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Molecular phylogeny of *Cypripedium* (Orchidaceae: Cypripedioideae) inferred from multiple nuclear and chloroplast regions

Ji-hong Li [a,h], Zhong-jian Liu [b], Gerardo A. Salazar [c], Peter Bernhardt [d], Holger Perner [e], Yukawa Tomohisa [f], Xiao-hua Jin [a], Shih-wen Chung [g], Yi-bo Luo [a,b,*]

[a] State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China
[b] The National Orchid Conservation Center, Shenzhen 518114, China
[c] Departamento de Botánica, Instituto de Biología, Universidad Nacional Autónoma de México, Apartado Postal 70-367, 04510 Mexico, D.F., Mexico
[d] Department of Biology, St. Louis University, St. Louis, MO 63103, USA
[e] Huanglong Administration of National Scenic Spot, Huanglong Sichuan, China
[f] Tsukuba Botanical Garden, National Museum of Nature and Science, 1-1, Amakubo 4, Tsukuba 305-0005, Japan
[g] Department of Life Science, National Taiwan University, 1, Roosevelt Road, Sec. 4, Taipei 106, Taiwan
[h] Graduate University of Chinese Academy of Sciences, Beijing 100039, China

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ABSTRACT

A molecular analysis was performed on 56 taxa in the orchid genus *Cypripedium* using nrDNA ITS and five chloroplast regions (*trnH-psbA*, *atpI-atpH*, *trnS-trnfM*, *trnL-F* spacer, and the *trnL* intron). The genus *Cypripedium* was confirmed as monophyletic. Our data provided strong support for monophyletic grouping of eight infrageneric sections (*Subtropica*, *Obtusipetala*, *Trigonopedia*, *Sinopedilum*, *Bifolia*, *Flabelinervia*, *Arietinum*, and *Cypripedium*) defined in earlier taxonomic treatments, and paraphyletic grouping of two sections (*Irapeana* and *Retinervi*). Within the genus *Cypripedium*, the first divergent lineage consisted of two Mesomaerican species, and subsequently the *Cypripedium debile* lineage from eastern Asia was split. Our study did not support the notion that two Asian species (*Cypripedium subtropicum* and *Cypripedium singchii*) were closely related to either Mesoamerican *Cypripedium irapeanum* or North American *Cypripedium californicum*, as indicated by previous interpretations based on morphological evidences. In addition, one pair of vicariant species, *Cypripedium plectrochilum* (eastern Asia) and *Cypripedium arietinum* (North America), unique to section *Arietinum*, was confirmed. Furthermore, within the monophyletic section *Cypripedium* two previously recognized subsections, *Cypripedium* and *Macrantha*, were shown to be paraphyletic. Our results suggested that this section split into two groups based on distribution (North America vs. Eurasia) instead of such previously used, morphological traits as flower color, and the shape of the lips (labellum) and lateral petals.

1. Introduction

The genus *Cypripedium* has a far wider distribution and more vegetative and floral variations than the four remaining genera in the subfamily Cypripediodeae, Orchidaceae (van der Pijl and Dodson, 1966; *sensu* Cribb, 1997). *Cypripedium* consists of approximately 50 species of terrestrial herbs found in woodland and meadow habitats, from sea level to middle-montane elevations. These species are distributed through subtropical to temperate latitudes of the Northern Hemisphere excluding northern Africa (Cribb, 1997; Averyanov, 2000; Chen and Cribb, 2005; Perner, 2008). Eastern Asia and North America represent two centers of diversity for this genus. At least 38 species occur in Eastern Asian region, and about 30 species in southwestern China (Cribb, 1997; Chen and Cribb, 2005). North America (including Mexico) with about 16 species, is the second and smaller center of diversity (Cribb, 1997).

