

# Phylogenetic placement of the enigmatic orchid genera *Thaia* and *Tangtsinia*: Evidence from molecular and morphological characters

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**Abstract** The phylogenetic position of two enigmatic Asian orchid genera, *Thaia* and *Tangtsinia*, were inferred from molecular data and morphological evidence. An analysis of combined plastid data (*rbcL*+*matK*+*psaB*) using Bayesian and parsimony methods revealed that *Thaia* is a sister group to the higher epidendroids, and tribe Neottieae is polyphyletic unless *Thaia* is removed. Morphological evidence, such as plicate leaves and corms, the structure of the gynostemium and the micromorphology of pollinia, also indicates that *Thaia* should be excluded from Neottieae. *Thaieae*, a new tribe, is therefore tentatively established. Using Bayesian and parsimony methods, analyses of combined plastid and nuclear datasets (*rbcL*, *matK*, *psaB*, *trnL-F*, ITS, *Xdh*) confirmed that the monotypic genus *Tangtsinia* was nested within and is synonymous with the genus *Cephalanthera*, in which an apical stigma has evolved independently at least twice. The tribe Neottieae (excluding *Thaia*) is strongly supported as monophyletic based on molecular analyses and divided into three clades as follows: *Palmorchis* diverged earliest; *Cephalanthera* (including the former *Tangtsinia*) is sister to the remaining of the genera; and *Aphyllorchis* is immediate sister to *Limodorum*, forming a third clade with *Neottia* Guett. (including *Listera*) and *Epipactis*.

**Keywords** molecular phylogeny; morphology; Neottieae; Orchidaceae; *Tangtsinia*; *Thaia*

**Supplementary Material** The alignment file is available in the Supplementary Data section of the online version of this article (<http://www.ingentaconnect.com/content/iapt/tax>).

## ■ INTRODUCTION

Neottieae Lindl. is a small tribe of orchids, totalling about 100 species, that is distributed primarily in the temperate and subtropical zones of the Northern Hemisphere (Dressler, 1981; Bateman & al., 2005). Despite its small size, the tribe shows both an unusually large amount of morphological variation and characters, such as gynostemium with erect or suberect anther as well as soft and friable pollinia, which are viewed as primitive in Orchidaceae. These features have caused difficulties in the delineation of tribes and genera and determination of their phylogenetic relationships (Bateman & al., 2005). Neottieae has been assigned to different subfamilies of Orchidaceae: Orchidoideae (e.g., Dressler, 1981) or Epidendroideae s.l. (e.g., Dressler, 1993; Cameron & al., 1999) or were treated as a separate subfamily Neottioideae (e.g., Dressler & Dodson, 1960; Garay, 1972; Szlachetko, 1995). Between 6 and 11 genera were at times recognised in Neottieae: *Aphyllorchis* Blume, *Cephalanthera* Rich., *Diplandrorchis* S.C. Chen, *Epipactis* Zinn, *Holopogon* Kom. & Nevski, *Limodorum* Boehm., *Listera* R. Br., *Neottia* Guett., *Palmorchis* Barb. Rodr., *Tangtsinia* S.C. Chen, and *Thaia* Seidenf. (Burns-Balogh & al., 1987; Dressler, 1993; Bateman & al., 2005; Chen & al., 2009).

All phylogenetic analyses to date have focused on the genera *Cephalanthera*, *Epipactis*, *Limodorum*, *Neottia* (including *Listera*), and *Palmorchis* (Freudenstein & al., 2004; Bateman & al., 2005; Roy & al., 2009). Other genera, especially the monotypic *Tangtsinia* and *Thaia*, have never been rigorously tested using molecular data. This contributed to systematic problems, such as the questionable monophyly of Neottieae and the uncertain phylogenetic relationships and evolution of morphological characters within this tribe.

*Thaia* was first described as holomycotrophic herbs endemic in Thailand (Seidenfaden, 1975). The genus has suffered a tortured systematic history. Dressler (1981) first placed it in Neottieae but then treated it as one of his 14 “misfit and leftover” orchid genera (Dressler, 1993). Burns-Balogh & al. (1987) excluded it from Neottieae and tentatively included it in Vanilleae, whereas Bateman & al. (2005) placed it in Neottieae. During our fieldwork in southern Yunnan, southwestern China in 2010, *Thaia* was discovered as a new record in China. Contrasting with the original description as saprotrophic (see Seidenfaden, 1975), *Thaia* is an autotrophic green plant with corms subtending 2–4 leaves, and often a large inflorescence (Fig. 1). Moreover, autotrophic green plants of *Thaia saprophytica* Seidenf. were discovered in Thailand in recent collections (Roy & al., 2009).



**Fig. 1.** Illustration of *Thaia saprophytica*: **A**, habit; **B**, inflorescence; **C**, lateral view of flower; **D**, front view of flower; **E**, sepals, petals and lip; **F**, lip and gynostemium; **G**, gynostemium; **H**, anther cap; **I**, lateral view of gynostemium; **J**, pollinia. — Drawn by Yingbao Sun.

*Tangtsinia* is a controversial genus, originally described by Chen (1965) based on the horizontal stigma terminating the gynostemium, the presence of five staminodes, and the erect median anther positioned above the stigma. Dressler (1981) suggested that it is a peloric mutant of *Cephalanthera*, which was later followed by most orchidologists (e.g., Rudall & Bateman, 2002; Bateman & al., 2005; Bateman & Rudall, 2006). Burns-Balogh & al. (1987) suggested that *Tangtsinia* is similar in certain respects to *Diplandrorchis* in having a horizontal stigma and a median anther situated above the stigma. However, these hypotheses remain to be tested using molecular data. During our fieldwork in northern Yunnan, China, we discovered a holomycotrophic species of *Cephalanthera*, *C. humilis* X.H. Jin, that possesses an actinomorphic perianth and a horizontal stigma on top of the gynostemium (Jin & al., 2011), providing a good opportunity to test the systematic position of *Tangtsinia* and thereby its morphological evolution.

The aims of the present study were (i) to determine the phylogenetic relationships of *Thaia* and *Tangtsinia* using chloroplast *rbcL*, *matK*, and *psaB*, and (ii) to discuss the relationships within Neottieae using evidence from both molecular (chloroplast *rbcL*, *matK*, *psaB*, *trnL-F*, and nuclear ITS, *Xdh*) and morphological data.

