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Phylogenetic analysis of *Paeonia* sect. *Moutan* (Paeoniaceae) based on multiple DNA fragments and morphological data

[1,2]Xuan ZHAO [1,2,3]Zhi-Qin ZHOU* [2]Qi-Bing LIN [2]Kai-Yu PAN [1]Ming-Yang LI

[1] (*College of Horticulture and Landscape Architecture, Southwest University,* Chongqing 400716, China)

[2] (*State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences,* Beijing 100093, China)

[3] (*Citrus Research Institute, Chinese Academy of Agricultural Sciences,* Chongqing 400712, China)

Abstract Tree peony, being crowned the title "King of Flowers" in China, is of great medicinal, ornamental, and economic values. In the present study, the phylogeny of the wild tree peony species (section *Moutan*, *Paeonia*, Paeoniaceae), represented by twelve accessions collected from all eight species in the section, was investigated based on the DNA sequence in five DNA fragments from both nuclear (*Adh*1A, *Adh*2 and *GPAT*) and chloroplast (*trn*S-*trn*G *and rps*16-*trn*Q) genomes, as well as morphological characters. Both maximum parsimony (MP) and Bayesian inference of phylogeny (BI) trees were reconstructed based on the combined data of the DNA sequences and morphological data, respectively. The MP and BI trees have the similar topology, and the sect. *Moutan* clearly branched into two clades. One clade consists of two species, *P. delavayi* and *P. ludlowii*, corresponding to the subsect. *Delavayanae*, and another clade is composed of other six species. Within the second clade, the six species can be divided into three subclades consisting of *P. rockii* and *P. decomposita*, *P. jishanensis* and *P. qiui*, *P. suffruticosa* and *P. ostii*, respectively. Among the three subclades, *P. jishanensis*/*P. qiui* is most closely related to *P. suffruticosa*/*P. ostii*. These results provide up to date the clearest picture of the phylogeny of wild tree peony species in the sect. *Moutan*.

Key words chloroplast DNA, morphological character, nuclear DNA, *Paeonia* sect. *Moutan*, phylogeny.

The cultivated tree peonies have been used as medicine for over 2000 years in China and 500 years in Europe, respectively (Foster & Yue, 1992), and now cultivated all over China and the temperate regions of the world for their medicinal, ornamental, and economic values (Hong & Pan, 1999a; Lan et al., 2002).

Tree peony belongs to the section *Moutan* DC. of the genus *Paeonia* L. (Paeoniaceae) (Stern, 1946; Pan, 1979). In addition to this woody group, there are two herbaceous sections in this genus, sect. *Paeonia* and sect. *Onaepia* (Stern, 1946; Pan, 1995). The genus *Paeonia* consists of more than 30 species, distributed widely in the temperate region of the world (Pan, 1995). The section *Onaepia* has only two species endemic to North America, whereas sect. *Paeonia* comprises about 22 species found in Europe, North-west Africa and Asia, spreading from Portugal and Morocco to Japan (Stern, 1946; Pan, 1995). The sect. *Moutan,* following a recent classification system of Hong & Pan (1999a), contains eight species and three of them each have two subspecies, distributed in southwestern, central and northern region of China (Hong & Pan, 1999a).

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* Authors for correspondence. E-mail: <zqzhoubj@yahoo.com>.

In the past decade, important progress has been made in the taxonomy of tree peony (Xi, 1984; Hong et al., 1988; Hong & Pan, 1999a; Zhou, 2006). However, although the interspecific relationships of wild tree peonies have been previously investigated using morphological data (Hong, 1997a; Zhou et al., 2003), molecular marker (Zou et al., 1999), and DNA fragments from different genomes (Sang et al., 1995, 1997a, b; Sang & Zhang, 1999; Ferguson & Sang, 2001; Tank & Sang, 2001; Lin et al., 2004; Zhao et al., 2004), the species phylogeny of the sect. *Moutan* is still poorly understood. Especially, the interspecific relationship among the six species (*P. suffruticosa, P. ostii, P. jishanesis, P. qiui, P. rockii,* and *P. decomposita*) remained unresolved (Hong & Pan, 1999b; Lin et al., 2004; Zhao et al., 2004).

Using DNA molecular marker and gene sequences from both nuclear and cytoplasmic genomes has had an enormous impact on studies of plant phylogenetics and systematics (Sang, 2002). However, the problem of the reconstruction of phylogeny of taxa at lower taxonomic level, especially the closely related species, remains unresolved (Sang, 2002; Grob et al., 2004). For examples, nuclear ribosomal DNA regions, especially the internal transcribed spacers (ITS1 and ITS2) are applied extensively for phylogeny reconstruction at lower

