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MOLECULAR PHYLOGENETIC EVIDENCE FOR THE ORIGIN OF A DIPLOID HYBRID OF *PAEONIA* (PAEONIACEAE)[1]

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There is growing evidence that hybridization not only by means of allopolyploidy but also at the homoploidy level was a major driving force of plant diversification. While allopolyploidy is known to be a common mode of speciation in *Paeonia* (Paeoniaceae), hybrid speciation at the diploid level needs further evaluation. *Paeonia anomala* was previously considered to be an interspecific hybrid but with an unknown ploidy level. In this study *P. anomala* is identified as a diploid ($2n = 10$). With increased sampling of populations and molecular markers, we showed that *P. anomala* is a homoploid hybrid that originated from a cross between *P. veitchii* and *P. lactiflora*. Five populations of *P. anomala* were sequenced for the following molecular markers: the *matK* gene and two intergenic spacers, *psbA-trnH* and *rps16-trnQ*, of the chloroplast genome; the internal transcribed spacers (ITS) of nuclear ribosomal DNA; and three low-copy nuclear genes, *Adh1*, *Adh2*, and *Gpat*. The populations of *P. anomala* were grouped together with *P. veitchii* on the ITS and *Gpat* phylogenies but with *P. lactiflora* on the chloroplast phylogeny. Sequence polymorphism was found at the *Adh1* and *Adh2* loci within individuals of *P. anomala*. These polymorphic sequences were grouped with *P. veitchii* and *P. lactiflora*, respectively. Phenetic analysis indicated that *P. anomala* is morphologically similar to *P. veitchii*. Phenotypic evolution resulting from the combination of two diverged genomes might have occurred primarily at the physiological level and allowed *P. anomala* to adapt to geographic regions different from those of its parents.

Key words: fluorescent in situ hybridization; homoploid hybrid; meiosis; *Paeonia anomala*; phenetic analysis; phylogeny; speciation.

Recent genetic studies of homoploid hybridization in sunflowers have indicated that the combination of diverged genomes allowed hybrid species to establish in novel environments (Rieseberg et al., 2003). The breakthrough in the understanding of the genetic mechanisms of homoploid hybrid speciation encourages accelerated progress toward phylogenetic documentation of diploid hybrids. The theoretical and experimental challenges in reconstructing evolutionary histories of homoploid hybridization deserve greater attention from plant systematists.

Discerning between allopolyploidization and homoploid hybridization requires different strategies of phylogenetic analyses. Reconstruction of evolutionary origins of allopolyploids is relatively straightforward because diverged nuclear alleles from both parents are usually maintained in the hybrid genome as different loci. Molecular cloning and phylogenetic analyses of the parental alleles or homoeologous loci in the allopolyploids together with the genes from the extant diploid relatives have led to the reconstruction of allopolyploidy (Small et al., 1998; Cronn et al., 1999; Sang and Zhang, 1999).

Difficulties in reconstructing homoploid hybridization arise when one of the parental alleles becomes fixed at the majority of nuclear loci of the hybrid. The more ancient the hybrid species, the larger the number of loci at which the fixation could have occurred through genetic drift. Fixation can be accelerated by population bottlenecks and frequent inbreeding. As a result, a hybrid species forms a sister group with one of the parents on a nuclear gene phylogeny. The hope to reconstruct homoploid hybridization, especially an ancient one, often comes from the observation of incongruent positions of the hybrid between multiple gene phylogenies (Rieseberg and Soltis, 1991; Wendel and Doyle, 1998; Sang and Zhong, 2000).

Several challenges remain in this approach to reconstruct homoploid hybridization. First, the incongruence may be caused by factors other than hybridization, such as lineage sorting, especially when the parental species of the hybrid shared a short history of common ancestry (Doyle, 1997). Second, we still lack an adequate number of nuclear markers for most plant groups. The fewer the nuclear phylogenies, the weaker the statistical power to detect hybridization. Third, an asymmetric fixation of parental alleles, possibly as a result of a backcross with one of the parents, also reduces the chance of finding phylogenetic incongruence. These complicating factors continue to challenge our ability to reliably identify a homoploid hybrid and most likely lead to an underestimation of the frequency of this mode of speciation.

Here we report multiple lines of evidence supporting the origin of a diploid hybrid species of *Paeonia* L. *Paeonia anomala* L. (previously named *P. sinjiangensis* K. Y. Pan; Hong and Pan, 2004) was previously hypothesized to be a hybrid because of incongruence between the chloroplast DNA (cpDNA) and nuclear ribosomal DNA (nrDNA) phylogenies of *Paeonia* (Sang et al., 1997). This species, with an unknown ploidy level at the time, formed a sister group with diploid species *P. veitchii* Lynch on the nrDNA ITS phylogeny, but formed a sister group with *P. lactiflora* Pall. in the cpDNA *matK* phylogeny. The subsequent study using *Adh* gene sequences revealed that the individual of *P. anomala* had sequence polymorphism for both *Adh1* and *Adh2*, providing further support for a hybrid origin (Sang and Zhang, 1999). Because a

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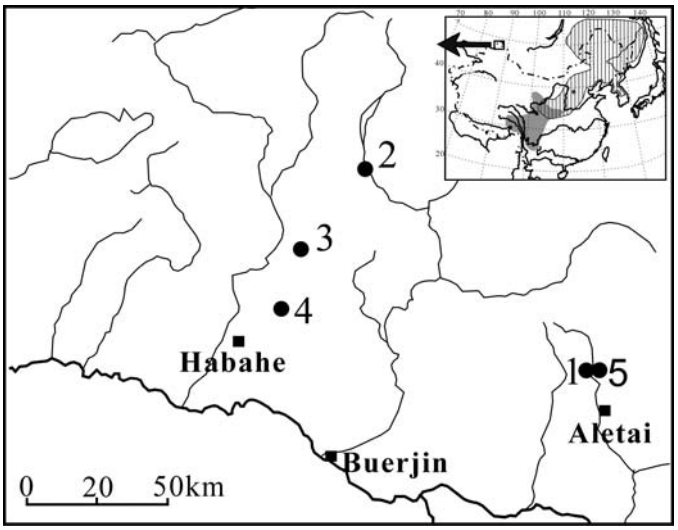


