

Speciation through homoploid hybridization between allotetraploids in peonies (*Paeonia*)

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Phylogenies of *Adh1* and *Adh2* genes suggest that a widespread Mediterranean peony, *Paeonia officinalis*, is a homoploid hybrid species between two allotetraploid species, *Paeonia peregrina* and a member of the *Paeonia arietina* species group. Three phylogenetically distinct types of *Adh* sequences have been identified from both accessions of *P. officinalis*, of which two types are most closely related to the two homoeologous *Adh* loci of the *P. arietina* group and the remaining type came from one of the two *Adh* homoeologs of *P. peregrina*. The other *Adh* homoeolog of *P. peregrina* was apparently lost from the hybrid genome, possibly through backcrossing with the *P. arietina* group. This is a documentation of homoploid hybrid speciation between allotetraploid species in nature. This study suggests that hybrid speciation between allotetraploids can occur without an intermediate stage of genome diploidization or a further doubling of genome size.

Hybridization is a widely documented mode of speciation in flowering plants (1). Two models of hybrid speciation, homoploid hybrid speciation and allopolyploidization, have been described (2). Although examples of allopolyploidy are found throughout angiosperms, conformed cases of homoploid hybrid species are rare (3). The rarity of homoploid hybrid species may be due to a combination of factors such as hybrid sterility, hybrid breakdown, difficulty of evolving reproductive isolation in sympatry, and difficulties in unambiguous identification of homoploid hybrid species (2, 4).

Documented examples of homoploid hybrid species in nature have so far been limited to diploids (3). However, there are no theoretical reasons why this mode of speciation could not occur among polyploid species. In fact, the classic experimental demonstration of this mode involved tetraploid species of *Gilia* (5, 6). Here we report a natural example of a homoploid hybrid species, *Paeonia officinalis*, that has arisen following hybridization between allotetraploid peony species.

The genus *Paeonia* comprises approximately 35 species of shrubs and perennial herbs distributed in disjunct areas of the northern temperate region (7). The Mediterranean region accommodates nearly 20 herbaceous *Paeonia* species, of which two-thirds are tetraploids ($2n = 20$). Although previous cytogenetic studies suggested that the majority of the tetraploid species were allotetraploids (8, 9), the origin of the putative allotetraploids had not been reconstructed until recent analyses of DNA sequences, in particular sequences of low-copy nuclear gene *Adh* (10–12).

The *Adh* genes constitute a small gene family with two to three loci in diploid angiosperms (13). This is one of the best-studied low-copy nuclear gene families in plants and has been used for phylogenetic inference of interspecific relationships in a number of flowering plant groups (14–17). Peonies have two *Adh* genes, *Adh1* and *Adh2*, that were duplicated before diversification of the genus *Paeonia*. Previous phylogenetic analyses indicated that each of the *Adh1* and *Adh2* genes, except for the *Adh2* of *Paeonia veitchii*, is orthologous among the diploid *Paeonia* species (16). Phylogenetic analyses of *Adh* gene sequences have led to the reconstruction of origins of several allotetraploid *Paeonia* species (12). In this study, *Adh* phylogenies provided evidence for

the homoploid hybrid origin of *P. officinalis* from two allotetraploid parental species.

Materials and Methods

The same accessions used in the previous phylogenetic studies of *Paeonia* (11) were included in this study. *Paeonia officinalis*2 was a new accession collected from Serra S. Antonio, Italy. Classification of *Paeonia* species included in this study is as follows: section *Paeonia* includes diploid species *P. anomala*, *P. cambessedesii*, *P. lactiflora*, *P. mlokosewitschi*, *P. tenuifolia*, and *P. veitchii*, and tetraploid species *P. arietina*, *P. humilis*, *P. officinalis*, *P. parnassica*, and *P. peregrina*; section *Oneapia* includes *P. californica*; section *Moutan* includes *P. lutea*, *P. rockii*, *P. suffruticosa*, and *P. szechuanica*.