taxonomic levels. ITS sequences, however, have their own problems when applied to phylogenetic studies, such as extensive length variations between copies, paralogy problems, and/or lack of resolving power (Grob et al., 2004). The mitochondrial *nad*1 intron 2 has been successfully used in studying population structure and phylogenetic relationships among closely related taxa (Gugerli et al., 2001). But for their low substitution rates and high levels of gene rearrangements, plant mitochondrial genes have been regarded as less useful for systematic studies than animal mtDNA (Sanjur et al., 2002). The chloroplast genome shares many features with animal mtDNA, such as the conserved gene order and their high levels of sequence variation in the noncoding parts of the genome (Provan et al., 2001). Therefore, the cpDNA sequences were widely used in systematic studies. Single- or low-copy nuclear genes have high rates of substitution. The phylogenetic utility of this kind of genes has been investigated in various plant taxa, such as granule-bound starch synthase (GBSSI or waxy) in Poaceae (Mason-Gamer et al., 1998), vicilin in Sterculiaceae (Whitlock & Baum, 1999), malate synthase in Arecaceae (Lewis & Doyle, 2001), and alcohol dehydrogenase (*Adh*) in *Oryza* (Ge et al., 1999; Guo & Ge, 2005). Especially, the single-copy nuclear gene glyceraldehyde 3-phosphate dehydrogenase (G3pdh) was successfully used in the phylogeographic study of Cassva and its close relatives (Olsen & Schaal, 1999). Furthermore, just as Zhang & Hewitt (2003) has pointed out that because of its general higher polymorphism and the ease of designing primer in the flanking coding sequences (normally conserved among closely related taxa), intron will be probably the most useful nuclear markers in the near future. The potential utility of the intron of low-copy nuclear genes in phylogenetic reconstruction at lower taxonomic levels has been explained completely (Grob et al., 2004).

Adh is a metabolic enzyme responsible for the interconversion of ethanol and acetaldehyde (Freeling & Bennett, 1985). *Adh* gene family usually has two or three loci in a broad array of angiosperm species (Clegg et al., 1997). The DNA divergence and molecular evolution of this gene family have been investigated in a broad array of plants, such as Brassicaceae (Koch et al., 2000), *Gossypium* (Small et al., 1998), *Paeonia* (Sang et al., 1997b), *Pinus banksiana* (Perry & Furnier, 1996), and Poaceae (grass) (Gaut, 1999; Ge et al., 1999; Guo & Ge, 2005). In the genus *Paeonia*, *Adh* gene family has three members, *Adh*1A, *Adh*1B and *Adh*2, among them *Adh*1B was merely found in sect. *Moutan* (Sang et al., 1997b). Sang et al. (1997b) reconstructed the phylogeny of 11 putative nonhybrid species and investigated the *Adh* gene evolution in the genus *Paeonia*. Their study showed that *Adh* gene is better than ITS and *mat*K genes in resolving the interspecific relations among the species in the genus *Paeonia*. However, in this study the species of section *Moutan* were poorly sampled. Similar to *Adh* gene, Glycerol-3-phosphate acyltransferase (*GPAT*) is an essential enzyme and has been used for phylogenetic analysis, which is utilized in the catalysis of the initial step of glycerolipid synthesis in the cells of all higher organisms (Nishida et al., 1993). In plant cell, there are three types of *GPAT*s that differ in their subcellular location (chloroplasts, mitochondria and cytoplasm) and substrate specificity (Tank & Sang, 2001). Among the genes coding for different *GPAT*s, the nuclear-encoded chloroplast-expressed *GPAT* gene has been found to be single-copy in several distantly related angiosperm families (Tank & Sang, 2001). In the genus *Paeonia*, Tank & Sang (2001) investigated the DNA variation in nuclear-encoded chloroplast-expressed *GPAT* gene, and successfully reconstructed the phylogeny of 13 *Paeonia* species. Their study revealed that the *Paeonia GPAT* gene contains a large intron of more than 2 kb with a high level of DNA variation rate (Tank & Sang, 2001).

Although many genes have been used effectively to address the systematic problems, the information from single gene usually was insufficient to resolve the problem of interspecific relationship of sect. *Moutan* (Sang et al., 1997a, b; Sang & Zhang, 1999; Lin et al., 2004; Zhao et al., 2004). In contrast, combined analysis of multiple data sets has become an effective way to increase the resolving power and the reliability of phylogenetic reconstruction (Hillis & Huelsenbeck, 1995; Wendel & Doyle, 1998; Ge et al., 1999; Cronn et al., 2002; Guo & Ge, 2005; James et al., 2006).

In the present study, we reconstruct the phylogeny of the sect. *Moutan* using five gene fragments from both nuclear (*Adh*1A, *Adh*2, and *GPAT*) and chloroplast (*trn*S-*trn*G and *rps*16-*trn*Q) genomes, as well as morphological characters. Our objective is to clarify unresolved problems concerning interpecific relationship within *Paeonia* sect. *Moutan*.

1 Material and methods

1.1 Material

In this study, twelve accessions represent eight wild species of *Paeonia* sect. *Moutan*. *Paeonia*

lactiflora was sampled as outgroup, which belongs to *Paeonia* sect. *Paeonia*. All vouchers are deposited at the Herbarium, Institute of Botany, Chinese Academy of Sciences, Beijing, China (PE). The details of the sampled species, accession numbers or vouchers and origins are listed in Table 1. Some sequences of *GPAT* gene (AY016249), *Adh* gene (AF009042, AF009043, AF009044, AF009049, AF009058, AF009060, AF009061 and AF009068), and *rps*16-*trn*Q gene (DQ313804) were downloaded from GenBank. All the sequences produced in this paper are deposited in GenBank (GenBank Accession Number: EF520815–EF520868).