Fig. 1. Map of distribution and collection location. Numbers indicate the collection localities of the populations of *Paeonia anomala* in Table 1. Distribution of the putative parents, *P. veitchii* (shaded) and *P. lactiflora* (lined), is shown.

similar pattern of *Adh* sequence polymorphism has been found for several allotetraploid species of *Paeonia* (Sang and Zhang, 1999), we speculated that *P. anomala* was an allotetraploid.

To test the hypothesis, we conducted a field investigation of *P. anomala* in the Aletai area in northwestern China. To our surprise, chromosome counts indicated that this is a diploid species. We thus examined the molecular phylogenies of four gene markers studied previously, with a larger population sample. In addition, we sequenced two new cpDNA regions to improve the support of the cpDNA phylogeny and a new single-copy nuclear marker, the *Gpat* gene (Tank and Sang, 2001), to provide an independent assessment of the nuclear phylogenies. We also studied the morphology and cytogenetics of the hybrid and the closely related diploid species. The data were brought together to bear on questions concerning the origin, evolution, and phylogenetic reconstruction of a homoploid hybrid.

MATERIALS AND METHODS

Plant materials—First, note the recent nomenclatural changes for some of the *Paeonia* species studied here. *Paeonia sinjiangensis* has been renamed *P. anomala*, and *P. anomala* var. *intermedia* (C. A. Meyer) O. & B. Fedtsch. is now called *P. intermedia* C. A. Meyer (Hong and Pan, 2004). For morphological analysis, we focus on *P. anomala*, its putative parents, *P. veitchii* and *P. lactiflora*, and the closely related species *P. intermedia* found to be nearly sympatric with *P. anomala*. Characters representing morphological variations among these species were measured from specimens either collected by us or previously deposited in the Herbarium (PE), Institute of Botany, the Chinese Academy of Sciences, Beijing. These include 17 specimens of *P. intermedia*, 12 of *P. anomala*, 20 of *P. lactiflora*, and 22 of *P. veitchii* (Appendix).

For molecular phylogenetic analyses, we sampled 11 individuals from five populations of *P. anomala* (Fig. 1, Table 1). Additional samples for the closely related species included two populations of *P. intermedia*, one population of *P. lactiflora*, and one population of *P. veitchii* (Table 1). Three of the populations of *P. anomala* collected were used for cytogenetic study. Voucher specimens have been deposited in the Herbarium (PE), Institute of Botany, the Chinese Academy of Sciences, Beijing.

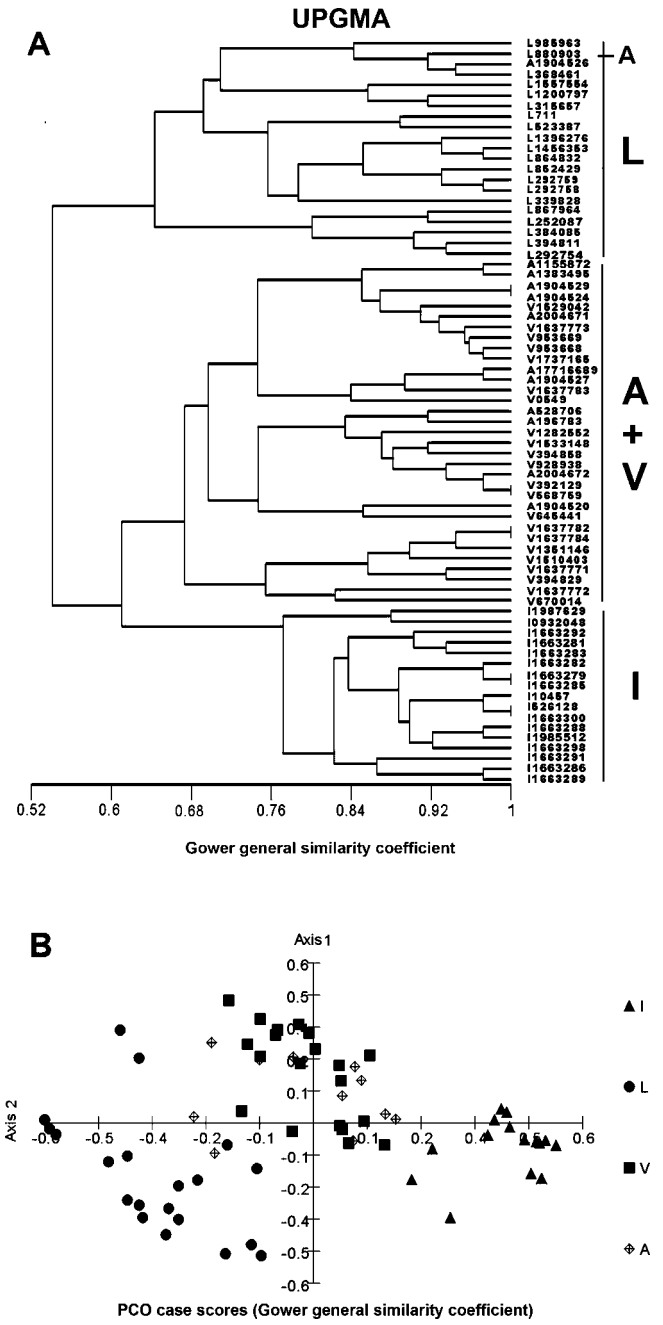


Fig. 2. Phenetic analysis of morphological characters. (A) Cluster analysis. Operational taxonomic units and characters used are described in Appendix. Abbreviations: L, *Paeonia lactiflora*; V, *P. veitchii*; A, *P. anomala*; I, *P. intermedia*. The scale at the bottom indicates the Gower general similarity coefficient. (B) Principal coordinate analysis. Axis 1 expresses 27.53% of the total variation, and axis 2 represents 15.94% of the total variation.

