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Yu SX, Liang Q, Luo H, Huang RQ, Li Y, Zhang YJ. Complete chloroplast genome of *Epimedium pinnatum* Fisch. ex DC. (Berberidaceae)[J]. *Plant Science Journal*, 2022, 40(1): 39-46

小檗科科尔切斯淫羊藿叶绿体全基因组研究

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摘 要: 小檗科科尔切斯淫羊藿(*Epimedium pinnatum* Fisch. ex DC.)是分布于高加索地区的一种具有观赏和药用价值的多年生草本植物。本研究首次报道了科尔切斯淫羊藿的叶绿体全基因组序列。结果显示: 科尔切斯淫羊藿叶绿体基因组全长为 156 155 bp, GC 含量为 38.82%; 由一个大的单拷贝区(LSC, 89 425 bp)、一个小的单拷贝区(SSC, 16 284 bp)和一对反向重复区(IRa 和 IRb, 25 223 bp)组成。叶绿体基因组包含 112 个基因, 其中蛋白质编码基因 78 个, tRNA 基因 30 个, rRNA 基因 4 个。系统发育分析结果表明, *Rhizophyllum* 亚属的科尔切斯淫羊藿与 *Epimedium* 亚属的 *Macroceras* 组最先聚为一支, 并与 *Epimedium* 亚属的 *Diphyllon* 组形成的一支分开。

关键词: 科尔切斯淫羊藿; 叶绿体基因组; 小檗科
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Complete chloroplast genome of *Epimedium pinnatum* Fisch. ex DC. (Berberidaceae)

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Abstract: *Epimedium pinnatum* Fisch. ex DC. (Berberidaceae) is a perennial herb with ornamental and medicinal value distributed in the Caucasus region. In this study, the Illumina NovaSeq 6000 sequencing system was used to assemble the complete chloroplast genome sequence of *E. pinnatum* for the first time. Results showed that the total length of the chloroplast genome was 156 155 bp with 38.82% GC content, comprising a large single-copy region (LSC, 89 425 bp), small single-copy region (SSC, 16 284 bp), and pair of inverted repeat regions (IRa and IRb, 25 223 bp). The chloroplast genome of *E. pinnatum* contained 112 genes, including 78 protein-coding genes, 30 transfer RNA (tRNA) genes, and four ribosomal RNA (rRNA) genes. Phylogenetic analysis showed that *E. pinnatum* subgen.

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Epimedium and sect. *Macroceras* was clustered on a branch, which was separated from the branch composed of species from sect. *Diphyllon*, subgen. *Epimedium.* These results suggest that further study is needed to clarify the natural system of *Epimedium* based on more data.

Key words: *Epimedium pinnatum*; Chloroplast genome; Berberidaceae

Epimedium is the largest perennial herbaceous genus of Berberidaceae and has important horticultural and medicinal value. It is widely and intermittently distributed along a narrow and extended zone (25° ~ 48° N, 5° ~ 143° E) from Japan to Algeria in North Africa. Except for a few species distributed in the Mediterranean, West Asia, Japan, and Russia, most *Epimedium* species are found in China[1, 2]. Pharmacological research has shown that *Epimedium* herbs are effective for improving erectile dysfunction (ED), cardiovascular function, and the immune system, and show anti-cancerous and anti-osteoporosis activities[3-7]. The main active ingredients in *Epimedium* plants are flavonoids, including icariin, epimedin A, epimedin B, and epimedin C[8]. Differences in the content and composition of these four flavonoid glycosides cause variations in the pharmacological effects of *Epimedium* plants[9-11]. *Epimedium* species are also ground cover and ornamental plants with high economic value due to the diversity in the color and shape of their flowers and leaves[12].

There are currently 62 recognized species of *Epimedium*, 52 of which are distributed in China[1, 13]. Based on the flower, leaf morphology, and geographical distribution, the genus is comprised of two subgenera, four sections, and four series[14]. As a member of subgen. *Rhizophyllum*, *Epimedium pinnatum* Fisch. ex DC. is easily recognized by a combination of its leaves with five, occasionally nine, conspicuous yellow petal - like inner sepals and minute brownish petals[14, 15]. However, based on molecular phylogenetics, the subgen. *Epimedium* and subgen. *Rhizophyllum* are not well supported[14]. Furthermore, *Epimedium* plants have abundant inter- and intra-species morphological variations and there are many challenges concerning its intragenetic phylogeny and species identification.

