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Short communication

A phylogenetic analysis of *Epimedium* (Berberidaceae) based on nuclear ribosomal DNA sequences

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1. Introduction

Epimedium L. (Berberidaceae) is a genus of the Old World. Members disperse from Japan to Algeria and mainly occur in eastern Asia and the Mediterranean lands (Stearn, 2002). Approximately 80% of the total species are found in central-southeastern China (Ying, 2001). Linnaeus recorded this genus and its type species *E. alpinum* in 1753. After that, Morren and Decaisne (1834); Fischer and Meyer (1846); Franchet (1886); Komarov (1908); and Stearn (1938, 2002) made monographic and systematic study of *Epimedium* (Table 1). Untill now about 50 species of *Epimedium* are recognized.

Corolla characteristics such as petal type, the form and relative size of the inner sepals and petals, and flower dimension are important characters used in the classification of *Epimedium* species. Morren and Decaisne (1834) established section *Macroceras* to accommodate species with large flowers and section *Microceras* for species with small flowers. This treatment was followed by Fischer and Meyer (1846). However, this arrangement becomes inadequate if extended to the many other species now known (Stearn, 1938, 2002). Petals of *Epimedium* species present a substantial variation. It has been postulated that flat petal is the ancestral type and it evolved by projecting outwards at basal portion with or without reduction of lamina (Stearn, 1938; Ying, 2002). However, if petal evolves in a continuous way or along different routes is quite controversial.

The number of leaves borne on the flowering stem is used to classify *Epimedium* species (Franchet, 1886; Komarov, 1908; Stearn, 1938, 2002). Fischer and Meyer (1846) established section *Rhizophyllum* based on *E. pinnatum* with leafless inflorescence. Franchet (1886) established section *Gymnocaulon* for species with leafless flowering stems and section *Phyllocaulon* for species with one or two leaves on flowering stems. Then, *Monophyllon*, *Diphyllon*, and *Polyphyllon* are established to accommodate species having one stem-leaf, two stem-leaves, and several stem-leaves (Komarov, 1908; Stearn, 1938). However, this character is not consistent in some species, such as *E. sagittatum*, *E. leptorrhizum*, and *E. elongatum* (Stearn, 1938).

Recently, geographical distribution and C-banding of chromosomes are incorporated to classify *Epimedium* species (Stearn, 1938, 2002). A natural arrangement of *Epimedium* species has become a big challenge as more new species are found. It is necessary to investigate the phylogenetic relationship of *Epimedium* species.

Random amplified polymorphic DNA (RAPD) and PCR-restriction fragment length polymorphism (RFLP) have been used to characterize Japanese *Epimedium* species (Nakai et al., 1996). Internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA and 5S rRNA gene spacer sequences have been used for examining relationships within genus (Kim et al., 2004a,b; Roser et al., 2001). In this study, we explore ITS sequences of nuclear ribosomal DNA and 5S rRNA gene spacer sequences to study the phylogenetic relationship of *Epimedium* species.

2. Materials and methods

A total of 22 *Epimedium* species were collected and used in the DNA analysis (Table 2). According to the

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Table 1
Systematics of *Epimedium* proposed by different researchers

Morren and Decaisne (1834)	Fischer and Meyer (1846)	Franchet (1886)	Komarov (1908)	Stearn (1938)	Stearn (2002)
Section *Microceras*	Section *Microceras*	Section *Gymnocaulon*	Section *Gymnocaulon*	Section *Rhizophyllum*	Subgenus *Rhizophyllum*
Section *Macroceras*	Section *Macroceras*	Section *Phyllocaulon*	Section *Phyllocaulon*	Section *Phyllocaulon*	Subgenus *Epimedium*
	Section *Rhizophyllum*		Series Monophylla	Subsection *Monophyllon*	Section *Diphyllon*
			Series Acerantha	Series Microcerae	Series Campanulatae
			Series Diphylla	Series Macrocerae	Series Davidianae
			Series Polyphylla	Subsection *Aceranthus*	Series Dolichocerae
				Subsection *Diphyllon*	Series Brachycerae
				Series Dolichocerae	Section *Macroceras*
				Series Brachycerae	Section *Polyphyllon*
				Subsection *Polyphyllon*	Section *Epimedium*
				Series Elongatae	
				Series Elatae	

classification of Stearn (2002), sampled species covered three sections of subgenus *Epimedium*, including section *Epimedium* from Mediterranean lands, section *Macroceras* from Japan, and section *Diphyllon* from China. Sampled Chinese species represented series *Davidianae*, series *Dolichocerae*, and series *Brachycerae*. *Vancouveria planipetala* Calloni was used as an outgroup. Voucher samples were stored in the Institute of Chinese Medicine, the Chinese University of Hong Kong.