Morphological analysis—Nine morphological characters that were taxonomically most informative for *P. anomala* and the close relatives were chosen for the phenetic analysis (Appendix). The characters were ordered by recognizing intermediate states but were not assigned evolutionary directions. The Gower general similarity coefficient (Gower, 1971) was used in cluster analysis. Unweighted pair-group method using arithmetic average (UPGMA) analysis and principal coordinate analysis (PCoA) were performed with the

TABLE 1. Sources of *Paeonia* materials used in the present study. All vouchers are in PE (Herbarium, Institute of Botany, the Chinese Academy of Sciences, Beijing).

Species Population	No. samples	Sources (vouchers)	GenBank accession numbers						
			ITS	*Gpat*	*Adh1*	*Adh2*	*matK*	*psbA-trnH*	*rps16-trnQ*
P. anomala									
Population 1*	3 (A-B-C)	Dadonggou, Aletai, Xinjiang, China (XJ008, XJ010, XJ019)	DQ313692	DQ313738–40	DQ313703–07 DQ313721–22	DQ313774–76 DQ313787–91	DQ313753	DQ313731	DQ313806
Population 2*	2 (A-B)	Hanasi, Burerjin, Xinjiang, China (XJ020, XJ026)	DQ313694	DQ313736–37 DQ313741	DQ313708–10	DQ313777; DQ313786 DQ313792–95	DQ313754	DQ313732	DQ313807
Population 3	2 (A-B)	Baihaba, Habahe, Xinjiang, China (XJ032, XJ033)	DQ313695	DQ313745–47	DQ313711–13	DQ313778–80 DQ313796; DQ313803	DQ313755	DQ313733	DQ313808
Population 4*	2 (A-B)	Habahe Co., Xinjiang, China (XJ034, XJ037)	DQ313693	DQ313742–44	DQ313714–17	DQ313781–83 DQ313797–99	DQ313756	DQ313724	DQ313809
Population 5	2 (A-B)	Xiaodonggou, Aletai, Xinjiang, China (XJ040, XJ048)	DQ313696	DQ313748–49	DQ313718–20	DQ313784–85 DQ313800–02	DQ313757	DQ313735	DQ313810
P. intermedia									
Population 1	1 (A)	Habahe Co., Xinjiang, China (XJ044)	DQ313697	—	DQ313701–02	DQ313760–63	DQ313751	DQ313729	DQ313811
Population 2	2 (A-B)	Xiaodonggou, Aletai, Xinjiang, China (INT-XJ)	DQ313698	—	DQ313699–700	DQ313764–66	DQ313752	DQ313730	DQ313812
P. lactiflora									
Population 1	1 (A)	Daqing Mt., Huhehaote, China (H04037)	—	—	DQ313726–28	DQ313772–73	—	—	DQ313804
P. veitchii									
Population 1	1 (A)	Qiaojia Co., Yunnan, China (H04032)	DQ313691	—	DQ313723–24	DQ313767–71	DQ313758	—	DQ313805

Note: * Population used for meiotic observation.

program MVSP (version 3.13b, Kovach Computing Services, Anglesy, Wales, UK).

Molecular experiments—Total DNA was isolated from silica-gel-dried leaves using the CTAB method (Doyle and Doyle, 1987). Conditions for the polymerase chain reaction (PCR) were reported previously (Sang et al., 1995, 1997; Tank and Sang, 2001). PCR products were purified using a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Buckinghamshire, UK). PCR products of nuclear genes were cloned with the pGEM-T Easy System (Promega Corporation, Madison, Wisconsin, USA). At least 16 clones with correct insertion (determined by digestion with *Eco*RI) were screened through comparison of their sequences generated from one of the PCR primers. All distinct clones were sequenced in both directions. Sequencing was done in an ABI377 automated DNA sequencer using ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA) and on MegaBACE1000 automated DNA sequencer using DYEnamic ET Dye Terminator Sequencing Kit (Amersham Biosciences).

Phylogenetic analyses—DNA sequence alignments were done with CLUSTAL X (Thompson et al., 1997), followed by manual adjustment. Parsimony, as implemented in PAUP* version 4.0b10 (Swofford, 2002), was used to infer phylogenies based on nucleotide substitutions in aligned sequences. Section *Mutan* of *Paeonia* was chosen as the outgroup (Sang et al., 1997). Additional diploid species *P. japonica* (Makino) Miyabe & Takeda, *P. obovata* Maxim., and *P. tenuifolia* L. were included in analyses to encompass the diversity within section *Paeonia*.

Heuristic searches were performed with 1000 (cpDNA and ITS) or 100 (*Adh1*, *Adh2*, *Gpat*) random addition sequence replicates and the tree-bisection-reconnection (TBR) branch swapping and MULTREES option. Bootstrap analysis was carried out with 1000 replicates of heuristic search with TBR branch swapping, ACCTRAN optimization, and random taxon addition MaxTree was set at 500 for the *Adh1*, *Adh2*, and *Gpat* data sets).

Bayesian analyses for topology estimation were carried out using MrBayes version 2.0 (Huelsenbeck and Ronquist, 2001). Modeltest 3.06 (Posada and Crandall, 1998) was used to determine appropriate models of sequence evolution for all data sets (Table 2). One cold and three incrementally heated

TABLE 2. Phylogenetic information for DNA sequence data sets.