## ■ MATERIALS AND METHODS

**Taxon sampling.** — For the subfamily-wide analysis, 132 genera (Appendix 1), representing all tribes of subfamily Epidendroideae, were included in the broad plastid (*rbcL*+*matK*+*psaB*) analyses to assess the tribal position of *Thaia saprophytica* and *Tangtsinia nanchuanica* S.C. Chen. *Phragmipedium longifolium* (Rchb. f. & Warsc.) Rolfe of subfamily Cypripedioideae, plus *Disa tripetaloides* (L. f.) N.E. Br., *Diuris sulphurea* R. Br., *Orchis quadripunctata* Cirillo ex M. Tenore, and *Spiranthes spiralis* (L.) Chevall of subfamily Orchidoideae were used as outgroups.

In the first analysis of the broad plastid dataset, *Tangtsinia* was nested within Neottieae (see Results). A second series of analyses focused on tribe Neottieae. A total of 11 species of the tribe (following Bateman & al., 2005) were sampled to further pinpoint the placement of *Tangtsinia* using molecular data (chloroplast *rbcL*, *matK*, *psaB*, and *trnL-F* plus nuclear ITS and *Xdh*). In addition, the new species bearing its stigma on top of its column, *Cephalanthera humilis* (Jin & al., 2011), was included in the analysis. *Tropidia* Lindl. of subfamily Epidendroideae was used as outgroup. Voucher information and GenBank accession numbers are listed in Appendix 2.

**Molecular data.** — Total genomic DNA was extracted from silica gel-dried leaves of living plants using the modified CTAB procedure of Doyle & Doyle (1987). The *rbcL*, *matK*, *trnL-F*, and ITS DNA regions were amplified using *rbcL*-1F and 724R (Muasya & al., 1998), *matK*-390F and *matK*-1326R (Cuenoud & al., 2002), *trnL*F (e) and *trnL*F (f) (Taberlet & al., 1991), and ITS-1 and ITS-4 (Baldwin, 1992), respectively. Sequences were processed on an ABI 3730 DNA analyser (Applied Biosystems, ABI, Carlsbad, California, U.S.A.). Three new sequences of

*rbcL*, four new sequences of *matK*, three of *trnL-F*, and one of ITS were generated in this study (Appendices 1, 2).

**Phylogenetic analyses.** — For DNA sequences, Clustal X v.1.83 (Thompson & al., 1997) was used to obtain an initial alignment, followed by manual adjustment with BioEdit v.7.1.3 (Hall, 1999). Phylogenetic analyses for each matrix were carried out using maximum parsimony (MP) and Bayesian inference (BI) methods in PAUP\* v.4.0b10 (Swofford, 2003) and MrBayes v.3.0b4 (Ronquist & Huelsenbeck, 2003), respectively.

For MP analyses, heuristic searches were conducted with 1000 replicates of random addition, one tree held at each step during stepwise addition, tree-bisection-reconnection (TBR) branch-swapping, MulTrees in effect, and steepest descent off. All characters were unordered and equally weighted, and gaps were coded as missing data. To assess node support, bootstrap analyses (Felsenstein, 1985) were performed using 1000 replicates with 10 random taxon additions and heuristic search options.

For BI analyses, each DNA region was assigned its own model of nucleotide substitution as determined by the Akaike information criterion (AIC) in Modeltest v.3.06 (Posada & Crandall, 1998). Four chains of the Markov Chain Monte Carlo (MCMC) were run, sampling one tree every 1000 generations for 3,000,000 starting with a random tree. Majority-rule (>50%) consensus trees were constructed after removing the burn-in period samples (the first 20% of the sampled trees).

**Macromorphology and micromorphology.** — Gross morphological data were obtained during fieldwork and specimens were deposited in PE and HITBC. Pollinia for micromorphological examination were taken from fully open flowers, observed under a Nikon SNZI1000 microscope and imaged using a Nikon DXM 1200 F digital camera.

To further examine the taxonomic status of *Tangtsinia*, we optimised the stigma position onto the cladogram from the BI analysis using MacClade v.4.06 (Maddison & Maddison, 2003).

## ■ RESULTS

**Broad analyses of subfamily Epidendroideae.** — The dataset included chloroplast *rbcL* (1362 sites), *matK* (1662 sites), and *psaB* (1666 sites) genes for 132 taxa. The dataset of the three combined plastid DNA sequences consisted of 4690 characters, of which 2953 (~63%) were constant and 962 (~21%) were parsimony-informative. Parsimony analyses generated 900 maximally parsimonious trees (MPTs) of 5338 steps, with a consistency index (CI) of 0.449 and a retention index (RI) of 0.632. Bayesian trees were congruent with MP trees except the least well supported nodes. Within subfamily Epidendroideae, the ‘higher’ Epidendroideae formed a monophyletic group with strong Bayesian (PP = 100) but weaker parsimony support (BS = 64). The ‘lower’ Epidendroideae divided into several clades and were paraphyletic relative to the higher Epidendroideae (Fig. 2).

The monotypic genus *Thaia* was sister to the ‘higher’ epidendroids in BI analysis (PP = 100) and had moderate parsimony support (BS = 74; Fig. 2). The monotypic genus *Tangtsinia* was nested within tribe Neottieae with strong support



**Fig. 2.** Phylogenetic relationships of subfamily Epidendroideae based on combined *rbcL*, *matK*, and *psaB* plastid data. Numbers at nodes are Bayesian posterior probabilities and bootstrap percentages ( $\geq 50\%$ ), respectively. A dash (-) indicates that a node is not supported in the analysis.



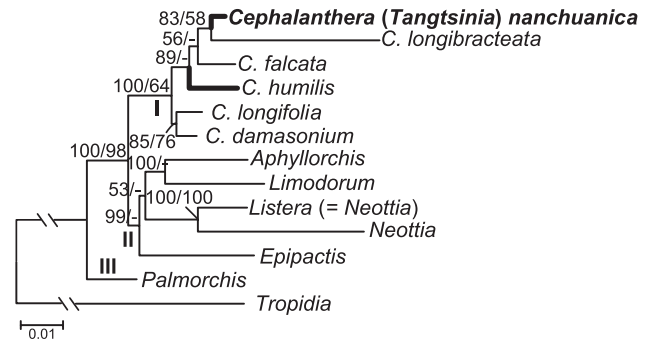
values (PP = 100, BS = 94), and the monophyly of tribe Neottieae was recognised strongly (PP = 98) in the Bayesian tree but less so in the parsimony analyses (BS = 56; Fig. 2). Characters of particular interest are discussed in detail below.