DNA extraction followed a standard CTAB protocol (18). *Adh1* and *Adh2* genes were amplified with the gene-specific primers: primers AdhF2 and Adh1R for the *Adh1* gene and primers Adh2F and Adh2R for the *Adh2* gene (12). PCR was conducted under reaction conditions reported previously (16). PCR products were cloned into plasmids by using TOPO TA cloning kits (Invitrogen). At least 15 clones with the correct insert (determined by digestion with *EcoRI*) were screened for each PCR. *Adh1* clones were screened by comparing restriction fragments of *EcoRV* and *AseI*, and *Adh2* clones were digested with *HaeIII* and *AseI*. All clones that were unique were sequenced in both directions. Sequencing was completed on an ABI373 automated sequencer using a DYEnamic ET Terminator Cycle Sequencing Premix Kit (Amersham Pharmacia). Sequences were edited in SeqEd (Perkin-Elmer Applied Biosystems) and aligned manually. Clones that clearly resulted from PCR recombination (16, 19) were not included in the analyses.

Parsimony, as implemented in PAUP* Version 4.0 (20), was used to infer phylogenies based on nucleotide substitutions in aligned sequences. Parsimony analyses were performed by heuristic search with TBR branch swapping, MULPARS option, ACCTRAN optimization, and 100 random addition replicates for the *Adh* data sets. Bootstrap analyses (21) were carried out with 1,000 replications of heuristic search with simple taxon addition and “maxtrees” set to 500. The trees were rooted between section *Moutan* and the other two sections based on intersectional relationships determined previously (16). The Kishino-Hasegawa test (22) was used to compare topologies of the *Adh1* and *Adh2* trees as well as the likelihood of the most parsimonious solutions to alternative hypotheses of relationships within each tree. The model of sequence evolution for both *Adh1*

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and *Adh2* data sets was HKY + G, as determined by MODELTEST Version 3.0 (23).

Results

The aligned sequences of the *Adh1* gene were 1214-bp long, of which 162 nucleotide sites were variable and 83 were phylogenetically informative. The *Adh2* data set contained 1,186 nucleotide sites, of which 204 sites were variable and 175 were phylogenetically informative. Analysis of the *Adh1* data set yielded 11 equally most parsimonious trees with a tree length of 196, a consistency index (CI) of 0.88, and a retention index (RI) of 0.93. One of the most parsimonious trees was randomly chosen and shown in Fig. 1A. Analysis of the *Adh2* data set yielded 312 equally most parsimonious trees with a tree length of 427, CI of 0.79, and RI of 0.85. A randomly chosen *Adh2* tree is shown in Fig. 1B.

Two distinct types of sequences of *Adh1* and *Adh2* genes were cloned from three closely related tetraploid species, *P. arietina*, *P. humilis*, and *P. parnassica* (Fig. 1). One type, which forms a well supported monophyletic group with diploid species *P. anomala* and *P. tenuifolia*, is designated as type B. The other type, consisting of only pseudogenes for *Adh2*, is designated as type A. The A-type *Adh2* sequence was not recovered from *P. humilis* probably because of mutations at the PCR priming sites of the pseudogene (12). These three tetraploid species are referred to here as the *P. arietina* group. Forcing A and B-type sequences of the *P. arietina* group to be monophyletic was rejected by both the *Adh1* ($P = 0.04$) and *Adh2* ($P = 0.002$) data based on the Kishino–Hasegawa test (22).

Two distinct types of *Adh1* and *Adh2* sequences were also identified from both accessions of *P. peregrina* (Fig. 1). One type is nested within the B clade of the *Adh* phylogenies. The other type is clustered with diploid species *P. cambessedesii* and *P. mlokosewitschi* in the C clade. Forcing type B and C sequences of *P. peregrina* into a monophyletic group was rejected by the *Adh1* ($P < 0.0001$) and *Adh2* ($P < 0.0001$) data.

In the B clade of the *Adh1* phylogeny, sequences of the *P. arietina* group form a strongly supported clade which is further designated as B₁. The sequences of *P. peregrina* and a diploid species *P. tenuifolia* form another clade designated as B₂. Therefore, the *P. arietina* group has A and B₁ types of *Adh1* sequences, and *P. peregrina* has B₂ and C types. On the *Adh2* tree, however, B-type sequences of the *P. arietina* group or *P. peregrina* do not form their own groups (Fig. 1B).

Three types of *Adh1* sequences were cloned from both accessions of *P. officinalis*, and fell into three strongly supported clades, A, B₁, and C (Fig. 1A). On the *Adh2* phylogeny, the accession *P. officinalis2* also has the A, B, and C types (Fig. 1B). Only the A and B types of *Adh2* sequences were recovered from *P. officinalis1*.

