Evolution of Alcohol Dehydrogenase Genes in Peonies (Paeonia): Phylogenetic Relationships of Putative Nonhybrid Species

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Alcohol dehydrogenase genes were amplified by PCR, cloned, and sequenced from 11 putative nonhybrid species of the angiosperm genus Paeonia. Sequences of five exons and six intron regions of the Adh gene were used to reconstruct the phylogeny of these species. Two paralogous genes, Adh1A and Adh2, were found; an additional gene, Adh1B, is also present in section Moutan. Phylogenetic analyses of exon sequences of the Adh genes of Paeonia and a variety of other angiosperms imply that duplication of Adh1 and Adh2 occurred prior to the divergence of Paeonia species and was followed by a duplication resulting in Adh1A and Adh1B. Concerted evolution appears to be absent between these paralogous loci. Phylogenetic analysis of only the Paeonia Adh exon sequences, positioning the root of the tree between the paralogous genes Adh1 and Adh2, suggests that the first evolutionary split within the genus occurred between the shrubby section Moutan and the other two herbaceous sections Oneupia and Paeonia. Restriction of Adh1B genes to section Moutan may have resulted from deletion of Adh1B from the common ancestor of sections Oneupia and Paeonia. A relative-rate test was designed to compare rates of molecular change among lineages based on the divergence of paralogous genes, and the results indicate a slower rate of evolution within the shrubby section Moutan than in section Oneupia. This may be responsible for the relatively long branch length of section Oneupia and the short branch length between section Moutan and the other two sections found on the Adh, ITS (nrDNA), and matK (cpDNA) phylogenies of the genus. Adh1 and Adh2 intron sequences cannot be aligned, and we therefore carried out separate analyses of Adh1A and Adh2 genes using exon and intron sequences together. The Templeton test suggested that there is not significant incongruence among Adh1A, ITS, and matK data sets, but that these three data sets conflict significantly with Adh2 sequence data. A combined analysis of Adh1A, ITS, and matK sequences produced a tree that is better resolved than that of any individual gene, and congruent with morphology and the results of artificial hybridization. It is therefore considered to be the current best estimate of the species phylogeny. Paraphyly of section Paeonia in the Adh2 gene tree may be caused by longer coalescence times and random sorting of ancestral alleles.

Introduction

Low-copy-number nuclear genes in plants (Okamura and Goldberg 1989) are a potentially rich source of information for phylogenetic studies. However, the phylogenetic utility of such genes remains underexplored due largely to the difficulties of distinguishing orthology from paralogy and detecting concerted evolution among members of a gene family (Sanderson and Doyle 1992). Chloroplast DNA (cpDNA) and nuclear ribosomal DNA (nrDNA) are the only widely used sequence data for phylogeny reconstruction in plants. Limited sources of independent gene phylogenies, however, may hamper our ability to obtain accurate species phylogenies when hybridization, lineage sorting, lateral gene transfer, or high homoplasy are involved. Conflicting cpDNA and nrDNA phylogenies have been reported in some plant groups (Solitís and Kuzoff 1995; Mason-Gamer and Kellogg 1996; Solitís, Johnson, and Looney 1996; Sang, Crawford, and Stuessy 1997) and in such cases it will be critical to obtain additional independent gene phylogenies and compare and combine them for stronger hypotheses of the species phylogenies (Hillis 1995).

In the present study, we cloned and sequenced alcohol dehydrogenase (Adh) genes in the angiosperm genus Paeonia (Paeoniaceae) to investigate the phylogenetic utility of a low-copy nuclear gene family at the interspecific level in plants and to better understand the evolution of peonies. Adh genes are among the best characterized nuclear protein-coding genes in plants. In the majority of flowering plants that have been studied, two to three Adh loci have been identified, each containing 10 exons and 9 introns (Gottlieb 1982; Dennis et al. 1985; Llewellyn et al. 1987; Trick et al. 1988; Wolyn and Jelenkovis 1990; Morton, Gaut, and Clegg 1996). In Arabidopsis and Arabis (Brassicaceae), however, a single Adh locus is present, which consists of seven exons and six introns (Chang and Meyerowitz 1986; Miyashita Innan, and Terauchi 1996). In Paeonia californica, two Adh loci were detected by enzyme electrophoresis (Zona et al. 1991).

Phylogenetic uses of Adh genes have been concerned with both high and low taxonomic levels. Sequences of amino acids derived from Adh gene sequences of vertebrates and plants were analyzed together using yeasts as outgroups (Yokoyama and Harry 1993). Sequences of Adh genes, including both exons and introns, have been used to resolve phylogenetic relationships within Droserophila (Jeffs, Holmes, and Ashburner 1994; Russo, Takezaki, and Nei 1995; Nurminsky et al. 1996). In plants, phylogenies of Adh genes and divergence rates were inferred based on exon sequences of representative grasses and palms (Gaut et al. 1996; Morton, Gaut, and Clegg 1996).

Key words: alcohol dehydrogenase, Adh, Paeonia, phylogeny, gene duplication, relative-rate test.

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Table 1  
Taxonomy and Sample Localities Paeonia Species Included in this Study, the Number of Clones Screened, and the Numbers of Adh1A, Adh1B, Adh2, and Recombined Clones Obtained

<table>
<thead>
<tr>
<th>Species</th>
<th>Sample Localities</th>
<th>Abbreviation</th>
<th>No. Screened</th>
<th>Adh1A</th>
<th>Adh1B</th>
<th>Adh2</th>
<th>Recombined</th>
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<tr>
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<td>P. szechuanica</td>
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<tr>
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<td>4</td>
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<td>ANO</td>
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<td>9a</td>
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<td>19</td>
<td>8a</td>
<td>6a</td>
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<tr>
<td>P. tenuifolia</td>
<td>Sofia, Bulgaria</td>
<td>TEN</td>
<td>11</td>
<td>7</td>
<td></td>
<td>4a</td>
<td></td>
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<tr>
<td>P. veitchii</td>
<td>Mt. Taibei, Shaanxi, China</td>
<td>VE11</td>
<td>6</td>
<td></td>
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<td>7</td>
<td>2</td>
<td></td>
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</tbody>
</table>

a Clones obtained from two PCR reactions.
b Clones of PCR products of the Adh2-specific primers (fig. 1).