**Analyses of tribe Neottieae.** — The analysis of the narrow *rbcL* data had 1356 characters, 28 of which were parsimony-informative. The analysis of the narrow *psaB* data had 1666 characters, 10 of which were parsimony-informative with no indels. The analysis of the narrow *matK* data had 1618 characters, 45 of which were parsimony-informative and there were no indels. The analysis of *trnL-F* data had 1391 characters, 15 of which were indels and 7 of which were parsimony-informative. The analysis of ITS data included 754 characters, 97 of which were parsimony-informative and 5 of which were indels. The analysis of *Xdh* data included 931 characters, 50 of which were parsimony-informative and 2 of which were indels. The aligned matrix of the combined three chloroplast data had 6031 sites, 90 of which were parsimony-informative. The aligned matrix of the two nuclear regions had 1685 sites, 147 of which were parsimony-informative. The combined matrix of the chloroplast and nuclear data had 7716 sites, 237 of which were parsimony-informative. The MP analysis produced 10 MPTs of 1469 steps, with a CI of 0.900 and RI of 0.616.

Bayesian analyses yielded trees with topologies that were consistent with those obtained from the MP analyses except for a few collapsed nodes in the strict consensus tree (Fig. 3). *Tangtsinia* was nested in the genus *Cephalanthera* and was identified as sister to *C. longibracteata* Blume with moderate support in the BI (PP = 83) and weak support in the parsimony analysis (BS = 58). *Cephalanthera falcata* (Thunb.) Blume was strongly supported as sister to *T. nanchuanica* plus *C. longibracteata* in the Bayesian analysis (PP = 56). *Cephalanthera humilis* was sister to the three species above (PP = 89). *Cephalanthera longifolia* (L.) Fritsch and *C. damasonium* (Mill.) Druce were sister species (PP = 85, BP = 76) and together diverged earliest within *Cephalanthera*. Morphological characters are discussed in detail below. Tribe Neottieae contains the three following clades (Fig. 3): *Palmorchis* diverged earliest (clade III); *Cephalanthera* (including the former *Tangtsinia*) is the second clade (clade I), and sister to the remaining genera; *Aphyllorchis* is sister to *Limodorum* together with *Neottia* (including *Listera*) and *Epipactis* (clade II).

**ITS of *Thaia saprophytica*.** — Comparison of the ITS sequence of *Thaia saprophytica* that we generated with the one published by Roy & al. (2009) indicates that the two ITS sequences were identical except at one site and hence were 99.86% similar. This comparison confirms the accuracy of both the sequence generated herein and that of Roy & al. (2009) for green specimens of *Thaia saprophytica*.

**Morphology of *Thaia saprophytica* (Figs. 1, 4, 5).** — *Thaia saprophytica* is a 50–150 cm tall plant. It is generally autotrophic, but in certain cases it may be mycoheterotrophic. The 4- to 5-noded depressed cylindrical corms are 4.0–4.5 cm in diameter and 4–5 cm long, enclosed by leaf sheaths. Roots arise from the nodes of the corms and are hairy. The 3–5 plicate leaves are lanceolate to elliptic and acuminate to caudate at the apex, and wither in winter. Leaf articulation is absent.



**Fig. 3.** Phylogenetic relationships of tribe Neottieae based on the combined plastid and nuclear data. Numbers at nodes are Bayesian posterior probabilities and bootstrap percentages ( $\geq 50\%$ ), respectively. A dash (-) indicates that a node is not supported in the analysis. I, II and III represent the three clades of Neottieae. Bold lines indicate species with a terminal stigma.

The blade is 14–17 cm long and 5.5–6.5 cm wide. The petioles form a 20–30 cm long pseudostem with 2–3 tubular sheaths at the base. The inflorescence arises from the top of the corm with the leaves. The flowers are resupinate and do not open fully. The lateral sepals form a minute mentum with the column foot. The lip attaches to the column foot, with two lamellae extending from the base to the centre. The gynostemium is more or less curved, with a pair of semi-circular wings at the base. A tongue-shaped callus extends from the base of the stigmatic area. The rostellum projects forwards at an angle of approximately 45°. The anther is suberect, with a two-celled depressed-triangular anther cap. There are two sectile pollinia, whose exine is striato-reticulate.

## DISCUSSION

**The identity of *Thaia saprophytica*.** — *Thaia saprophytica* was originally described as mycoheterotrophic (see Seidenfaden, 1975; Bateman & al., 2005). However, autotrophic green plants were discovered both in China and Thailand, and the ITS sequence comparison indicated that only one site differs between the nrDNA ITS sequence of the autotrophic Chinese plants of *Thaia* included in this study and the sample from Thailand. The flowers of the “autotrophic” and “saprotrophic” forms are identical, and it appears that the herbarium specimens DB9176 and *Beuselkom 3518* were fertile parts of autotrophic green plants. We suspect that either the vegetative parts were overlooked during the collection of the type material of *Thaia saprophytica* or it grows in dry areas and therefore its leaves had withered by flowering time. It may also be that “autotrophic” and “saprotrophic” forms are two different taxa, although they are identical in flower morphology. Within tribe Neottieae, several green orchids acquire organic carbon both from their mycorrhizal fungi and from photosynthesis (Bidartondo & al., 2004; Selosse & al., 2004; Julou & al., 2005). Numerous cases are known in *Epipactis* (Selosse & al., 2004; Selosse & Roy, 2009) and *Cephalanthera* (Pedersen & al.,



2009; Roy & al., 2009) in which typically autotrophic species contain mycoheterotrophic individuals (Bateman & al., 2005; Tranchida-Lombardo & al., 2010).

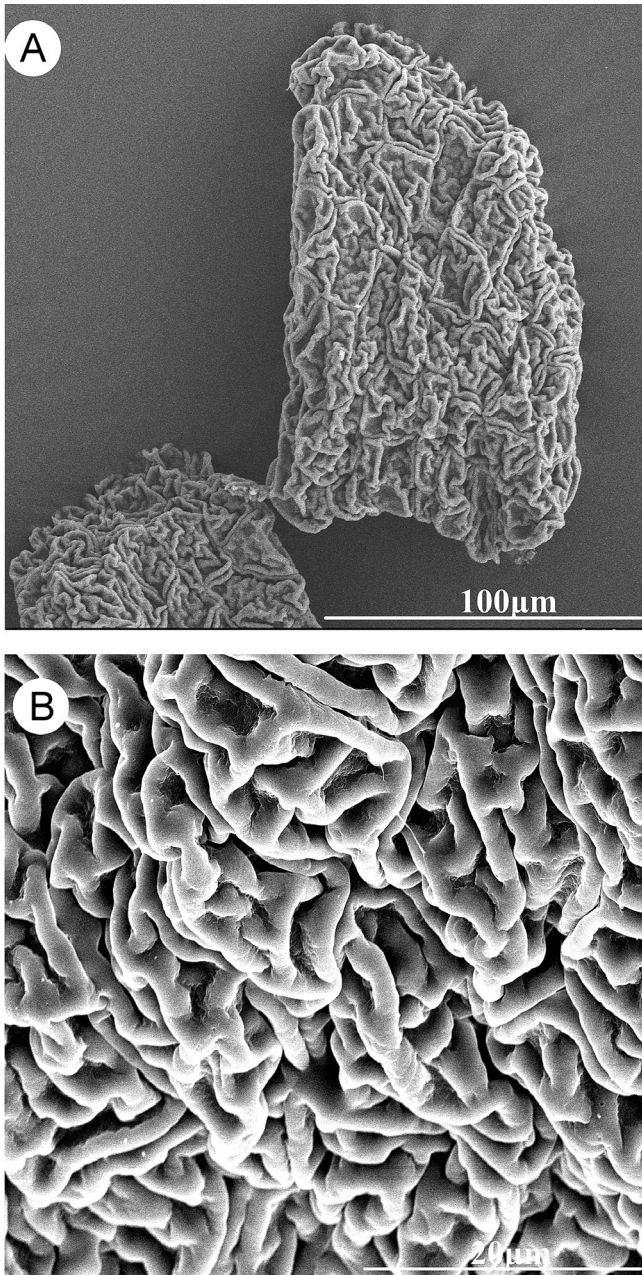
**Systematic position of *Thaia*.** — Using *Tropidia polystachya* (Sw.) Ames, *Nervilia shirensis* (Rolfe) Schltr., and *Vanilla planifolia* Andrews as outgroups, Roy & al. (2009) inferred that tribe Neottieae (including *Thaia*) is monophyletic but with weak support. In their study, *Thaia* occupied a basal position but with low support owing to the lack of chloroplast *trnS-G* and *rbcL* sequences in their matrix. In contrast, our study is based on three chloroplast loci (*rbcL*, *matK*, *psaB*) and broad taxonomic sampling within Epidendroideae. Our