Relationships in the B clade of the *Adh2* tree are obscured by a lack of resolution. This appears to be a result of dynamic gene duplication and deletion at the *Adh2* locus, as indicated by the presence of a larger number of clones, as well as pseudogenes. Nevertheless, creating a B₁ clade by forcing B-type sequences of the *P. arietina* group and *P. officinalis* (except for *P. officinalis1-26*) to be monophyletic was not rejected by the *Adh2* data ($P = 0.26$). Placing *P. officinalis1-26* within the B₁ clade, however, was rejected by the Kishino–Hasegawa test ($P = 0.013$). Including *P. officinalis1-26* with *P. peregrina* and *P. tenuifolia*, or the presence of a B₂ clade in the *Adh2* tree, was not rejected ($P = 0.22$). Therefore, the B₁ and B₂ types of *Adh2* sequences seem to be hidden by a lack of resolution in the *Adh2* phylogeny. The sequence *P. officinalis1-26* is most likely to represent the B₂ type, whereas the remaining *P. officinalis* sequences in the B clade are likely the B₁ type. Therefore, *P. officinalis2* has three types of *Adh2* sequences, A, B₁, and C, which are the same as the *Adh1*

types of both accessions. *P. officinalis1*, however, has A, B₁, and B₂ types of *Adh2* sequences.

Discussion

Both accessions of *P. officinalis* have three distinct types of *Adh1* sequences, of which A and B₁ types are most closely related to the *P. arietina* group. The previous cytogenetic evidence and molecular phylogenetic data suggested that the *P. arietina* group has an allotetraploid origin, and the A and B₁ types of *Adh1* sequences represent the two *Adh1* homoeologs derived from the diploid parents (12). The existence of an additional C type of *Adh1* gene in *P. officinalis* could be explained by two alternative hypotheses. One suggests that *P. officinalis* had the same allotetraploid origin as the *P. arietina* group, and the C-type sequence is a result of a gene duplication. The other considers *P. officinalis* to be a homoploid hybrid species with one parent being the *P. arietina* group that contributed the A- and B₁-type sequences and the other tetraploid parent that donated the C-type sequence.

The gene duplication hypothesis is very unlikely because it requires many additional assumptions. This hypothesis assumes that the gene was duplicated before diversification of the A and C clades, and one of the copies was subsequently deleted independently from all six other species except *P. officinalis* in the A and C clades (Fig. 1). Moreover, identification of the same three types of sequences, A, B₁, and C, at both *Adh1* and *Adh2* loci of *P. officinalis2* requires another hypothesis that the same pattern of gene duplication and deletion occurred between the two loci.

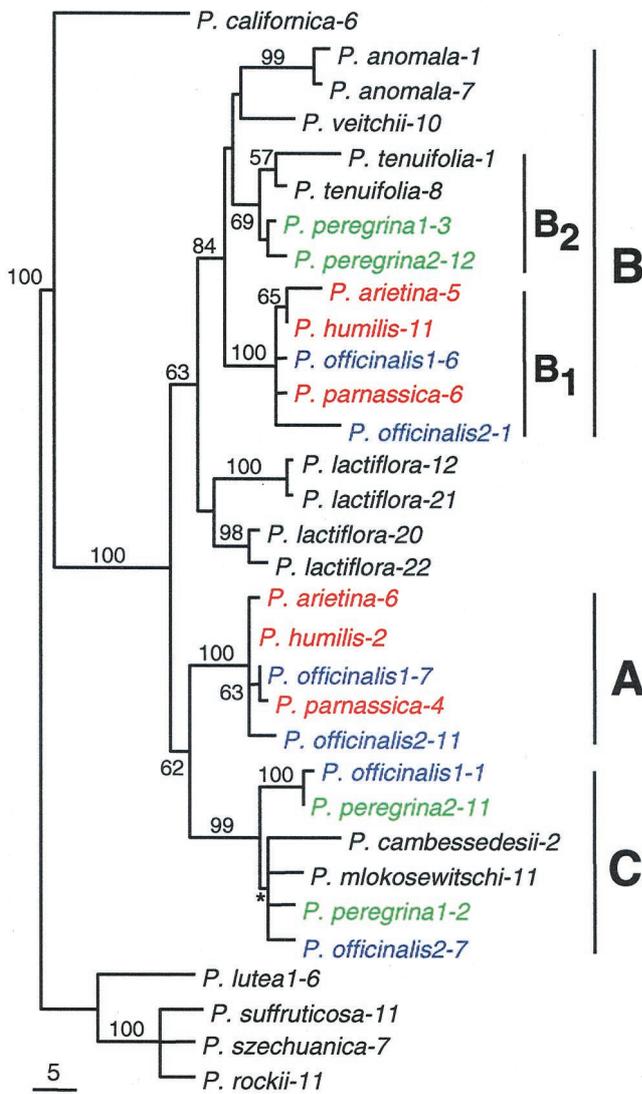
The hybridization hypothesis is favored not only because it requires fewer hypotheses, but also because both parents of the hybrid can be identified based on the *Adh* phylogenies. Whereas the *P. arietina* group is recognized as one of the parents of *P. officinalis*, the other parent is most likely *P. peregrina*. The C-type sequences of *P. officinalis* are closely related to those from *P. peregrina*. Particularly, the clone *P. officinalis1-1* forms a strongly supported sister group with *P. peregrina2-11* on the *Adh1* tree. In addition, the monophyly of all *P. peregrina* and *P. officinalis* clones in the C clade is among the most parsimonious solutions of the *Adh1* data. Formation of a monophyletic group of the C-type sequences of *P. officinalis2* and *P. peregrina* is not rejected by the *Adh2* data ($P = 0.088$).

