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 breadth of 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 superfamilies. Many genera, such as Amitostigma, Habenaria, Herminium, Platanthera, Persitylus and Ponerorchis, are not monophyletic. Habenaria is subdivided into two distinctly 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 Hemipiliosis with Hemipilia, Amitostigma and Neottianthe with Ponerorchis, Smithorchis and Plananthera, and Acerorchis and Neolindleya with Galearia, 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, Hsienthsua, and 41 new combinations are proposed based on these findings.

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1. Introduction

The subtribe Orchidinae consist of about 35 genera and 350–400 species, distributed mainly in Eastern Asia, the Mediterranean, and in several genera 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 (Douzer et al., 1999). However, Inda et al. (2010, 2012) indicated that Habenaria 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 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, Persitylus, Ponerorchis, and Smithorchis.
(Dressler, 1993; Lang, 1999; Pridgeon et al., 2001), while some true Platanthera species (Orchidaceae) 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, homoplasies 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 Ehmov, 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 Tsaiochris (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/oligo-typic genera, were not represented in previous molecular studies. Generic delimitation and systematic position of many genera in Orchidinae and Habenariinae from Asia, such as Acrorochis, Amrito-stigma, Hemipilia, Hemipiliopsis, Neolindelea, Neottianthe, Platanther-a, 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, Her/minium, 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 the 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
parsimony-informative. Table 1 summarizes the properties of each aligned data partition.

3.2. Phylogenetic analyses

The partition homogeneity test for plastid DNA + ITS shows there were no strongly supported incongruent results in the datasets (P = 0.17), therefore, we combined the datasets for simultaneous analyses.

Phylogenetic relationships based on the ITS data had a better resolution than the two combined plastid DNA data (results not shown here). Based on the combined ITS and plastid DNA data, our findings are consistent in the overall topology of the trees produced with maximum parsimony (MP) and Bayesian inference (BI) methods, except for a few of the collapsed nodes. Bootstrap values (BS) were often lower than the posterior Probability (PP) from the Bayesian analysis.

The BI topology from the combined dataset is chosen as the primary tree for discussion of phylogenetic relationships (Figs. 1 and 2; the MP strict consensus tree is not shown). Our results indicate that the tribe Orchideae can be divided into two well supported sister clades, Habenariinae (PP = 100, BS = 67) (Fig. 1) and Orchidinae (PP = 100, BS = 97) (Fig. 2). Within the Orchidinae, six of nine major clades (Clade I to IX) are well supported along the backbone of the Orchidinae, and many interrelationships among them are well-resolved (Fig. 1). Within the Habenariinae, nine major clades, Clade X to XVIII, can be recognized (Fig. 2). Our results show that many morphological delimited genera of the Asian Orchidinae in Pridgeon et al. (2001) and Chen et al. (2009) are not monophyletic, such as Hemipilia, Herminium, Platpanthera, and Ponerorchis.

Clade I (Fig. 1) comprises 42 species (PP = 89, BS < 50), most of which belong to Platanthera s.l., the remainders are from genera Herminium, Peristylium, and Smithorchis. The interrelationships within Clade I are not well resolved. Clade II comprises two species of Galeopsis, two monotypic genera, Acerorchis and Neolindleya, and one species of Aorthis with weak support (PP = 86) (Fig. 1). Clade III includes one species of Pseudorchis (Fig. 1). Clade IV consists of four species from Dactylorhiza and Gymnadenia with strong support (PP = 100, BS = 85) (Fig. 1). Clade V includes three species of Orchis s.s. with robust support (PP = 100, BS = 99) (Fig. 1). Clade VI (PP = 100, BS = 93) consists of two species of Ophrys and two species of Serapias with strong support (PP = 100, BS = 99) (Fig. 1).

Clade VII (Fig. 1) includes about 15 species from Amitostigma, Neottianthe, and Ponerorchis, and is subdivided into three well supported subclades: one subclade contains five species from Amitostigma and Ponerorchis; another subclade includes four species from Amitostigma and Neottianthe, including types of these two genera; and the third subclade includes four to six species of Amitostigma. Clade VIII (Fig. 1) comprises nine species, six from Hemipilia, two from two monotypic genera, Hemipilopsis and Tsaiorchis, and one from Ponerorchis, with weak support (PP = 81, BS = 56). Clade VIII can be divided into two subclades, one includes Tsaiorchis, and the other includes the remainder with strong support (PP = 100, BS = 97). Clade IX includes a species from the oligotypic genus Sirindhornia (Fig. 1).

