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# Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: Implications for biogeography and concerted evolution

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**ABSTRACT** **The internal transcribed spacers (ITS) of nuclear ribosomal DNA of 33 species of genus *Paeonia* (Paeoniaceae) were sequenced. In section *Paeonia*, different patterns of nucleotide additivity were detected in 14 diploid and tetraploid species at sites that are variable in the other 12 species of the section, suggesting that reticulate evolution has occurred. Phylogenetic relationships of species that do not show additivity, and thus ostensibly were not derived through hybridization, were reconstructed by parsimony analysis. The taxa presumably derived through reticulate evolution were then added to the phylogenetic tree according to additivity from putative parents. The study provides an example of successfully using ITS sequences to reconstruct reticulate evolution in plants and further demonstrates that the sequence data could be highly informative and accurate for detecting hybridization. Maintenance of parental sequences in the species of hybrid origin is likely due to slowing of concerted evolution caused by the long generation time of peonies. The partial and uneven homogenization of parental sequences displayed in nine species of putative hybrid origin may have resulted from gradients of gene conversion. The documented hybridizations may have occurred since the Pleistocene glaciations. The species of hybrid origin and their putative parents are now distantly allopatric. Reconstruction of reticulate evolution with sequence data, therefore, provides gene records for distributional histories of some of the parental species.**

Speciation via hybridization, particularly when combined with polyploidization, is an important evolutionary mechanism in plants (1). Reconstructing reticulate evolution, however, has been a remarkably challenging task. Although the application of molecular markers has greatly facilitated the detection of hybridization and the recognition of allopolyploids in many plant groups, difficulties remain, largely due to lack of understanding the complex dynamic of molecular evolution (2–5). Despite very few applications of sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA to studies of allopolyploid taxa, strikingly contrasting results have been reported. While in *Krigia* it was shown that both parental sequences of the ITS region have been maintained in an allopolyploid species (6), in cotton the sequences of allotetraploids have been homogenized to that of either parental diploid species due to concerted evolution (7). Further investigations, therefore, are needed to understand evolution of the ITS region after hybridization and polyploidization. Such understanding is particularly important because ITS sequences are becoming a widely used tool for phylogenetic reconstruction in plants (8).

The peonies (*Paeonia*) represent a group that appears to have undergone extensive reticulate evolution (9–11). The genus, comprising three sections and ≈35 diploid ($2n = 10$) and tetraploid species of shrubs and perennial herbs, occurs widely in disjunct areas of the northern temperate region (11–13). All the tetraploid species belong to herbaceous section *Paeonia*. The majority of the tetraploids occurring in the Mediterranean region has been suggested as allopolyploids (9, 10). The origins of the putative allotetraploids, however, remain unknown. The diploid species of this section have never been considered to be of hybrid origin.

In the present study, ITS sequences were used to reconstruct the phylogeny of *Paeonia*.† The sequence data indicate that multiple hybridization events have led to speciation at both diploid and tetraploid levels in section *Paeonia*. The results provide an example of using ITS sequences to detect relatively ancient reticulate evolution in plants. Reconstruction of reticulate evolution in peonies yields significant insights into their distributional histories. The possible mechanisms responsible for maintenance of complete or partial parental sequences in the species of hybrid origin are discussed.

## MATERIALS AND METHODS

Forty-five accessions of 33 *Paeonia* species, including all the well recognized ones in section *Paeonia*, were sequenced. For most species, fresh leaves used as sources of DNA were collected from natural populations in Bulgaria, China, Greece, and Spain. The remaining species were collected from The Royal Botanic Garden (Kew). Total DNA was isolated from leaf tissues by the CTAB method (14) and purified in CsCl/ethidium bromide gradients. Double-stranded DNA of the complete ITS region was amplified by 30 cycles of symmetric PCR using primers ITS-4 and ITS-5 of White *et al.* (15), but ITS-5 was modified to match sequences of the 18S gene in seed plants (ITS-5m, GGAAGGAGAAGTCGTAACAAGG). The amplification products were purified by electrophoresis through 1.0% agarose gel followed by use of Bioclean (United States Biochemical) (16). Purified double-stranded DNAs were used for sequencing reactions employing Sequenase version 2.0 (United States Biochemical), deoxyadenosine 5′-[α-[$^{35}$S]thio]triphosphate, and two forward (ITS-3 and ITS-5m) and two reverse (ITS-2 and ITS-4) primers (15, 16). The sequencing reaction products were separated electrophoretically in 6% acrylamide gel with wedge spacers for 3 hr at 1500 V. After fixation, gels were dried and exposed to Kodak XAR x-ray film for 24–48 hr. DNA sequences were aligned manually and read for both strains.