1.2 Methods

1.2.1 Morphological data The data set of morphology was taken from the matrix presented by Zhou et al. (2003). In the present study, we selected 12 samples, including yinpingmudan-HNSX, yinpingmudan-AHCH, jishanensis-ShXYA, jishanesis-SXJS, decomposita-MEK, rotundiloba-MX, ostii-HNNX, qiui-HBBK, taibaishanica-ShXMX, rockii-HNNX, ludlowii-ML1, and delavayi-XZLZ.

1.2.2 DNA isolation, amplification, and sequencing Total DNA was isolated from silica-gel dried leaves using the CTAB method as described by Doyle and Doyle (1987). Amplifications were performed in a Peltier Thermal Cycler (PTC-200, PE). The nuclear *Adh* and *GPAT* gene were amplified and sequenced as described by Lin et al. (2004) and Zhao et al. (2004), respectively. PCR cycling parameters for the two chloroplast genes (*trn*S-*trn*G and *rps*16-*trn*Q) were similar, with an initial 4 min at 70 ℃, followed by 2 cycles of 1 min at 94 ℃, 20 s at 52 ℃, and 1.5 min at 72 ℃; after that, 35 cycles of 20 s at 94 ℃, 20 s at 52 ℃, and 1.5 min at 72 ℃ were conducted, with a final extension time of 10 min at 72 ℃. All the amplifying and internal sequencing primers are listed in Table 2.

PCR products were electrophoresed on and excised from 1.5% agarose gel, then purified using DNA Purification Kit (Pharmacia). Sequencing was done on an ABI 377 (Applied Biosystems, Foster City, CA) or MegaBASE 1000 automatic DNA sequencer (Amersham Biosciences, Buckinghamshire, UK).

1.2.3 Data analysis Morphological characters were treated following Zhou et al (2003).

Sequences were aligned using ClustalX version 1.81 (Thompson et al., 1997) and refined manually. The partition-homogeneity test, implemented in PAUP* version 4.0b10 (Swofford, 2002), was used to evaluate the congruence between different data sets (Farris et al., 1995). Replicates were analyzed using the parsimony criterion, and branch swapping using tree bisection-reconnection (TBR) was performed and one tree held at each step during the stepwise addition. However, the test was not used as a criterion to decide whether we are allowed to combine data sets. Because there is no agreement on how to treat different data set, whether analyze them as a combined data set or analyze them separately one by one (Kluge, 1989; Miyamoto & Fitch, 1995; Farris et al., 1995);

Table 1 Source of materials

No.	Taxon	Voucher/ID	Origin
1	*Paeonia ludlowii* (Taylor & Stem) D. Y. Hong (大花黄牡丹)	S. L. Zhou (周世良) H02124	Nyingchi, Xizang, China (西藏林芝)
2	*P. delavayi* Franch. (滇牡丹)	S. L. Zhou (周世良) H02123	Nyingchi, Xizang, China (西藏林芝)
3	*P. decomposita* Hand.-Mazz. ssp. *decomposita* (四川牡丹)	Z. Q. Zhou (周志钦) H03001	Barkam, Sichuan, China (四川马尔康)
4	*P. decomposita* ssp. *rotundiloba* D. Y. Hong (圆裂四川牡丹)	D. Y. Hong et al. (洪德元等) H02016	Pengzhou, Sichuan, China (四川彭州)
5	*P. rockii* (S. G. Haw & Lauener) T. Hong & J. J. Li ex D. Y. Hong ssp. *rockii* (紫斑牡丹)	D. Y. Hong et al. (洪德元等) H02121 (H97015)	Neixiang, Henan, China (河南内乡)
6	*P. rockii* ssp. *taibaishanica* D. Y. Hong (太白紫斑牡丹)	D. Y. Hong et al. (洪德元等) H02122 (H97058)	Mt. Taibai, Shaanxi, China (陕西太白山)
7	*P. ostii* T. Hong & J. X. Zhang (凤丹)	D. Y. Hong & K. Y. Pan (洪德元, 潘开玉) H02106	Lushi, Henan, China (河南卢氏)
8	*P. suffruticosa* ssp. *yinpingmudan* D. Y. Hong, K.Y. Pan & Z. W. Xie (银屏牡丹)	K. Y. Pan & Z. W. Xie (潘开玉, 谢中稳) H02117 (H9701)	Chaohu, Anhui, China (安徽巢湖)
		D. Y. Hong (洪德元) H02118 (H97010)	Song Xian, Henan, China (河南嵩县)
9	*P. qiui* Y. L. Pei & D. Y. Hong (卵叶牡丹)	Z. Q. Zhou (周志钦) H02080	Lanzhou, Gansu, China (甘肃兰州)
10	*P. jishanensis* T. Hong & W. Z. Zhao (矮牡丹)	Z. Q. Zhou (周志钦) H02077	Lanzhou, Gansu, China (甘肃兰州)
		D. Y. Hong et al. (洪德元等) H02119 (H97066)	Yan'an, Shaanxi, China (陕西延安)