Paeonia consists of approximately 35 species placed in three sections, Moutan, Oneapia, and Paonia (Stern 1946; Pan 1979; Tzanoudakis 1983). Section Moutan contains five diploid shrubby species occurring in central and western China. Section Oneapia contains two diploid perennial herbaceous species endemic to Pacific North America. Section Paonia comprises approximately 28 diploid and tetraploid herbaceous species found in eastern Asia, central Asia, the western Himalayas, and the Mediterranean region. The genus has been placed in its own family, Paeoniaceae, and often in its own order, Paeoniales (Takhtajan 1969, 1987; Thorne 1992); its broader relationships within angiosperms have been controversial (Keefe and Moseley 1978; Melville 1983; Cronquist 1988), although it appears to be related to Crassulaceae based on rbcL and 18S ribosomal DNA sequences (Chase et al. 1993; Rice, Donoghue, and Olmstead 1997; Soltis et al. 1997). Recent studies using sequences of the internal transcribed spacer (ITS) region of nrDNA and the matK gene of cpDNA revealed complex reticulated evolution within section Paonia (Sang, Crawford, and Stuessy 1995, 1997).

Here we focus on reconstructing Adh gene trees for all of the putative nonhybrid species (Sang, Crawford, and Stuessy 1997). We have identified orthologous and paralogous Adh genes based on sequence divergence and phylogenetic analyses. Sequence divergence has also helped us determine whether concerted evolution of paralogous genes has occurred. We have taken advantage of Adh gene duplications for rooting purposes and to test relative rates of molecular divergence among peony lineages. Exon and intron sequences of the orthologous genes are analyzed together, and the resulting gene trees are compared with previous nrDNA and cpDNA phylogenies. A combined analysis of Adh, ITS, and matK sequences was conducted to estimate the species phylogeny.

Materials and Methods  
Amplification, Cloning, and Sequencing  
Total DNAs were isolated from leaf tissue using the CTAB method (Doyle and Doyle 1987) and purified in CsCl/ethidium bromide gradients. Leaves of most of the Paeonia species included in this study were collected from natural populations in Bulgaria, China, and the United States (table 1).

Two PCR primers, AdhF1 and AdhR1, were designed for maximal coverage of Adh genes using regions conserved across the eudicot families Brassicaceae, Fabaceae, Solanaceae, and Rosaceae (Chang and Meyerowitz 1986; Llewellyn et al. 1987; Ellison, Yu, and White 1990; Wolyn and Jelenkovis 1990). These primers amplify a large portion of the peony Adh genes (fig. 1). The PCR reactions were carried out under standard conditions with Taq DNA polymerase (Gibco BRL Life Technologies) on a GeneAmp PCR system 9600 (Perkin Elmer). The PCR reactions include the following cycles: (1) 94°C, 2 min; (2–5) 94°C, 45 s—55°C, 1.5 min—72°C, 1 min; (6–36) 94°C, 30 s—55°C, 40 s—72°C, 1 min; (37) 72°C, 5 min. The PCR products were examined with 1% agarose gel in TBE buffer.

PCR reactions using primers AdhF1 and AdhR1 produced a single band of approximately 1.4 kb for the peony DNAs that we were able to amplify (including some hybrid species). Direct sequencing of purified PCR products with either of these primers produced only about 200 bp of readable sequence. Based on sequences obtained from several species, two new primers, AdhF2
and AdhR2, were designed in the conserved regions and led to approximately 500 bp of readable sequence (fig. 1). When AdhF2 and AdhR2 were used as PCR primers, a single band was obtained which was much stronger than that obtained for the same species using primers AdhF1 and AdhR1. AdhF2 and AdhR2 also amplified the species that we could not amplify with AdhF1 and AdhR1. The results suggest that AdhF2 and AdhR2 match Adh sequences of peonies better than the first set of primers, and we used PCR products amplified with these two primers for cloning and subsequent sequencing.

PCR products were ligated with plasmids and transformed into E. coli competent cells, which were selected on the plates containing ampicillin and X-Gal (Original TA Cloning Kit, Invitrogen). Ten to 15 white E. coli clones were picked and cultured for isolating plasmids. Purified plasmid DNAs were digested with EcoRI and amplified with primers AdhF2 and AdhR2 to check whether they contain the correct inserts. The plasmids with correct inserts were sequenced by at least one primer, usually AdhR2 (reading three introns), to screen variation among clones. Clones with sequences determined to be different were sequenced for both strands in their entirety and included in the phylogenetic analyses. The locations and sequences of the PCR and sequencing primers used in this study are given in figure 1 (also see Results). Sequencing was conducted using ABI370A and ABI373A automated DNA sequencers with the Tag Cycle Sequencing DyeDeoxy Terminator reagents (Applied Biosystems). Sequences were edited with the program SeqEd and aligned manually. Sequences obtained in this study have been assigned GenBank accession numbers AF009041–AF009068.

Phylogenetic Analyses

Maximum parsimony, as implemented in PAUP 3.1.1 (Swofford 1993), was used to infer relationships based on nucleotide substitutions in aligned Adh sequences. Heuristic searches were performed using TBR branch swapping MULPAS on 100 starting trees derived using the random option in PAUP. Branch-and-bound was employed when there were less than 18 sequences in an analysis. ACCTRAN option was used for character optimization. Bootstrap analyses (Felsenstein 1985) were carried out with 500 replicates, using simple taxon addition. Nucleotide substitutions were weighted equally, and gaps were treated as missing information.