Variable nucleotide sites were analyzed by unweighted Wagner parsimony using PAUP version 3.1.1 (17). The shortest trees were searched by the branch-and-bound method, and character changes were interpreted with ACCTRAN optimization. Bootstrap analyses were carried out with 1000 replica-

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Abbreviation: ITS, internal transcribed spacer(s).

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U27670–U27697).

tions using TBR branch swapping of the heuristic search (18). Section *Oneapia* of *Paeonia* was chosen as the outgroup for the cladistic analysis of the genus for the following reasons: (*i*) *Paeonia* is so isolated systematically that choice of a proper outgroup from outside of the genus is not feasible for polarizing ITS variation within it; and (*ii*) evidence from morphology, biogeography, ITS sequences, and chloroplast DNA divergence suggests that the earliest evolutionary split probably occurred between section *Oneapia* and the rest of the genus (refs. 19 and 20; and T.S., unpublished data).

## RESULTS

**Variation in ITS Sequences and Phylogenetic Reconstruction.** ITS-1 of all species sequenced is 267 bp long. There is a 1-bp and a 3-bp insertion/deletion in ITS-2 between *Paeonia californica* of section *Oneapia* and the rest of the species, and thus ITS-2 is 220 bp for the former and 222 bp for the latter. Sequences of the 5.8S rRNA gene are identical for species sampled from the three sections and are 164 bp long. Percentage G+C content ranges from 54.3% to 56.6% in ITS-1 and from 57.2% to 59.5% in ITS-2, and it is 53.7% for 5.8S rDNA. The number of nucleotide sites showing fixed differences among all species is 29 in ITS-1 and 20 in ITS-2. No nucleotide substitutions were found among different populations of a species. Of 27 species and subspecies sequenced in section *Paeonia*, 15 of them show nucleotide additivity at the variable sites within the section, suggesting that they may have originated via hybridization (Figs. 1 and 2; refs. 6 and 8). Besides these clearly additive sites, very few ambiguous ones were found in all the sequences.

Since reticulate evolution cannot be reconstructed directly by parsimony analysis, only those species showing no additivity at the variable sites in their ITS sequences were included in the analysis. Two equally most-parsimonious trees (a consistency index of 0.927 and a retention index of 0.967) were obtained based on 47 variable sites found in these species, and a strict consensus tree (a consistency index of 0.911 and a retention index of 0.959) was generated. For the purpose of this paper, only relationships within section *Paeonia* are shown (Fig. 3).

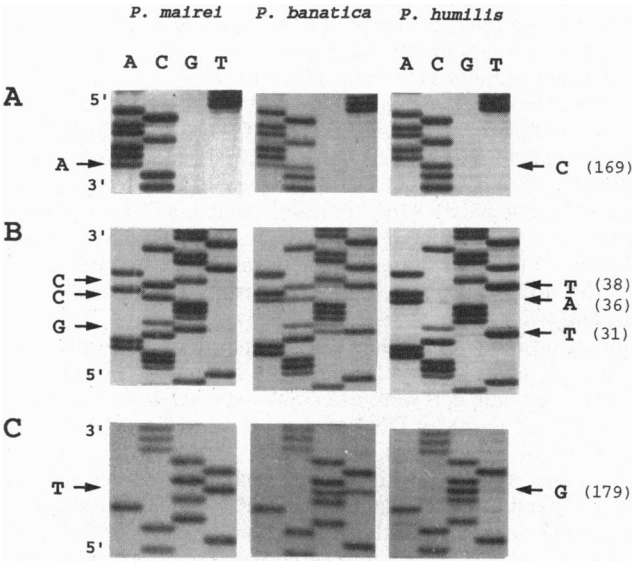


FIG. 1. Selected nucleotide sites that are variable between ITS sequences of *P. mairei* and *P. humilis* and show additivities in the sequence of *P. banatica*. Arrows indicate variable sites, and numbers indicate positions of sites in ITS-1 and ITS-2. Nucleotide sites in ITS-1 and ITS-2 are numbered separately from 5′ to 3′. (*A*) ITS-1. (*B* and *C*) ITS-2.