Table 2 The primers used for PCR amplification and sequencing

Primer	Sequence
*rps*16-*trn*Q	
*rps*16	5′- CGTTGCTTTCTACCACATCG
*trn*Q	5′- TTACTCGGAGGTTCGAATCC
*trn*Q -r*	5′- CCCTCCCTCACTTCATATTG
*trn*S-*trn*G	
*trn*S	5′- GCCGCTTTAGTCCACTCAGC
*trn*G	5′- GAACGAATCACACTTTTACCAC
*trn*G-r*	5′- TCGTCAGGGAACTTAACGAG

* represents the internal primer for sequence.

moreover, it is legitimate to combine data sets although different sources of data may yield alternative phylogenetic results (Farris, 1997; Soltis et al., 1997).

Maximum parsimony (MP) analyses were conducted using PAUP* version 4.0b10 (Swofford, 2002). All characters were equally weighted, gaps were treated as missing, and character states were treated as unordered. Heuristic search was performed with MULPARS option, tree bisection-reconnection (TBR) branch swapping, and random stepwise addition with 1000 replicates. Topological robustness was assessed by bootstrap analysis with 1000 replicates using simple taxon addition (Felsenstein, 1985).

An appropriate nucleotide substitution model was determined using Modeltest version 3.06 (Posada & Crandall, 1998) for each data set. The models were chosen according to the Hierarchical Likelihood Ratio Test (LRT) and then used for subsequent Bayesian analysis. Bayesian inference (BI) was conducted using MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2001). One cold and three incrementally heated Markov Chain Monte Carlo (MCMC) chains were run for 1 million generations, with trees sampled every 100 generation, using random tree as its starting point and a temperature parameter value of 0.2 (the default setting of MrBayes). For each data set, MCMC runs were repeated twice as a safeguard against spurious results. The first 1000 trees were discarded as burn-in, and the remaining trees were used to construct Bayesian trees. Examination of the log-likelihoods and the observed consistency between runs suggested that the burn-in periods were sufficiently long enough for chains to have become stationary.

2 Results

2.1 Sequence characteristics

In the present study, the amplified sequences of *Adh*1 and *Adh*2 include four introns and five exons. The resulting fragment of *Adh*1A ranged in length from 1141 to 1167 bp with an aligned length of 1191 bp, among which 93 sites are variable and 23 sites are parsimony informative. The sequences of *Adh*2 vary from 1095 to 1149 bp in length. The final alignment of *Adh*2 is 1185 bp, with 135 sites variable and 31 sites informative. The amplified segment of *GPAT* gene is a big intron between exon 5 and 6. The sequence length varies from 1848 to 1916 bp, and the final alignment of *GPAT* is 1962 bp, including 144 variable sites and 54 informative sites. Among the three nuclear gene segments, the informative site of *GPAT* is the highest (2.75%), *Adh*2 is 2.62%, and *Adh*1A is the lowest (1.93%). Chloroplast genes *rps*16-*trn*Q and *trn*S-*trn*G have less variable and informative sites than the nuclear genes, with the informative sites 1.06% and 0.97%, respectively. The characteristics of each sampled sequence are presented in Table 3.

Each data set was identified to fit a corresponding model using Modeltest ver. 3.06 (Posada & Crandall, 1998). Nuclear genes *Adh*1A, *Adh*2, and *GPAT* have the same evolutionary model HKY+G, while the models of chloroplast genes *rps*16-*trn*Q and *trn*S-*trn*G are F81.

2.2 Phylogenetic analysis

Due to limited information and low resolution of single gene data set or morphological data set (data not shown), in the present study, we only analyzed data in combined data sets. Based on the information of chloroplast genes, the phylogenetic analysis of the combined data set (*rps*16-*trn*Q and *trn*S-*trn*G, P=1.000 for partition homogeneity tests) suggests that these species can be divided into two clades: one clade includes *P. ludlowii* and *P. delavayi*; the second clade consists of other six species (data not shown). The Bayesian analysis generates a similar topology, with only a few differences in bootstrapping support (BS) and Bayesian posterior probability for some clades (data not shown).

Three nuclear genes (hereafter represented as data1, P<0.001), three nuclear genes plus two chloroplast genes (hereafter represented as data2, P<0.001), and all DNA sequence data with morphological data (hereafter represented as data3, P<0.001), were analyzed one by one (Table 4). The data1 generated two most parsimonious trees (Fig. 1), the data2 generated one (Fig. 2), and one most parsimonious tree (Fig. 3) was obtained from the data3.