Total DNA isolation, polymerase chain reaction (PCR), PCR product cloning, and sequencing were carried out as described (Sun et al., 2004). The whole ITS regions (including ITS1, 5.8S, and ITS2) were amplified and sequenced by using primers ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) and ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) (White et al., 1990). Primer N18L18 (5′-AAG TCG TAA CAA GGT TTC-3′) (Wen and Zimmer, 1996) was used in ITS sequencing occasionally. 5S rRNA gene spacers were amplified and sequenced by using the primers S-1 (5′-GGA TCC GTG CTT GGG CGA GAG TAG TA-3′) and AS-1 (5′-GGA TCC TTA GTG CTG GTA TGA TCG CA-3′) (Carles et al., 2001; Sun et al., 2004). 5S PCR product was first cloned and two clones with insert were sequenced for every individual sample.

Multiple alignment of DNA sequences was performed using 'Clustal_W' with default parameters. The data matrix was available upon request. Phylogenetic analysis was performed with PAUP* version 4.0 b 10 (Swofford, 2003) and PAUPRat (Sikes and Lewis, 2001). The most parsimonious trees were searched by the method of "Parsimony Ratchet" (Nixon, 1999). Gaps were treated as missing state. Twenty independent Ratchet searches were executed uninterruptedly. Two hundred Ratchet iterations were performed in every Ratchet search and 15% of characters were perturbed in every Ratchet iteration with uniform weights. In bootstrap analysis, 1000 replications were performed with heuristic search strategy and simple addition sequences.

3. Results and discussion

The whole ITS region (including ITS1, 5.8S, and ITS2) was found to be 683 bp in length in the *Epimedium* species, and 657 bp in *V. planipetala*. A matrix of 683 positions was obtained from multiple alignment of the ITS sequences including the outgroup. Twenty-six variable sites were found among the *Epimedium* species and 11 variable sites were parsimony-informative. Sequence similarities of the ITS regions in the *Epimedium* species were from 98 to 100%.

Parsimony analysis of the ITS sequences found 195 optimal trees [tree length = 58; consistency index (CI) = 0.9310; CI excluding uninformative characters = 0.7647; retention index (RI) = 0.8889; and rescaled consistency index (RC) = 0.8276]. On the strict consensus tree (figure was not shown), *E. alpinum*, *E. pubigerum*, *E. koreanum*, *E. grandiflorum*, *E. sempervirens*, and *E. diphyllum* were resolved as a clade with bootstrap value of 89; *E. davidii* and *E. ogisui* of 63; *E. rhizomatosum*; and *E. pauciflorum* of 63. Two subclades were highly supportive: *E. pubigerum* and *E. alpinum* formed a subclade with bootstrap value of 64, *E. sagittatum* and *E. dolichostemon* formed a subclade with bootstrap value of 83.

PCR product of 5S region was about 300 bp in all sampled *Epimedium* species. Two clones of this PCR product were sequenced for every individual species. Among the 300 bp, 57 bp were coding sequences of the 5S rRNA gene, the others were sequences of 5S rRNA gene spacer. 5S rRNA gene spacer was from 222 to 245 bp in length, the shortest was in *E. diphyllum* and the longest was in *E. alpinum*. One deletion of 21 bp was found in both clones of *E. diphyllum*, one insertion of 3 bp (AAA) was found in both clones of *E. pauciflorum*. The sequences of 5S rRNA gene spacers were A + T rich, with average A + T content of 70%.

A matrix of 310 positions was obtained from multiple alignment of the 5S sequences including the outgroup. Sequence similarities of two clones from the same *Epimedium* species were from 94% in *E. chlorandrum* to