**Additivity of Nucleotides and Reticulate Evolution.** Five *Paeonia* species—*P. banatica, P. russi, P. emodi, P. sterniana*, and *P. peregrina*—show perfect or almost perfect additivity at all sites that are variable between two or three other species or species groups (Figs. 1 and 2), suggesting strongly that these five species were derived through hybridization (6, 8).

*P. banatica*, a tetraploid, may have originated from hybridization between *Paeonia mairei* and ARI-TEN (*Paeonia arietina, Paeonia humilis, Paeonia officinalis*, or *Paeonia tenuifolia*, or their common ancestor) because it combines different nucleotides at all sites that are variable between the putative parents (Fig. 2). At each of the additive sites—4, 7, 8, 9, 12, 13, 14, 15, 16, 17, 19, and 20 of *P. banatica*—1 of the 2 nucleotides is a synapomorphy of ARI-TEN, which clearly indicates ARI-TEN to be a parent of *P. banatica*. At each of the remaining additive sites 2 and 6, 1 of the nucleotides is a synapomorphy for *P. mairei* and JAP & OBO (*Paeonia japonica* or *Paeonia obovata* or their common ancestor), suggesting that either of them may be the other parent of *P. banatica*. The possibility of JAP & OBO being the parent can be ruled out because, at sites 7 and 8, their synapomorphies do not contribute to the additivity. Likewise, *P. russi*, also a tetraploid, shows additivity at all sites but site 12 that are variable between *Paeonia lactiflora* and *P. mairei* and thus may have been derived through hybridization between these two species.

The ITS sequence of the diploid *P. emodi* shows additivity at sites that are variable between *P. lactiflora* and VEI & XIN (*Paeonia veitchii* or *Paeonia xinjiangensis* or their common ancestor). At site 5, however, *P. emodi* combines G and T, but its putative parents, *P. lactiflora* and VEI & XIN, have the same nucleotide, T. This may imply that the T is not the synapomorphy for *P. lactiflora* and VEI & XIN but a parallelism. Hybridization between them may have occurred before the substitution from G to T occurred in one of the parents.

*P. sterniana* appears to combine the ITS sequences of *P. lactiflora*, VEI & XIN, and *P. mairei*; at site 12, *P. sterniana* has nucleotides G, T, and A, which apparently came from each of these three parental sequences, respectively. At the remaining sites, additivity is seen for only two different nucleotides because two of the three putative parental sequences have the same nucleotides at these sites. This suggests that *P. sterniana* may have been derived from hybridization either between *P. emodi* and *P. mairei* or among the three putative parents in other orders of occurrence. It is, however, difficult to assess the mechanism of maintaining three different sequences in *P. sterniana* because its chromosome number is unknown.

Compared with the above four species, the tetraploid *P. peregrina* combines less perfectly two types of sequences. At all the variable sites, *P. peregrina* contains the sequence of ARI-TEN, suggesting that ARI-TEN is one of the parents. The other parent of *P. peregrina* is less clear, although the most likely candidate is *Paeonia anomala* because it could have contributed to additivity at sites 4, 8, 12, and 20 of *P. peregrina*. At additive site 13 of *P. peregrina*, the same nucleotide C found in *P. anomala* and ARI-TEN must be the result of a homoplasious substitution, as judged from the phylogeny (Fig. 3), and thus it does not conflict with the hypothesis. One apparent conflict comes from the additivity of C and T at site 18, where plesiomorphy C exists in the two putative parents.

Unlike the 5 species described above, the last 10 species and subspecies in Fig. 2 (denoted as BRT-WIT), show a similar type of nucleotide additivity and only in ITS-1. Their sequences, however, cannot be interpreted directly as a combination between any two extant species in the section. Nevertheless, the additivity appears to be a partial combination between sequences of *P. lactiflora* and JAP & OBO in ITS-1. A possible process producing such a pattern of additivity in this group of species will be discussed later.