The topologies of the trees generated from the different combined data sets are significantly congruent with each other. *Paeonia* sect. *Moutan* is divided into two major clades, consistent with the above results based on combined chloroplast sequence data. The first clade consists of *P. ludlowii* and *P. delavayi*;

Table 3 Informative parameters for each DNA fragment

DNA fragment	Aligned length	variable site (%)	Informative site (%)	GC (%)	Model	Nst	Rate
*rps*16-*trn*Q	1229	40 (3.25)	13 (1.06)	29.85	F81	1	equal
*trn*S-*trn*G	512	6 (1.17)	5 (0.97)	30.01	F81	1	equal
*Adh*1A	1191	116 (9.74)	23 (1.93)	39.75	HKY+G	2	gamma
*Adh*2	1185	166 (14.00)	31 (2.62)	40.68	HKY+G	2	gamma
GPAT	1962	198 (10.09)	54 (2.75)	35.18	HKY+G	2	gamma
morphological character	25	24 (96.00)	22 (88.00)	–	–	–	–
Summary	7309	664 (9.08)	194 (2.65)	–	–	–	–

Table 4 Results conducted by partition homogeneity test for various combinations of the data sets

Combined data sets	PHT value
*rps*16-*trn*Q + *trn*S-*trn*G	1.000
*Adh*1A + *Adh*2 + *GPAT*	0.001
*Adh*1A + *Adh*2 + *GPAT* + *rps*16-*trn*Q + *trn*S-*trn*G	0.001
*Adh*1A + *Adh*2 + *GPAT*+ *rps*16-*trn*Q + *trn*S-*trn*G + morphology	0.001

the second clade includes other six species. Within the second clade, *P. rockii* is closely related to *P. decomposita*; while other four species form another subclade, and *P. jishanensis*/*P. qiui* and *P. suffruticosa*/*P. ostii* was sister to each other. Although the topologies are congruent between the three different combined data sets, there are some differences among them: (1) in *P. jishanensis*/*P. qiui* clade, two samples of *P. jishanensis* grouped in the data2 and data3, but not in data1; (2) within *P. rockii*/*P. decomposita* clade, two subspecies of *P. rockii* grouped in the data1 and the data3, but not in the data2. In addition, it is also apparently that the BS value is increasing with the addition of separate data set to combined data set. For each node of the data1, the average BS is 82% (Fig. 1). The average BS for each node of phylogeny derived from the data2 was 85% (Fig. 2), while that of data3 get an average support of 91.4% (Fig. 3). The trend of Bayesian posterior probability is the same as that of BS.

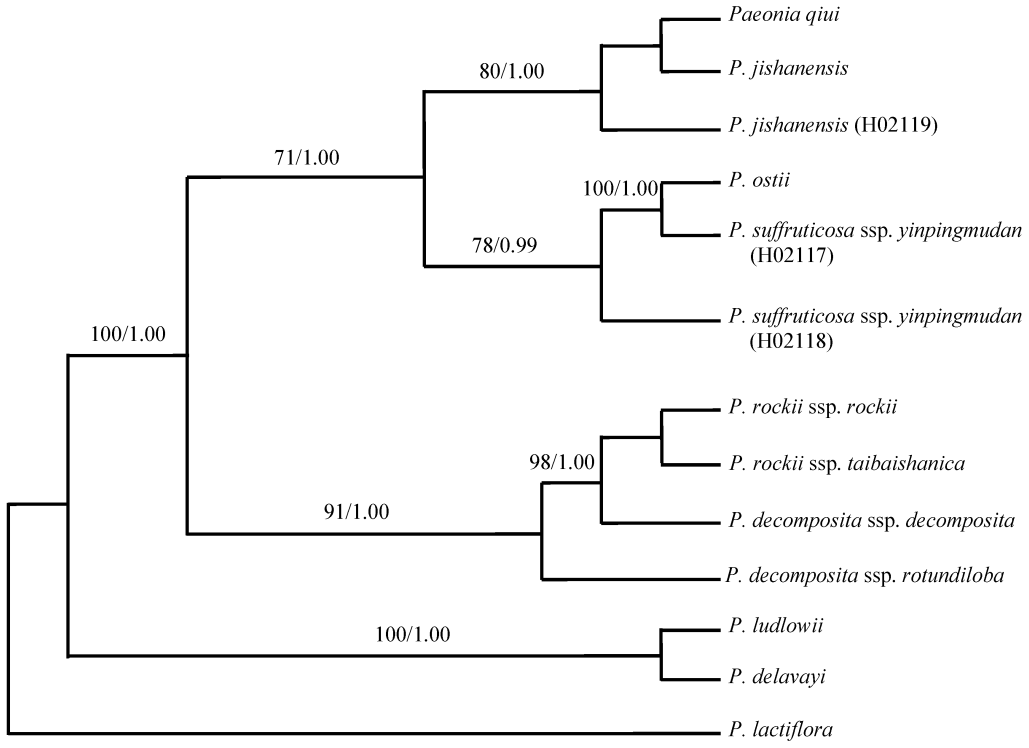

Fig. 1. The 50% majority-rule consensus tree of two MP trees based on *Adh*1A, *Adh*2 and *GPAT* (Tree length = 559, *CI* = 0.8927, *RI* = 0.7196). The numbers near branches are bootstrap percentages followed by Bayesian posterior probabilities.

