Bull. Natl. Mus. Nat. Sci., Ser. B, 38(1), pp. 11–17, February 22, 2012

The Phylogeny of Japanese *Enkianthus* species (Ericaceae)

Chie Tsutsumi* and Yumiko Hirayama

Department of Botany, National Museum of Nature and Science,
Amakubo 4–1–1, Tsukuba, Ibaraki 305–0005, Japan
* E-mail: tsutsumi@kahaku.go.jp

(Received 16 November 2011; accepted 28 December 2011)

Abstract The genus *Enkianthus* consists of 12–17 species, most of which are distributed in China and Japan. We performed molecular phylogenetic analyses for *Enkianthus* species of Japan, using nuclear ITS, chloroplast *matK* with *trnK* intron, and *trnS-trnG* spacer regions. The result suggested that *Enkianthus* was separated into two groups; i.e., sect. *Enkianthus* with umbellate inflorescences and a clade of sect. *Andromedina*, sect. *Enkiantella* and sect. *Meisteria* with racemose inflorescences. It also inferred that the ancestral *Enkianthus* seems likely to be shrubs with tubular or urceolate corollas and 4–5-apertured pollen, whereas the more derived species may possess campanulate corollas and 3-apertured pollen.

Kew words : *Enkianthus*, ITS, *matK*, molecular phylogeny, *trnSG*.

Introduction

The genus *Enkianthus* Lour. (Ericaceae) is a genus of shrubs or small trees and often used as ornamental plants in the gardens. The genus consists of 12–17 species (Anderberg, 1994; Kron *et al.*, 2002; Fang and Stevens, 2005). All of the species are distributed from the Himalayas to Japan (Fang and Stevens, 2005) and most of them are in China and Japan. Recent molecular phylogenetic studies showed that *Enkianthus* is sister to all other representatives of Ericaceae s.l. including Empetraceae, Epacridaceae, Monotropaceae and Pyrolaceae (Kron and Chase, 1993; Kron, 1996; Morton *et al.*, 1996; Anderberg *et al.*, 2002; Kron *et al.*, 2002). A cladistic analysis using morphological, anatomical, embryological and cytological characters suggested monophyly of the genus (Anderberg, 1994). The species of *Enkianthus* are distinct from the other Ericaceae in several characters, such as embryological features and morphologies of anther, pollen, seed and hair (reviewed by Kron *et al.*, 2002).

The species of *Enkianthus* differ in leaf texture, inflorescences structure, corolla shape, and morphologies of anther, pollen and seed (Anderberg, 1994; Kron *et al.*, 2002). Infrageneric relationships of *Enkianthus* were studied by Anderberg (1994), who proposed a classification comprising four sections. Sect. *Enkianthus* has more or less coriaceous leaves, tubular or urceolate flowers, umbellate inflorescences, 4–5-apertured pollen, glabrous pedicels and smooth anthers (except for *E. serotinus* Chun et Fang with papillose anthers). Sect. *Andromedina* shares several features with sect. *Enkianthus* but is distinct in racemose inflorescences and non-winged seeds. Sect. *Meisteria* is characterized by racemose inflorescences, 3-apertured pollen, campanulate corollas, and a papillose corolla surface, and sect. *Enkiantella* by racemose inflorescences (except for *E. pauciflorus* Wils. with solitary flowers), 3-apertured pollen, campanulate corollas, conspicuously winged and lamellate seeds, and anthers without a prolonged connective. Anderberg (1994) also suggested that sect. *Enkianthus* is sister to sect. *Andromedina*, then they are sister to sect. *Meisteria*, and sect.

Enkiantella is firstly diverged group in the genus based on morphology. Therefore, the ancestral *Enkianthus* seems to have deciduous leaves, racemose inflorescences, campanulate corollas, and winged seeds, whereas the more derived species likely possess coriaceous leaves, umbellate inflorescences, flowers with tubular or urceolate corollas, and non-winged seeds (Anderberg, 1994). The classification was supported by a pollen morphological study (Golam Sarwar and Takahashi, 2007). The molecular phylogeny of the genus, however, remains to be examined.