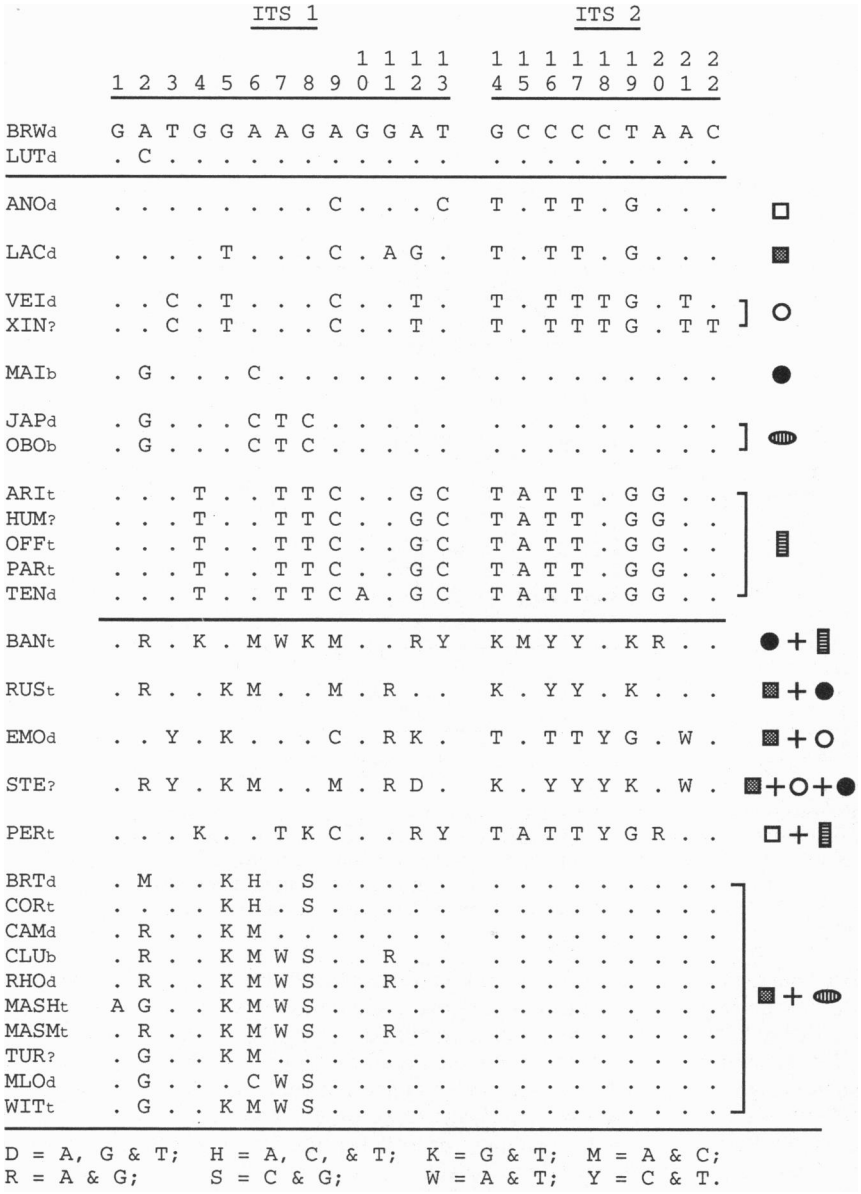


FIG. 2. All variable nucleotide sites among ITS sequences of taxa of *Paeonia* section *Paeonia*. Abbreviations of taxa of this section are as follows: *P. anomala* (ANO), *P. arietina* (ARI), *P. banatica* (BAN), *P. broteri* (BRT), *P. cambessidesii* (CAM), *P. clusii* (CLU), *P. coriacea* (COR), *P. emodi* (EMO), *P. humilis* (HUM), *P. japonica* (JAP), *P. lactiflora* (LAC), *P. mairei* (MAI), *P. mascula* ssp. *hellenica* (MASH), *P. mascula* ssp. *mascula* (MASM), *P. mlokosewitschi* (MLO), *P. officinalis* (OFF), *P. obovata* (OBO), *P. parnassica* (PAR), *P. peregrina* (PER), *P. rhodia* (RHO), *P. russi* (RUS), *P. sterniana* (STE), *P. tenuifolia* (TEN), *P. turcica* (TUR), *P. veitchii* (VEI), *P. wittmanniana* (WIT), and *P. xinjiangensis* (XIN). *Paeonia brownii* (BRW) and *Paeonia lutea* (LUT) representing section *Oneapis* and section *Moutan*, respectively, are used as outgroups for comparison. Dots indicate sequence matches to the first taxon. Site numbers 1–24 correspond to the actual sites: ITS-1, 34, 49, 74, 97, 108, 131, 138, 139, 169, 202, 226, 227, and 231; ITS-2, 31, 36, 38, 82, 99, 179, 189, 190, and 207, respectively. d, Diploid; t, tetraploid; b, both diploid and tetraploid populations known; ?, ploidy level unknown. Symbols on the right represent genotypes of parental species and their combinations in the species of hybrid origin.