molecular results indicate that *Thaia* is strongly supported as sister to the higher epidendroid clade (Fig. 2). Instead of *Thaia*, Prutsch & Schill (2000) suggested that *Cephalanthera* provides a link between the primitive and derived Epidendroideae on the basis of micromorphological, anatomical, and ultrastructural observations of the stigma of several species of Neottieae. However, this hypothesis was not supported by the subsequent phylogenetics studies (such as Freudenstein & al., 2004). It appears that the resemblance of the stigma between *Cephalanthera* and *Phaius* Lour. is due to convergence.

The exclusion of *Thaia* from Neottieae is also supported by our morphological data. The pollen micromorphology of *Thaia* is significantly different from that of Neottieae. The exine of all known genera of Neottieae is reticulate (Burns-Balogh & al., 1987), whereas it is striato-reticulate in *Thaia*. The gross morphological characters of *Thaia*, such as robust corms, plicate leaves, the pseudostem formed by petioles, the curved gynostemium with a short foot, mentum, lip attached to the foot, the special appendix below the stigma area and the depressed-triangular anther cap (Fig. 1), all suggest that *Thaia* should be excluded from Neottieae. Our broad molecular analyses also strongly support the exclusion of *Thaia* from tribe Neottieae; instead, *Thaia* is resolved as the sister group of the monophyletic higher Epidendroideae. Based on both molecular and morphological evidence, a new tribe, Thaieae, is tentatively established below.

***Tangtsinia* belongs within *Cephalanthera*.** — Chen (1965) argued that *Tangtsinia* differs markedly from *Cephalanthera* in having actinomorphic flowers with the lip similar to the petals, the stigma located on top of the gynostemium, and the flower having five staminodes (Chen, 1965, 1982; Chen & Gale, 2009). However, most orchidologists have treated the plant as a peloric mutant of *Cephalanthera* (e.g., Dressler, 1981; Burns-Balogh & al., 1987; Bateman & Rudall, 2006). Our results here indicate that *Tangtsinia* is indeed nested within *Cephalanthera*, with strong support in our molecular data (Fig. 3), and that a horizontal stigma has therefore evolved independently at least twice in *Cephalanthera* (Fig. 3). Moreover, *Tangtsinia* and *Cephalanthera* share many morphological characters, such as creeping rhizomes, a terminal inflorescence and an erect gynostemium with a sub-erect/erect anther and naked granular-farinaceous pollinia. Hence, it is preferable to treat *Tangtsinia* as a synonym of *Cephalanthera* rather than a distinctive endemic genus.

**Relationships within the tribe Neottieae.** — In molecular studies using *rbcL* and *matK*, Freudenstein & al. (2004) proposed that tribe Neottieae was sister group to other lower Epidendroideae, but this was poorly supported due to limited sampling. Our analyses indicated that tribe Neottieae is well-supported within lower Epidendroideae based on the *rbcL*, *matK* and *psaB* analyses (Fig. 2). These findings are consistent with the molecular analysis by Cameron & al. (1999). Tribe Neottieae (largely following Bateman & al., 2005, but excluding *Thaia*) was divided into three clades (Fig. 3). Within clade I, the monophyly of *Cephalanthera* (including *Tangtsinia*) was well supported, and relationships were identified by the molecular data as discussed above. Clade II contains four genera,



**Fig. 4.** Pollinium exine of *Thaia saprophytica*: **A**, shape of pollinium; **B**, detailed surface view.





**Fig. 5.** Habit of *Thaia saprophytica*: **A**, plant with inflorescence, pseudo-stem, and corms; **B**, middle sepal; **C**, lateral sepal; **D**, petal; **E**, lip; **F**, gynostemium.

i.e., *Neottia* (including *Listera*), *Aphyllorchis*, *Limodorum*, and *Epipactis*. Chase & al. (2003) suggested that *Listera* and *Neottia* should be combined which was also followed by others (Bateman & al., 2005; Chen & al., 2009). *Aphyllorchis* and *Limodorum* were sister groups (Roy & al., 2009) with strong support, sharing several morphological characters (Bateman & al., 2005). *Epipactis* was sister to these two groups. *Palmorchis* was the earliest divergent clade (clade III; Fig. 3).

## ■ CONCLUSIONS

*Thaia* and *Tangtsinia* are two Asian endemic genera of Orchidaceae with controversial systematic positions. In this study, we performed two phylogenetic analyses: the first analysis used the chloroplast *rbcL*, *matK*, and *psaB* sequences to broadly place *Thaia* and *Tangtsinia* within subfamily Epidendroideae, and the second analysis used six loci (*rbcL*, *matK*, *psaB*, *trnL-F*, ITS, *Xdh*) to further clarify their phylogenetic relationships and the taxonomic status of *Tangtsinia*. The molecular analyses strongly support *Thaia* as sister group of higher Epidendroideae, and *Tangtsinia* as a group nested within *Cephalanthera*. Both results are supported by morphological evidence.

## ■ TAXONOMIC TREATMENT

**Thaieae** X.H. Jin & X.G. Xiang, **tr. nov.** — Figures 1, 4, 5.

Tribus nova Neottiis affinis, sed depressa cylindrata cormo, plicatis foliis, petiolis fascibus pseudocaulis, gynostemium curva et semirotundatis alis ad basim.

