of Orchidinae were largely based on sampling from the Mediterranean area (Bateman et al., 2003, 2009; Douzery et al., 1999; Soliva et al., 2001; Inda et al., 2012), and/or mainly utilized a single DNA marker (ITS) (Bateman et al., 2003, 2009; Douzery et al., 1999; Soliva et al., 2001), some conclusions and results were weakly supported or even without statistical support, and many taxonomic problems remain unresolved. Despite the high diversity of Orchidinae and Habenariinae in Eastern Asia, many taxa, especially those from monotypic/oligotypic genera, were not represented in previous molecular studies. Generic delimitation and systematic position of many genera in Orchidinae and Habenariinae from Asia, such as *Aceratorchis*, *Amitostigma*, *Hemipilia*, *Hemipiliopsis*, *Neolindleya*, *Neottianthe*, *Platanthera*, *Ponerorchis*, *Smithorchis*, and *Tsaiorchis*, are not or little known, and as a result many taxonomical suggestions and proposals remain to be tested (Bateman et al., 2003, 2009; Chen et al., 2009; Pridgeon et al., 2001). For a better understanding of the generic delimitation within Orchidinae and of the interrelationships among major clades within Orchidinae, it is desirable to base the analyses on multiple DNA markers and a denser sampling across many systematically difficult genera and their allies from Asia.

In the present study, phylogenetic relationships were inferred using three DNA markers (plastid *matK*, *rbcL* and nuclear ITS sequence), with 153 samples representing 146 species of Orchidinae and selected Habenariinae, such as *Androcorys*, *Habenaria*, *Herminium*, and *Peristylus*, mainly from Asia, with the aims of (1) increasing our understanding of the generic delimitation within Orchidinae and Habenariinae; (2) reconstructing the phylogenetic interrelationships within Orchidinae.

2. Materials and methods

2.1. Taxon sampling

There are about 32 genera of Orchideae distributed in Asia, out of which about ten genera are endemic (Chen et al., 2009; Dressler, 1993; Jin et al., 2012; Pedersen et al., 2002; Pridgeon et al., 2001). In order to represent the taxonomic diversity of Orchideae in Asia, 153 accessions of 145 species in 31 genera, including 27 genera and 103 species from Asia (about 84% of Asian genera and 25% of Asian species), were included in this study. Additionally, several genera and some species from Africa, Europe, and South America, including *Bonatea*, *Gennaria*, *Habenaria*, *Ophrys*, *Pseudorchis*, and *Serapias*, were used to broaden the sampling of Orchideae in our analyses. Since previous results indicated that Orchideae is closely related to tribe Diseae (Bytebier et al., 2007; Douzery et al., 1999; Pridgeon et al., 2001), two species of *Disa*, *Disa tripetaloides* and *Disa uniflora*, were used as outgroups. The voucher information and the GenBank accession numbers used in this study are listed in Table A.1.

2.2. DNA extraction, amplification and sequencing

Total genomic DNA was isolated from silica-gel-dried materials using a Plant Genomic DNA Kit (Beijing Biomed Co., LTD, Beijing, China). For this study, two plastid markers (the coding gene *matK*, *rbcL*) and the nuclear ribosomal DNA internal transcribed spacers (ITS) were used. The PCR and sequencing primers for *matK*, *rbcL*, and ITS are listed in Table A.2. The selected DNA regions were amplified by using a standard polymerase chain reaction (PCR). The sequencing reactions were performed by 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 ITS data and the combined plastid dataset (*matK*, *rbcL*) was tested using the incongruence length difference (ILD) (Farris et al., 1995), implemented in PAUP v4.0b10 (Swofford, 2002). Following Cunningham (1997), no cases of strongly supported incongruence were detected ($P = 0.17$), therefore, we combined ITS data and the plastid dataset (*matK*, *rbcL*) in SequenceMatrix v1.7.8 (Vaidya et al., 2011) to perform further phylogenetic analyses.

The phylogenetic analyses for each matrix were performed using the maximum parsimony (MP) in PAUP v4.0b10 (Swofford, 2002) and Bayesian inference (BI) in MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) on CIPRES Science Gateway Web server (Old MrBayes on XSEDE 3.1.2) (Miller et al., 2010).

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, a model for sequence evolution for each matrix was determined by using ModelTest v3.7 (Posada and Crandall, 1998) under the Akaike information criterion. For the BI analyses, two separate four Markov chain Monte Carlo (MCMC) analyses were run, with 10,000,000 generations and sampling every 1000 generation. Majority rule (>50%) consensus trees were constructed after removing the “burn-in period” samples (the first 25% of the sampled trees).

3. Results

3.1. Sequences and alignment

In this study, 215 new sequences were obtained. Sequence lengths were as follows: 825 bp for ITS region, 1254 bp for *rbcL*, 1870 bp for *matK*. The combined alignment of ITS and plastid regions comprised 3949 bp, 24% of which were