The species presumably derived through reticulate evolution were added to the cladogram according to the combinations of putative parental sequences (Fig. 3).

## DISCUSSION

*Paeonia* section *Paeonia* has long been a taxonomically difficult group, presumably because of reticulate evolution, which has obscured morphological distinctions among divergent species (10). Although it has been suggested that the majority of tetraploid species in the Mediterranean region are allotetraploids, their origins have not been resolved from morphological and cytogenetic data (9, 11). The present study, using ITS sequences, documents a number of speciations via hybridization at both diploid and tetraploid levels. The parentages of five hybrid species—*P. banatica*, *P. russi*, *P. emodi*, *P. sterniana*, and *P. peregrina*—were effectively identified. The results show clearly that sequence data can be highly informative in detecting the origin of hybrids.

Although allotetraploidy is common in flowering plants (1, 2), the evolutionary significance of diploid hybrid speciation has remained unanswered and recent molecular studies have both confirmed and refuted earlier reports of stabilized diploid hybrid species (21–24). Thus, the documentation of a stabilized diploid hybrid species, *P. emodi*, which is now allopatric with its parents is particularly noteworthy.

**Biogeographical Implications.** The current disjunct distributions between the species of hybrid origin and their putative parents is intriguing (Fig. 4). This is particularly true for those species that now occur only in eastern Asia but are the parents of hybrid species in the Mediterranean region. Such disjunct patterns suggest that hybridization may be relatively ancient. Stebbins (10) hypothesized that the hybridization events that gave rise to the allotetraploids in the Mediterranean region began at the middle or end of the Pliocene and continued during Pleistocene glaciations. This hypothesis is plausible because the parental species were likely sympatric when they occupied refugia in the Mediterranean region during Pleistocene glaciation (25, 26). Furthermore, documentation in this study of multiple hybridizations in the Mediterranean region also suggests that the hybridization events may have occurred during different glaciations.

Another significant biogeographic implication of detecting hybridization in section *Paeonia* is that the eastern Asiatic species—such as *P. lactiflora*, *P. mairei*, *P. japonica*, and *P. obovata*—must have had much broader distribution ranges, including the Mediterranean region where they hybridized. Their distributional ranges probably began to shrink as they