Historically, the morphological boundaries of this genus are based on only two diagnostic characters. *Cypripedium* spp. are the only "slipper" orchids in the subfamily bearing both plicate leaves and unilocular ovaries with parietal placentation (Cox et al., 1997; Cribb, 1997). While the genus *Cypripedium* is regarded as monophyletic based on the morphological data (above) and DNA sequences (Cox et al., 1997; Cribb, 1997; Eccarius, 2009), the previous molecular phylogenies of this genus have left a number of unresolved questions due to incomplete samples. As fresh specimen material of pivotal species were unavailable for previous phylogenetic analyses such studies were based on unbalanced samplings from eastern Asia compared to more comprehensive collections derived from the Western Hemisphere and Western

* Corresponding author at: State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China. Fax: +86 010 82590843.
E-mail address: luoyb@ibcas.ac.cn (Y.-b. Luo).

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Europe (Cox et al., 1997). In particular, earlier studies lacked information on the DNA sequence analysis of a key species, *Cypripedium subtropicum* (Cox et al., 1997; Eccarius, 2009). This enigmatic and, until recently, unavailable species, was known from a single collection in Tibet, China. Habit, inflorescence and column structure in *C. subtropicum* resembled three Mesoamerican-South American taxa, *Mexipedium xerophyticum*, *Cypripedium irapeanum* and *Selenipedium* spp. (Rosso, 1996; Chen and Lang, 1986; Cribb, 1997). Consequently, the uncertain position of *C. subtropicum* within the phylogeny blurred borders between genera within the subfamily Cypripedioideae (Chen, 1983; Chen and Lang, 1986; Cox et al., 1997; Cribb, 1997). Indeed, the taxonomic borders of nearly every genus described within this subfamily have been debated since the second half of the 19th century (Atwood, 1984). The positions of eastern Asian *Cypripedium debile* and North American *Cypripedium californicum* within the genus *Cypripedium* also varied between studies (Cox et al., 1997; Eccarius, 2009).

We argue that this uncertainty regarding the precise relationship between *C. subtropicum* and *C. irapeanum* in previous studies likely inhibited accurate reconstruction of phylogenetic relationships within the genus *Cypripedium*, and between *Cypripedium* and other genera within the subfamily, Cypripedioideae. We also argue that incomplete sampling of species from eastern Asia raises questions about earlier interpretations by taxonomists that some Eurasian species are sister species of some North American species with parallel morphologies (Chen, 1983; Chen and Lang, 1986; Cox et al., 1997; Cribb, 1997).

For this study, we have focused on improved sampling of *Cypripedium* to facilitate a more accurate reconstruction of phylogenetic relationships. We have included the first published sequences of the key species, *C. subtropicum* and its close ally *Cypripedium singchii* (Liu and Chen, 2009), and also collected specimens from 46 of the 50 described *Cypripedium* spp. (excluding known recurrent hybrids), with an emphasis on expanded representation of eastern Asian taxa. Based on sequences of five cpDNA regions (*trnH-psbA*, *atpI-atpH*, *trnS-trnfM*, *trnL-F* spacer, and the *trnL* intron) and the internal transcribed spacer region of nuclear ribosomal DNA (nrDNA ITS), we provide a well-supported phylogenetic resolution for the intra-generic placement of *C. subtropicum* and *C. singchii*, and a robust generic phylogeny for *Cypripedium* within the Cypripedioideae.

2. Materials and methods

2.1. Ingroup sampling and outgroup selection

The specimens analyzed in this study were selected to maximize all infrageneric groups following the classification by Cribb (1997) and Perner (2008), and to represent the full distributional range of the genus *Cypripedium* in the Northern Hemisphere. Specimens were obtained from either cultivated or wild-collected plants. Taxonomy and nomenclature followed Cribb (1997) and Perner (2008). We obtained 67 samples representing 46 of the currently recognized 50 species excluding the rare *Cypripedium dickinsonianum*, *Cypripedium ludlowii*, *Cypripedium elegans* and *Cypripedium cordigerum*. Based on previous molecular analyses on orchid taxa (see Cox et al., 1997; Cameron et al., 1999; Chase et al., 2003; Górniak et al., 2010), five species assigned to the remaining four genera in the subfamily Cypripedioideae (*Mexipedium*, *Paphiopedium*, *Phragmipedium* and *Selenipedium*) were also included in this study, and additionally six species now classified as members of the subfamilies Apostasioideae (*Apostasia*, *Neuwiedia*) and Vanilloideae (*Vanilla s.s.*) were used to represent outgroups. A list of the taxa analyzed, including information on voucher specimens and GenBank accessions, is given in Table 1.