Analysis I

Sequences of five Adh exons of peony species and other angiosperms were aligned (without gaps) and analyzed using monotoc sequences for rooting purposes. Adh sequences of the following taxa were obtained from GenBank: Arabidopsis thaliana (M12196), cotton (Gossypium hirsutum; U49061), apple (Malus domestica; Z48234), strawberry (Fragaria ananassa; X15588), tobacco (Nicotiana tabacum; X81853), petunia (Petunia hybrida; X54106), tepary bean (Phaseolus acutifolius; Z23171), pea (Pisum sativum; X06281), white clover (Trifolium repens; X14826), maize (Zea mays; Adh1, X04049; Adh2, X02915), and barley (Hordeum vulgare; Adh1, X12732; Adh2, X12733; Adh3, X12734).

Analysis II

In previous phylogenetic analyses of Paeonia using ITS and cpDNA sequences (Sang, Crawford, and Stuessy 1995, 1997), gene trees were rooted along the longest branch, thereby separating section Oneapia from the other two sections. This approach was necessitated by the great distance of any potential outgroups. Analysis I (above), which included distantly related outgroups, did not help resolve the root within Paeonia (see below). Nevertheless, it did suggest that the duplication giving rise to the Adh1 and Adh2 genes occurred before the diversification of Paeonia. Analysis II was designed to include only Paeonia Adh genes, with trees rooted between the two paralogous genes (see Discussion for references on this approach). Since introns could not be aligned between the two paralogous genes, only exon sequences were analyzed.

Analysis III

In order to obtain better resolution of interspecific relationships, both exons and introns of the orthologous genes, Adh1 and Adh2, were analyzed separately.

![Diagram of the Adh gene in peonies](image)

**Fig. 1.**—Diagram of the Adh gene in peonies. Shaded boxes represent the exons amplified and sequenced, with sizes indicated above by the number of base pairs; open boxes represent the remaining exons of Adh genes as documented in other eudicots. The line below the gene represents the region sequenced and used in phylogenetic analyses. Arrows indicate the locations and directions of the PCR and sequencing primers: AdhF1, 5'-TACTCTTGGAAGCYYAGC-3'; AdhF2, 5'-CCTCGCATATTTGTCACAGAAG-3'; AdhF3, 5'-TCAAGGATTATTGGTGTTGA-3'; AdhF4, 5'-TCACACCAATTTCCTTG-3'; AdhR1, 5'-CCCTTTGCTCTCATCCTC-3'; AdhR2, 5'-GGGACACCACAAGTTACTG-3'; AdhR3, 5'-CTGTCACACCAAATTTCCCTTG-3'; AdhR4, 5'-TCAACAATATAATCCCTTG-3'; AdhR5, 5'-AGAACCACACAACTTATCAAG-3'; AdhR6, 5'-CATCCTCTGCAAAAGATA-3'; AdhR7, 5'-CATCCTCTGCAACAAAGATA-3'; AdhR1, 5'-CATCCTCTGCAACAAAGATA-3'; AdhR2, 5'-GAATTAGAACAACCTCGT-3'; AdhR3, 5'-TGGGTTCCTGGATTTG-3'; AdhR4, 5'-CATCCTCTGCAACATCATG-3'. AdhR1 is an Adh1-gene-specific primer. AdhBF and AdhBR are Adh1B-gene-specific primers. AdhF2 and AdhR2 are Adh2-gene-specific primers.
on the results of Analysis II and additional information on rates of evolution (see below) the trees were rooted between section Moutan and the other two sections. Several pseudogenes, identified based on deletions in the exons, were also included in these analyses.

**Templeton Test**

Incongruence among Adh1A, Adh2, ITS, and matK data sets was tested using the Templeton test (Templeton 1983; Larson 1994). This test was chosen because it can identify potentially conflicting individual nodes among trees while entire data sets are compared (Mason-Gamer and Kellogg 1996). Only the 11 species included in this study were retained in ITS and matK data sets (Sang, Crawford, and Stuessy 1997), and Adh1A and Adh2 data sets were reduced by randomly choosing a single clone representing a species in each data set.

**Combined Analysis**

Since the Templeton test implies a significant incongruence between the Adh2 data set and each of the other data sets but not among Adh1A, ITS, and matK, a combined analysis of these three data sets was conducted to better estimate the species phylogeny (de Queiroz, Donoghue, and Kim 1995; Huelsenbeck, Bull, and Cunningham 1996). However, we also conducted a combined analysis all four data sets, Adh1A, Adh2, ITS, and matK.

**Comparisons of Sequence Divergence**

Sequence divergences for all nucleotides, for synonymous sites, and for nonsynonymous sites were estimated using Jukes-Cantor corrections (Jukes and Cantor 1969) as calculated by MEGA 1.02 (Kumar, Tamura, and Nei 1993). Pseudogenes were not included in comparisons of sequence divergence.

When concerted evolution occurs between paralogous genes, it is expected that sequence divergence between paralogues will be significantly lower within than among taxa (Dover 1982; Arthheim 1983; Hughes 1995). Concerned evolution between the paralogous genes of *Paeania* was examined (1) by comparing average sequence divergence within species and among species within each section, and (2) by comparing average sequence divergence within each section and among the three sections.