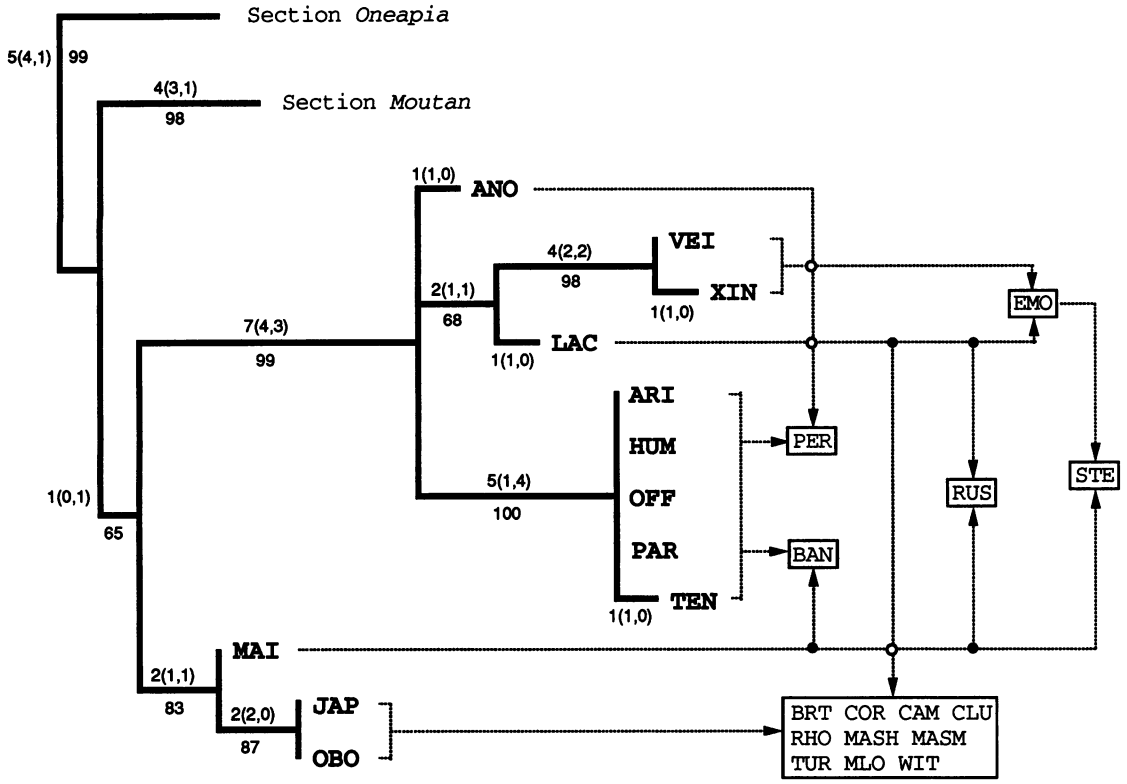


FIG. 3. Left portion of figure represents strict consensus tree of two equally most-parsimonious trees of species of divergent origin in *Paeonia* generated from variable sites of ITS sequences, and only interspecific relationships of section *Paeonia* are shown. Numbers above lines are the number of nucleotide substitutions for a branch followed by the number of transitions and transversions in parentheses. Numbers below branches give percentage occurrence of a group in 1000 bootstrap replications. Reticulate evolution is demonstrated by dotted lines in the right portion of the figure. Solid circles indicate connection between two lines; open circles indicate that two lines cross but do not connect. Abbreviations of species in section *Paeonia* are the same as in the legend to Fig. 2.

were replaced by their hybrids in the Mediterranean region during Pleistocene climatic changes in Europe (26–28). During the reduction of distributional ranges, *P. lactiflora*, VEI & XIN, and *P. mairei* may have become sympatric in the Himalayas and hybridized to produce *P. emodi* and *P. sterniana* (Fig. 4). Therefore, reconstruction of reticulate evolution in section *Paeonia* provides gene records for the historical distributional ranges of these eastern Asiatic species. This could be an effective approach for studying historical plant biogeography, especially in those groups, such as *Paeonia*, where hybridization occurred but no fossil record is available.

**Concerted Evolution.** Documentation of Pleistocene hybridization in peonies using ITS sequences obviously raises a question of whether concerted evolution has operated in this group. Concerted evolution, via gene conversion or unequal crossing-over, can homogenize different parental genomes in a hybrid so that only one parental genome type may be seen in the hybrid (7, 29, 30). In peonies, concerted evolution apparently is operating given the high homogeneity of ITS sequences within a species—i.e., very few ambiguous nucleotide sites (except additivity resulting from hybridization) were found in the sequences obtained directly from a PCR pool, and no nucleotide substitutions were detected among populations of a species (31, 32). However, concerted evolution clearly has not homogenized the parental ITS sequences in the five species of hybrid origin discussed above. Several factors may be responsible for changing the tempo of concerted evolution (32). Vegetative reproduction is likely to be a reason for maintenance of parental ITS sequences in the hybrids (8). Frequent reproduction via rhizomes in peonies may prolong generation time significantly, which, in turn, could slow rates of concerted evolution (33–36). If this were the case, it might be expected that the long generation time would also slow the rate of nucleotide substitution (37–39). This is most likely to be true because no unique substitutions were found in any of the five species of clear hybrid origin (Fig. 2), despite the fact that they may be 1 million years old (40). The other factors that may affect the tempo of concerted evolution, however, cannot be assessed here because we do not know the number, chromosomal localities, or genetic interaction of rDNA arrays in peonies (27, 28, 41).