Chloroplasts (cp), which are photosynthetic organelles found in the cells of plants, contain a circular double-stranded genome that is maternally inherited in most angiosperms. Therefore, chloroplast genomic sequencing in plants has great scientific significance. Plant chloroplast genomes show a highly conserved quadripartite structure with two inverted repeat sequences (IR) dividing the entire circular chloroplast gene components into a large single-copy (LSC) and small single-copy (SSC) region[16]. Chloroplast genomes encode approximately 110 to 130 genes, including self-replication genes and photosynthesis genes[17]. Chloroplast genomes are of moderate size with a relatively conserved structure and considerable genetic information, and thus are suitable for genomic research. Microsatellite and phylogenetic analyses of chloroplast genomes can help clarify genomic divergence and identify different species. Simple-sequence repeat (SSR) molecular markers of plant chloroplast genomes are a simple and stable technology, which can be used to analyze intra- and extra-species variation[18]. In this study, we sequenced the whole chloroplast genome of *E. pinnatum* and analyzed its genomic characteristics. This is the first report of a complete chloroplast genome of an *Epimedium* species distributed in the Mediterranean region. Phylogenetic relationships of *Epimedium* species with published chloroplast genomes were re-constructed to provide basic genetic information for taxonomic and

phylogenetic studies of *Epimedium*.

1 Materials and Methods

1.1 Plant material and DNA extraction

For this study, *E. pinnatum* samples were collected from Darrell Probst's Garden Vision *Epimediums* of Massachusetts, America. A voucher specimen *Yanjun Zhang 679* (HIB) was deposited at the Herbarium of Wuhan Botanical Garden, Chinese Academy of Sciences (HIB). We collected 5 g of young leaves, which were stored in a dark environment at 4℃ for 48 h. Initial separation was conducted at 4℃ and total genomic DNA was extracted from the fresh leaves of *E. pinnatum* using the modified CTAB method[19].

1.2 Genome sequencing, assembly, and annotation

The libraries were sequenced on the Illumina NovaSeq 6000 platform. Clean reads were assembled using GetOrganelle v1.7.2a with the *E. koreanum* Nakai chloroplast genome (GenBank: NC_029943) as a reference[20]. The chloroplast genome was annotated using CPGAVAS v2, followed by careful manual correction[21]. The chloroplast genome sequence of *E. pinnatum* was submitted to the NCBI database under accession number MW446247. The genome file in GenBank format was imported into Organellar Genome DRAW (http://ogdraw.mpimapgolm.mpg.de/) to draw a circle map of the chloroplast genome.

1.3 Genomic characteristic analysis

Genomic characteristics, including genome length, gene content, base composition, and GC content, were analyzed using Geneious v9.0.2 and DNAStar Lasergene v7.1[22, 23]. Repeat sequences and SSRs were analyzed using the whole-genome sequence of *E. pinnatum*. REPuter software was used to search the forward repeat (F), reverse repeat (R), complement repeat (C), and palindromic repeat (P) sequences[24]. Hamming distance was set to 3, minimal repeat size was set to 30 bp, and maximum computed repeats was set to 90 bp. MiSA (MIcroSAtellite identification tool) was used to search SSRs[25, 26]. The minimum repeat number thresholds of mononucleotide, dinucleotide, trinucleotide, tetranucleotide, pentanucleotide, and hexanucleotide repeats were set to 10, 5, 4, 3, 3, and 3, respectively.

1.4 Phylogenetic analysis

Phylogenetic analysis was carried out using the complete chloroplast sequences of *E. pinnatum*, including 32 reported *Epimedium* species and two species of other genera in Berberidaceae, with *Ranzania japonica* (T. Ito ex Maxim.) T. Ito used as an outgroup. The sequences were aligned using MAFFT v7 and adjusted with BioEdit v7.0.9[27, 28]. A maximum-likelihood (ML) tree was constructed with raxmlGUI1.5b v8.2.10[29, 30]. Bootstrap for each clade was set to 1000 replicates and only branches with bootstrap values above 70% were identified as strongly supported clades[31, 32]. The genome sequence data that support the findings of this study are openly available in GenBank under accession number MW4462471. The associated BioProject, SRA, and Bio-Sample accession numbers are PRJNA715033, SRR13983935, and SAMN18323759, respectively.