We have made use of gene duplication to test relative rates of molecular evolution (fig. 2). If a duplication resulting in two paralogous genes (1 and 2) occurred prior to the divergence of two taxa (A and B) (fig. 2A), sequence divergence between genes A1 and A2 ($d_{A1,A2}$) should be equal to the divergence between B1 and B2 ($d_{B1,B2}$)---that is, $d_{A1,A2} - d_{B1,B2} = 0$---if molecular evolution occurred at the same rate in taxa A and B and concerted evolution between paralogous genes 1 and 2 was absent. The variance of ($d_{A1,A2} - d_{B1,B2}$) is equal to the variance of ($d_{A1,A2}$) plus the variance of ($d_{B1,B2}$) minus twice the covariance of ($d_{A1,A2}$, $d_{B1,B2}$). The covariance of ($d_{A1,A2}$, $d_{B1,B2}$) equals the variance of $x$, where $x$ is the estimated length of the interior branch on the unrooted tree for the four genes (fig. 2B), which equals half of $d_{A1,A2} + d_{B1,B2} - d_{A1,B1} - d_{A2,B2}$ (Wu and Li 1985; M. Sanderson, personal communication). The variance of $x$ equals $P(1 - P)[L(1 - P/0.75)^2]$, where $P = 0.75(1 - e^{-0.75})$ and $L$ is the number of sites compared (Li and Tanimura 1987).

**Results**

**Adh Genes in Paeonia**

The number of clones screened and the number of clones found for each paralogous Adh gene in each species are shown in table 1. Two types of sequences, which we have called Adh1 and Adh2, were discovered in peony species: the introns of these two forms are highly diverged and could not be aligned. After the first round of cloning and screening, only Adh1 clones were obtained from *P. anomala* or *P. tenuifolia*, and only Adh2 clones were found in *P. brownii*. Adh1- and Adh2-specific primers were then designed based on the obtained sequences in the intron regions of the other species to amplify these three species (fig. 1). Adh2 genes were cloned successfully from *P. anomala* and *P. tenuifolia* using the Adh2-specific primers (Adh2F and Adh2R). Amplification of the Adh1 gene of *P. brownii* using the Adh1-specific primers (Adh1R) with AdhF2 still failed. This was probably due to poor quality of the template DNA; the DNA of *P. brownii* was isolated from dry specimens, while fresh leaves were used for the rest of the species.

Two types of Adh1 genes were cloned from the species of the shrubby section Moutan. The type that is also found in the rest of genus is designated as Adh1A, and the type that is limited to section Moutan is called Adh1B. To better establish the distribution of the Adh1B...
gene in peonies, a pair of Adh1B-specific primers, Adh1BF and Adh1BR (fig. 1), were designed and used in PCR and DNAs of all species included in the study. The Adh1B gene was amplified for all the studied individuals belonging to section Moutan, but from none of the species belonging to the other two sections. The Adh1B locus, therefore, appears to be present only in section Moutan. Both Adh1A and Adh1B genes were cloned from the same individual of P. suffruticosa subsp. spontanea (table 1). The absence of Adh1A clones for DEL and LUT2 and of Adh1B clones for LUT1, ROC2, and SZE is most likely due to screening an insufficient number of clones. An exhaustive search for these genes was not carried out, because it does not appear to be critical for understanding paralogy and orthology of Adh genes or interspecific relationships of the peony species in this study.

Four clones, two from P. delavayi, and one each from P. rockii and P. veitchii, have combined sequences of Adh1 and Adh2 genes. To determine whether these resulted from amplification of genomic interlocus re-combinations, a combination of Adh1- and Adh2-specific primers, Adh2F and Adh1R, was used to amplify the genomic DNAs of these three species. None of the PCR reactions yielded any bands, implying that the recombined sequences we obtained were an artifact of particular PCR reactions (Bradley and Hillis 1997). We, therefore, urge caution in interpreting sequences of PCR products of low-copy-number nuclear genes.

Adh2 pseudogenes were cloned from P. delavayi, P. lactiflora, and P. suffruticosa subsp. spontanea. The pseudogenes found in P. delavayi and P. lactiflora have a 1-bp deletion in the 324-bp exon (at different sites). A 2-bp deletion in this exon is found in the pseudogene of P. suffruticosa subsp. spontanea. These deletions resulted in the stop codons in the exon. Both the pseudogene and the normal Adh2 gene were cloned from an individual of P. delavayi and an individual of P. suffruticosa subsp. spontanea. Only the pseudogene was found in P. lactiflora after screening 11 Adh2 clones, which implies that the normal Adh2 gene is either absent from this individual plant or could not be amplified for some unknown reasons.

Phylogenetic Analyses

Analysis I, including exon sequences of all peony Adh clones and other angiosperm Adh genes, generated 181 most-parsimonious trees; the strict consensus of these is shown in figure 3. The resolved and well-supported clades within eudicots include Paeonia, Rosaceae (including apple and strawberry), Fabaceae (including pea, teppary bean, and white clover), and Solanaceae (including tobacco and petunia). A clade containing cotton and Arabidopsis is more weakly supported (72% bootstrap). Relationships among these major clades, however, are not supported by bootstrap values higher than 50%, despite the relatively long branch lengths evident in figure 3.

All peony Adh sequences form a single clade, within which there are two major groups corresponding to Adh1 and Adh2. This result implies that a duplication event occurred prior to the diversification of the peonies. The Adh1A and Adh1B clades split at the base of the Adh1 clade. Most other relationships within peonies, except for the monophyly of section Oneapia, are poorly resolved in this analysis.