Data set	Total length	No. variable char.	No. informative char.	No. of trees	Length of trees	CI	RI	Appropriate model
cpDNA								
Combined data	2409	78	56	2	86	0.92	0.95	F81+G
matK	1452	35	24	1	36	0.97	0.98	F81+G
psbA-trnH	297	13	12	1	13	1.0	1.0	F81
rps16-trnQ	660	30	20	3	32	0.97	0.98	F81
Nuclear regions								
ITS	634	39	26	4	46	0.91	0.96	K80+G
Adh1	1208	256	97	500	339	0.81	0.85	HKY+G
Adh2	1197	402	213	500	587	0.76	0.88	HKY+G
Gpat	2746	530	272	500	634	0.89	0.95	HKY+G

Note: Consistency index (CI) excludes uninformative characters. Char. = characters, RI = retention index.

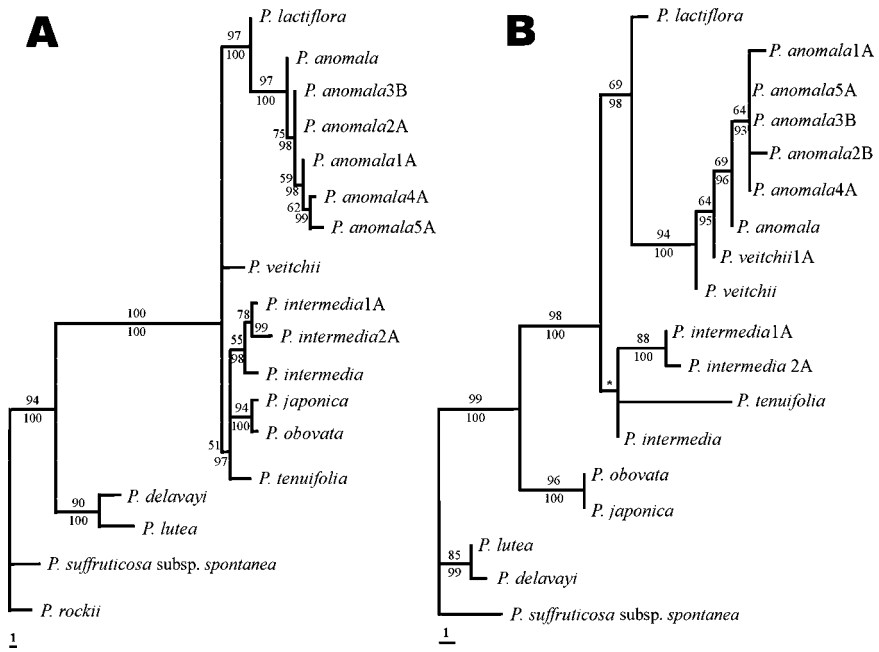


Fig. 3. The cpDNA and ITS phylogenies for *Paeonia anomala, P. veitchii, P. lactiflora, P. intermedia,* and the putative nonhybrid species of *Paeonia*. (A) cpDNA tree obtained from a combined analysis of *matK*, *rps16-trnQ,* and *psbA-trnH* sequences. (B) ITS tree. Branch lengths are proportional to number of nucleotide substitutions (scales represent one substitution). Numbers above branches represent bootstrap percentages greater than 50%; numbers below branches represent posterior probability multiplied by 100 and greater than 70. Asterisks denote clades that collapse in the strict consensus tree. See Tables 1 and 2 for accession and phylogenetic information.

Markov Chain Monte Carlo (mcmc) chains were run each for 1 100 000 generations and were sampled every 1000 generations. For all analyses, the first 300 samples from each run were discarded as burn-in to ensure the chains reached stationarity. Phylogenetic inferences were based on those trees sampled after generation 300 000.

The three cpDNA regions were combined for phylogenetic analysis. The potential recombination of low-copy nuclear genes was tested using the modified version of the program GENECONV version 1.81 (http://www.math.wustl.edu/~sawyer/geneconv; Sawyer, 1989). The global permutation *P* value was estimated using BLAST-like global scores obtained from 10 000 replicates. Recombination was considered to be present if $P < 0.05$. The possibility of gene conversion was also tested.

Cytogenetics—For meiotic studies, young floral buds were fixed in Carnoy's solution (absolute ethanol : acetic acid = 3 : 1) and then stored in 70% alcohol at 20°C. The samples for microscopic observation were prepared using squashing methods and stained with modified Carbol fuchsin (Hong et al., 1988). For each individual, more than two anthers were studied for a meiotic stage, and at least 100 cells were recorded. The micrographs were taken using a Leitz (Wetzlar, German) Orthoplan microscope with a 100× oil lens. All negatives were scanned into a computer with a Scan Wit 2720S (Acer, Taiwan, China) scanner at a resolution of 2700 dots per inch. The scanned images were processed by using Photoshop (version 6.0, Adobe, San Jose, California, USA). The procedures for fluorescent in situ hybridization (FISH) were described in Zhang and Sang (1999). The relative length of fragments (L_f, the absolute length of fragment divided by the absolute length of the long arm of chromosome 1) was calculated to estimate inverted segments in *P. anomala*.

TABLE 3. Maximum BLAST-like scores from analysis of three low-copy nuclear gene sequences with the program GENECONV version 1.81.

Gene	Fragment	Max score	Sim *P*-value	S.D.s above sim. mean
Gpat	Inner	4.526	0.3011	0.37
	Outer	3.744	0.0848	1.37
Adh1	Inner	4.361	0.1174	1.21
	Outer	3.367	0.1277	1.32
Adh2	Inner	8.147	0.0032	3.66
	Outer	6.649	0.0027	4.34

Note: Max score is Maximum BLAST-like score; Sim. *P*-value = Simulated *P*-value that is calculated from the simulation of 10 000 random permutations of the alignment. Recombination is detected when $P < 0.05$.