Autotrophic plants, or in certain cases mycoheterotrophic. Corm multi-noded. Leaf petioles forming pseudostem. Inflorescence arising from the top of the corm, dense and many-flowered, racemose. Gynostemium more or less curved, with a tongue-shaped callus extending from the base of the stigmatic area; base of gynostemium extending, forming a short mentum with lateral sepals; rostellum projecting forwards; anther suberect, two-celled, and acuminate. Pollinia two, sectile, exine striato-reticulate.

Type: *Thaia* Seidenf. in Bot. Tidsskr. 70(1): 73. 1975.

Distribution: Thailand (Provinces of Phayao, Loei and Kancharaburi) and China (Yunnan Province, Mengla County, Menglun).

***Cephalanthera nanchuanica*** (S.C. Chen) X.H. Jin & X.G. Xiang, **comb. nov.** ≡ *Tangtsinia nanchuanica* S.C. Chen in Acta Phytotax. Sin. 10(3): 194, fig. 39. 1965 – Type: CHINA. Sichuan, Nanchuan, Jinfoshan, 970 m, 3 May 1964, Chen S.C. & Lang K.Y. 2119 (PE!).

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**Appendix 1.** Taxa and GenBank accession numbers for the *rbcL*, *matK*, and *psaB* sequences in phylogenetic analysis of subfamily Epidendroideae. A dash (–) indicates missing data; an asterisk (\*) denotes sequences obtained in this study, and the remaining sequences are from GenBank. The superscripts 1–38 refer to the literature (see bottom of Appendix 2) or GenBank sources.