In Japan, six species and two varieties of *Enkianthus* are recognized (Yamazaki, 1993). According to the section concept sensu Anderberg (1994), *Enkianthus perulatus* (Miq.) C.K.Schneid. is assigned to sect. *Enkianthus, E. nudipes* (Honda) Ueno and *E. subsessilis* (Miq.) Makino are assigned to sect. *Andromedina*, *E. campanulatus* (Miq.) G.Nicholson, and *E. sikokianus* (Palib.) Ohwi and *E. cernuus* (Siebold et Zucc.) Makino belong to sect. *Meisteria. Enkianthus perulatus* is distributed in Japan and Taiwan, although plants in Taiwan are sometimes treated as a distinct species, *E. taiwanianus* Ying. The other five species are endemic to Japan. In this study, the molecular phylogeny of the six Japanese *Enkianthus* species was analyzed using nuclear ITS regions and two chloroplast DNA regions, *matK* with *trnK* intron and *trnS-trnG* spacer. *Enkianthus quinqueflorus* Lour. distributed in China to Vietnam, and *Clethra* species, one of sister candidates to *Enkianthus*, were also analyzed in this study.

Materials and Methods

Leaf samples of seven *Enkianthus* species were collected in the field or from cultivated plants in the Tsukuba Botanical Garden, National Museum of Nature and Science (Table 1). *Clethra barbinervis* Siebold et Zucc. was also analyzed (Table 1). DNA was extracted from fresh or silica-gel-dried material using a QUIAGEN DNeasy Mini Kit (QUIAGEN, Valencia, CA) following the manufacturer's instruction.

The internal transcribed spacer regions of 18S-26S nuclear ribosomal DNA (ITS), and two chloroplast regions, *matK* (the maturase-encoding gene) with *trnK* intron, and *trnS-trnG* spacer, were analyzed. Primers for amplification were 17SE (Sun *et al.*, 1994) and '26SE' (Topik *et al.*, 2005, modified 26SE in Sun *et al.*, 1994) in ITS region, and trnK-3914F and trnK-2R (Johnson and Soltis, 1994) in *matK* with internal primers MK-F1 (Koi *et al.*, 2008), matK-E728F (5′-ATAGATCTCAGCAACATGAC-3′), matK-E1166F (5′-CATCCATCTGGAAATCTTGG-3′), matK-E780R (5′-GTGGATATATGAAGTCATGTTGCTG-3′), and matK-E1391R (5′-TTGGCACTATGGTATCGAACT-3′), and trnS (GCU) and trnG (UCC) in *trnS-trnG* spacer (Hamilton, 1999). PCR for ITS region was performed using a DNA thermal cycler (Perkin-Elmer 9700, Applied Biosystems, Foster, CA) with *LA Taq* DNA polymerase (TaKaRa Bio, Tokyo, Japan) in 35 denaturation, annealing, and elongation cycles (30 sec at 95℃, 30 sec at 55℃ and 90 sec at 72℃) with a final elongation step (5 min at 72℃). PCR for *matK* with *trnK* intron and *trnS-trnG* spacer regions was conducted with *Ex Taq* DNA polymerase (TaKaRa Bio, Tokyo, Japan) in 35 denaturation, annealing, and elongation cycles (30 sec at 95℃, 30 sec at 55℃ and 90 sec at 72℃) with a final elongation step (7 min at 72℃). The PCR products were purified with ExoSAP-IT (USB corporation, Cleveland, OH) following the manufacturer's instruction. Sequencing was conducted using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The sequences obtained were assembled using Seqman II (Dnastar, Madison, WI). The assembled sequences were aligned by Clustal X program (Thompson *et al.*, 1997) and then aligned manually. In addition to the sequences analyzed, several registered sequences of *Enkianthus* and *Cyrilla racemiflora* L. (Cyrillaceae) in GenBank were added for phylogenetic analyses.