RESULTS

Morphology—In the UPGMA and PCoA analyses, the three diploid species, *P. intermedia*, *P. lactiflora*, and *P. veitchii*, were morphologically distinct, and *P. intermedia* and *P. veitchii* were more similar to each other than to *P. lactiflora* (Fig. 2A–B). All but one specimen of *P. anomala* was intermixed with those of *P. veitchii* in the results of both analyses.

Molecular phylogenies—Parsimony and Bayesian analyses yielded phylogenetic trees with very similar topologies for each

TABLE 4. Frequency of chromatid bridge and fragment abnormalities in *Paeonia anomala* during meiosis at anaphase.

Population: Sample	Frequency			
	No. pmc observed	Bridge + fragment (%)	Bridge only (%)	Fragment only (%)
1A: XJ012	104	7.69	4.81	8.65
2A: XJ021	629	1.75	1.75	2.55
2B: XJ027	441	6.8	6.58	4.77
4A: XJ034	412	25.24	8.5	16.02

Note: pmc = pollen mother cells.

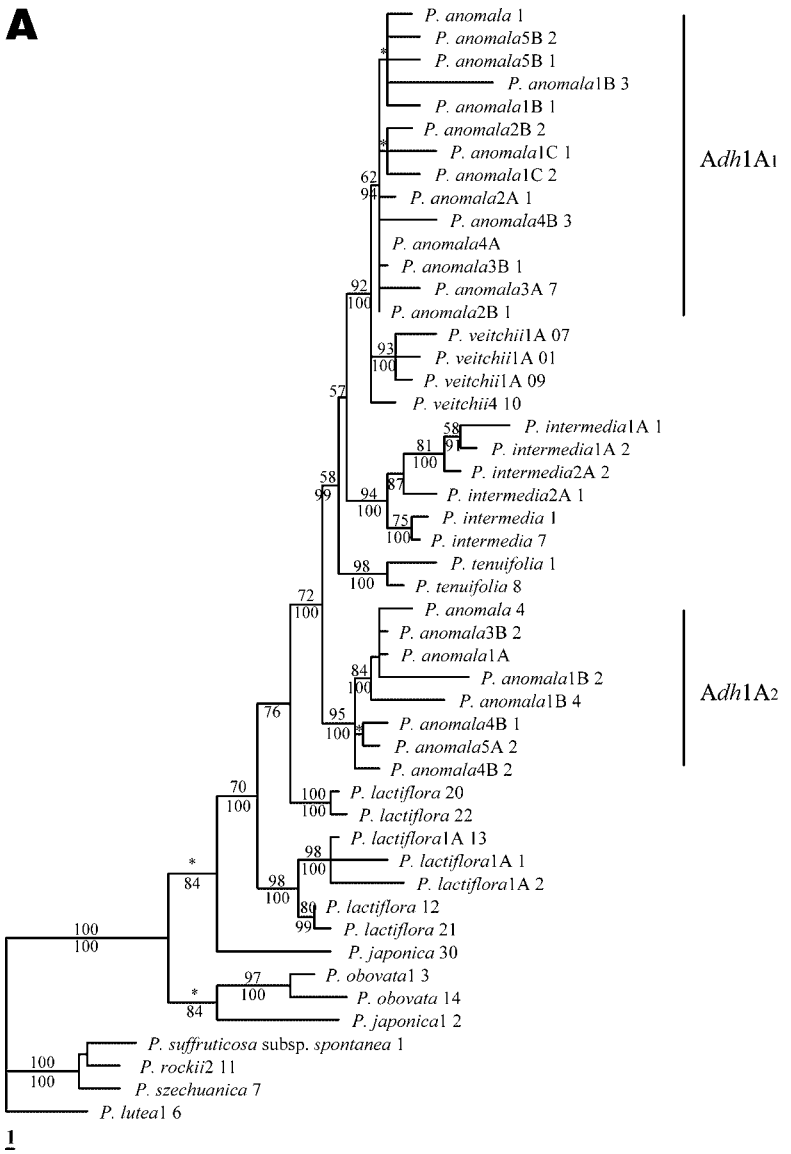


Fig. 4. Low-copy nuclear gene phylogenies for *Paeonia* taxa. (A) *Adh1* tree. (B) *Adh2* tree. (C) *Gpat* tree. Branch lengths are proportional to number of nucleotide substitutions (scales represent one substitution). Numbers above branches represent bootstrap percentages greater than 50%; numbers below branches represent posterior probability multiplied by 100 and greater than 70. Asterisks denote clades that collapse in the strict consensus tree. See Tables 1 and 2 for accession and phylogenetic information.

data set. The statistics for the phylogenetic analyses were summarized in Table 2. For each data set, a randomly chosen parsimonious tree was presented, with branches collapsed on the strict consensus indicated (Figs. 3–4). Bootstrap values greater than 50% and posterior probabilities greater than 0.70 were on the corresponding branches.

In the cpDNA and ITS phylogenies, all sampled populations of *P. anomala* formed a monophyletic group (Fig. 3). As found in our previous study, *P. anomala* grouped with *P. lactiflora* on the cpDNA tree but with *P. veitchii* on the ITS phylogenetic tree.

In the *Adh1* phylogeny, the cloned sequences of *P. anomala* formed two diverged clades, with one (*Adh1*A_1) being a sister group of *P. veitchii* and the other (*Adh1*A_2) closely related to *P. lactiflora* (Fig. 4A). Also, the clones on the *Adh1*A_2 clade had the same three indels of *P. lactiflora*. Four individuals had both types of sequences: one individual of population 1 (1B), one individual of population 3 (3B), one individual of population 4 (4B), and the individual studied previously. Two individuals from population 1 and 5 (1A and 5A) had only one type of *Adh1* sequence closely related to that of *P. lactiflora*. The remaining six individuals, 1C, 2A, 2B, 3A, 4A, and 5B, had only the *P. veitchii* type of sequence.