Clade X includes four species, two of Androcorys and two of Porolabium with strong support (PP = 100, BS = 100) (Fig. 2). Clade XI consists of three species from Platanthera, i.e., Platanthera kallitridis group, with strong support (PP = 100, BS = 100) (Fig. 2). Clade XII includes one species from Herminium, H. lanceum. Clade XIII (PP = 100, BS = 100) includes six species, two from Peristylium, and four from Herminium (Fig. 2). An unidentified species of Herminium is sister to clades X to XIII, with weak support for the sister group (Fig. 2). Clade XIV (PP = 100, BS = 100) includes two terminals from one species, Ponerorchis chrysea (Fig. 2). Clade XV includes nine species, eight from Asian Habenaria and one from Pecteilis, with strong support (PP = 100, BS = 99) (Fig. 2). Clade XVI includes seven species, six from Peristylium, and one from Platanthera, with substantial support (PP = 100, BS = 84) (Fig. 2). Clade XVII includes 24 species of Habenaria from Africa, Asia and Southern America, and two species of Botansea from Africa with some support (PP = 100, BS = 59) (Fig. 2). Clade XVIII includes two monotypic genera from Europe and Asia, Gennaria and Nuyangia, with strong support (PP = 100, BS = 100) (Fig. 2).

4. Discussion

4.1. An overview of phylogenetics of Orchideae

With broader sampling and more DNA markers, our results confirmed the earlier findings that Orchidinae (PP = 100, BS = 97) (Fig. 1) and Asian Habenariinae (PP = 100, BS = 67) (Fig. 2) are two well resolved sister groups. However, our results indicated that the morphological distinctions, such as the types of stigma lobes, between these two subtribes are problematic (see discussion of each clade). Inda et al. (2012) indicated that Habenariinae is paraphyletic, and several genera from Africa, such as Stenoglottis, Satyrium, Cynorkis, and Holothrix, were resolved as successive sister to Orchidinae + Asian Habenariinae. Batista et al. (2013) showed that the Habenariae clade (formed by Habenaria s.l. + Cynorkis + Stenoglottis) is sister to the Orchidinae clade formed by (Orchis + Platpanthera). Given the complex taxonomy of Orchideae, it would be premature to make any firm conclusion about these two subtribes. More importantly, our results showed that many previous findings of interrelationships within Orchideae based on molecular phylogenetics were strongly supported, and many unknown or overlooked phylogenetic relationships have been detected. Asian Orchidinae is subdivided into two superclades (Fig. 1). Superclade A includes Dactylorhiza, Galeapis, Gymnadenia, Orchis s.s., and Platanthera s.l. (PP = 87, BS = 58). Superclade B includes Amitostigma, Hemipilia, Neottianthe and Sirindhornia (PP = 100). Clade VI, formed by Ophrys and Serapias, is suggested as a sister to Superclade A with strong support, and Sirindhornia is resolved as sister to the rest of Superclade B with weak support (PP = 100) (Fig. 2).
Fig. 1. Phylogenetic tree obtained by Bayesian analysis of the combination of ITS and plastid regions, showing the detailed relationships of subtribe Orchidinae. Numbers above the branches indicate posterior probabilities (PP) and bootstrap percentages (BS). ‘–’ indicates node is not supported in the analysis. ‘/C3’ indicates node is with support value 100%.
Asian Habenariinae is subdivided into three moderately to strongly supported superclades (Fig. 2). Superclade C (PP = 98), consisting of Androcorys, Porolabium, Herminium, Ponerorchis chrysea, Platanthera latilabris group, and some species of Habenaria, occur mostly in montane to alpine regions in Asia. Superclade D (PP = 100, BS = 84), comprising Peristylus, occur mainly in tropical and subtropical Asia with a few species extending into alpine regions. Superclade E (PP = 100), including Habenaria from Asia, Africa and the Neotropics, African Bonatea, and two monotypic genera from Europe and Asia, Gennaria and Nujiangia, mainly occurs in tropical regions with a few species extending into subtropical mountains. The clade formed by Gennaria and Nujiangia is resolved as sister to the remainder in Superclade E.

As Orchidinae is well-represented in Europe, the generic delimitation and classification of many genera around the Mediterranean have been thoroughly studied, for example, Orchis and its alliance by Aceto et al. (1999), Ophrys by Soliva et al. (2001), Serapias by Belluscio et al. (2008), Dactylorhiza by Devos et al. (2006) and
Box et al. (2008). Recently, molecular phylogenetics of Habenaria from Neotropics has been investigated based on comprehensive sampling from Neotropics (Batista et al., 2013). Hence, in our analyses, sampling of genera or species from the Africa, Europe, and Neotropics is mainly used as placeholders for these taxa. Their phylogeny will not be discussed here because our focus is on phylogenetic analyses on Asian taxa.