2.2. DNA extraction, PCR amplification, and sequencing

Total DNA was extracted from silica-gel dried leaves with the TIANamp plant DNA Kit (Tiangen, China) according to the instructions of the manufacturer. The primers used for amplification and sequencing of each individual region were ITS5/ITS4 (Baldwin, 1992) for ITS, c/d for the *trnL* intron and e/f for the *trnL-F* intergeneric spacer (Taberlet et al., 1991), *trnH* (Tate and Simpson, 2003)/*psbA* (Sang et al., 1997) for the *trnH-psbA* intergenic spacer, *trnS*/*trnfM* for the *trnS-trnfM* intergenic spacer (Demesure et al., 1995), and *atpI*/*atpH* for the *atpI-atpH* intergenic spacer (Shaw et al., 2007). PCRs were performed in a total reaction (50 μl) containing 10 ng of template DNA, 5 μl of 10× reaction buffer with $MgCl_2$, 50 mM of each dNTP, 0.5 U of Ex Taq DNA polymerase (Takara Biotechnology, Japan) and 0.2 mM of each primer (Sangon Biotechnology, China). The thermal cycler programme consisted of an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 52–55 °C (depending on the annealing temperature of specific primers), 1 min at 72 °C and a final extension at 72 °C for 10 min. The PCR products were purified by using the DNA Fragment Quick Purification/Recover Kit (Dingguo, China), following the manufacturer's protocol prior to sequencing. Sequencing reactions were performed using the dye-terminator cycle-sequencing ready-reaction kit following the manufacturer's protocol, and analyzed on an ABI 3730 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Each fragment was sequenced for both strands. For DNA sequences added newly in this study, their 5′ and 3′ ends were identified using Cypripediodeae or Orchidaceae sequences already available in Genebank (http://www.ncbi.nlm.nih.gov). New sequences have been deposited in GenBank under the Accession Numbers JF796853—JF797170, JF825972—JF825978, FR720327—FR720330, and FR851209—FR851227.

2.3. Phylogenetic analyses

All sequences were aligned using ClustalX v.1.83 (Thompson et al., 1997). With the use of BioEdit (Hall, 1999) manual adjustments were made by inserting gaps to improve the alignments. The analyses excluded two difficult-to-align regions in ITS, representing 51 sites, one poly T region and one difficult-to-align region in *trnL* [UAA] 3′ exon–*trnF* [GAA] intergenic spacer, encompassing 84 positions, two difficult-to-align regions in *trnH-psbA* intergenic spacer, involving 168 sites, one poly A region in *atpI-tpH* intergenic spacer, including 33 sites, one difficult-to-align region in *trnS-trnfM* intergenic spacer, containing 20 positions. Some unavailable sequences in combined data analyses were treated as missing. Sequence alignments and Nexus formatted files are available upon request from the corresponding author.

The homogeneities across the five cpDNA fragments, and between nrDNA ITS data and the combined cpDNA dataset (*trnL* intron, *trnL* [UAA] 3′ exon–*trnF* [GAA] intergenic spacer, and *trnH-psbA*, *atpI-atpH*, *trnS-trnfM* intergenic spacers) were tested using the incongruence length difference (ILD) test (Farris et al., 1995), as implemented in PAUP* v4.0b10 (Swofford, 2003). The ILD test was conducted with 1000 replicates, each with 10 random addition sequence replicates, TBR branch swapping, and keeping no more than 100 trees per random addition replicate. Following Cunningham (1997), a significance level of $P = 0.01$ was adopted for this test. Also in the present study, congruence between datasets was assessed by comparison of topology and support values of strict consensus trees of data partitions. This "hard incongruence" test was performed by directly comparing visually the support and resolution of each of the clades in the separate analyses with a higher bootstrap percentage (BP) and posterior probability (PP) than BP > 75 and PP > 90 (Wiens, 1998; Norup et al., 2006).