Table 2
Taxa used in DNA analysis

Taxa	Systematic arrangement according to Stearn (2002)	Voucher	Source of material	GenBank Accession Nos.	
				ITS	5S rRNA gene spacer
E. davidii Franch.	Section *Diphyllon* Series Davidianae	ICM-2004-2553	Plant Delights Nursery, USA	AY362414	AY362374 AY362375
E. epsteinii W.T. Stearn	Section *Diphyllon* Series Davidianae	ICM-2004-2555	Plant Delights Nursery, USA	AY362417	AY362380 AY362381
E. ogisui W.T. Stearn	Section *Diphyllon* Series Davidianae	ICM-2004-2562	Heronswood Nursery, USA	AY362425	AY362396 AY362397
E. pauciflorum K.C. Yen	Section *Diphyllon* Series Davidianae	ICM-2004-2564	Heronswood Nursery, USA	AY362428	AY362402 AY362403
E. simplicifolium T.S. Ying	Section *Diphyllon* Series Dolichocerae	ICM-2004-2550	Institute of Chinese Materia Medica in Guizhou, China	AY362420	AY362386 AY362387
E. franchetii W.T. Stearn	Section *Diphyllon* Series Dolichocerae	ICM-2004-2551	Plant Delights Nursery, USA	AY362412	AY362370 AY362371
E. rhizomatosum W.T. Stearn	Section *Diphyllon* Series Dolichocerae	ICM-2004-2552	Plant Delights Nursery, USA	AY362415	AY362376 AY362377
E. brachyrrhizum W.T. Stearn	Section *Diphyllon* Series Dolichocerae	ICM-2004-2554	Plant Delights Nursery, USA	AY362411	AY362368 AY362369
E. chlorandrum W.T. Stearn	Section *Diphyllon* Series Dolichocerae	ICM-2004-2557	Plant Delights Nursery, USA	AY362418	AY362382 AY362383
E. acuminatum Franch.	Section *Diphyllon* Series Dolichocerae	ICM-2004-2561	Heronswood Nursery, USA	AY362423	AY362392 AY362393
E. wushanense T.S. Ying	Section *Diphyllon* Series Dolichocerae	SUNYE-GZ-02	Guizhou, China	AY362421	AY362389 AY362388
E. leptorrhizum Stearn	Section *Diphyllon* Series Dolichocerae	SUNYE-GZ-03	Guizhou, China	AY362419	AY362384 AY362385
E. brevicornu Maxim.	Section *Diphyllon* Series Brachycerae	ICM-2004-2549	Institute of Chinese Materia Medica in Beijing, China	AY362429	AY362404 AY362405
E. pubescens Maxim.	Section *Diphyllon* Series Brachycerae	ICM-2004-2556	Plant Delights Nursery, USA	AY362416	AY362378 AY362379
E. sagittatum Maxim.	Section *Diphyllon* Series Brachycerae	ICM-2004-2559	Heronswood Nursery, USA	AY362427	AY362400 AY362401
E. dolichostemon W.T. Stearn	Section *Diphyllon* Series Brachycerae	ICM-2004-2560	Heronswood Nursery, USA	AY362424	AY362394 AY362395
E. diphyllum Lodd.	Section *Macroceras*	PDN-02840	Plant Delights Nursery, USA	AY362409	AY362364, AY362365
E. grandiflorum Morr.	Section *Macroceras*	PDN-03518	Plant Delights Nursery, USA	AY362410	AY362366, AY362367
E. koreanum Nakai	Section *Macroceras*	ICM-2004-2558	Plant Delights Nursery, USA	AY362413	AY362372, AY362373
E. sempervirens Nakai ex Maekawa	Section *Macroceras*	ICM-2004-2563	Heronswood Nursery, USA	AY362426	AY362398, AY362399
E. alpinum L.	Section *Epimedium*	PT-1200	Paradise Centre, UK	AY362422	AY362390, AY362391
E. pubigerum Morr. and Decne.	Section *Epimedium*	PDN-02959	Plant Delights Nursery, USA	AY362408	AY362362, AY362363
V. planipetala Calloni		H.H. Schmidt and L. Woodruff 658	Missouri Botanical Garden, USA	AY667154	AY667152, AY667153

99% in *E. franchetii*, *E. rhizomatosum*, *E. leptorrhizum*, *E. wushanense*, *E. simplicifolium*, *E. dolichostemon*, *E. sagittatum*, *E. brevicornu*, and *E. alpinum*. One hundred and twenty-five variable sites were found among the *Epimedium* species and 81 variable sites were parsimony-informative. All the parsimony-informative sites were located in the 5S rRNA gene spacers.