Two diverged types of *Adh2* sequences were also identified for *P. anomala*, with one forming a monophyletic group with *P. veitchii* and the other being closely related to *P. lactiflora* (Fig. 4B). All sampled individuals of *P. anomala* had these two types of *Adh2* sequences.

On the *Gpat* phylogeny, all individuals of *P. anomala* formed a strongly supported monophyletic group (Fig. 4C). It

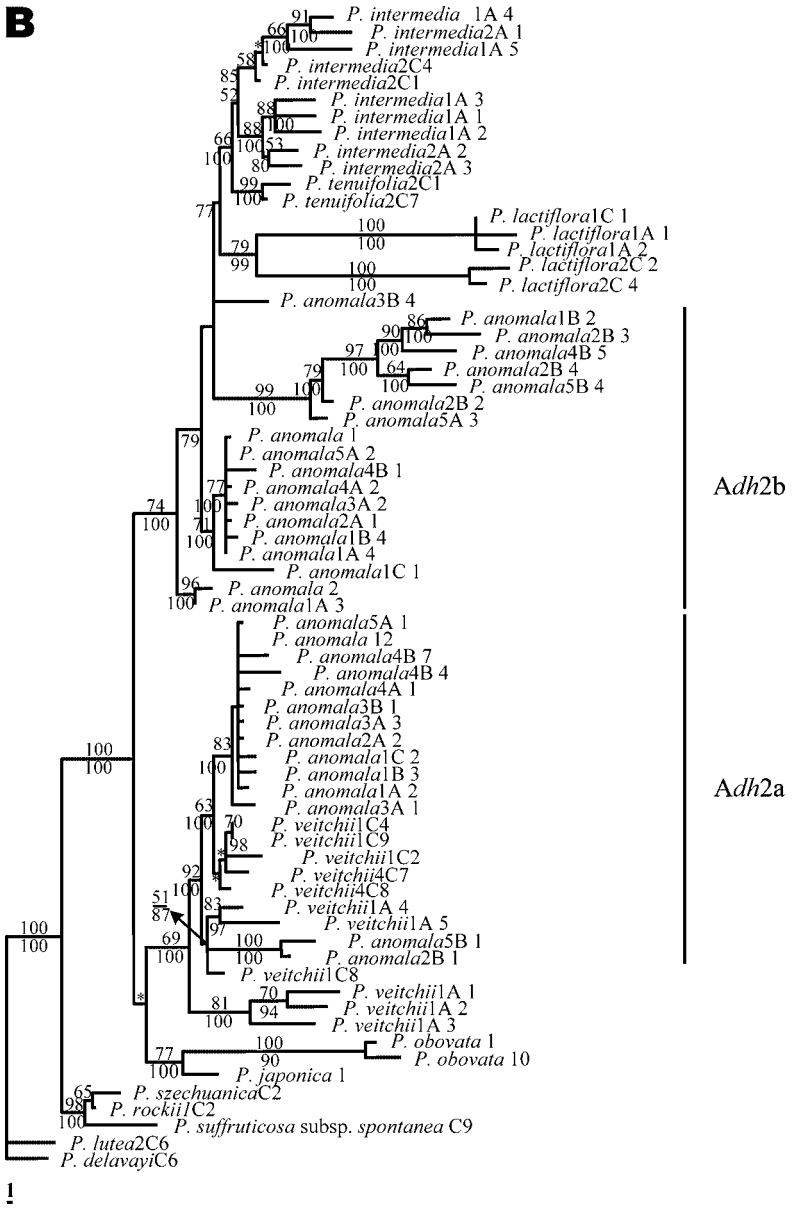


Fig. 4. Continued.

was then grouped with *P. veitchii* and *P. intermedia* in an unresolved trichotomy in the strict consensus, with bootstrap support of 90%.

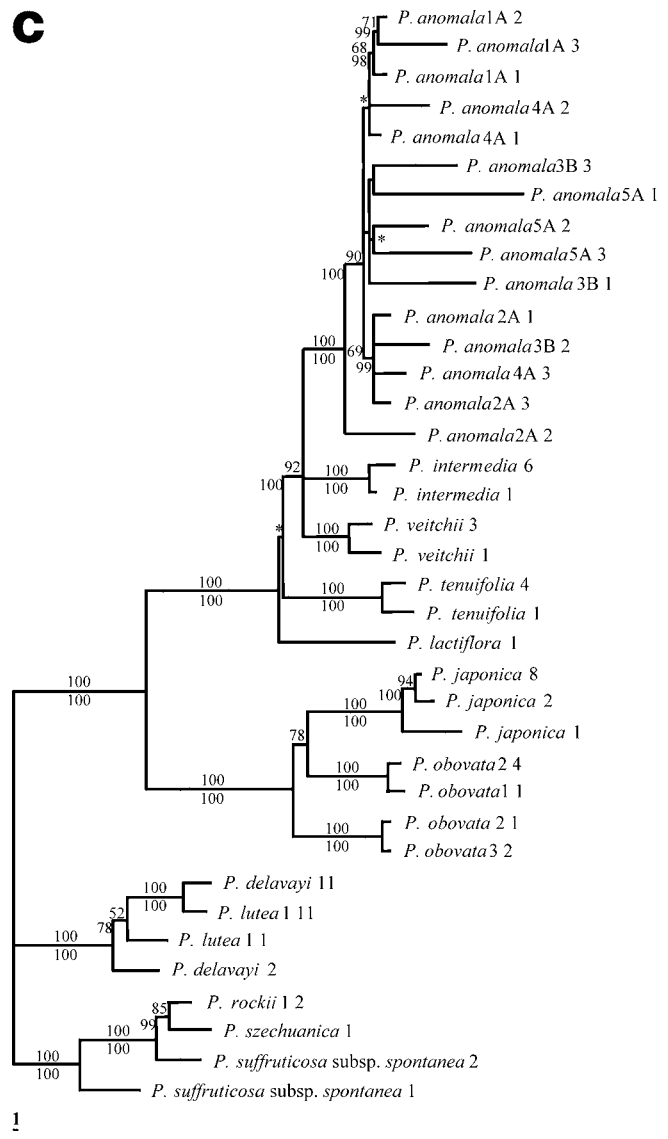
The global permutation *P* values (simulated *P* values) of three low-copy nuclear gene sequences of *P. anomala* were shown in Table 3. No recombination was detected for *Gpat* or *Adh1*, whereas it was detected for *Adh2*. The *Adh2* recombinants identified were only from *P. anomala*. These sequences were then excluded from the phylogenetic analysis.