Phylogenetic analyses were performed by Bayesian analyses and maximum parsimony (MP). Bayesian analyses were performed by MrBayes 3.1.2 (Huelsenbeck and Ronquist,

Table 1. Species used in molecular phylogenetic analysis, sources and GenBank accession no. of ITS, *matK* with *trnK* intron and *trnS-trnG* spacer regions.

Species	Source	GenBank accession no.		
		ITS	*matK*	*trnSG*
Sect. *Meisteria*				
E. campanulatus (Miq.) G.Nicholson	Cultivated in Tsukuba Botanical Garden; *Tsutsumi s.n.*	AB697666	AB697674	AB697682
E. campanulatus (Miq.)*1	from Genbank	AF091940	U61344	GU176714
E. campanulatus (Miq.)*2	from Genbank	AF133752		
E. sikokianus (Palib.) Ohwi	Cultivated in Tsukuba Botanical Garden (Origin; Kochi, Japan); *Tsutsumi s.n.*	AB697667	AB697675	AB697683
E. cernuus (Siebold et Zucc.) Makino f. *cernuus*	Cultivated in Tsukuba Botanical Garden; *Tsutsumi s.n.*	AB697668	AB697676	AB697684
Sect. *Andromedina*				
E. nudipes (Honda) Ueno	Shinshiro, Aichi, Japan; *Tsutsumi Ho3-20100514*	AB697669	AB697677	AB697685
E. subsessilis (Miq.) Makino	Cultivated in Tsukuba Botanical Garden (Origin; Tochigi, Japan); *Tsutsumi s.n.*	AB697670	AB697678	AB697686
Sect. *Enkianthus*				
E. perulatus (Miq.) C.K.Schneid.	Cultivated in Tsukuba Botanical Garden; *Tsutsumi s.n.*	AB697671	AB697679	AB697687
E. quinqueflorus Lour.	Cultivated in Tsukuba Botanical Garden (Origin; Hong Kong); *Tsutsumi s.n.*	AB697672	AB697680	AB697688
E. quinqueflorus Lour.*	from Genbank	FJ378572		FJ357264
Sect. *Enkiantella*				
E. chinensis Franch.*	from Genbank	FJ378571		FJ357263
Clethraceae				
Clethra barbinervis Siebold et Zucc.	Kagoshima Pref. Japan; *Tagane & Fuse* (*TNS763118*)	AB697673	AB697681	AB697689
Cyrillaceae				
Cyrilla racemiflora L.*	from Genbank	AF396236	AJ429282	

* Asterisks show the data derived from registered sequence in Genbank.

2001; Ronquist and Huelsenbeck, 2003). MrModeltest 2.0 (Nylander, 2004) was used to determine the nucleotide substitution model for Bayesian analysis. Bayesian searches were conducted by mcmc with four chains over one million generations for each of ITS, *matK* with *trnK* intron, *trnS-trnG* spacer regions, and over two million generations for combined data, sampling every 100 generations. Trees obtained before stationary generations were discarded as burn-in trees and the rest of trees were used to calculate posterior probabilities. Maximum parsimony (MP) inference was conducted with PAUP* 4.0b10 (Swofford, 2002). The bases that could not been identified were treated as unknown (N), and gaps were treated as missing data. Bootstrap values were calculated from 1000 pseudo-replicates, each with 100 random additions replicates involving TBR branch swapping. *Clethra barbinervis* and/or *Cyrilla racemiflora* were used as outgroups (Anderberg *et al.*, 2002; Soltis *et al.*, 2011).

Homogeneity among the different datasets was calculated with the ILD test (Farris *et al.*, 1995; Johnson and Soltis, 1998) as implemented in PAUP. The ILD tests were performed between two of the three datasets. The test was conducted by heuristic search with 100 replicates, using 10 random additions with parsimony-uninformative characters excluded. All ILD-tests for different partitions tested indicated that *p*-values were higher than 0.05 (the lowest *p*-value was 0.09 between ITS and *matK* with *trnK* intron), suggesting that the datasets were not incongruent to be combined.