4.2. Phylogeny and generic delimitation

4.2.1. Platanthera (Clade I, and XVI)

Platanthera consists of about 100–150 species and is among the larger genera in Orchideae, however, generic delimitation of Platanthera is unclear. Luer (1975) suggested that several previous recognized genera, such as Blephariglottis, Lacera, Limorchis, and Tulotis, should be treated as sections within Platanthera, and Dressler (1993) suggested that Platanthera is close to Dactylorhiza–Galearis alliance instead of Habenaria. These suggestions have been supported by recent results of molecular studies (Bateman et al., 2003, 2009; Hapeman and Inoue, 1997). Bateman et al. (2009) broadened Platanthera to include more genera, such as Diphyllax, Piperia, and Tsaorchis. Based on seed micromorphology, however, Camarra et al. (2008) stated that Limorchis should be separated from Platanthera as a distinct genus.

Our analyses do not only support most of these conclusions, but also indicate that Platanthera s.l. is polyphyletic and subdivided into three distant related groups that belong to two subtribes, Platanthera clade (Clade I, Orchidinae), Platanthera latilabris group (Clade XI, Habenariinae), and Platanthera biermanniana (included in Clade XVI, Habenariinae). The Platanthera clade (Clade I, Orchidinae) is not strongly supported and the interrelationships within the genus are not well resolved. However, the Platanthera clade is well characterized by morphological characters, such as fleshy rootstock (no tuber), leaves basal to cauline and grading into foliaceous bracts, sheathing at base, lip spurred, column short and truncate, anther broad, loculi more or less separated by connective, stigmas two and more or less divergent at the base of the entrance of spur.

Platanthera latilabris group, consisting of three closely related species, P. clavigera, P. edgeworthii, P. latilabris (Fig. 3a), form an unambiguous clade deeply nested within Habenariinae. Species of this clade are characterized by ovoid to globose tubers, densely flowered inflorescence, linear and fleshy lip with a callus at base, stigma lobes more or less stalked, extending at base of lip (Fig. 3a). Hooker (1890) treated these species as members of Habenaria, Szlachetko and Kras (2006) transferred these species into the Neotropical genus, Habenella. Our results indicated that Platanthera latilabris group and Habenella (included in Clade XVII) are two quite distantly related groups. Instead, Platanthera latilabris group are resolved as sister to Androcorys plus Porolabium with weak support (PP = 92, BS = 56).

Platanthera biermanniana resembles species of Peristylus in gross morphology. Our analyses show that P. biermanniana is deeply nested within Peristylus with robust support, which is congruent with morphological characters, such as cylindrical-ovoid tuber, 3-lobed lip, short spur, anther with very narrow connective.

Several species of Herminium (H. carnosilabre and H. angustilabre), Peristylus (P. nematocalox), and the monotypic Smithorchis (S. calceoliformis) are deeply nested within Platanthera s.l. These species are restricted in alpine region with elevation ranging from 3500 to 4300 m. The taxonomy has been complicated by these species having minute flower (the smallest in Orchidinae) and a greatly reduced column which is occupied almost entirely by the anther. Most genera of Asian Orchideae were distinguished on the basis of floral characters, especially of the column (Lang, 1999; Prigédon et al., 2001). Our observations and morphological comparison established that these species have oblong to fusiform rootstock (which are near horizontal in S. calceoliformis), entire and spurred lip, naked viscidium, and a relative obvious anther connective, which supports the transfer of these species into Platanthera.

Dozens of Platanthera species from the Himalayas, such as P. bakeriana, P. lepto caulon, P. roseoinctia, and others, have two projecting stigmatic lobes (stalked stigma lobes) and an entire lip (Fig. 3b). Dutrie (1906), Hooker (1890), King and Pantling (1896, 1898), and Tuyama (1966, 1971, 1975) treated these species as Habenaria, while Kraenzlin (1901), Lindley (1835), Lang (1998, 1999), and Soó (1929) placed them in Platanthera. Lang (1998) even proposed a subgenus, subgen. Stigmatosae, to contain these species. Our results indicate that stalked stigma lobes have evolved at least twice in Platanthera, one in P. yodongensiis (Jin et al., 2013), the other in P. exelliana and its relative species. This renders the morphological distinction between Habenariinae and Orchidinae, which is mainly based on the morphology of the stigma, problematic.