Cytogenetic analyses—Cytogenetic observations revealed that *P. anomala* is a diploid species ($2n = 10$; Fig. 5). In situ hybridization located major 18S rDNA sites near the telomeres of eight of the 10 mitotic chromosomes.

From observations of various meiotic stages, we found abnormal chromosomal pairing at anaphase, including chromatid bridges and fragments, which occurred frequently in all sampled individuals. The number and frequency of the bridges and fragments are summarized in Table 4. Of four individuals observed, 2A had a relatively low frequency of abnormality (~6%) compared with those in the remaining samples (18%–50%, Table 4).

DISCUSSION

The hybrid origin of *P. anomala* was initially hypothesized on the basis of its incongruent position between nrDNA ITS and cpDNA *matK* phylogenies. Because only one individual of *P. anomala* was included in the study and the support for the sister relationship of *P. anomala* and *P. lactiflora* was relatively low (68% bootstrap) on the chloroplast *matK*



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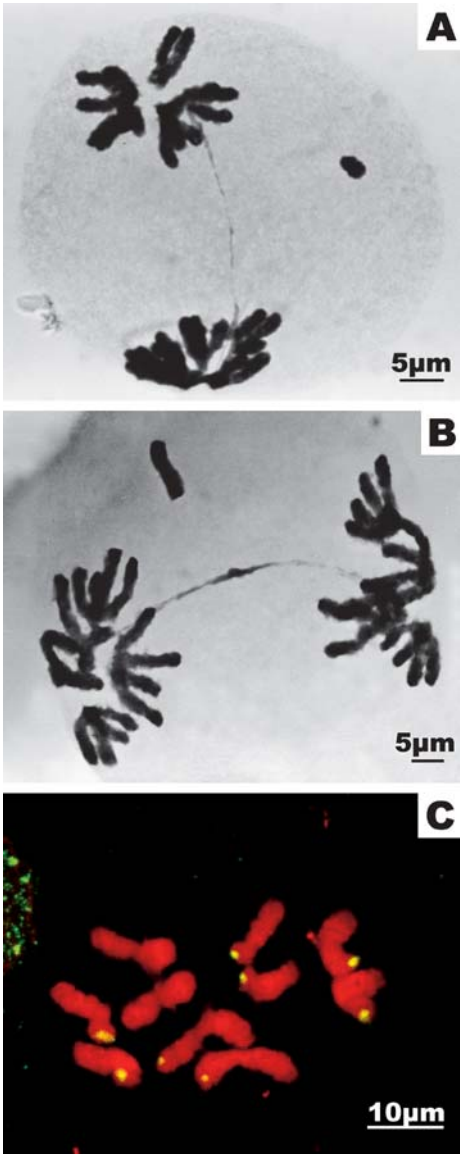

Fig. 5. Cytogenetic data and pollen viability of *Paeonia anomala*. (A–B) Chromatid bridge and fragment at anaphase I in XJ021 (bar = 5 μm). (C) Fluorescent in situ hybridization (FISH) localization of 18S ribosomal DNA (yellow-green) on anaphase I chromosomes of XJ021 (bar = 10 μm).

phylogeny, the immediate requirement for testing the hypothesis was to increase population sampling and obtain additional cpDNA sequences.

A field study was conducted in the Aletai area, and five additional populations of *P. anomala* were sampled. In the chloroplast genome, sequencing two additional fast-evolving regions yielded useful phylogenetic information. The fact that all populations of *P. anomala* formed monophyletic groups on the ITS and cpDNA phylogenies and the bootstrap value for the sister relationship between *P. anomala* and *P. lactiflora* increased to 97% in the cpDNA tree further supports the hypothesis of hybrid speciation. It is clear that *P. anomala* has a chloroplast genome more similar to that of *P. lactiflora* than to *P. veitchii*. Additionally, in situ hybridization showed that the number of 18S rDNA sites (eight) was also more similar to *P. lactiflora* (seven major and one minor site) than to *P. veitchii* (10 major sites) (Fig. 5C; Zhang and Sang, 1999).

The *Adh2* phylogeny provides stronger support for the hybrid origin of *P. anomala* than does the *Adh1* phylogeny. Each individual of *P. anomala* sampled in this study has two diverged *Adh2* sequences. One type of sequence forms a monophyletic group with *P. veitchii*, and the other is closely related to *P. lactiflora*, although it did not resolve into a monophyletic group. These two types of *Adh2* sequences likely represent two loci rather than two alleles of a locus because the chance of randomly sampling only heterozygous individuals from 13 individuals of five populations is very low ($P = 0.0001$, assuming half the individuals in the populations are heterozygous).