The unusual pattern of nucleotide additivity displayed in the ITS sequences of the species group BRT-WIT makes it less straightforward to interpret the origins of these species. They apparently combine nucleotides, to different degrees, at six of eight sites in ITS-1 that are variable between *P. lactiflora* and JAP & OBO. In ITS-2, however, they have the same sequence as JAP & OBO. One possible explanation for this pattern of additivity is that these species originated through hybridization between JAP & OBO and an extinct species with ITS-1 sequences similar to that of *P. lactiflora* and with ITS-2 sequences identical to JAP & OBO. It is very unlikely, however, that such a species ever existed according to phylogenetic reconstruction and the corresponding patterns of nucleotide substitutions in section *Paeonia* (Figs. 2 and 3). We thus propose the alternative hypothesis that the species group BRT-WIT was derived through hybridization between *P. lactiflora* and JAP & OBO (Fig. 3), and some of the additive sites, including all in ITS-2 and two (sites 9 and 12) near the 3′ end of ITS-1, have been homogenized by concerted evolution. The pattern of uneven occurrence of homogenization fits with the model of gradients of gene conversion (42, 43). According to this model, certain regions within a gene are more likely to undergo gene conversion than others. The suggested mechanism is that gene conversion is initiated by formation of heteroduplexes, which can move in either direction and be

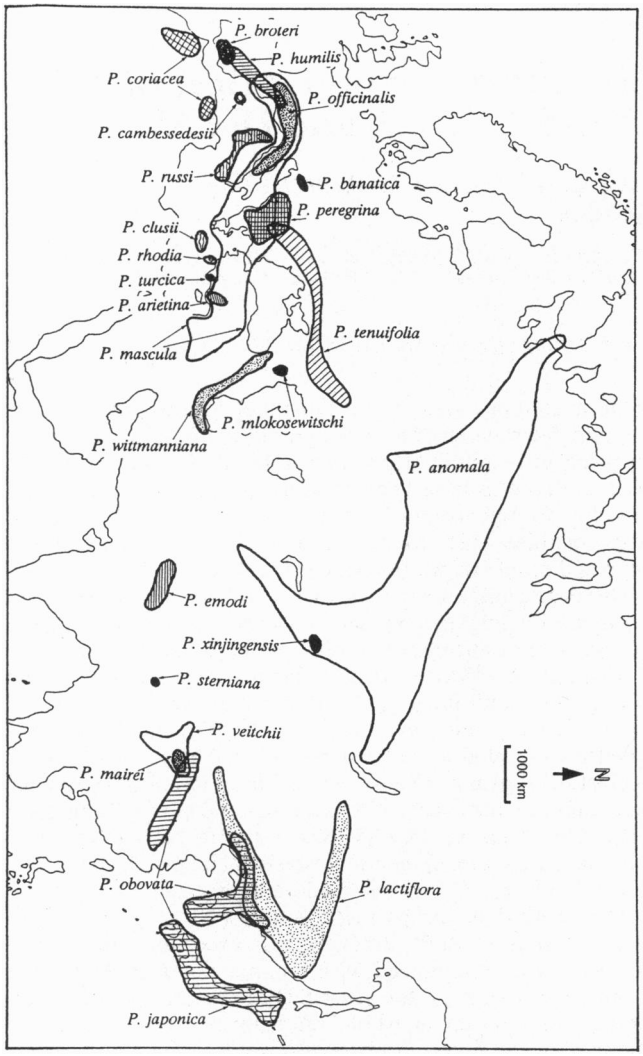


FIG. 4. Distribution of species of *Paeonia* section *Paeonia* in Eurasia.

terminated randomly (43). In the present case, there may be hot spots for the initiation of heteroduplex formation somewhere in the 26S rRNA gene, and from these spots gene conversion starts and migrates into the ITS region. Based on this hypothesis, the observed pattern of homogenization in ITS-2 and near the 3′ end of ITS-1 is predicted. The question of whether this process is common for ITS regions in flowering plants is subject to further investigation and could be addressed by the accumulation of similar observations in more plant groups.

In conclusion, this study provides an example of the successful use of ITS sequence data to reconstruct reticulate evolution in plants and demonstrates that sequence data can be highly informative and accurate for detecting hybridization. On the other hand, concerted evolution may lead to obscured patterns of nucleotide additivity in the ITS sequences of hybrids, and thus caution should be exercised when applying these data to phylogenetic studies.

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