4.2.2. Aceratorchis, Aorchis, Galearis, and Neolindleya (Clade II)

Both Aceratorchis (Fig. 3c) and Neolindleya are little-known monotypic genera. Bateman et al. (2009) considered Aceratorchis to be a peloric form of Galearis. Neolindleya was previously considered as Gymnadenia (Bateman et al., 2003), then it was separated as a distinctive genus and sister to Galearis (Bateman et al., 2009; Efimov et al., 2009). Prigédon et al. (2001) considered Aorchis as congeneric with Galearis. In our phylogenetic analyses, Aceratorchis, Aorchis, Galearis and Neolindleya form a weak supported clade (PP = 86) (Fig. 1), while Aceratorchis and Neolindleya were resolved as successive sister to Galearis and Aorchis. These four genera typically share a stolon-like rhizome, anther loculi with distinctrostellum between them, concave stigma, and viscidium enclosed in bursicle. Considering the weak support of the clade, we tentatively proposed to subsume all other three genera into Galearis. However, this needs to be tested by further studies.

4.2.3. Amitostigma, Neottianthe, and Ponerorchis (Clade VII)

The generic borders between Ponerorchis and Amitostigma are unclear due to the difficulty of distinguishing the supposed diagnostic characters, such as appendages of column and the bursicle, and several species have been transferred back and forth between these genera. However, it is nevertheless somewhat unexpected that Amitostigma, Neottianthe and Ponerorchis are nested together and form a moderately supported group (Fig. 1). This clade mainly occurs in Eastern Asia, with one or two species extending into the northern temperate zone. Members of this clade share an ovoid tuber, slender plants with 1–3 slightly fleshy leaves, flowers secund along rachis, dorsal sepal and petals forming a hood, the base of lip more or less connecting the column, column short, lateral appendages obvious, parallel anther cells, stigma lobes situated and confluent under rostellum. The subclade formed by three species of Neottianthe (including the type, Neottianthe cuculata) and Amitostigma gracile (type of Amitostigma) is strongly supported and well characterized by morphological characters, such as leaves basal, elliptic to ovate, petals and three sepal forming a hood, lip papillose. Ponerorchis is said to differ from Amitostigma and Neottianthe by having a viscidium enveloped in a bursicle (see Prigédon et al., 2001). Our results indicate that a bursicle has independently evolved four times in Orchideae (Clade II, VII, VIII, XIV). Moreover, our fieldwork established that the presence of a bursicle is difficult to determine even in living specimens, and some species of Amitostigma, such as A. monanthum (Fig. 3g), A. yuann (Fig. 3h), do have bursicles. It is almost impossible to assign some species, such as Amitostigma yuannum (Fig. 3h), Ponerorchis nana (Fig. 3i), and A. farreri, to Amitostigma or Ponerorchis,
based on morphological characters. On these grounds, we propose to unite *Amotostigma* and *Neottianthe* into *Ponerorchis.*

4.2.4. Hemipilia, Hemipiliopsis and Tsaiorchis (Clade VIII)

Both *Hemipiliopsis* (Fig. 3d, e) and *Tsaiorchis* (Fig. 3f) are little-known and narrowly distributed monotypic genera. Garay and Kettredge (1985) placed *Tsaiorchis neottianthoides* (= *Habenaria keiskeoides*) in *Amotostigma,* Pridgeon et al. (2001) treated *Tsaiorchis* as *Diphylax.* Bateman et al. (2009) positioned *Tsaiorchis neottianthoides* in *Platanthera.* Bateman et al. (2003) stated that the monotypic *Hemipiliopsis* is closely related to *Ponerorchis brevicalcarata,* and suggested these two species should be assigned to *Hemipilia.*

In our analyses, *Hemipilia* is nested within *Hemipilia* with robust support (PP = 100, BS = 97), while *Tsaiorchis* is resolved as sister to *Hemipiliopsis* plus *Hemipilia* with weak support. These results agree with morphological characters. On the one hand, members of this clade (Clade VIII) typically share a distinctly elongate and erect rostellum. On the other hand, *Tsaiorchis* differs greatly from *Hemipiliopsis* and *Hemipilia* both in vegetative and floral characters. *Hemipiliopsis* and *Hemipilia* are characterized by cylindrical to ovoid-globose tubers, leaf solitary, basal, prostrate fleshy, more or less distinctly spotted, inflorescence with purple spots (Fig. 3d, e), stigma lobes confluent. In contrast to these, *Tsaiorchis* (Fig. 3f) is characterized by hairy and horizontally extending rhizomes, one to two chartaceous and green leaves, inflorescence green, two lateral appendages longer than anther, rostellum elongate, stigma lobes separate, linear and extending at the base of lip. Although Luo and Chen (2003) established *Hemipiliopsis* on the basis of its less developed rostellum and the shape of spur, *Hemipiliopsis* is characterized by the well-developed and erect rostellum as long as half of anther locus (Fig. 3e). The sister taxon of *Hemipiliopsis,* *Ponerorchis brevicalcarata,* fits well in *Hemipilia.* In addition, *Hemipiliopsis* is sympatric with *Hemipilia.* Based on these findings, we propose to broaden *Hemipilia* to include *Hemipiliopsis,* and maintain *Tsaiorchis* as a distinctive genus.