For *Adh1*, although two types of sequences have been cloned from *P. anomala*, they are not as highly diverged from each other as are the *Adh2* types and are found in fewer than half the sampled individuals of *P. anomala*. We cannot determine whether the two types represent two loci or two alleles of a

locus. In any case, however, the *Adh1* data are consistent with the hypothesis of the hybrid origin of *P. anomala* from diploid species closely related to *P. veitchii* and *P. lactiflora*.

The results here demonstrate how a diploid hybrid could maintain the sequence polymorphism derived from parental species. The polymorphic sequences may result from duplicated loci or diverged alleles from the parents at the time of hybridization. It is also possible that parental alleles ended up in the different chromosomal locations in a hybrid genome as a result of chromosomal rearrangement after homoploid hybridization. Chromosomal inversion heterozygotes, seen as the formation of bridges and fragments in meiosis, of *P. anomala* could provide the mechanism for the maintenance of the parental alleles (Fig. 5A, B).

A genetic analysis of nuclear gene sequences in an F_2 population between two individuals with known genotypes can determine whether the polymorphic sequences represent different loci or alleles at the same locus. We obtained a few F_1 seeds but have not developed F_2 populations because of the long generation time of peonies (3–4 yr from seed to flowering). In any event, our results indicate that while phylogenetic incongruence has provided the primary source of evidence for homoploid hybrid speciation, sequence polymorphism of single- or low-copy nuclear genes can potentially serve as another line of evidence.

Among four nuclear genes sampled from *P. anomala*, only the *P. veitchii* type of sequence was found for the nrDNA and *Gpat* genes, suggesting the *P. veitchii* alleles have been fixed at these loci. The fixation of the ITS sequence may have resulted from concerted evolution among nrDNA sites near telomeres of eight chromosomes consistent with the previous findings in allotetraploid species of *Paeonia* (Zhang and Sang, 1999).

It has been recently demonstrated that new adaptation could arise from homoploid hybridization through transgressive segregation (Rieseberg et al., 2003). The populations of *P. anomala* have so far been unambiguously identified in the Aletai area of northwestern China. The distribution of the species may extend farther north but certainly not south, given the careful documentation of *Paeonia* populations in those areas in China (Hong and Pan, 2004). The distribution of this species thus does not overlap with its parents, *P. veitchii* and *P. lactiflora*, which are found in southern and eastern regions (Fig. 1). The phenology of these regions is distinct, suggesting that a novel adaptation has most likely resulted from the hybridization.

The high level of morphological similarity between *P. anomala* and *P. veitchii* may be explained by a backcross with *P. veitchii* or by the dominance of the *P. veitchii* alleles for the morphological traits. The novel adaptive traits of *P. anomala* in comparison to *P. veitchii* may be primarily physiological rather than morphological. From the morphological point of view, it is reasonable or even more appealing to treat *P. veitchii* and *P. anomala* as conspecific taxa (Hong and Pan, 2004). The questions of how to classify hybrids and how to effectively reflect the corresponding phylogenetic, physiological, and ecological information in a practically useful classification still need to be addressed.

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APPENDIX. Data matrix for morphological characters for specimens of *Paeonia anomala, P. veitchii, P. lactiflora,* and *P. intermedia* deposited in the Herbarium (PE), Institute of Botany, the Chinese Academy of Sciences, Beijing under the numbers listed. Morphological characters: 1 = root type: carrot-shaped (0); fusiform to tuberous (1). 2 = leaf pubescence: glabrous on both sides (0); sparsely pubescent on one side (1); sparsely pubescent on both sides (2). 3 = leaflet width (cm): <0.5 (0); 0.5–1 (1); 1–1.5 (2); 1.5–2 (3); >2 (4). 4 = leaflet length (cm): <5 (0); 5–10 (1); >10 (2). 5 = no. flowers per shoot: 1 (0); 2 or more (1). 6 = no. undeveloped flower buds per shoot: 0 (0); 1 or more (1). 7 = no. bracts (surrounding sepals): 1 (0); 2 (1); 3 (2); 4 (3). 8 = no. non-caudate sepals: 0 (0); 1 (1); 2 (2); 3 (3); 4 (4). 9 = carpel pubescence: glabrous (0); tomentose (1).

Taxon Specimen	Character 1 2 3 4 5 6 7 8 9	Taxon Specimen	Character 1 2 3 4 5 6 7 8 9
P. intermedia		*P. veitchii*	
1663289	112100221	670014	010011011
1663298	111100041	645441	020001120
1663300	110000141	0549	021100210
1663283	121100131	568759	021101131
1663281	121100241	1737165	002200121
1663292	122100041	394829	011110011
1663286	111100221	928938	021101111
1663291	113200121	1011353	011100031
1985512	110100131	394858	021201101
0932048	121100230	392129	021101131
526128	110000141	1529042	002100121
1987629	111100140	1533148	022201121
10457	110000131	1510403	021110101
1663285	100100141	1351146	022210121
1663279	100100141	1282552	012101111
1663282	100100131	953668	002200111
1663288	111100131	1637771	011110121
P. lactiflora		953669	002200101
292754	024211340	1637773	001200111
339828	004201131	1637784	021210111
292758	004101130	1637772	021111111
315657	004210140	1637782	021210111
864832	004100120	1637783	021100301
292759	004101120	*P. anomala*	
384085	024211120	2004671	002200021
252087	024211131	2004672	022101131
1200797	004210110	1383495	011200141
867964	024211101	196783	012201211
368461	024200120	1155872	011200131
523387	014111230	1904520	022101220
394811	024211230	528706	023201211
711	014101230	1904526	023200110
880903	023100120	1904524	012200211
1396276	004200130	1904529	014200211
1557554	004210300	1904527	022100221
852429	004201130	17716689	022100211
1456353	014100130		
985963	024210121		