Parsimony analysis of the 5S sequences got 201 optimal trees (tree length = 218; CI = 0.8899; CI excluding uninformative characters = 0.8452; RI = 0.9667; and RC = 0.8602). On the 50% majority-rule consensus tree (Fig. 1), *V. planipetala* showed very close relationship to *Epmedium*. This is consistent to Kim et al. (2004a,b), who found *Epimedium* and *Vancouveria* have a strong sister relationship. *E. pubigerum*, *E. alpinum*, *E. diphyllum*, *E. grandiflorum*, *E. sempervirens*, and *E. koreanum* were resolved as a clade with bootstrap value of 100. Within this clade, *E. alpinum* and *E. pubigerum* formed

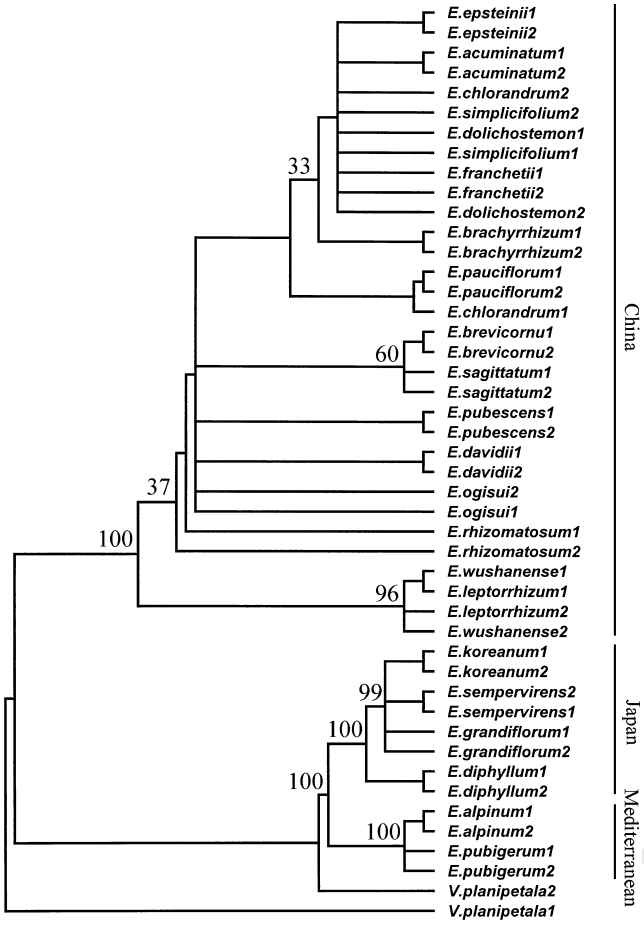

Fig. 1. Fifty percent majority-rule consensus of the 201 equally maximum-parsimony (MP) trees generated from MP analysis on the 5S data (tree length = 218; CI = 0.8899; CI excluding uninformative characters = 0.8452; RI = 0.9667; and RC = 0.8602). Numbers above branches are bootstrap values.

a subclade with bootstrap value of 100. The two species have one biternate stem leaf and distribute in Mediterranean lands. The former is found in southern Europe and the latter in northern Asia Minor. *E. diphyllum*, *E. grandiflorum*, *E. sempervirens*, and *E. koreanum* formed another subclade with bootstrap value of 100. They possess one leaf on the flowering stem and distribute in Japan. *E. diphyllum* with flat spurless petals was found to diverge from *E. koreanum*, *E. sempervirens*, and *E. grandiflorum*.

The rest 16 *Epimedium* species distributed in China were resolved as a clade with bootstrap value of 100. Within this clade, *E. wushanense*, *E. leptorrhizum*, and *E. rhizomatosum* diverged first, *E. wushanense* and *E. leptorrhizum* formed a subclade supported by bootstrap value of 96. *E. ogisui*, *E. davidii*, *E. pubescens*, *E. sagittatum*, *E. brevicornu*, *E. chlorandrum*, and *E. pauciflorum* separated secondly, *E. sagittatum* and *E. brevicornu* formed a subclade with bootstrap value of 60. *E. brachyrrhizum*, *E. dolichostemon*, *E. franchetii*, *E. simplicifolium*, *E. chlorandrum*, *E. acuminatum*, and *E. epsteinii* were clustered as a heterogeneous subclade with a weak bootstrap value of 33.

E. wushanense, *E. leptorrhizum* and *E. rhizomatosum*, *E. acuminatum*, *E. simplicifolium*, *E. brachyrrhizum*, *E. franchetii*, and *E. chlorandrum* have long-spurred flowers without or almost no lamina to the petal, which is longer or much longer than the inner sepal. *E. davidii*, *E. ogisui*, *E. pauciflorum*, and *E. epsteinii* have elongated spur with a conspicuous lamina at the base of the petal, which is longer than or almost equal to the inner sepal. *E. pubescens*, *E. sagittatum*, *E. brevicornu*, and *E. dolichostemon* have smaller flowers, the lamina and the spur have reduced and formed a small pouch, which is shorter than the inner sepal. However, 5S data did not draw a clear route for petal evolution. Chinese species with different types of the petal are nested together.