4.2.5. Sirindhornia (Clade IX)

*Sirindhornia* is a recently established and morphologically distinctive genus. There are three species and they are mainly distributed in border regions in Thailand, Myanmar, and China. However,
the systematic position of Sirindhornia has been disputed. Pedersen et al. (2002) stated that Sirindhornia is close to Hemipilia, Ponerorchis and reminiscent of Orchis. Instead, Chen et al. (2009) included Sirindhornia monophiphya (type of Sirindhornia) in Ponerorchis.

In our phylogenetic analyses, Sirindhornia is unambiguously resolved as sister to the remaining genera, including Hemipilia, Tsaorchis, in Superclade B, which is supported by morphological characters of Sirindhornia. Sirindhornia is characterized by the elongated tuber, solitary and fleshy leaf, leaf convolute and not spreading, stem and ovary papillo–pubescent, ciliate bract, lip spurred, stigma lobes concave, and viscidium enveloped in a bursicle (Fig. 3). In addition, species of Sirindhornia typically grow in lime–stone regions, and flower from April to June, which is one to two months earlier than other Orchidinae from Eastern Asia. Based on these findings, we recognize Sirindhornia as a distinct genus.

4.2.6. Androcorys, Herminium, and Porolabium (Clade X, XI, XII, and XIII)

Morphologically, it is difficult to determine the systematic positions of Androcorys, Herminium, and Porolabium in Orchidaceae due to the relative small flowers, short column occupied almost by anther, greatly reduced stigma and rostellum (Pridgeon et al., 2001). Pridgeon et al. (2001) proposed to broaden Androcorys to include some members of Herminium with spurred lip; Bateman et al. (2003) suggested that some temperate members of Peristylus should be transferred to Herminium. In our analyses, Androcorys, Herminium, and Porolabium are deeply embedded within Habe- narinaceae, and Herminium is not monophyletic. Some misplaced members have to be excluded and assigned to other genera, while some species currently residing in other genera need to be transferred to it.

Herminium lanceum is resolved as immediate sister to groups formed by clade X plus XI with moderate support (PP = 99, BS = 50). Our observations indicate that H. lanceum differs from other species of Herminium by having parallel anther loculi, one pulvinate stigma swelling at the base of the column, and transverse rostellum, while most Herminium (including the type, H. monor- chis) have more or less divergent anther loculi, two stalked stigma lobes extending along the base of column, and a cylindrical rostellum.

A little known species of Herminium is resolved as immediate sister to clade X, XI, XII and XIII with weak support. Our morpho- logical studies show that this entity is similar to Herminium and shares many morphological characters, such as ovoid tubers, visci- dium involute and hornlike, two stalked stigma lobes extending at the base of column. This entity differs from Herminium by having parallel anther loculi with a long (as long as half of anther locus) and slender appendage between them. More molecular data and a better understanding of the morphological characters are needed to elucidate the phylogeny of this little known entity.

Tang and Wang (1940) separated Porolabium from Herminium on the basis of having a lip with two pores. Pridgeon et al. (2001) stated that the rostellum of Porolabium is identical to Herminium; Pearce and Cribb (2002) suggested that Androcorys ophioglossoides resembles Porolabium biporus in morphological characters. Our results indicate that Porolabium is nested within Androcorys with strong support, and these two genera are sympatric. We found that Androcorys and Porolabium both have a pulvinate stigma at the base of the column. There are several divergent taxonomic options to classify Herminium and its alliance into one or more monophyletic groups. One is to broaden Herminium to include Androcorys and its alliance (Clade X, XI, XII, XIII). Another is to recognize each clade in this alliance (Clade X, XI, XII, XIII) as a distinctive genus. However, with the currently available sampling and weak support interrelationships among these clades, it would be premature to make any firm decision.

4.2.7. Ponerorchis chrysea (Habenaria chrysea, Orchis chrysea, Clade XIV)

Ponerorchis chrysea (Fig. 3h) may have the largest flowers in Orchidinae, however, its systematic position is poorly understood. Schlechter (1924) treated Habenaria chrysea as a member of Orchis s.l., Soó (1966) transferred it to Ponerorchis, Hunt (1971) included it in Chusua. In our analyses, Ponerorchis chrysea is resolved as sister to the entity comprised of Clades X–XIII, with moderate support (PP = 100, BS = 69) (Fig. 2). Most members of these clades have basal and green leaf (or leaves), yellow, yellowish or green flowers, which strikingly differ from Ponerorchis. Our detailed morphologi- cal examination indicate that Ponerorchis chrysea is a quite out of place in Ponerorchis on account of several morphological charac- ters, such as leaves basal, sheaths long and clasping peduncle, floral bracts similar to leaf and enclosing the spur, pedicel longer than ovary, anther connective elongate and drawn out, three slightly convex stigma lobes confluent under rostellum. Some of these characters, such as pedicel longer than ovary, connective elongate and drawn out, are even unique in Orchideae in Eastern Asia. On these grounds, we propose to establish a new genus, Hsenshuia, to accommodate this taxon.