Analysis II, involving peony exon sequences only, yielded six most-parsimonious trees; the strict consensus of these is shown in figure 4 (rooted between Adh1 and Adh2). In the Adh1A clade, species of the herbaceous sections Oneapia and Paeonia form a monophyletic group supported by a 72% bootstrap value, suggesting that the root of the genus may be near the shrubby section Moutan. In the Adh2 clade, however, the relation-
FIG. 4.—The strict consensus tree of six most-parsimonious trees of length 192 (consistency index = 0.86; retention index = 0.97) based on sequences of five exons of the cloned Adh1 and Adh2 genes of peonies. Branch lengths are proportional to the number of nucleotide substitutions (scale represents two substitutions). The branch between Adh1 and Adh2 genes is reduced; its length is given in parentheses. All other numbers are bootstrap percentages above 50%.

ship among sections is not clearly resolved, although section Oneapia is supported as monophyletic. Despite low bootstrap support for a link between any two of the sections, the highest bootstrap value of 36% supports the relationship of sections Oneapia and Paeonia; this compares to 17% for Oneapia and Moutan and 0.1% for Moutan and Paeonia.

Analysis III (Adh1A and Adh2 analyzed separately, with introns added) provided better resolution of relationships within Paeonia (fig. 5A and B). Monophyly of section Oneapia, section Moutan, and the subsections Delavayanae and Vaginatae of section Moutan is strongly supported. The monophyly of section Paeonia is also strongly supported on the Adh1A tree. These results are concordant with the relationships previously obtained based on ITS and matK sequences (fig. 6A and B; Sang, Crawford, and Stuessy 1997). Discordance among these four gene trees is found within section Paeonia. In particular, section Paeonia is paraphyletic in the Adh2 tree; sequences from two populations of P.
**veitchii** form their own clade, which is a sister group to the clade containing section *Oneaplia* and the remaining species of section *Paeonia*. Discordance is also found between the *Adh1A* and ITS trees: *P. lactiflora* and *P. veitchii* are sister groups on the ITS tree (supported by 81% bootstrap), whereas *P. anomala*, *P. tenuifolia*, and *P. veitchii* form a monophyletic group in the *Adh1A* trees (supported by 81% bootstrap).

The Templeton test was conducted to determine whether the discordance among gene trees is significant (table 2). When the *Adh2* topology (section *Paeonia* paraphyletic) is used as the constraint topology, parsimony analysis of the *Adh1A*, ITS, and *matK* data sets are each seen to be significantly worse. In contrast, when the relationships of section *Paeonia* found in the ITS tree are used as the constraint topology, parsimony analysis of the *Adh1A* data set is not significantly worse. A combined analysis of *Adh1A*, ITS, and *matK* data sets resulted in the single most-parsimonious tree shown in figure 7A. The single most-parsimonious tree generated from the combined analysis of all four data sets is shown in figure 7B. These two trees are better resolved than any individual gene trees but differ by the positions of *P. veitchii* and *P. lactiflora*.

It is noteworthy that clones of each species are united on the *Adh1A* trees (fig. 5A). On the *Adh2* trees, however, the clones of each species are seen to be directly linked for only half of the species (fig. 5B). Two clones from one population of *P. rockii* (ROC1-1 and ROC2-19) form a clade with *P. szechuanica*, while two clones from another population of *P. rockii* (ROC1-2 and ROC1-3) form a trichotomy with this clade and *P. suffruticosa* subsp. *spontanea*. Clones from the same population of *P. lutea* (LUT2-2 and LUT2-6) are also grouped together but do not form a monophyletic group with the LUT1-8 clone from a different population. In *P. californica*, clones from two populations (CAL1-7 and CAL2-2) form a well-supported group, while a second clone (CAL2-20) from one of the two populations appears as the sister group of a clade containing *P. californica* and *P. brownii*.

### Sequence Divergence

We compared average sequence divergence between *Adh1A* and *Adh2* genes within and among species within each section, as well as within and among sections (table 3). Likewise, we compared *Adh1A* and *Adh1B* genes within and among species within section *Moutan*. Within each section, average overall sequence divergence of *Adh1A* and *Adh2* genes is slightly higher within species than among them, suggesting that concerted evolution between the two paralogous loci has not occurred within species after the divergence of each section (Dover 1982; Hughes 1995). Almost identical average sequence divergence between *Adh1A* and *Adh2* genes is found within sections and among sections, suggesting that concerted evolution has not occurred within sections and among sections.
any section following the divergence of the three sections. Average sequence divergence between Adh1A and Adh1B is very similar within species and among species in section Moutan, again implying that concerted evolution has not occurred between these two paralogous loci.

The average divergence between Adh1A and Adh2 genes is highest in section Oneapia and lowest in section Paeonia.

### Table 3

Average Percent Divergence (with the range) of Exon Sequences Between Two Pairs of Paralogous Genes (Adh1A and Adh2, Adh1A and Adh1B) and Exon and Intron Sequences of Each of the Adh1A and Adh2 Genes at all (\(d\)), Synonymous (\(d_s\)), and Nonsynonymous (\(d_a\)) Sites