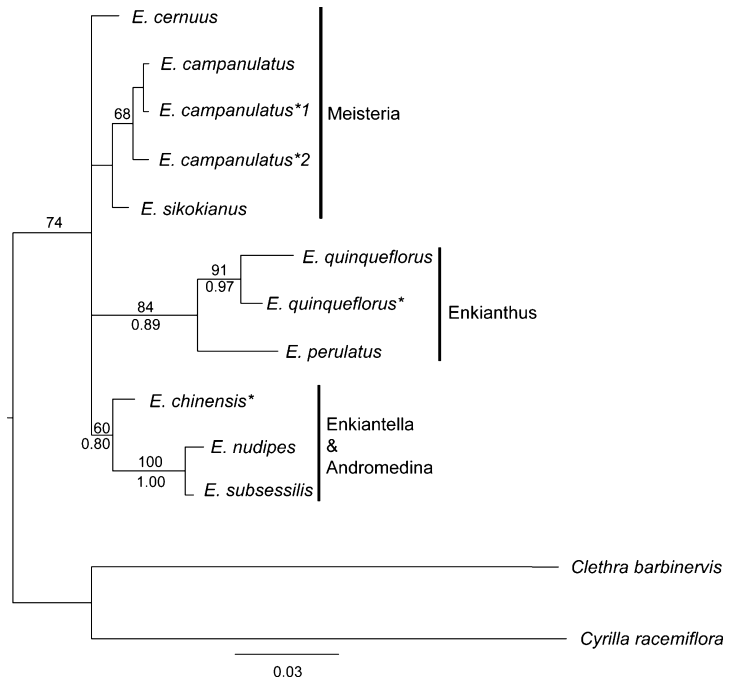

Fig. 1. Consensus tree by Bayesian analysis based on nuclear ITS sequence dataset. Figures below branches indicate posterior probabilities ($p \geqq 0.8$) calculated by Bayesian analysis and those above branches indicate maximum parsimony bootstrap values (> 50).

Results

The molecular phylogenetic trees deduced from each of the nuclear ITS region, the *matK* with *trnK* intron sequences, and the *trnS-trnG* spacer region showed that all the species of *Enkianthus* examined formed a monophyletic group (Figs. 1–3). The results showed that each of the three sections (sect. *Enkianthus*, sect. *Andromedina*, sect. *Meisteria*) formed a clade in all the trees except sect. *Meisteria* in the ITS tree. Supports were high of the sect. *Andromedina* in all the trees, of the sect. *Meisteria* in the trees of *matK* with *trnK* intron and the *trnS-trnG* spacer (including *E. chinensis* in the *trnS-trnG* spacer tree), and of the sect. *Enkianthus* in the *matK* tree. In the trees deduced from the two chloroplast regions, sect. *Enkianthus* was firstly diverged from the other species of *Enkianthus*, and sect. *Meisteria* was sister to sect. *Andromedina*, although the infrageneric relationships remained unsolved in the ITS tree. The molecular phylogenetic positions of *Enkianthus chinensis* in sect. *Enkiantella* differed in trees; in the ITS tree it formed a clade with the species of sect. *Andromedina*, and it was nested in the clade of sect. *Meisteria* in the *trnS-trnG* spacer tree.

Deduced phylogenies based on the combined datasets of the nuclear ITS and two chloroplast regions also showed that the genus *Enkianthus* was monophyletic with high supports (Fig. 4). The tree indicated that sect. *Enkianthus* was firstly separated from the remaining species of *Enkianthus*, and sect. *Meisteria* including *E. chinensis* was sister to the clade comprising the species of sect. *Andromedina*. Those relationships were supported by high credibilities.