Diagnosis: Tuber subglobose. Stem 2 leaved. Leaves green, basal, Inflorescence 1-flowered. Floral bracts foliaceous, sheathing, enclosing the spur. Pedicel longer than ovary, anther connective elongate and drawn out; rostellum protruding with two arms; three slightly convex stigma lobes confluent under rostellum.

Morphological evidence: Morphologically, Hsenshuia is similar to Ponerorchis s.l. by sharing subglobose tuber, three-lobed lip with a distinct spur, rostellum protruding with two arms, but differs from the latter by having foliaceous bracts sheathing and enclosing the spur, pedicel longer than ovary, anther connective elongate and drawn out. Hsenshuia can be readily distinguished from Herminium s.l. by its foliaceous bracts, elongate anther connective, three slightly convex stigma lobes confluent under rostellum.

Molecular evidence: In the current study, the combined analyses of the datasets of three markers (two chloroplast, one nuclear) shows moderate support for a sister group relationships of Hsens- suia with Herminium s.l. (including Androcorys, Herminium, Porolabi- um and other species) (PP = 100, BS = 69).

Habit and Distribution: Growing in humid alpine grassland and shrub between 3600 and 4200 m in the Eastern Himalayas, includ- ing China (NW Yunnan, SE to S Xizang), and Bhutan.

Etymology: Hsenshuia is named in honor of Prof. Hsen–Hsu Hu, the renowned Chinese scientist who was a pioneer and founder of modern botany in China.

4.2.8. Habenaria and its alliance (Clade XV, XVII, and XVIII)

Habenaria is one of the largest genera of orchid family and widespread across the tropical and subtropical regions of the world (Pridgeon et al., 2001). There is much debate about the generic delimitation and infrageneric systems (see Batista et al., 2013; Pridgeon et al., 2001). Bateman et al. (2003) stated that Habenaria is polyphyletic and needs to be dismantled into many monophyletic genera. On the other hand, Batista et al. (2013) indicated that Neotropical Habenaria is monophyletic and closely related to some African congeners, and suggested that a revision of the infrageneric system rather than an extensive generic fragmentation is most appropriate. In our phylogenetic analyses with 36 sampled species from Asia, Africa and the Neotropics, Habenaria is resolved into two
unambiguously supported groups: Clade XV and clade XVII (Fig. 2). Clade XV includes nine Asian species mainly distributed in subtropical region, such as *H. dentata*, *H. rhodocheila* and *Pecteilis gigantea*, and is sister to the predominantly alpine *Herminium* s.l. clade (Clades X–XIII) and *Ponerorchis chrysea*. Clade XVII, mainly including African and South American *Habenaria*, and *Bonatea*, is resolved as sister to two monotypic genera, *Nujiangia* and *Gennaria*. It is interesting to note that five Asian *Habenaria* are nested within Clade XVII. Asian tropical *Habenaria* stenopetala is resolved as sister to the African-Neotropical *H. heyneanna* plus *H. foliosa* with strong support (PP = 100, BS = 100). Asian alpine *H. intermedia* is resolved as sister to African montane *H. praestans* with strong support (PP = 100, BS = 100); both belong to *Habenaria* sect. *Mutipartitae*.

Two monotypic and morphologically isolated genera in *Habenariinae*, *Gennaria* and *Nujiangia*, were uncovered as sister to African and American *Habenaria* with strong support. These two genera share several morphological characters, such as two alternate leaves, densely flowered inflorescence, 3-lobed lip with a short spur, auricles longer than anther, and a convex stigma (Jin et al., 2012).

Although with limited sampling of *Habenaria* and its alliance from Africa and Neotropics, our phylogenetic analyses indicate that the taxonomy of *Habenaria* is even more complicated than previously thought. It is clear that as currently defined *Habenaria* is patently polyphyletic. There have been several highly divergent taxonomic proposals to delimit *Habenaria* to be monophyletic. One is the generic fragmentation of *Habenaria* into many genera, such as *Bonatea*, *Habenella*, and many others; another is to broaden *Habenaria* to include nearly all genera, such as *Androcorys*, *Herminium*, and *Peristylus*, in *Habenariinae*. The third is to redefine clade XVII as *Habenaria* s.s., with typification of *Habenaria macroceratitis*, while clade XV is segregated as a distinct genus. With the currently

Fig. 4. Plant of *Hsenhsua chrysea*. (a) habit of *Hsenhsua chrysea*; (b) front view of flower; (c) bract; (d) dorsal sepal, lateral sepal, petal and lip; (e) lateral view of flower (bract removed); (f) lateral view of longitudinal section of column and upper part of ovary. (Drawn by Yunxi Zhu.)
available work it is premature to make any firm decision. Future work with more comprehensive sampling and more molecular data will undoubtedly provide new insights into the taxonomy of Habenaria.