**INGROUP:** *Acanthephippium mantinianum* Linden & Cogn., AF074100<sup>1</sup>, AF263618<sup>2</sup>, AY380927<sup>3</sup>; *Acineta chrysantha* R. Br., AF074102<sup>1</sup>, AF263619<sup>2</sup>, AY380929<sup>3</sup>; *Aerangis* sp., AF074103<sup>1</sup>, AY368389<sup>4</sup>, AY380931<sup>3</sup>; *Aeranthus* sp., AF074104<sup>1</sup>, AY368390<sup>4</sup>, AY380932<sup>3</sup>; *Angraecum* sp., AF074106<sup>1</sup>, AF263621<sup>2</sup>, AY380933<sup>3</sup>; *Aplectrum hyemale* (Muhl. ex Willd.) Nutt., FJ445516<sup>5</sup>, EU266416<sup>6</sup>, AY380935<sup>3</sup>; *Arethusa bulbosa* L., AF264154<sup>7</sup>, AF263624<sup>2</sup>, AY380938<sup>3</sup>; *Arpophyllum giganteum* Hartw. ex Lindley, AF074110<sup>1</sup>, AF265485<sup>8</sup>, AY380939<sup>3</sup>; *Arundina graminifolia* (D. Don) Hochr., AF074111<sup>1</sup>, EF079333<sup>9</sup>, AY380940<sup>3</sup>; *Aspasia lunata* Lindl., FJ534160<sup>10</sup>, EF079200<sup>3</sup>, FJ534283<sup>10</sup>; *Bifrenaria harrisoniae* (Hook.) Rchb. f., AF074112<sup>1</sup>, EF065567<sup>9</sup>, AY380941<sup>3</sup>; *Bletia catenulata* Ruiz & Pav., AF518024<sup>11</sup>, AY121720<sup>12</sup>, AY380942<sup>3</sup>; *Bletilla striata* Rchb. f., AF074114<sup>1</sup>, EF079331<sup>9</sup>, AY380943<sup>3</sup>; *Bracthia andina* Rchb. f., FJ534236<sup>10</sup>, FJ565088<sup>13</sup>, FJ534358<sup>10</sup>; *Brassia arcuigera* Rchb. f., FJ534192<sup>10</sup>, AF350601<sup>14</sup>, FJ534314<sup>10</sup>; *Bulbophyllum lobbii* Lindley, AF074115<sup>1</sup>, AY121740<sup>12</sup>, AY380944<sup>3</sup>; *Cadetia taylori* (F. Muell.) Schltr., D58406<sup>15</sup>, EF079346<sup>9</sup>, AY380945<sup>3</sup>; *Calanthe* sp., AF264159<sup>7</sup>, AF263632<sup>2</sup>, AY380947<sup>3</sup>; *Calypso bulbosa* (L.) Oakes, AF264162<sup>7</sup>, EF525689<sup>16</sup>, AY380950<sup>3</sup>; *Capanea superflua* Lindley, FJ534139<sup>10</sup>, FJ563840<sup>13</sup>, FJ534262<sup>10</sup>; *Catasetum expansum* Rchb. f., AF074121<sup>1</sup>, AF263637<sup>7</sup>, AY380951<sup>3</sup>; *Cattleya* sp., AF074122<sup>1</sup>, AY263638<sup>7</sup>, AY380952<sup>3</sup>; *Caucaea phalaenopsis* (Linden & Rchb. f.) N.H. Williams & M.W. Chase, FJ534221<sup>10</sup>, FJ565012<sup>13</sup>, FJ534343<sup>10</sup>; *Cephalanthera damasonium* (Mill.) Druce, AF074123<sup>1</sup>, AY368396<sup>4</sup>, –; *Chysis bractescens* Lindl., AF074126<sup>1</sup>, EF079351<sup>9</sup>, AY380956<sup>3</sup>; *Chytroglossa marileoniae* Rchb. f., FJ534244<sup>10</sup>, FJ565112<sup>13</sup>, FJ534366<sup>10</sup>; *Cischioclema dasyandra* (Rchb. f.) Dressler & N.H. Williams, FJ534248<sup>10</sup>, FJ565125<sup>13</sup>, FJ534370<sup>10</sup>; *Coelia* sp., AF518055<sup>11</sup>, AY121743<sup>12</sup>, AY380966<sup>3</sup>; *Coelogyne cristata* Lindley, AF074133<sup>1</sup>, AF263644<sup>7</sup>, AY380967<sup>3</sup>; *Comparettia falcata* Poepp. & Endl., FJ534237<sup>10</sup>, FJ565090<sup>13</sup>, FJ534359<sup>10</sup>; *Cymbidium* sp., AY368356<sup>4</sup>, AF470470<sup>17</sup>, AY380978<sup>3</sup>; *Dendrobium* sp., AB519784<sup>18</sup>, AF448863<sup>19</sup>, AY380983<sup>3</sup>; *Dendrochilum* sp., AF264164<sup>7</sup>, AF263215<sup>7</sup>, AY380984<sup>3</sup>; *Diaphananthe rutila* (Rchb. f.) Summerh., AF074147<sup>1</sup>, AY368403<sup>4</sup>, AY380985<sup>3</sup>; *Dilomilis montana* (Sw.) Summerh., AF074150<sup>1</sup>, AF263765<sup>7</sup>, AY380987<sup>3</sup>; *Dressleria* sp., AF074153<sup>1</sup>, EF079265<sup>9</sup>, AY380991<sup>3</sup>; *Earina* sp., AF074155<sup>1</sup>, EF079336<sup>9</sup>, AY380993<sup>3</sup>; *Elleanthus* sp., AF074156<sup>1</sup>, AF263658<sup>7</sup>, AY380994<sup>3</sup>; *Epidendrum* sp., AF518060<sup>11</sup>, AF263781<sup>7</sup>, AY380996<sup>3</sup>; *Epipactis* sp., FJ454877<sup>20</sup>, AF263659<sup>7</sup>, AY380998<sup>3</sup>; *Eria* sp., AF074164<sup>1</sup>, AF263660<sup>7</sup>, AY381004<sup>3</sup>; *Fernandezia ionanthera* (Rchb. f. & Warsz.) Schltr., FJ534219<sup>10</sup>, FJ565010<sup>13</sup>, FJ534341<sup>10</sup>; *Galeandra devoniana* Lindl., AF074171<sup>1</sup>, AY368408<sup>4</sup>, AY381011<sup>3</sup>; *Glomera* sp., AB586467<sup>21</sup>, AY121742<sup>12</sup>, AY381013<sup>3</sup>; *Gomesa gomezoides* (Barb. Rodr.) Pabst, FJ534154<sup>10</sup>, AF350632<sup>14</sup>, FJ534277<sup>10</sup>; *Granthophyllum auriculatum* (Vell.) Docha Neto, FJ534200<sup>10</sup>, FJ565155<sup>13</sup>, FJ534322<sup>10</sup>; *Helcia* sp., FJ534148<sup>10</sup>, EF079229<sup>9</sup>, FJ534336<sup>10</sup>; *Hintonella mexicana* Ames, FJ534195<sup>10</sup>, DQ315890<sup>14</sup>, FJ534317<sup>10</sup>; *Hirtzia benzingii*, FJ534242<sup>10</sup>, FJ565102<sup>13</sup>, FJ534364<sup>10</sup>; *Hofmeisterella eumicroscopica* (Rchb. f.) Rchb. f., FJ534238<sup>10</sup>, FJ565091<sup>13</sup>, FJ534360<sup>10</sup>; *Houlletia sanderi* Rolfe, AF074178<sup>1</sup>, AF239467<sup>22</sup>, AY381020<sup>3</sup>; *Houtleya heteroclitae* (Poepp. & Endl.) Garay, AF074179<sup>1</sup>, –; AY381021<sup>3</sup>; *Ionopsis minutiflora* (Dodson & N. Williams) Pupulin, FJ534024<sup>10</sup>, FJ565047<sup>13</sup>, FJ381024<sup>3</sup>; *Kegelella atropilosa* L.O. Williams & A.H. Heller, AF074181<sup>1</sup>, EF079232<sup>9</sup>, FJ534326<sup>10</sup>; *Leochilus inconspicuus* (Kraenzl.) M.W. Chase & N.H. Williams, FJ534197<sup>10</sup>, FJ564943<sup>13</sup>, FJ534319<sup>10</sup>; *Listera* sp. (= *Neottia*), AF074184<sup>1</sup>, AF521058<sup>11</sup>, AY381027<sup>3</sup>; *Lockhartia micrantha* Rchb. f., FJ534134<sup>10</sup>, FJ564691<sup>13</sup>, FJ534255<sup>10</sup>; *Lycaste* sp., AF074184<sup>1</sup>, AF239438<sup>22</sup>, AY381028<sup>3</sup>; *Lycormorium squalidum* (Poepp. & Endl.) Rchb. f., AF074186<sup>1</sup>, AF368414<sup>4</sup>, AY381029<sup>3</sup>; *Macradenia rubescens* Barb. Rodr., FJ534181<sup>10</sup>, FJ564839<sup>13</sup>, FJ534303<sup>10</sup>; *Macroclinium* sp., FJ534191<sup>10</sup>, FJ564931<sup>13</sup>, FJ534313<sup>10</sup>; *Malaxis spicata* Sw., AF074188<sup>1</sup>, AY368415<sup>4</sup>, AY381031<sup>3</sup>; *Masdevallia* sp., AF518040<sup>11</sup>, AF265446<sup>8</sup>, AY381032<sup>3</sup>; *Meiracyllium trinatum* Rchb. f., AF074192<sup>1</sup>, EF079319<sup>9</sup>, AY381037<sup>3</sup>; *Mesospinidium horichii* I. Bock, FJ534185<sup>10</sup>, FJ564861<sup>13</sup>, FJ534307<sup>10</sup>; *Miltonia regnellii* Rchb. f., FJ534193<sup>10</sup>, AF239491<sup>22</sup>, FJ534315<sup>10</sup>; *Monophyllorchis* sp., AF074195<sup>1</sup>, EF065603<sup>9</sup>, AY381040<sup>3</sup>; *Neofinetia falcata* (Thunb.) H.H. Hu, AF074197<sup>1</sup>, EF655782<sup>23</sup>, AY381041<sup>3</sup>; *Neottia nidus-avis* Rich., AY368364<sup>4</sup>, EF079303<sup>9</sup>, –; *Nephelephyllorchis* sp., AF264170<sup>7</sup>, AF263674<sup>24</sup>,