P. peregrina was previously considered to be an allotetraploid based on cytogenetic evidence and sequence additivity in the internal transcribed spacers of nuclear ribosomal DNA (9, 10). Identification of the B and C types of sequences of *Adh1* and *Adh2* genes from both accessions of *P. peregrina* strongly supported its allotetraploid origin. These results, thus, suggest that *P. officinalis* is a homoploid hybrid species derived from two allotetraploid parents.

To better understand the origin of *P. officinalis*, it is necessary to further characterize the genome types of the allotetraploid parents. According to the *Adh1* phylogeny, one of the diploid parents of the *P. arietina* group was from a basal lineage B₁ of the B clade. The other parent was from the A clade, which is not closely related to any diploid species. The diploid parents of *P. peregrina* are closely related to *P. tenuifolia* of the B₂ clade and *P. cambessedesii* and *P. mlokosewitschi* of the C clade. On the *Adh2* phylogeny, the monophyly of B-type *Adh2* sequences of *P. peregrina* and *P. tenuifolia*, which corresponds to the B₂ clade of the *Adh1* tree, was not rejected by the Kishino–Hasegawa test. Therefore, the *Adh* gene phylogenies suggested that *P. arietina* group has a genome type AAB₁B₁, and that *P. peregrina* has a genome type B₂B₂CC.

All diploid species of section *Paeonia* that are not of hybrid origin are included in this study (11). The remaining four diploid species of the section that are not included in this analysis had cloned sequences falling into distinct clades on *Adh1* or *Adh2* phylogeny (data not shown), supporting possible hybrid origins