4.2.9. Peristylus (Clade XVI)

Peristylus is a widespread genus in Asia with about 70 species (Pridgeon et al., 2001), however, it is almost neglected in previous studies of Orchidaceae. There is some debate on its distinction from Habenaria. Seidenfaden (1977) proposed to define Peristylus on the basis of a set of characters, such as size of floral parts, the position of stigma lobes and caudicles. Pridgeon et al. (2001) stated that Peristylus typically have pulvinate stigmatic swellings at the base of the column and adnate to the base of the lip. Comber (2001) also suggested a set of morphological characters to define Peristylus. In our phylogenetic analyses, Peristylus is resolved as an unambiguously supported clade within Habenariinae, and immediate sister to the group formed by Clades X to XV (Fig. 2). Some misplaced species of Peristylus, such as P. nematocaulon and P. coeclerter, are nested within Platanthera or Herminium, whereas at least one misplaced Platanthera with trilobed lip and ovoid tubers, namely P. biermanniana, is nested within Peristylus. Geographically, Peristylus is mainly distributed in Asia in tropical to subtropical regions, while Platanthera and Herminium mainly occur in alpine regions. In our phylogenetic analyses, the currently delimited Peristylus s.s. is strongly supported by molecular evidence and is more uniform in morphological characters, characterized by having ovoid to globose tubers, flowers small, dorsal sepal and petals forming a hood, lip trilobed and spurred, spur shorter than ovary, and pulvinate stigmas adnate to the base of lip. Therefore, we suggest to make some intergeneric transfers to retain Peristylus as a monophyletic genus which mainly includes members from tropical and subtropical regions.

4.3. Biogeography

Orchidaceae are mostly distributed in northern temperate or alpine regions, however, recent studies indicate that the widespread tropical genus Brachycorythis is sister to the remaining Orchidaceae (Bateman et al., 2003; Inda et al., 2010). There are two centers of biodiversity of Orchidaceae. One is the Mediterranean Region, the other is the Pan-Himalayas (including Himalayas and the Hengduan Mountains) (Pridgeon et al., 2001; Lang, 1998). Inda et al. (2012) stated that Orchidaceae have diversified in the Mediterranean Region in the last 15 Million years. Many taxa of Superclade A, such as Himantoglossum, Ophrys, Pseudorchis and Serapias, are predominantly Mediterranean (Pridgeon et al., 2001). On the other hand, many genera in Superclade A, such as Platanthera and its alliance, have their biodiversity centers in Pan-Himalayas (Chen et al., 2009; Inda et al., 2012; King and Pantling, 1898; Lang, 1998; Pridgeon et al., 2001). Bateman et al. (2003) and Inda et al. (2012) showed that Platanthera and its alliance are deeply nested within the Mediterranean Region group. Our results indicate that Clade III, IV, V, and VI (the Mediterranean Region group) are resolved as successive sister to Platanthera and its alliance. Members of Superclade B, including Hemipilia, Ponerorchis, Sirindhornia and Tsaiorchis, are mostly restricted to mountain regions in Eastern Asia, especially the Pan-Himalayas. Tropical/subtropical genera, such as Sirindhornia and Tsaiorchis, are resolved as successive sister to alpine taxa (Fig. 1).

In contrast to Orchidaceae, Habenariinae are widespread in tropical/subtropical regions around the world and only a few species occur in alpine regions. Recent studies indicate that tropical African genera, such as Cynorkis and, Stenoglottis, are basal groups in the subtribe (Bateman et al., 2003; Batista et al., 2013; Inda et al., 2010, 2012). Our results indicate that alpine groups, such as Herminium and its alliance (including Clade X, XI, XII, XIII, and XIV, most endemic in Pan-Himalayas), are deeply nested within Habenariinae (Fig. 2).

Taken together, it seems that the tropical ancestral group of Orchidaceae radiated in the Mediterranean region in the last 15 million years and in the Pan-Himalayas in the last 8 million years (Geographical history of Pan-Himalayas see Shi et al., 1998; Biogeography of Pan-Himalayas see Sun, 2002a, 2002b).