2 Results

2.1 Genomic characteristics

The chloroplast genome of *E. pinnatum* contained 156 155 bp with a GC content of 38.82% and included a large single-copy (LSC) region (89 425 bp) and small single-copy (SSC) region (16 284 bp), which were separated by a pair of inverted repeat sequence regions (IRa and IRb, 25 223 bp each) (Fig.1). GC content was 37.33%, 33.20%, and 43.29% in the LSC, SSC, and IR regions, respectively (Table 1). GC

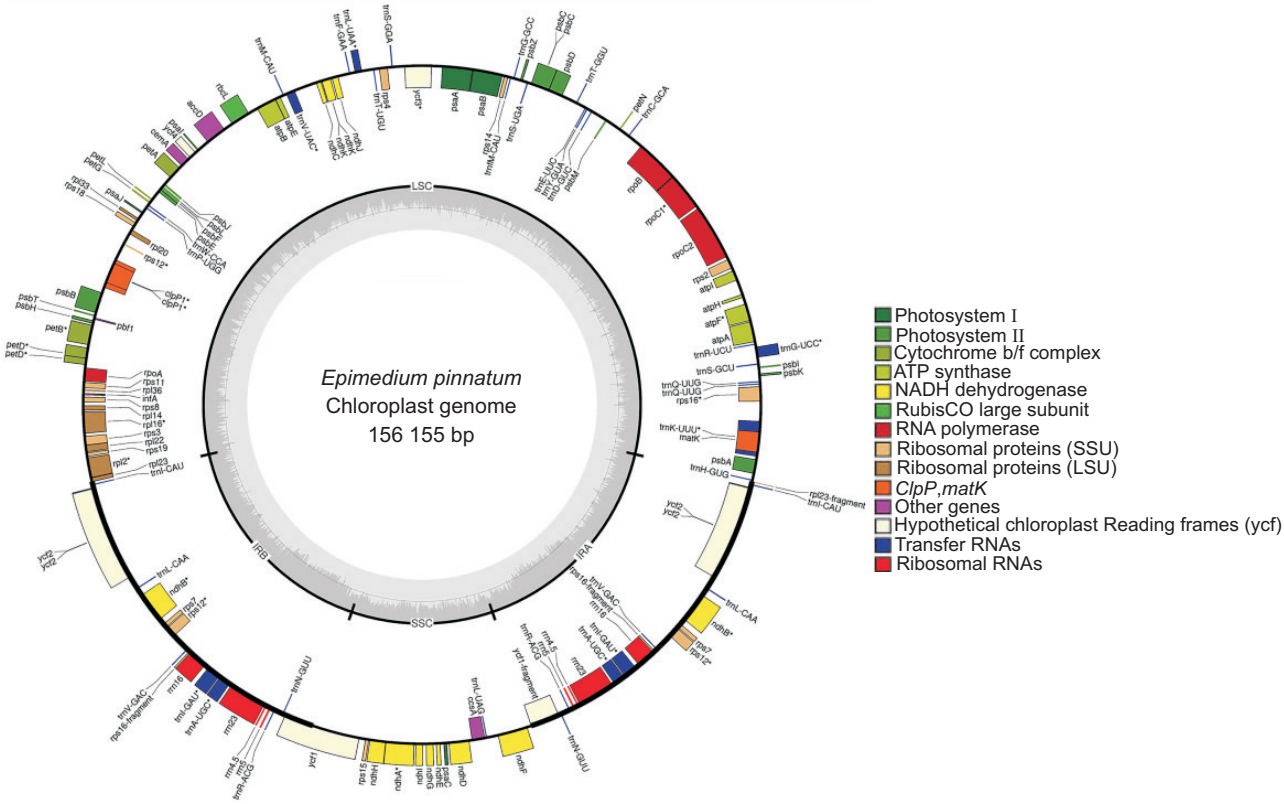


Fig. 1 Chloroplast genome map of *Epimedium pinnatum*

Table 1 GC content in complete chloroplast genome of *Epimedium pinnatum*

Quadripartite region	GC content / %
LSC	37.33
SSC	33.20
IRa	43.29
IRb	43.29
All	38.82

content was higher in the IR region than in the LSC and SSC regions because the four ribosomal RNAs (rRNAs) were all located in the IR region.