A. *Adh1*



B. *Adh2*

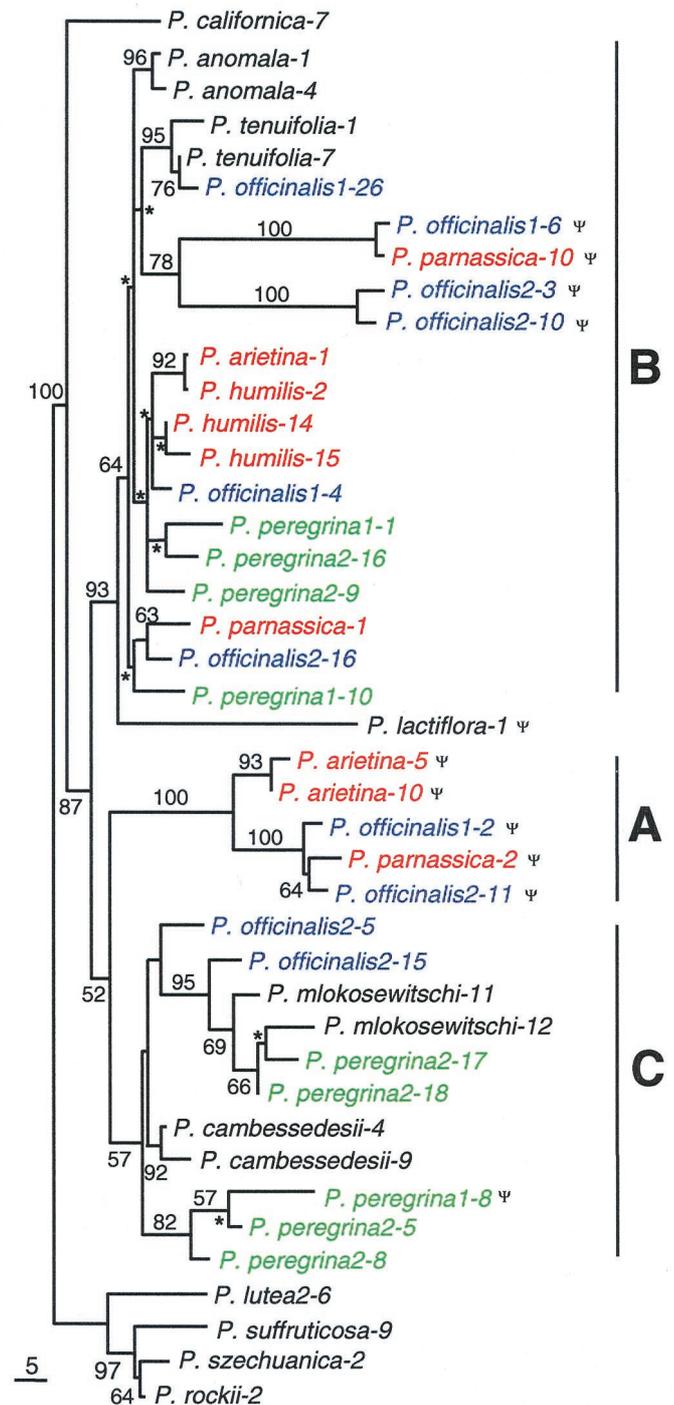


Fig. 1. *Adh* gene phylogenies of *Paeonia*. Diploids are in black, and tetraploids are in color: green, *P. peregrina*; blue, *P. officinalis*; red, *P. arietina* species group. (A) One of 11 most parsimonious *Adh1* trees chosen at random (tree length = 196, 114 without uninformative characters; consistency index = 0.88, 0.79 without uninformative characters; retention index = 0.93). (B) One of 312 most parsimonious *Adh2* trees chosen at random (tree length = 427, 291 without uninformative characters; consistency index = 0.79, 0.69 without uninformative characters; retention index = 0.85). Numbers associated with branches are bootstrap percentages above 50%. Branch lengths are proportional to the number of nucleotide substitutions (scale represents five substitutions). Asterisks denote clades that collapse in the strict consensus. Pseudogenes are identified with Ψ . The A, B (B_1 , B_2), and C sequence types are indicated. A number following a species name represents an accession number. A number following a hyphen represents a clone number.

of these species (11). Furthermore, none of the excluded diploid species is more closely related to the diploid progenitors of the *P. arietina* group or *P. peregrina* than the ones included in this

study. Therefore, the lack of a diploid species in the A clade is most likely due to the extinction of the diploid parent of the *P. arietina* group.

Given the inferred genome types of two allotetraploid parents, AAB₁B₁ and B₂B₂CC, the F₁ homoploid hybrid should have a genome type AB₁B₂C. The sampled accessions of *P. officinalis*, however, have maintained only three of the four homoeologs for each *Adh* gene. For the *Adh1* gene, both accessions have the A and B₁ types from the *P. arietina* group and the C type from *P. peregrina*. For the *Adh2* gene, the accession *P. officinalis2* has also maintained the A, B₁, and C types, whereas *P. officinalis1* possesses A, B₁, and B₂ types. In an attempt to isolate all *Adh* loci through PCR cloning, more than 30 clones of each *Adh* gene were screened from two runs of PCR for each accession of *P. officinalis*. In most cases, screening 15 clones is sufficient to identify both homoeologous *Adh* loci from an allotetraploid peony (ref. 12 and unpublished data). Furthermore, a C-type *Adh2*-specific primer, Adh2C (5'-CTTCTCTTTGATCTA-ATAAGT), was designed and used together with the reverse *Adh2*-specific primer Adh2R to amplify this genes from both accessions of *P. officinalis*. The C-type *Adh2* gene was amplified from *P. officinalis2* but not from *P. officinalis1*, indicating that this type of the *Adh2* gene is indeed absent from *P. officinalis1*.