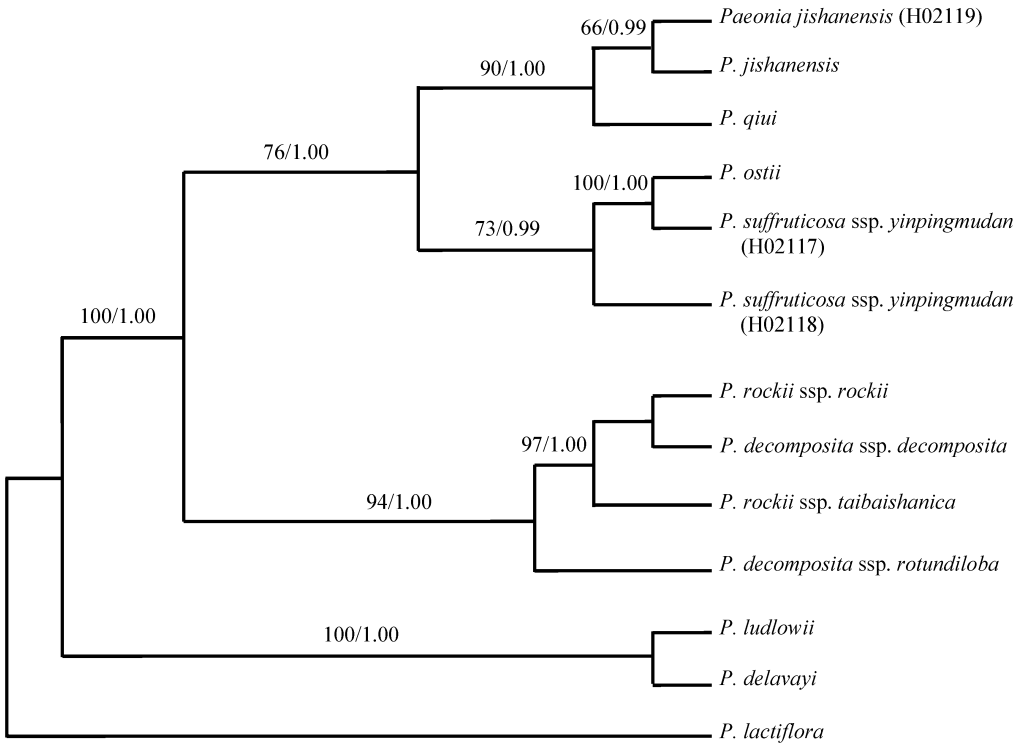

Fig. 2. Single MP tree based on *rps*16-*trn*Q, *trn*S-*trn*G, *Adh*1A, *Adh*2 and *GPAT* (Tree length = 613, *CI* = 0.8907, *RI* = 0.7220). The numbers near branches are bootstrap percentages followed by Bayesian posterior probabilities.

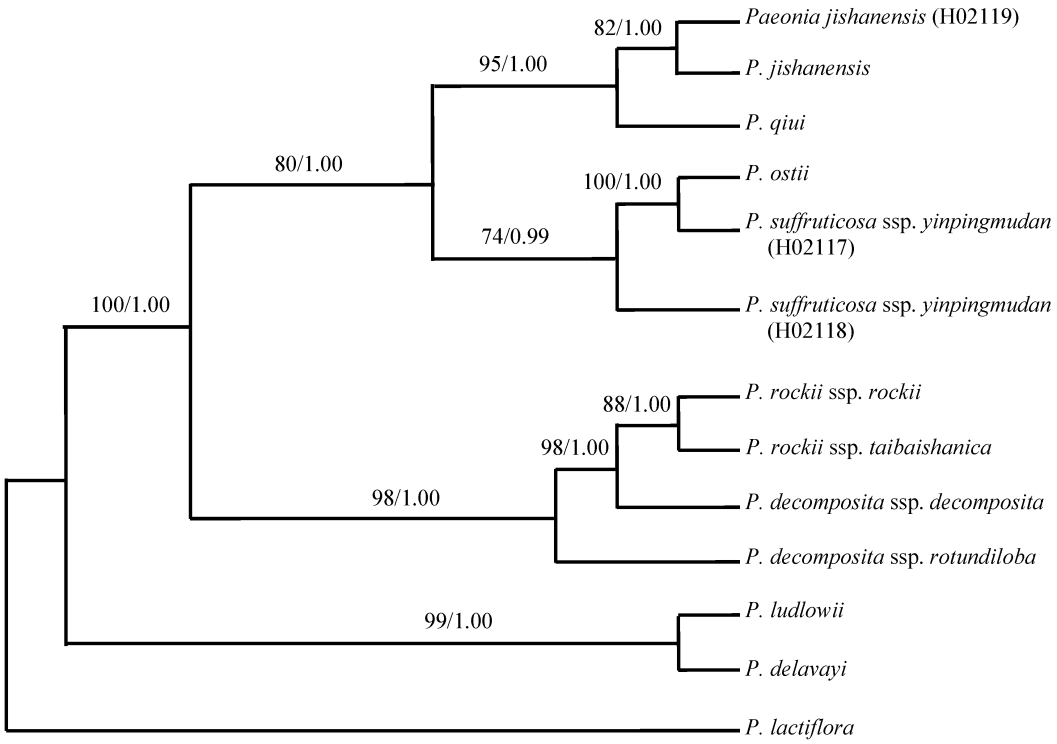

Fig. 3. One MP tree based on *rps*16-*trn*Q, *trn*S-*trn*G, *Adh*1A, *Adh*2, *GPAT* and morphological data (Tree length = 679, *CI* = 0.8616, *RI* = 0.6846). The numbers above branches are bootstrap percentages followed by Bayesian posterior probabilities.