A total of 112 genes were identified from the chloroplast genome of *E. pinnatum*, including 78 protein-coding genes, 30 transfer RNA (tRNA) genes, and four rRNA genes (Table 2). There were 16 double-copy genes (*ndhB*, *rps7*, *rps12*, *rrn4.5*, *rrn5*, *rrn16*, *rrn23*, *trnA-UGC*, *trnI-CAU*, *trnI-GAU*, *trnL-CAA*, *trnN-GUU*, *trnQ-UUG*, *trnR-ACG*, *trnV-GAC*, and *ycf2*) in the genome. One split gene (*rpl23*) was found at the boundary of the LSC and IRb regions, with 276 bp in the LSC region and 33 bp in the IRb region. The overlapping sections of *PsbC-psbD*, *atpE-atpB*, and *rps3-rpl22* were 53, 4 and 16 bp, respectively. Intron-exon structure analysis indicated that nine protein-coding genes (*rps16*, *rpoC1*, *petB*, *petD*, *rpl2*, *rpl16*, *atpF*, *ndhA*, and *ndhB*) and six tRNA genes (*trnK-UUU*, *trnG-UCC*, *trnL-UAA*, *trnV-UAC*, *trnI-GAU*, and *trnA-UGC*) had one intron, while three genes (*ycf 3*, *clpP*, and *rps12*) contained two introns.

2.2 Microsatellite analysis

In total, 89 repeat sequences were found in the chloroplast genome of *E. pinnatum*. No reverse or complement repeats were found in the genome, but 44 forward and 45 palindromic repeats were detected. The lengths of these repeat sequences ranged from 31 – 131 bp, though most were in the range of 30 – 50 bp (Table 3).

Table 2　Gene content in complete chloroplast genome of *Epimedium pinnatum*

Category	Gene group	Name of gene
Self-replication	Ribosomal proteins (SSU)	*rps2*, *rps3*, *rps4*, *rps7*, *rps8*, *rps11*, *rps12*, *rps14*, *rps15*, *rps16*, *rps18*, *rps19*
	Ribosomal proteins (LSU)	*rpl2*, *rpl14*, *rpl16*, *rpl20*, *rpl22*, *rpl23*, *rpl32*, *rpl33*, *rpl36*,
	Ribosomal RNAs	*rrn4.5*, *rrn5*, *rrn16*, *rrn23*
	RNA polymerase	*rpoA*, *rpoB*, *rpoC1*, *rpoC2*
	Transfer RNAs	*trnA-UGC*, *trnC-GCA*, *trnD-GUC*, *trnE-UUC*, *trnF-GAA*, *trnfM-CAU* *trnG-GCC*, *trnG-UCC*, *trnH-GUG*, *trnI-CAU*, *trnI-GAU*, *trnK-UUU*, *trnL-UAG*, *trnL-UAA*, *trnL-CAA*, *trnM-CAU*, *trnN-GUU*, *trnP-UGG*, *trnQ-UUG*, *trnR-ACG*, *trnR-UCU*, *trnS-GGA*, *trnS-GCU*, *trnS-UGA*, *trnT-GGU*, *trnT-UGU*, *trnV-GAC*, *trnV-UAC*, *trnW-CCA*, *trnY-GUA*
Photosynthesis	Photosystem Ⅰ	*psaA*, *psaB*, *psaC*, *psaI*, *psaJ*
	Photosystem Ⅱ	*psbA*, *psbB*, *psbC*, *psbD*, *psbE*, *psbF*, *psbH*, *psbI*, *psbJ*, *psbK*, *psbL*, *psbM*, *psbN*, *psbT*, *psbZ*
	NADH dehydrogenase	*ndhA*, *ndhB*, *ndhC*, *ndhD*, *ndhE*, *ndhF*, *ndhG*, *ndhH*, *ndhI*, *ndhJ*, *ndhK*
	Cytochrome b/f complex	*petA*, *petB*, *petD*, *petG*, *petL*, *petN*
	ATP synthase	*atpA*, *atpB*, *atpE*, *atpF*, *atpH*, *atpI*
	RubisCO large subunit	*rbcL*
Other genes	Maturase	*matK*
	Hypothetical chloroplast Reading frames (ycf)	*ycf1*, *ycf2*, *ycf4*, *ycf3*
	protease	*clpP*
	Envelope membrane protein	*cemA*
	Subunit of acetyl-CoA carboxylase	*accD*
	C-type cytochrome synthesis gene	*ccsA*