<table>
<thead>
<tr>
<th></th>
<th>(d)</th>
<th>(d_s)</th>
<th>(d_a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adh1A-Adh2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within section Moutan . . . . . .</td>
<td>8.21 (7.19–8.92)</td>
<td>34.01 (29.60–39.07)</td>
<td>1.88 (1.54–2.58)</td>
</tr>
<tr>
<td>Within species . . . . . . . . .</td>
<td>8.21 (7.53–8.92)</td>
<td>34.06 (31.66–36.95)</td>
<td>1.86 (1.54–2.16)</td>
</tr>
<tr>
<td>Among species . . . . . . . . .</td>
<td>8.12 (7.19–8.92)</td>
<td>33.74 (29.60–39.07)</td>
<td>1.82 (1.54–2.37)</td>
</tr>
<tr>
<td>Within section Oneapia(^a)</td>
<td>9.36 (9.27–9.45)</td>
<td>36.50 (35.66–37.79)</td>
<td>2.74 (2.37–3.01)</td>
</tr>
<tr>
<td>Within species . . . . . . . . .</td>
<td>9.39 (9.27–9.45)</td>
<td>36.24 (35.66–36.92)</td>
<td>2.83 (2.59–3.01)</td>
</tr>
<tr>
<td>Among species . . . . . . . . .</td>
<td>9.21 (9.27–9.27)</td>
<td>37.30 (36.80–37.79)</td>
<td>2.48 (2.37–2.58)</td>
</tr>
<tr>
<td>Within section Paeonia . . . . .</td>
<td>8.68 (7.88–8.90)</td>
<td>33.55 (30.64–39.13)</td>
<td>2.55 (2.16–3.42)</td>
</tr>
<tr>
<td>Within species . . . . . . . . .</td>
<td>8.99 (8.40–9.63)</td>
<td>34.88 (33.50–36.92)</td>
<td>2.64 (2.16–3.21)</td>
</tr>
<tr>
<td>Among species . . . . . . . . .</td>
<td>8.80 (8.05–8.80)</td>
<td>33.79 (31.70–39.13)</td>
<td>2.64 (2.16–3.42)</td>
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<tr>
<td>Within three sections . . . . . .</td>
<td>8.58 (7.19–8.90)</td>
<td>33.87 (29.60–39.13)</td>
<td>2.35 (1.54–3.21)</td>
</tr>
<tr>
<td>Among three sections . . . . . .</td>
<td>8.56 (7.98–8.98)</td>
<td>33.54 (27.60–41.23)</td>
<td>2.39 (1.74–3.31)</td>
</tr>
<tr>
<td>Adh1A-Adh1B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within section Moutan . . . . . .</td>
<td>5.30 (4.82–5.66)</td>
<td>17.97 (16.42–20.43)</td>
<td>1.87 (1.64–2.27)</td>
</tr>
<tr>
<td>Within species . . . . . . . . .</td>
<td>5.15 (4.82–5.32)</td>
<td>17.45 (16.44–18.16)</td>
<td>1.81 (1.64–1.95)</td>
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<tr>
<td>Among species . . . . . . . . .</td>
<td>5.21 (4.99–5.49)</td>
<td>17.46 (16.42–19.50)</td>
<td>1.88 (1.64–2.27)</td>
</tr>
<tr>
<td>Adh1(^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exons . . . . . . . . . . . . . .</td>
<td>1.36 (0.31–2.37)</td>
<td>3.64 ± 0.89(^d)</td>
<td>0.69 ± 0.23(^d)</td>
</tr>
<tr>
<td>Introns . . . . . . . . . . . . .</td>
<td>4.68 (1.09–7.30)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Adh2(^c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exons . . . . . . . . . . . . . .</td>
<td>2.14 (0.47–3.67)</td>
<td>7.53 ± 1.34(^d)</td>
<td>0.39 ± 0.17(^d)</td>
</tr>
<tr>
<td>Introns . . . . . . . . . . . . .</td>
<td>4.54 (0.52–6.47)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\) Since there are only two species in section Oneapia, and only Adh2 genes were cloned from P. brownii, within-species divergence is represented by divergence within only P. californica, and divergence between Adh2 of P. brownii and Adh1A of P. californica is calculated as among-species divergence.

\(^b\) One clone is randomly chosen to represent one of nine species from which Adh1A genes were cloned.

\(^c\) One clone is randomly chosen to represent one of 10 species from which Adh2 genes were cloned.

\(^d\) Standard errors of \(d_s\) and \(d_a\) were calculated by MEGA, and \(d_s\) is significantly higher in Adh2 than in Adh1 (\(p < 0.05\)), while \(d_a\) is not significantly different between Adh1 and Adh2 (t-test; Kumar, Tamura, and Nei 1993).
tion Moutan, and the ranges in divergence between species of these two groups do not overlap (table 3). The average divergence value for section Paeonia is intermediate, but the range of divergence overlaps with those of the other two sections. Our relative-rate test comparing divergences between section Oneapia and section Moutan included species of the two sections from which we had cloned both AdhIA and Adh2 genes; the highest and lowest divergence values within each species are included in the test (table 4). The results indicate that the highest divergence value for P. californica is not significantly higher than the divergence values of the paralogous loci in P. lutea and P. suffruticosa subsp. spontanea, but both the higher and lower divergence values in P. californica are higher (P < 0.1 or 0.05) than those of P. rockii and P. szechuanica. Divergence of the two genes was not compared by the relative-rate test between section Paeonia and the other two sections owing to the overlap in the ranges of divergence.

Discussion

Our discovery that there are two major paralogous genes, AdhIA and Adh2, present in peonies and an additional gene, AdhIB, in the shrubby species is in agreement with the findings that two to three Adh loci usually exist in angiosperms (Gottlieb 1982; Morton, Gaut, and Clegg 1996). Identification of two major loci, AdhIA and Adh2, in P. californica is also concordant with the results of enzyme electrophoresis for this species (Zona et al. 1991).

The phylogenetic analysis of five Adh exon sequences of peonies along with a variety of other angiosperms (e.g., genes labeled Adh1 in different groups may not be homologous).

Based on our phylogenetic analyses, the duplication giving rise to AdhIA and AdhIB occurred later from an ancestral Adh1 gene member. Finding AdhIB only in section Moutan leads to two alternative explanations: (1) a duplication of AdhIA and AdhIB genes occurred only in the ancestral lineage of section Moutan; or (2) a duplication of AdhIA and AdhIB genes occurred in the ancestor of the entire peony clade followed by subsequent loss of the AdhIB locus from the other two sections. Our finding that AdhIB genes form the sister group of all AdhIA genes (figs. 3 and 4) is best explained by the second hypothesis. Sequence divergence data also appear to support this explanation. Under the first hypothesis we would expect sequence divergence between AdhIA and AdhIB within section Moutan to be lower than the divergence of AdhIA genes between section Moutan and either of the other two sections unless AdhIB genes evolved very rapidly in section Moutan, perhaps under selection. The observed sequence divergence between AdhIA and AdhIB within section Moutan (averaging 5.30%), however, is much higher than AdhIA sequence divergence between this section and the other two sections (averaging 1.54%). Synonymous substitutions are almost 10 times higher than nonsynonymous substitutions between AdhIA and AdhIB genes in section Moutan (table 2), which suggests that strong positive selection has not been a major factor during the evolution of AdhIB genes (Hughes and Nei 1988, 1989). Taken together, these arguments favor the hypothesis of an early duplication followed by a loss (fig. 8).