Discussion

The present results showed that the phylogenetic relationships of mainly Japanese *Enkianthus* species by the nuclear ITS and two chloroplast regions. The infrageneric classification was

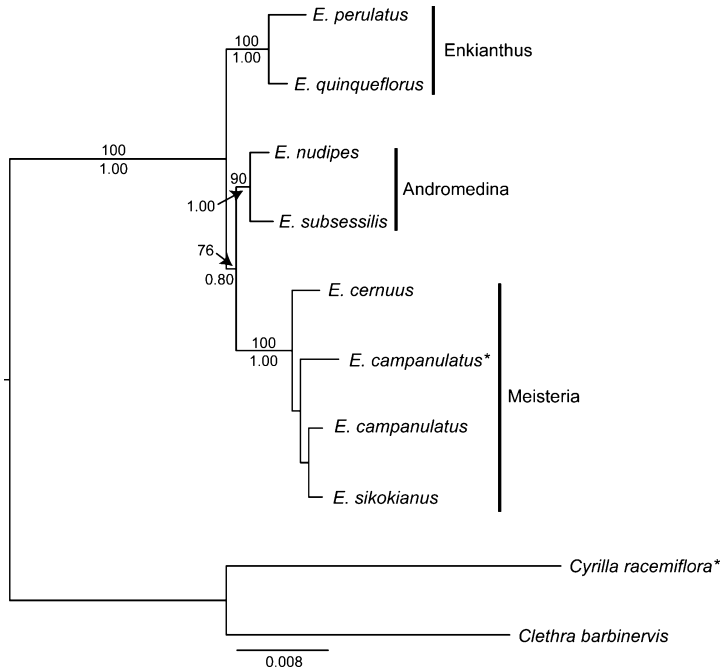

Fig. 2. Consensus tree by Bayesian analysis based on sequence dataset of chloroplast *matK* with *trnK* intron. Figures below branches indicate posterior probabilities (p≧0.8) calculated by Bayesian analysis and those above branches indicate maximum parsimony bootstrap values (＞50).

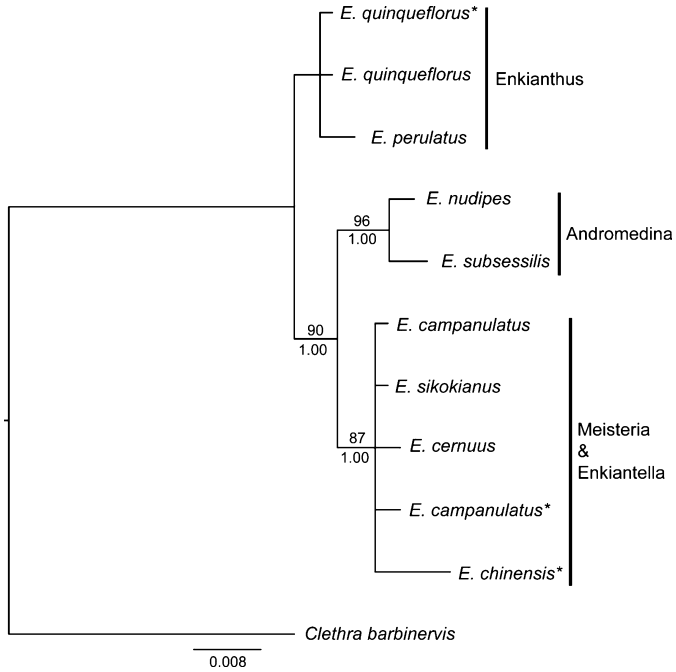

Fig. 3. Consensus tree by Bayesian analysis based on chloroplast *trnS-trnG* spacer sequence dataset. Figures below branches indicate posterior probabilities (p≧0.8) calculated by Bayesian analysis and those above branches indicate maximum parsimony bootstrap values (＞50).