Table 3　Length of repeat sequences

Sequence length / bp	Amount
30 – 50	72
51 – 70	8
71 – 90	3
91 – 110	4
111 – 130	0
>130	2

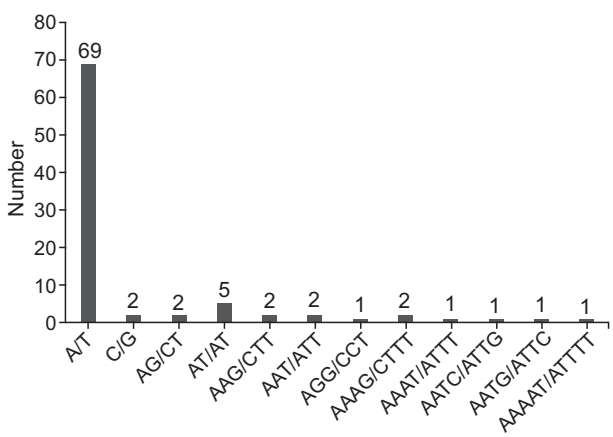
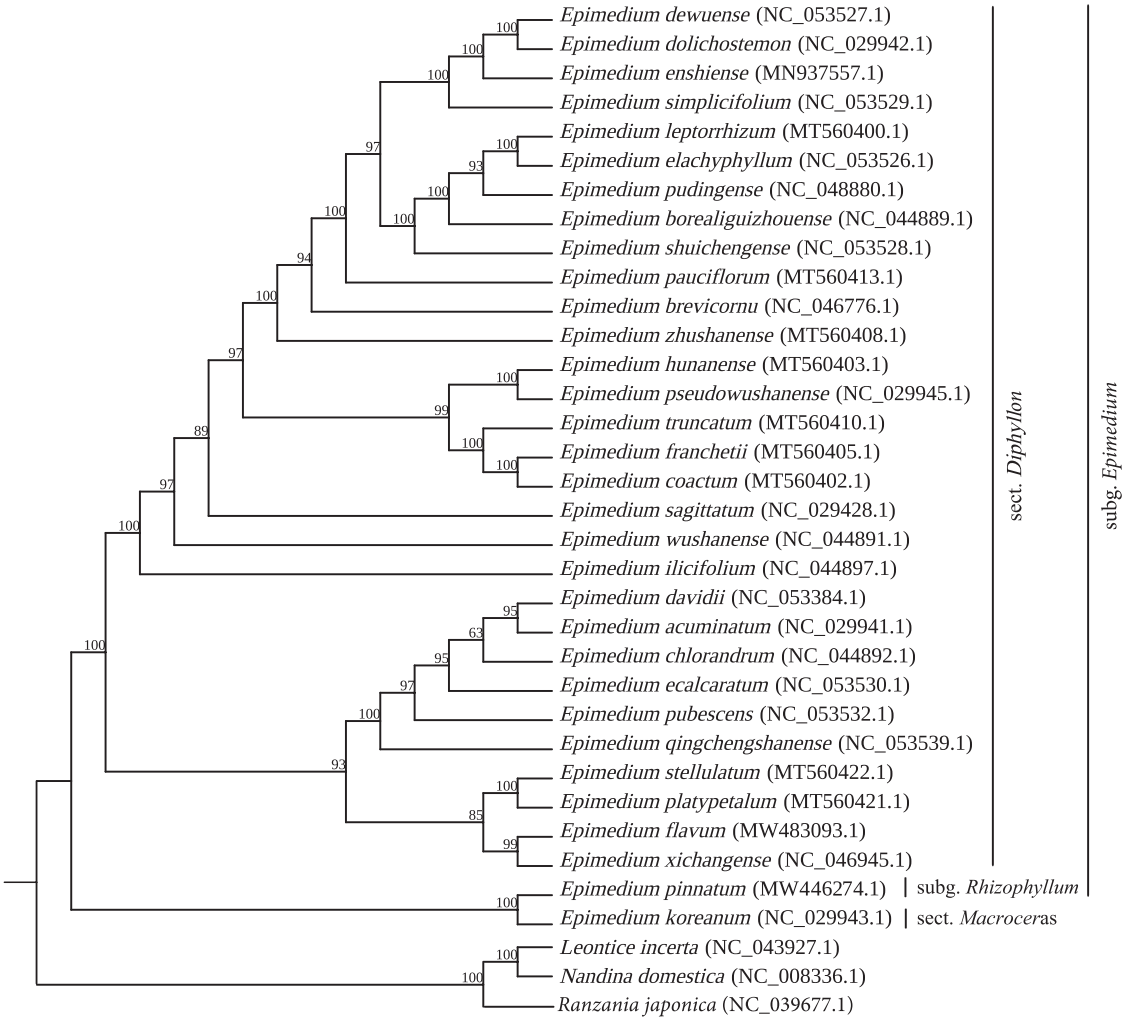