## Appendix 1. Continued.

AY381043<sup>3</sup>; *Nervilia bicarinata* (Blume) Schltr., AF074199<sup>1</sup>, AY368420<sup>4</sup>, AY381044<sup>3</sup>; *Nohawilliamsia orthostates* (Ridl.) M.W. Chase & Whitten, FJ534189<sup>10</sup>, FJ563950<sup>13</sup>, FJ534311<sup>10</sup>; *Notylia ecuadorensis* Schltr., FJ534208<sup>10</sup>, FJ564961<sup>13</sup>, FJ534330<sup>10</sup>; *Notyliopsis beatrix* P. Ortiz., FJ534234<sup>10</sup>, FJ565086<sup>13</sup>, FJ534356<sup>10</sup>; *Odontoglossum sanguineum* (Rchb. f.) Dalström, FJ534145<sup>10</sup>, FJ564985<sup>13</sup>, FJ534268<sup>10</sup>; *Oliveriana brevilibia* (C. Schweinf.) Dressler & N.H. Williams, FJ534174<sup>10</sup>, EF079202<sup>9</sup>, FJ534296<sup>10</sup>; *Oncidium* sp., FJ534144<sup>10</sup>, EF079216<sup>9</sup>, FJ534352<sup>10</sup>; *Ornithocephalus dressleri* (Toscano) Toscano & Dressler, FJ534233<sup>10</sup>, FJ565086<sup>13</sup>, FJ534355<sup>10</sup>; *Otoglossum harlingii* (Stacy) N.H. Williams & M.W. Chase, FJ534207<sup>10</sup>, AF433018<sup>14</sup>, FJ534329<sup>10</sup>; *Pachyphyllum crystallinum* Lindl., FJ534232<sup>10</sup>, FJ565077<sup>13</sup>, FJ534354<sup>10</sup>; *Palmorchis trilobulata* L.O. Williams, AF074206<sup>1</sup>, AJ310052<sup>4</sup>, AY381051<sup>3</sup>; *Phaius* sp., AF074210<sup>1</sup>, AF263676<sup>7</sup>, AY381053<sup>3</sup>; *Phalaenopsis aphrodite* Rchb. f., AY916449<sup>26</sup>, EU256324<sup>25</sup>, NC007499<sup>26</sup>; *Phreatia* sp., AF074214<sup>1</sup>, AY368425<sup>4</sup>, AY381056<sup>3</sup>; *Phymatidium fulcifolium* Lindl., FJ534183<sup>10</sup>, FJ563942<sup>13</sup>, FJ534305<sup>10</sup>; *Plectrophora cultrifolia* (Barb.Rodr.) Cogn., FJ534213<sup>10</sup>, FJ564979<sup>13</sup>, AY381059<sup>3</sup>; *Pleurothallis* sp., AF074217<sup>1</sup>, AF265456<sup>8</sup>, AY381059<sup>3</sup>; *Podochilus cultratus* Lindl., AF074218<sup>1</sup>, AY121738<sup>12</sup>, AY381060<sup>3</sup>; *Polyotidium huebneri* (Mansf.) Garay, FJ534201<sup>10</sup>, FJ563960<sup>13</sup>, FJ534323<sup>10</sup>; *Polystachya pubescens* (Lindl.) Rchb. f., AF074222<sup>1</sup>, AY368426<sup>4</sup>, AY381064<sup>3</sup>; *Prosthechea abbreviata* (Schltr.) W.E. Higgins, AF518063<sup>11</sup>, AF263757<sup>12</sup>, –; *Pseudolaelia vellozicola* (Hoehne) Porto & Brade, AF518057<sup>11</sup>, EF079384<sup>9</sup>, –; *Psychopsis sanderi* (Rolfe) Lückel & Braem, FJ534141<sup>10</sup>, FJ564712<sup>13</sup>, FJ534264<sup>10</sup>; *Pterostemma antioquiense* F. Lehm. & Kraenzl., FJ534188<sup>10</sup>, FJ563948<sup>13</sup>, FJ534310<sup>10</sup>; *Raycadenco ecuadorensis* Dodson, FJ534249<sup>10</sup>, FJ565127<sup>13</sup>, FJ534371<sup>10</sup>; *Rhynchostele londesboroughiana* (Rchb. f.) Soto Arenas & G.A. Salazar, FJ534157<sup>10</sup>, AF350609<sup>14</sup>, FJ534280<sup>10</sup>; *Rodriguezia batemanii* Poepp. & Endl., FJ534211<sup>10</sup>, FJ564975<sup>13</sup>, FJ534333<sup>10</sup>; *Rossioglossum* sp., FJ534135<sup>10</sup>, FJ563263<sup>13</sup>, FJ534258<sup>10</sup>; *Rudolfiella* sp., FJ534212<sup>10</sup>, FJ564977<sup>13</sup>, FJ534334<sup>10</sup>; *Saundersia paniculata* Brade., FJ534155<sup>10</sup>, FJ564734<sup>13</sup>, FJ534278<sup>10</sup>; *Schunkea vierlingii* Senghas, FJ534178<sup>10</sup>, FJ563933<sup>13</sup>, FJ534300<sup>10</sup>; *Seegeriella pinifolia* Senghas, FJ534177<sup>10</sup>, FJ564829<sup>13</sup>, FJ534299<sup>10</sup>; *Sobralia macrantha* Lindley, AF074228<sup>1</sup>, AF263681<sup>12</sup>, AY381076<sup>3</sup>; *Solenidium portillae* Dalström & Whitten, FJ534206<sup>10</sup>, FJ564956<sup>13</sup>, FJ534328<sup>10</sup>; *Spathoglottis pacifica* Rchb. f., AY381134<sup>3</sup>, AY368429<sup>4</sup>, AY381077<sup>3</sup>; *Stanhopea ecornuta* Lem., AF074230<sup>1</sup>, AF239445<sup>22</sup>, AY381079<sup>3</sup>; *Stellilabium pogonostalis* (Rchb. f.) Garay & Dunst., AF074213<sup>1</sup>, AF239488<sup>22</sup>, AY381080<sup>3</sup>; *Sutrina garayi* Senghas, FJ534176<sup>10</sup>, FJ564828<sup>13</sup>, FJ534298<sup>10</sup>; *Systemoglossum acuminatum* Ames & C. Schweinf., FJ534165<sup>10</sup>, AF350607<sup>14</sup>, FJ534287<sup>10</sup>; *Tangtsinia nanchuanica* S.C. Chen, Jin 9783, Chongqing, China (PE), JN706687<sup>8</sup>, JN706689<sup>8</sup>, –; *Thaia saprophytica* Seidenf., Jin 10463, Yunnan, China (PE), JN706687<sup>8</sup>, JN706690<sup>8</sup>, –; *Telipogon obovatus* Lindl., FJ534239<sup>10</sup>, FJ565093<sup>13</sup>, FJ534361<sup>10</sup>; *Thecostele alata* (Roxb.) Parish & Rchb. f., AY368371<sup>4</sup>, AY368431<sup>4</sup>, –; *Thunia* sp., AF074233<sup>1</sup>, AY121731<sup>12</sup>, AY381083<sup>3</sup>; *Tipularia discolor* (Pursh) Nuttall, AF074234<sup>1</sup>, AF263685<sup>12</sup>, AY381084<sup>3</sup>; *Tolunnia calochila* (Cogn.) Braem., FJ534158<sup>10</sup>, FJ564796<sup>13</sup>, FJ534281<sup>10</sup>; *Trichocentrum lindenii* (Brongn.) M.W. Chase & N.H. Williams, FJ534223<sup>10</sup>, FJ565028<sup>13</sup>, FJ534345<sup>10</sup>; *Trichoceros antennifer* (Humb. & Bonpl.) Kunth, FJ534226<sup>10</sup>, FJ564953<sup>13</sup>, FJ534348<sup>10</sup>; *Trichopilia fragrans* (Lindl.) Rchb. f., FJ534229<sup>10</sup>, FJ565053<sup>13</sup>, FJ534351<sup>10</sup>; *Trichotomia ferox* Blume, AF074235<sup>1</sup>, AY368432<sup>4</sup>, AY381085<sup>3</sup>; *Trizexis falcata* Lindl., FJ534151<sup>10</sup>, FJ563850<sup>13</sup>, FJ534274<sup>10</sup>; *Tropidia* sp., AF074237<sup>1</sup>, AF263686<sup>12</sup>, AY381087<sup>3</sup>; *Vitekorchis excavata* (Lindl.) Ramowicz & Szlach., FJ534240<sup>10</sup>, FJ565094<sup>13</sup>, FJ534362<sup>10</sup>; *Warmingia zamorana* Dodson, FJ534184<sup>10</sup>, FJ563944<sup>13</sup>, FJ534306<sup>10</sup>; *Xerorchis amazonica* Schltr., AF074244<sup>1</sup>, AF263688<sup>12</sup>, AY381096<sup>3</sup>; *Zelenkoa onusta* (Lindl.) M.W. Chase & N.H. Williams., FJ534196<sup>10</sup>, FJ564942<sup>13</sup>, FJ534318<sup>10</sup>; *Zygostates apiculata* (Lindl.) Toscano, FJ534243<sup>10</sup>, FJ565111<sup>13</sup>, FJ534365<sup>10</sup>. **OUTGROUP:** *Disa tripetaloides* (L. f.) N.E. Br., AF074151<sup>1</sup>, DQ415011<sup>27</sup>, AY380988<sup>3</sup>; *Diuris sulphurea* R. Br., AF074152<sup>1</sup>, AF263655<sup>12</sup>, AY380990<sup>3</sup>; *Orchis quadripunctata* Cirillo ex Ten., AF074203<sup>1</sup>, AY368385<sup>4</sup>, AY381048<sup>3</sup>; *Phragmipedium longifolium* (Rchb. f. & Warsc.) Rolfe, AF074212<sup>1</sup>, AY918831<sup>28</sup>, AY381055<sup>3</sup>; *Spiranthes spiralis* (L.) Chevall., AJ542434<sup>29</sup>, AJ543918<sup>29</sup>, FJ571255<sup>30</sup>.