A stable homoploid hybrid species can be formed through recombinational speciation. According to this model, backcrossing or interbreeding among partially sterile F₁ individuals may give rise to certain novel genotypes that have restored fertility and established at least partial reproductive isolation from the parental species (24, 25). The model has been rigorously tested at the genomic level through comparison of linkage maps of a homoploid hybrid species *Helianthus anomalus* and its diploid parents (26–29). Understanding of the genetic outcome of recombinational speciation at individual nuclear loci came from comparison of isozyme, RAPD, AFLP, and ISSR profiles of diploid hybrids and their parents (1, 30, 31). A homoploid hybrid species tends to have a combination of alleles and/or loci that are specific to either parent. However, it would be more difficult to identify a homoploid hybrid of allotetraploids by using these molecular markers because of the complex genome composition and possible gene silencing and deletion in both hybrid and parental species. The lack of suitable markers may have impeded the identification of this type of homoploid hybrid speciation in the past. Phylogenetic analysis of low-copy nuclear gene sequences, which has proven an effective approach to reconstruct allotetraploidization (12, 15, 32–34), may also contribute to the future documentation of homoploid hybrid species derived from allotetraploid parents.

It is probably impossible to predict genome composition of a homoploid hybrid species between allotetraploid parents even though the parental genome types are known. A tetraploid hybrid that integrates four sets of more or less diverged diploid genomes may have to undergo extensive genome reorganization. This process coupled with segregation, gene deletion (due to genetic redundancy), and possible backcrossing is most likely to yield a variety of combinations of the homoeologs from the allotetraploid parents in the hybrid genome. *P. officinalis* con-

tains *Adh* genes from three of the four types of the genes from both parents. In all cases, the *P. officinalis* accessions have both *Adh* homoeologs (A and B₁) from the *P. arietina* group and one of the homoeologs (B₂ in *P. officinalis1* or C in *P. officinalis2*) from *P. peregrina*. The other *Adh2* homoeolog of *P. peregrina* was lost from the hybrid genome possibly through backcrossing with the *P. arietina* group.

Previous cytogenetic studies found that *P. officinalis* had abnormal chromosomal pairing at the meiotic metaphase I where many univalents and some multivalents were observed (35, 36). This suggests that the homoploid hybrid genome is not yet completely stabilized, and thus has not recovered full fertility (2). Apparently, *P. officinalis* is a well established species both morphologically and ecologically. It is among the most widely distributed and most abundant peony species in the Mediterranean region. Having two similar copies of the B genome, B₁ and B₂, in the F₁ generation may have facilitated meiotic chromosomal pairing and helped the initial establishment of the hybrid population. Vegetative reproduction through rhizomes in peonies may also have facilitated the expansion of the hybrid populations despite potentially low fertility of hybrid individuals. Integration of genes from multiple genomes provided genetic and phenotypic variation that might facilitate the colonization of new habitats (4).

Future investigations of population and reproductive biology of *P. officinalis* should help determine hybrid fertility as well as other genetic and ecological factors involved in hybrid speciation. Increase in sampling of *P. officinalis* populations and the number of nuclear loci examined will contribute to a better characterization of this type of hybrid speciation at both populational and genomic levels.

It has been suggested that up to 70% of angiosperms are polyploids (37). Many angiosperms may have gone through several cycles of allopolyploidization followed by diploidization that results in a reduction in genome size through gene silencing or other means of genome rearrangement (38–40). This study suggests that hybrid speciation between allotetraploids can occur without an intermediate stage of genome diploidization given that the A, B, and C types of *Adh1* sequences are not silenced (at least at the level of gene integrity; the expression status of *Adh* copies was not examined). Furthermore, homoploid hybrid speciation between allotetraploids allows an integration of multiple diploid genomes without a further doubling of genome size, and consequently a greater opportunity of reunion and interaction of diverged diploid genomes during the evolution of flowering plants. The frequency of this type of hybrid speciation during angiosperm evolution remains unknown, and awaits to be assessed especially based on low-copy nuclear gene phylogenies.

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