Fig. 2　Number of different types of SSR motifs

In total, 89 SSRs were identified in the complete chloroplast genome sequence, including 10 in compound formation and one containing sequences. There were four types of SSRs, including 71 mononucleotides, seven dinucleotides, five trinucleotides, and six tetranucleotides. Most SSRs were a combination of A – T or T – A (Fig.2).

2.3　Phylogenetic analysis

The phylogenetic ML tree was constructed based on the complete chloroplast genome of 35 species, with *R. japonica* as an outgroup (Fig.3). The phylogenetic tree showed that *E. pinnatum* of subgen. *Rhizophyllum* was separated from other *Epimedium* species of subgen. *Epimedium*. All species of subgen. *Epimedium* were clustered into a clade with high support,



Numbers above lines represent ML bootstrap values.

Fig. 3 Phylogenetic maximum-likelihood (ML) tree based on complete chloroplast genome of 35 species, with *Ranzania japonica* as an outgroup

including a clade with *E. koreanum* of sect. *Mac-roceras* and a clade consisting of species of sect. *Diphyllon*. The two other species in Berberidaceae were separated from all *Epimedium* species.

3 Discussion

In this study, we sequenced the complete chloroplast genome of *E. pinnatum*, which is the first for an *Epimedium* species distributed in the Mediterranean region. The complete *E. pinnatum* chloroplast genome was conserved, with a typical quadripartite structure. Gene content, gene order, IR-boundaries, base composition, and GC content were all highly conserved compared with chloroplast genomes of other reported *Epimedium* species[13, 15]. A-T content in the total SSRs was 77.5%, which is because the A-T bond has one less hydrogen bond than the G-C bond and is easier to disconnect[33]. In plant genomes, SSR sites are highly variable and can be used as molecular markers in research.

The updated classification system of *Epimedium* was constructed based on morphological, chromosomal, and geographical characteristics. Notably, *Epimedium* was divided into subgen. *Epimedium* and subgen. *Rhizophyllum*, with subgen. *Epimedium* divided into ser. *Campanulatae*,

ser. *Davidianae*, ser. *Brachycerae*, and ser. *Dolichocerae*[14]. Subgen. *Rhizophyllum* is recognized by its leafless flower-stalk and subgen. *Epimedium* is recognized by its cauline leaves[14, 15]. Based on internal transcribed spacer (ITS), *trnK-matK* sequence, *atpB-rbcL* spacer sequence variation, and amplified fragment length polymorphism (AFLP) data, previous molecular phylogenetic studies consistently support subgen. *Rhizophyllum* and the four series as five monophyletic branches; however, subgen. *Rhizophyllum* and subgen. *Epimedium* are not well supported[34-36]. In the present study, we assembled the first chloroplast genome of subgen. *Rhizophyllum* and conducted phylogenetic analyses based on chloroplast genome sequences of *Epimedium* reported in the present and previous studies[13, 15]. Results showed that *Epimedium* clustered into a monophyletic group with high support. In *Epimedium*, sect. *Macroceras* of subgen. *Epimedium* was most closely related to subgen. *Rhizophyllum*, not with sect. *Diphyllon* of subgen. *Epimedium*.

The chloroplast genome sequences of sect. *Polyphyllon* and sect. *Epimedium* have not been reported, and our phylogenetic tree did not cover all *Epimedium* sections. Thus, additional chloroplast genomes of *Epimedium* are needed to connect taxonomic and phylogenetic studies and improve species identification.

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