5. Main conclusions

Based on three DNA markers (plastid matK, rbcl, and nuclear ITS), morphological characters and comprehensive sampling, our current study greatly advances our understanding of the phylogeny of Orchidaceae and Asian Habenariinae. Orchidaceae are subdivided into two sister groups: one is Superclade A + Clade VI (including Gymnadenia, Ophrys, Orchis and its alliance, and Platanthera and its alliance) with diversity centers in the Mediterranean region and Pan-Himalayas, the other is Superclade B (Hemipilia, Ponerorchis, Sirindhornia, and Tsaiorchis) and is mainly distributed in Eastern Asia. Habenaria is subdivided into two distant related groups: one group mainly distributed in tropical and subtropical Asia, the other group (including type of Habenaria) is widespread in both Old and New Tropics. Elements of alpine regions are deeply nested within each tropical/subtropical group. Many genera, such as Herminium, Platanthera, Peristylus and Ponerorchis, are not monophyletic. Many previously undetected phylogenetic relationships, such as clades formed by Ponerorchis chrysea, the Platanthera latilabris group, Sirindhornia, and Tsaiorchis, are well supported by both molecular and morphological evidence. We propose to combine Hemipiliopsis with Hemipilia, to broaden Ponerorchis to include Ammitostigma and Neottianthe, to combine Aorchis, Acerorchis, and Neolindleya with Galearis, and to establish a new genus to accommodate Ponerorchis chrysea. Tsaiorchis and Sirindhornia are recognized as two distinctive genera. A nomenclatural revision is provided in the Appendix A. At the same time, the relationships of many groups, such as Herminium and its alliance (including Androcorys, Herminium, Platanthera latilabris group, and Porolabium) and Galearis and its alliance (including Acerorchis, Aorchis, Galearis, and Neolindleya) remain unresolved. Further work based on broader sampling and more markers is needed.

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Appendix A

A.1. Nomenclatural revision

   (1) Galearis camtschatica (Cham.) X.H. Jin, Schuit. et W.T. Jin, comb. nov.
   Basionym Orchis camtschatica Cham., Linnaea 3: 27 (1828).

2. Hemipilia Lindl.
   Synonym.
   Hemipiliopsis Y.B. Luo et S.C. Chen, syn. nov.

3. Peristylus Blume.
(3) Peristylus biermannianus (King et Pantl.) X.H. Jin, Schuit. & W.T. Jin, comb. nov.  
Synonym.
Smithorches Tang et F.T. Wang, syn. nov.  
(4) Platanthera angustilabris (King et Pantl.) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
(5) Platanthera calciciformis (W.W. Sm.) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
Basionym Herminium carnosilabre X.H. Jin, Comb. nov.  
Basionym Platanthera carnosilabris (Tang et F.T. Wang) X.H. Jin, Comb. nov.
(6) Platanthera chlrr  (34) Ponerorchis secundiflora  
Basionym Amitostigma simplex  
(7) Ponerorchis alpestris (Fukuy.) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
Basionym Amitostigma amplexifolium X.H. Jin, Comb. nov.  
Basionym Platanthera amplexifolia (Tang et F.T. Wang) X.H. Jin, Comb. nov.
(9) Ponerorchis basifoliata (Finet) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
Basionym Amitostigma bifolium X.H. Jin, Comb. nov.  
Basionym Platanthera bifolia (Tang et F.T. Wang) X.H. Jin, Comb. nov.
Basionym Peristylus bicolor X.H. Jin, Comb. nov.  
Basionym Platanthera bicolor (Tang et F.T. Wang) X.H. Jin, Comb. nov.
(13) Ponerorchis caerulea (Schltr.) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
Basionym Gymnadenia caerulea (Schltr.) X.H. Jin, Comb. nov.  
(14) Ponerorchis caerulea var. calcicola (W.W. Sm.) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
(16) Ponerorchis dolichocentra var. calcicola (W.W. Sm.) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
(18) Ponerorchis faberi (Rolfe) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
(19) Ponerorchis farreri (Schltr.) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
(21) Ponerorchis gracilis (Blume) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
(22) Ponerorchis keiskei (Finet) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
(23) Ponerorchis kinoshitae (Makino) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
(24) Ponerorchis lepida (Rchb. f.) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
(26) Ponerorchis monantha (Finet) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
(30) Ponerorchis parciflora (Finet) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
Basionym Peristylus parciflorus f. parciflorus Finet, Rev. Gén. Bot. 13: 525 (1902) (as 'parceflorus').
(31) Ponerorchis physoceras (Schltr.) X.H. Jin, Schuit. et W.T. Jin, comb. nov.  
(34) Ponerorchis secundiflora (Hook. f.) X.H. Jin, Schuit. et W.T. Jin, comb. nov.
References