3 Discussion

3.1 Phylogenetic relationships among species of *Paeonia* sect. *Moutan*

In previous studies, due to the incomplete sampling or limited informative characters (Zou et al., 1999; Yu et al., 1998; Tank & Sang, 2001), the phylogenetic relationships of *Paeonia* sect. *Moutan* is ambiguous. Recently, both morphological (Zhou et al., 2003) and molecular (Lin et al., 2004; Zhao et al., 2004) evidence was used to investigate the phylogenetic relationship of *Paeonia* sect. *Moutan* based on the new classification system proposed by Hong and Pan (1999a). These studies made some impressive advances, but interspecific relationship of *Paeonia* sect. *Moutan* is still not resolved because of the limited phylogenetic information (Sang et al., 1997a, b; Zhou et al., 2003; Lin et al., 2004; Zhao et al., 2004). For the first time, in this study, we use both multiple genes and morphological characters to reconstruct the phylogeny of *Paeonia* sect. *Moutan*.

There are eight wild species in *Paeonia* sect. *Moutan*, distributed in a wide range from Southwest to North China (Pan, 1995; Hong & Pan, 1999a). Within the eight species, *P. ludlowii* and *P. delavayi* are distributed in the west of Mt. Daxueshan, while other six species grow in the east of Mt. Daxueshan (Hong & Pan, 1999a). Based on the morphological characters, *P. ludlowii* is similar to *P. delavayi*, their flowers are terminal and axillary, disc fleshy and short. In contrast, the flowers of other six species are solitary, disc leathery (Hong, 1997b; Hong & Pan, 1999a). Recently, phylogenetic analyses suggested that *Paeonia* sect. *Moutan* is composed of two clades, one clade includes *P. ludlowii* and *P. delavayi*, another clade includes other six species (Zhou et al., 2003; Lin et al., 2004; Zhao et al., 2004). These results are correspondent to the treatment of two subsections by Stern (1946). In the present study, our results (Figs. 1–3) support the two-subsection treatment, namely, subsect. *Delavayanae* and subsect. *Vaginatae*.

The phylogenetic relationship of subsect. *Vaginatae* has been in dispute in the past several decades. After analyzing the morphological characters, Hong and Pan (1999b) treated five species as the complex *P. suffruticosa*, including *P. rockii*, *P. jishanensis*, *P. qiui*, *P. suffruticosa*, and *P. ostii*, due to the entirety disc leathery, whereas *P. decomposita* is distinguished from them by its partially disc leathery. Zhou et al. (2003) used twenty-five morphological characters to study the phylogenetic relationships of eight wild species of *Paeonia* sect. *Moutan*, and the result indicated that *P. decomposita* grouped with the complex *P. suffruticosa* and located at the basal position of this clade, thus supported the treatment of the complex *P. suffruticosa*. But in the recent DNA sequence analyses, Lin et al. (2004) and Zhao et al. (2004) found that *P. decomposita* was grouped with *P. rockii*, and other four species formed a clade with higher BS. Other previous studies based on molecular markers also got the similar results (Sang et al., 1997a, b; Sang & Zhang, 1999; Zou et al., 1999; Tank & Sang, 2001). In the present study, the combined analysis of molecular and morphological data supports that *P. decomposita* is grouped with *P. rockii*, and other four species formed another clade (Fig. 3). Therefore, we can conclude that *P. decomposita* is closely related to *P. rockii* comparing to other four species in subsect. *Vaginatae*.

Paeonia decomposita and *P. rockii* have two subspecies, respectively. In previous studies, the relationships among the four subspecies conflicted with each other (Hong et al., 1996; Zhou et al., 2003; Lin et al., 2004; Zhao et al., 2004). Our results suggest that *P. decomposita* ssp. *rotundiloba* was sister to *P. decomposita* ssp. *decomposita* and two subspecies of *P. rockii* (Figs. 1–3). Therefore, we can conclude that *P. decomposita* ssp. *decomposita* is closely related to *P. rockii*. Comparing the morphological characters, *P. decomposita* ssp. *rotundiloba* has 2–5 carpels, usually 3 or 4, while *P. decomposita* ssp. *decomposita* has a stable number (5) of carpel (Hong & Pan, 1999a). Therefore, both morphological and molecular evidence supports that *P. decomposita* ssp. *decomposita* is closely related to *P. rockii*.