Epimedium species are well-known medicinal plants. Five species of this genus, *Epimedium brevicornu*, *E. sagittatum*, *E. wushanense*, *E. pubescens*, and *E. koreanum* are listed as source plants of Chinese medicine 'Ying Yang Huo' in the latest edition of Chinese Pharmacopoeia (The State Pharmacopoeia Commission of P.R. China, 2000). More than 10 species have been recorded for medicinal use (Ying, 2001). In this study, low sequence variation is found in the Chinese *Epimedium* species. Limiting large-scale exploitation is essential to protect genetic diversity of these medicinal plants.

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References

Carles, M., Lee, T., Moganti, S., Lenigk, R., Tsim, K.W.K., Ip, N.Y., Hsing, I.-M., Sucher, N.J., 2001. Chips and Qi: microcomponent-based analysis in traditional Chinese medicine. Fresenius J. Anal. Chem. 371, 190–194.

Fischer von, F.E.L., Meyer, C.A., 1846. Sertum Petropolitanum. St. Petersburg.

Franchet, A., 1886. Sur les especes du genre *Epimedium*. Bulletin de la Société Botanique de France 33, 38–41 pp. 103-116.

Kim, Y.-D., Kim, S.-H., Landrum, L.R., 2004a. Taxonomic and phytogeographic implication from ITS phylogeny in *Berberis* (Berberidaceae). J. Plant Res. 117, 175–182.

Kim, Y.-D., Kim, S.-H., Kim, C.H., Jansen, R.K., 2004b. Phylogeny of Berberidaceae based on sequences of the chloroplast gene ndhF. Biochem. Syst. Ecol. 32, 291–301.

Komarov, V.L., 1908. Revisio critica specierum generis *Epimedium* L. Trudy Imperatorskago S.-Petersburgskago Botanicheskago Sada 29, 125–151.

Morren, C., Decaisne, J., 1834. Observations sur la flore du Japon suivies de la monographie du genre *Epimedium*. Annales des Sciences Naturelles 11, Botanique 2, 347–361.

Nakai, R., Shoyama, Y., Shiraishi, S., 1996. Genetic characterization of Epimedium species using random amplified polymorphic DNA (RAPD) and PCR-restriction fragment length polymorphism (RFLP) diagnosis. Biol. Pharm. Bull. 19, 67–70.

Nixon, K.C., 1999. The Parsimony Ratchet, a new method for rapid parsimony analysis. Cladistics 15, 407–414.

Roser, M., Winterfeld, G., Grebenstein, B., Hemleben, V., 2001. Molecular diversity and physical mapping of 5S rDNA in wild and cultivated oat grasses (Poaceae: Aveneae). Mol. Phylogen. Evol. 21, 198–217.

Sikes, D.S., Lewis, P.O., 2001. PAUPRat: a tool to implement Parsimony Ratchet searches using PAUP. Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs.

Stearn, W.T., 1938. *Epimedium* and *Vancouveria* (*Berberidaceae*), a monograph. J. Linn. Soc. Bot. 51, 409–555.

Stearn, W.T., 2002. The genus *Epimedium* and other herbaceous berberidaceae. Timber Press, Portland, Oregon pp. 3-42, 174-176.

Sun, Y., Fung, K.P., Leung, P.C., Shi, D.W., Shaw, P.C., 2004. Characterization of medicinal *Epimedium* species by 5S rRNA gene spacer sequencing. Plant Med. 70, 287–288.

Swofford, D.L., 2003. PAUP*: phylogenetic analysis using parsimony and other methods, version 4.0 b 10. Sinauer Associates, Sunderland, MA.

The State Pharmacopoeia Commission of P.R. China, 2000. In: Pharmacopoeia of The People's Republic of China (English Edition), vol. I. Chemical Industry Press, Beijing, China, p. 107.

Wen, J., Zimmer, E.A., 1996. Phylogeny and biogeography of *Panax* L. (the ginseng genus, *Araliaceae*): inference from ITS sequences of nuclear ribosomal DNA. Mol. Phylogenet. Evol. 6, 166–177.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal RNA genes for phylogenetics. In: Innis, M., Gelfand, D., Sninsky, J., White, T. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, CA, pp. 315–322.

Ying, T.S., 2001. Epimedium. In: Ying, T.S., Chen, T.C. (Eds.), Flora Reipublica Popularis Sinica. Science Press, Beijing, pp. 262–300.

Ying, T.S., 2002. Petal evolution and distribution patterns of *Epimedium* L. (Berberidaceae). Acta Phytotax. Sin. 46, 481–489.