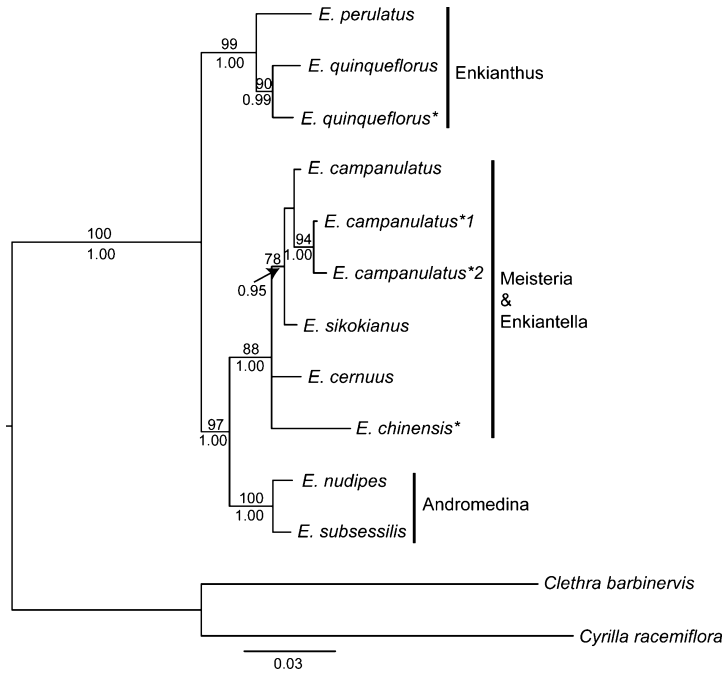

Fig. 4. Consensus tree by Bayesian analysis based on combined sequence datasets of ITS, *matK* with *trnK* intron and *trnS-trnG* spacer. Figures below branches indicate posterior probabilities ($p \geqq 0.8$) calculated by Bayesian analysis and those above branches indicate maximum parsimony bootstrap values (> 50).

generally consistent with Anderberg (1994), except for sect. *Enkiantella*, whose samples were not well analyzed in this study (Figs. 1–4). The previous cladistic analysis based on the morphological characters suggested that sect. *Enkianthus* was sister to sect. *Andromedina*, then they were sister to sect. *Meisteria*, and sect. *Enkiantella* was firstly diverged group from the remaining species (Anderberg, 1994). The present results were, however, incongruent to the topology derived from the previous cladistic analysis. Our tree deduced from combined datasets showed that sect. *Enkianthus* with umbellate inflorescences was separated from the other sections with racemose inflorescences (Fig. 4). The tree also indicated that sect. *Andromedina* was sister to sect. *Meisteria* including *E. chinensis* with 3-apertured pollen, hairy anthers, and campanulate corollas, although sect. *Andromedina* shares a number of character states with the species of sect. *Enkianthus*. Therefore, the ancestral *Enkianthus*, like sect. *Enkianthus*, seems to be shrubs with tubular or urceolate corollas and 4–5-apertured pollen, while campanulate corollas and 3-apertured pollen seem likely to be apomorphic.

Anderberg (1994) noted the similarities of *E. nudipes* and *E. subsessilis*, both of which differ mainly in the indumentum of the calyx and the pedicel, while Ueno (1950) suggested that they are distinct by mount of crystals in the leaf mesophylls. The molecular differences between the two species were three substitutions in ITS regions, seven substitutions, one insertion and three variations of poly-(N) in *matK* with *trnK* intron, and two insertions in *trnS-trnG* spacer. It suggests that the two species seem likely to be genetically differentiated. Further, the taxonomic treatments of *E. campanulatus* and *E. sikokianus* varied by scientists. Anderberg (1994) treated it as one species with variations. Yamazaki (1993) opined that *E. sikokianus* is a distinct species from *E. campanulatus* by the long racemes and short pedicels with many flowers, and that *E. campanulatus* consists of three varieties, var.

campanulatus, var. *palibinii* and var. *longilobus*, which differed from each other in corolla color and sizes of corolla and lobes. The molecular differences between *E. campanulatus* and *E. sikokianus* were not identical in the three regions examined; two substitutions in ITS region, four substitutions in *matK* with *trnK* intron, and one insertion in *trnS-trnG* spacer. It needs further analysis to clarify the cause of these.