In summary, *Paeonia* sect. *Moutan* can be divided into two subsections, the subsect. *Delavayanae* and subsect. *Vaginatae*. Subsection *Delavayanae* consists of two species, *P. delavayi* and *P. ludlowii*; subsect. *Vaginatae* includes other six species. Within the subsection *Vaginatae*, *P. rockii* is closely related to *P. decomposita*; while other four species form a clade, and *P. jishanensis*/*P. qiui* and *P. suffruticosa*/*P. ostii* were sister to each other.

3.2 The significance of the analysis based on combined data

The primary aim of phylogenetic analysis is to infer the relationship among species, namely species tree. As we know, gene tree derived from single gene is difficult to give a correct species tree for the limited information at lower taxonomic group (Doyle & Gaut, 2000). In addition, different data sets usually generate different phylogenetic relationships (Doyle & Gaut, 2000), such as among different molecular data sets or between molecular data set and morphological

character data. Therefore, using the combined genes data set to infer phylogenetic relationship has become a widely accepted trend (Hillis & Huelsenbeck, 1995; Wendel & Doyle, 1998; Ge et al., 1999; Cronn et al., 2002; Guo & Ge, 2005; James et al., 2006).

Up to now there are three commonly used methods to deal with the multiple data sets. Firstly, always combine the data (Kluge, 1989). In particular, all of the available taxa (e.g. fossil and living) should be combined in a phylogenetic analysis, as well as all of the available characters. Kluge (1989) believes the total evidence solution is sought because it maximizes the "informativeness" and "explanatory power" of the character data used in the analysis. Secondly, analyze different data set separatly (Miyamoto & Fitch, 1995). That means analyzing the different data independently. Thirdly, conditional data combination. Namely, partitions were subjected to a statistical test of "homogeneity": Heterogeneous data partitions are those that result in significantly different estimates about phylogeny (differences beyond that expected from sampling error) when analyzed separately (Farris et al., 1995). If the test result is non-significant, then the data can be combined. The three methods have their respective advantages, and the third method, conditional data combination, essentially was the intermediate between other two methods. The criterion of the PHT value does not have a common agreement and the theoretical basis of PHT is not mature (Cunningham, 1997); moreover, it is believed that it is legitimate to combine data sets although different sources of data may yield alternative phylogenetic results (Farris, 1997; Soltis et al., 1997). Therefore, in the present study, we combined the data and analyzed them together even some combined data sets have a lower value of partition-homogeneity test.

In this study, the information of single gene and combined chloroplast genes is too limited to clarify the phylogenetic relationship among all the species in *Paeonia* sect. *Moutan*. With the addition of separate data set to the combined data set, the number of trees reconstructed reduced to only single one, and some ambiguous clade was resolved clearly (Figs. 1–3). At the same time, the average support of BS and Bayesian posterior probability for each node is improved apparently. Consequently, based on the comparison of the phylogenetic relationship resulted from different combined data sets, it is apparent that the combined data sets of all partitions give the most robust systematic relationships among species of *Paeonia* sect. *Moutan*.

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基于多基因序列和形态性状的牡丹组种间关系

[1,2]赵 宣 [1,2,3]周志钦* [2]林启冰 [2]潘开玉 [1]李名扬

[1](西南大学园林园艺学院 重庆 400716)

[2](系统与进化植物学国家重点实验室, 中国科学院植物研究所 北京 100093)

[3](中国农业科学院柑桔研究所 重庆 400712)

摘要 牡丹被认为是中国的国花, 具有很高的医学、观赏和经济价值。野生牡丹被认为是栽培牡丹的野生祖先, 因此弄清牡丹组的种间亲缘关系具有重要的理论和实践意义。由于受到信息量的限制, 根据单基因数据或形态数据往往无法对牡丹组的种间关系得到明确的结果。本研究用12份样品代表野生牡丹组(*Paeonia* section *Moutan* DC., Paeoniaceae) 8个种, 利用包括核基因(*Adh*1A、*Adh*2和*GPAT*)和叶绿体基因(*trn*S-*trn*G和*rps*16-*trn*Q)的DNA序列以及形态性状的多套数据来探讨野生牡丹的种间关系。合并分析得到具高支持率的牡丹组物种间的系统发育关系。结果表明, 芍药属牡丹组8个野生种分为两个亚组, 即肉质花盘亚组subsect. *Delavayanae*和革质花盘亚组subsect. *Vaginatae*。肉质花盘亚组包括滇牡丹*P. delavayi*和大花黄牡丹*P. ludlowii*; 革质花盘亚组包括其余6个种。革质花盘亚组中, 四川牡丹*P. decomposita* ssp. *decomposita*和紫斑牡丹*P. rockii* ssp. *rockii*关系密切; 卵叶牡丹*P. qiui*和矮牡丹*P. jishanensis*关系密切; 银屏牡丹*P. suffruticosa* ssp. *yinpingmudan*与凤丹*P. ostii*关系密切, 并且后两个分支为姊妹群。

关键词 叶绿体基因; 形态性状; 核基因; 芍药属牡丹组; 系统发育