**Appendix 2.** Taxa, voucher identification and GenBank accession numbers for molecular analyses (*rbcl*, *psaB*, *matK*, *trnL-F*, ITS, *Xdh*) of the tribe Neottieae. A dash (–) indicates missing data; an asterisk (\*) denotes sequences obtained in this study, and the remaining sequences are from GenBank. The superscripts 1–38 refer to the literature or GenBank sources.

**INGROUP:** *Aphyllorchis gollanii* Duthie, SET-ET 540, Tibet, China (PE), –, –, JN706691<sup>8</sup>, JN706695<sup>8</sup>, –, –, *Aphyllorchis* sp., FJ454873<sup>20</sup>, –, –, FJ454866<sup>20</sup>, GU004475<sup>31</sup>; *Cephalanthera damasonium* (Mill.) Druce, AF074123<sup>1</sup>, –, AY368396<sup>4</sup>, AJ409381<sup>24</sup>, AY833027<sup>32</sup>, –; *Cephalanthera falcata* (Thunb.) Blume, –, –, AB568427<sup>33</sup>, –; *Cephalanthera humilis* X.H. Jin, JIN 10379, Yunnan, China (PE), JN706688<sup>8</sup>, –, JN706692<sup>8</sup>, JN706694<sup>8</sup>, –, –; *Cephalanthera longibracteata* Bl., HM640549<sup>24</sup>, AY380953<sup>3</sup>, HM640666<sup>34</sup>, –, –, –; *Cephalanthera longifolia*, FJ454875<sup>20</sup>, –, –, AY146447<sup>35</sup>, –; *Epipactis* sp., –, AY380998<sup>3</sup>, AF263659<sup>7</sup>, AF519922<sup>11</sup>, –, GU004476<sup>31</sup>; *Limodorum abortivum* L., AF074206<sup>1</sup>, –, –, AY351378<sup>36</sup>, GU004474<sup>31</sup>; *Listera* sp. (= *Neottia*), AF074184<sup>1</sup>, AY381027<sup>3</sup>, AF263668<sup>7</sup>, AF519920<sup>11</sup>, FJ694841<sup>37</sup>, GU004473<sup>31</sup>; *Neottia nidus-avis* Rich., AF368364<sup>4</sup>, –, AY368419<sup>4</sup>, –, AY351383<sup>36</sup>, GU004472<sup>31</sup>; *Palmorchis* sp., –, AY381051, AJ310052, AJ409435, –, GU004471<sup>31</sup>; *Tangtsinia nanchuanica* S.C. Chen, Jin 9783, Chongqing, China (PE), JN706686<sup>8</sup>, –, JN706689<sup>8</sup>, JN706693<sup>8</sup>, JN706696<sup>8</sup>, –. **OUTGROUP:** *Tropidia* sp., AF074237<sup>1</sup>, AY381087<sup>3</sup>, AF263686<sup>12</sup>, –, EF490674<sup>38</sup>, GU004477<sup>31</sup>.

Sources: 1. Cameron & al. (1999); 2. Goldman & al. (2001); 3. Cameron (2004); 4. Freudenstein & al. (2004); 5. Barrett & Freudenstein (2009); 6. Eum & al. (2008); 7. Goldman & al. (2001); 8. Pridgeon & al. (2001); 9. Gorniak & Szlachetko (unpub.); 10. Whitten & al. (unpub.); 11. Van de Berg & al. (2005); 12. Van de Berg (unpub.); 13. Chase & al. (2009); 14. Williams & al. (2001); 15. Yukawa & al. (unpub.); 16. Freudenstein & Senyo (2008); 17. Van de Berg & al. (2002); 18. Asahina & al. (unpub.); 19. Teng & al. (unpub.); 20. Roy & al. (2009); 21. Tobe & al. (unpub.); 22. Whitten & al. (2000); 23. Kocyan & al. (2008); 24. Kores & al. (2001); 25. Chang & al. (2006); 26. Chang & al. (unpub.); 27. Bytebier & al. (2007); 28. Whitten & al. (2005); 29. Salazar & al. (2003); 30. Alvarez-Molina & Cameron (2009); 31. Gorniak & al. (2010); 32. Julou & al. (2005); 33. Sakamoto & al. (unpub.); 34. Kin & al. (2010); 35. Moscone & al. (unpub.); 36. Bernardos & al. (2004); 37. Roy & Selosse (unpub.); 38. Zurbriggen & al. (2008).