Acknowledgments

We thank A. Ebihara, K. Fuse, T. Muramatsu and S. Tagane for providing the materials and/or help during the field trips. We also gratefully thank A. Uehara for technical assistance and M. Kato for useful comments on the manuscript. This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

References

Anderberg, A. A. 1994. Cladistic analysis of *Enkianthus* with notes on the early diversification of the Ericaceae. Nordic Journal of Botany 14: 385–401.

Anderberg, A. A., Rydin, C. and Källersjö, M. 2002. Phylogenetic relationships in the order Ericales s.l.: Analyses of molecular data from five genes from the plastid and mitochondrial genomes. American Journal of Botany 89: 677–687.

Farris, J. S., Källersjö, M., Kluge, A. G. and Bult, C. 1995. Testing significance for incongruence. Cladistics 10: 315–319.

Golam Sarwar, K. M. and Takahashi, H. 2006. Pollen morphology of *Enkianthus* (Ericaceae) and its taxonomic significance. Grana 45: 161–174.

Hamilton, M. D. 1999. Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. Molecular Ecology 8: 521–523.

Huelsenbeck, J. P. and Ronquist, F. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755.

Johnson, L. A. and Soltis, D. E. 1994. *MatK* DNA sequences and phylogenetic reconstruction in Saxifragaceae s. str. Systematic Botany 19: 143–156.

Johnson, L. A. and Soltis, D. E. 1998. Assessing congruence: empirical examples from molecular data. *In*: Soltis, D. E., Soltis, P. S. and Doyle, J. J. (eds.) Molecular Systematics of Plants II. DNA Sequencing, pp. 297–348. Kluwer Academic Publishers, Boston.

Koi, S., Kita, Y. and Kato, M. 2008. *Paracladopus chanthaburiensis*, a new species of Podostemaceae from Thailand, with notes on its morphology, phylogeny and distribution. Taxon 57: 201–210.

Kron, K. A. 1996. Phylogenetic relationships of Empetraceae, Epacridaceae, Ericaceae, Monotropaceae, and Pyrolaceae: evidence from nuclear ribosomal 18S sequence data. Annals of Botany 77: 293–303.

Kron, K. A. and Chase, M. W. 1993. Systematics of the Ericaceae, Empetraceae, Epacridaceae and related taxa based upon *rbc*L sequence data. Annals of the Missouri Botanical Garden 80: 735–741.

Kron, K. A., Judd, W. S., Stevens, P. F., Crayn, D. M., Anderberg, A. A., Gadek, P. A., Quinn, C. J. and Luteyn, J. L. 2002. Phylogenetic classification of Ericaceae: molecular and morphological evidence. The Botanical Review 68: 335–423.

Morton, C. M., Chase, M .W., Kron, K. A. and Swensen, S. M. 1996. A molecular evaluation of the monophyly of the order Ebenales based upon *rbcL* sequence data. Systematic Botany 21: 567–586.

Nylander, J. A. A. 2004. MrModeltest 2.0. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala.

Ronquist, F. and Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Sun, Y., Skinner, D. Z., Liang, G. H. and Hulbert, S. H. 1994. Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribosomal DNA. Theoretical and Applied Genetics 89: 26–32.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. 1997. The Clustal X Windows Interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876–4882.

Topik, H., Yukawa, T. and Ito, M. 2005. Molecular phylogenetics of subtribe Aeridinae (Orchidaceae): insights from plastid *matK* and nuclear ribosomal ITS sequences. Journal of Plant Research 118: 271–284.

Ueno, J. 1950. On *Enkianthus*. A classification of the genus *Enkianthus* based upon the characters of pollen grains and of crystals in the leaf. Journal of the Institute of Polytechnics, Osaka City University. Ser. D, Biology 1: 55–62.

Yamazaki, T. 1993. Ericaceae. In: Iwatsuki, K., Yamazaki, T., Boufford, D. E. and Ohba, H. (eds.), Flora of Japan, Vol IIIa. Angiospermae Dicotyledoneae Sympetalae (a), pp. 6–63. Kodansha, Tokyo.