Using sequences of low-copy nuclear gene families as phylogenetic markers provides an opportunity for rooting trees when outgroups are unavailable or very highly diverged. An example of this application is the rooting of the entire tree of life (Gogarten et al. 1989; Iwabe et al. 1989; Brown and Doolittle 1995; Lawson, Charlebois, and Dillon 1996). Based on Analysis I, it is clear that peony Adh1 and Adh2 genes are more closely related to each other than they are to any other angiosperm Adh genes included in the analysis. When the root
is positioned between Adh1 and Adh2 sequences in Analysis II, a monophyletic group containing sections Oneapia and Paeonia is obtained in the Adh1A clade, implying that the root of the genus lies between section Moutan and the lineage containing the other two sections. Although the same result was not supported above the 50% bootstrap level within the Adh2 portion of the tree, support for the monophyly of Oneapia and Paeonia is higher than other possible relationships among the three sections. The observation that sectional relationships are less well resolved for Adh2 could be a function of longer coalescence times for Adh2 genes than for Adh1A genes (see below).

The view that the first evolutionary split occurred between section Moutan and the rest of the genus accords well with our hypothesis of a duplication followed by a deletion of Adh1B from sections Oneapia and Paeonia. When the peony tree is rooted between section Moutan and the other two sections, only one deletion of Adh1B is required; that is, in the common ancestor of sections Oneapia and Paeonia. Other rootings would require independent deletions of Adh1B from section Oneapia and section Paeonia.

Better understanding of the early evolution of Paeonia provides insights into morphological evolution and biogeography. The previous hypothesis of rooting—between the New World section Oneapia and the Old World sections—implied that the first evolutionary split within the genus may have been coupled with an intercontinental disjunct distribution, and that the herbaceous habit may well have been the ancestral condition from which the shrubby habit of section Moutan was derived.

The new view of rooting—between the woody section and the two herbaceous sections—allows that the ancestral habit of peonies may have been shrubby (depending on the habit of close outgroups) and implies that the Eurasia–North America disjunction occurred during early diversification of the herbaceous lineage.

Sequence divergence among the sections, however, appears to conflict with our rooting hypothesis if a molecular clock is assumed. Sequence divergence of Adh1A genes is highest between section Oneapia and the other two sections (average 2.03%) and lowest between section Moutan and the other two sections (average 1.55%). For Adh2 genes, the lowest divergence value is also between section Moutan and the other sections (average 2.27%), while the highest average sequence divergence is found between section Paeonia and the others (2.31%). These results are concordant with those based on ITS and matK coding sequences and the psbA-trnH intergenic spacer region of cpDNA. That is, studies of these sequences also show the lowest sequence divergence between section Moutan and other two sections and the highest divergence between section Oneapia and the others (Sang, Crawford, and Stuessy 1997).

If the first evolutionary split within peonies was between section Moutan and the other two sections, it would appear that rates of evolution either decreased in section Moutan or increased in the other sections, particularly section Oneapia. Relative rates of molecular divergence between sections Moutan and Oneapia can be gauged by examining the divergence of Adh1A and Adh2 in each section. If divergence rates have been the same in the two sections, and if concerted evolution has
not occurred, then the divergence of the two paralogous genes should be the same in each section (Wu and Li 1985). We used a relative-rate test based on the divergence of paralogous genes, Adh1A and Adh2, to assess whether paralogous genes diverged significantly more rapidly in section Oneapia than in section Moutan. The results indicate that rates have been slower in at least a part of section Moutan than in section Oneapia given that concerted evolution between Adh1A and Adh2 loci is not detected. Although it is not possible to carry out a similar relative-rate test for ITS or matK, lower sequence divergence in section Moutan appears to be correlated among nrDNA, cpDNA, and Adh genes. Such a correlation has been found in monocots, where synonymous substitution rates of both Adh genes and the chloroplast rbcl gene appear to be lower in palms than in grasses (Gaut et al. 1996). The longer generation time of palms was postulated by Gaut et al. (1996) to be responsible for their slower rate of molecular evolution. Generation time could also explain differences in rate within peonies, as the shrubby species of section Moutan are likely to have longer generation times than the herbaceous species of the other two sections.

Based on the foregoing conclusions, separate phylogenetic analyses of Adh1A and Adh2 genes including both exons and introns were carried out (Analysis III), and the individual trees were rooted between section Moutan and the other two sections. Relationships within Paeonia on each resulting gene tree are better resolved and, based on the Templeton test, generally congruent with each other and with the ITS and matK trees, except that section Paeonia is paraphyletic in Adh2 trees. The unusual position of P. veitchii on the Adh2 phylogeny may have been caused by either hybridization or random sorting of ancestral alleles. The hybridization hypothesis is less likely here given that no polymorphism has been found at the nrDNA, Adh1A, or Adh2 loci in P. veitchii, and the phylogenetic positions of this diploid species do not conflict among the ITS, cpDNA, and Adh1A trees. Although additional independent nuclear loci need to be examined to rule out the hybridization hypothesis, the lineage-sorting hypothesis is currently favored. It is possible, however, that the ancestral allele cloned from P. veitchii is still maintained in all or some other species of section Paeonia but was not discovered due to the rarity of the allele and/or the limited intraspecific sampling.

The tree resulting from the combined analysis of Adh1A, ITS, and matK sequences (fig. 7A) correlates better with morphology and the results of artificial hybridization experiments than the tree generated by the combined analysis of all four data sets (fig. 7B). Paeonia lactiflora has relatively broad leaflets and is placed in subsection Foliolatae, while the remaining three species have narrow leaflets and are placed in subsection Paeonia (Stern 1946). Artificial hybrids can easily be obtained from crosses among P. anomal a, P. veitchii, and P. tenui folia but are very difficult to obtain from hybridization between any of these three species and P. lactiflora (Saunders and Stebbins 1938). The sister group relationship of P. anomal a and P. tenui folia is supported by the production of only one terminal flower per stem (Stern 1946). Paeonia lactiflora, in contrast, has more than one flower on each stem; this is likely to be the ancestral state because it also occurs in section Oneapia and subsection Delavayanae of section Moutan. Paeonia veitchii seems to be intermediate on this character: some populations have one terminal flower and aborted flower buds on side branches (Pan 1979). Owing to the congruence of the tree based on the three data sets (fig. 7A) with other data we consider this to be the current best estimate of the species phylogeny, and on this basis we have reconstructed the Adh gene phylogenies shown in figure 8. Furthermore, in the case of considering the tree based on the four data sets to be the species phylogeny, a lineage-sorting hypothesis still has to be invoked to account for the paralogy of section Paeonia on the Adh2 phylogeny, and an additional hypothesis is needed to explain the incongruent relationships of P. lactiflora and P. veitchii between the Adh1 phylogeny and this hypothetical species phylogeny.

In comparison with the Adh1 tree, wherein alleles form monophyletic groups within each species, Adh2 alleles are not directly linked within five species or even within section Paeonia (fig. 5A and B). Adh2 genes appear to have longer coalescence times than Adh1A genes, which might also explain the higher divergence at synonymous sites in Adh2 (table 3). It is possible that longer coalescence times in Adh2 are a function of there being a larger number of copies than there arc of Adh1A (Kreitman 1991; Moore 1995). This is suggested by the observation that we found a larger number of distinct clones of the Adh2 gene in the majority of peony species. The number of distinct clones, however, may not precisely reflect the number of gene copies in the genome, because we cannot distinguish whether two Adh2 clones represent recently duplicated loci or heterozygous alleles at a single locus. Furthermore, small sequence differences among clones may be caused by Taq polymerase errors during PCR. Nevertheless, cloning both a normal Adh2 gene and a pseudogene from the same genome of P. delavayi and P. suffruticosus subsp. spontanea provides evidence for the previous existence of more than one Adh2 locus in these two species. Of the three Adh2 pseudogenes cloned in this study, two (1-AC-1 and DEL-10) are highly divergent from the normal Adh2 gene (fig. 5B), indicating that their origins may be quite ancient. In contrast, clone SPO-2 is even less diverged than the normal Adh2 clone of the same plant (SPO-9), which implies a recent origin of the pseudogene or a PCR error. Variation in copy numbers among different genes, and perhaps among species (as well as the occurrence of pseudogenes), is characteristic of the evolution of multigene families (Morton, Gaut, and Clegg 1996). In view of this dynamism, it is interesting to note that the copy number of the Adh gene family appears to be relatively stable in flowering plants, where two to three loci are usually found (Gottlieb 1982; Morton, Gaut, and Clegg 1996).

Phylogenetic inference at lower taxonomic levels (e.g., the interspecific level) in plants may be complicated by lineage sorting, hybridization, and/or limited resolu-
tion. A number of independent gene trees, therefore, are needed to represent the species tree (Pamilo and Nei 1988; Wu 1991; Hudson 1992; Maddison 1995). With respect to lineage sorting, it has been argued that the phylogeny of maternally inherited mitochondrial DNA has better chance of being congruent with the species tree than a nuclear gene phylogeny owing to a smaller effective population size and therefore shorter coalescence times (Moore 1995). The same argument may apply to chloroplast DNA phylogenies of the majority of flowering plants whose cpDNA is maternally inherited (Corriveau and Cloeman 1988; Mogensen 1996). Unlike mitochondrial DNA, however, sequences of cpDNA have rarely been used for phylogenetic inference at the species level owing to limited variation. For example, the matK coding region, which is the most rapidly evolving coding sequence known in cpDNA (Olmstead and Palmer 1994), seems to have evolved more slowly than Adh exons have in peonies (average divergence of 0.85% for the matK sequences of the 11 species; compare table 3). The non-coding regions of cpDNA also appear to be evolutionarily conserved and provided very limited resolution of interspecific relationships in Paeonia (Sang, Crawford, and Stuessy 1997). ITS sequences are now widely used phylogenetic markers at lower taxonomic levels in plants but also show limited variation in many cases (Baldwin et al. 1995).

These observations highlight the need to extend the sampling of low-copy-number nuclear genes in phylogenetic studies, particularly at lower taxonomic levels. It is noteworthy that the Adh1A gene tree for Paeonia is better resolved and better supported than the ITS and matK phylogenies (figs. 5 and 6). This may be due largely to the variable introns of the Adh genes, which are more variable than ITS sequences among the same species (average divergence of 3.11% for ITS; compare table 3). Likewise, Gottlieb and Ford (1996) demonstrated the phylogenetic utility of PgiC gene sequences in Clarkia (Onagraceae). Although it may be difficult in practice to identify paralogous and orthologous sequences, the extra effort required to make use of nuclear gene families may often be worth the effort. Knowledge of duplications in the evolutionary history of a gene can be useful in rooting phylogenetic trees and in assessing relative rates of molecular evolution.

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