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EVOLUTION OF TRIPLOIDY IN *APIOS AMERICANA* (LEGUMINOSAE) REVEALED BY GENEALOGICAL ANALYSIS OF THE HISTONE H3-D GENE

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Abstract.—Autotriploidy is normally considered to be maladaptive in plants because of its association with high levels of sterility. Nonetheless, triploid individuals are found in many plant species and play important roles in plant evolution, in particular as a first step toward tetraploid formation. However, few studies have addressed the evolutionary potential of triploid lineages, which may principally suffer from the impossibility of combining useful mutations in a single genome due to their low fertility. Therefore, triploids acquire genetic diversity only via recurrent evolution and somaclonal mutation. This study evaluates the potential of multiple origins of polyploidy as a source of genetic diversity in *Apios americana*, a North American legume that possesses both diploid and triploid populations. Ploidy level determination via flow cytometry shows that triploids are mainly restricted to the portion of eastern North America that was covered by ice during the Wisconsinan glaciation 18,000 years ago. This distribution implies that either selection or postglaciation colonization played a role in shaping this cytogeographic pattern. A haplotype network of the single copy nuclear histone H3-D gene reconstructed using statistical parsimony, together with single-strand conformational polymorphism analysis, shows that autotriploidy evolved at least three times in this species and that heterozygosity is high in triploids. The genetic diversity found in *A. americana* resulting from recurrent evolution and fixed heterozygosity increases the likelihood of producing successful genotypes and may give the opportunity for triploids to be better fit than diploids in new habitats. This suggests that triploid lineages can exhibit evolutionary potential of their own, and do not serve solely as a first step toward tetraploid formation.

Key words.—Cytogeography, haplotype network, multiple origins, phylogeography, polyploidy, statistical parsimony, triploidy.

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Autotriploidy has generally been thought to be an evolutionary dead-end, in part because of the low fertility of triploids caused by meiotic problems that result in aneuploid gamete formation. Yet, autotriploids produce euploid gametes (haploid, diploid, triploid), albeit in low rates, and are considered important in tetraploid formation (Harlan and deWet 1975; deWet 1980; Felber and Bever 1997; Ramsey and Schemske 1998; Burton and Husband 2000). Triploids also are involved in the incorporation of genetic diversity in polyploid complexes (Lord and Richards 1977; Lumaret and Barrientos 1990). Triploid individuals are found in diverse plant species (e.g., Les and Philbrick 1993; Takano and Okada 2002) and contribute important crops such as the banana, the unseeded watermelon, and some apple cultivars. A recent study also has demonstrated that triploid toads can reproduce sexually (*Bufo* complex; Stöck et al. 2002). However, in spite of their potential, the evolutionary significance of autotriploidy as an evolutionary lineage has rarely been addressed (however, see Haufler et al. 1985).

In contrast to sexually reproducing polyploids, the low fertility of triploids hinders the spread of beneficial traits since useful mutations occurring in separate individuals cannot be combined in a single genome via gene segregation and recombination. Therefore, these asexual lineages must contend with the initial genetic variation or with mutations that occur during its evolution (Otto and Whitton 2000). For triploid taxa, multiple origins constitute an important contribution to genetic diversity, and more origins means an increased probability of producing successful genotypes. To date, clear examples of recurrent autotriploid evolution in natural plant populations involve two ferns, *Cystopteris protrusa* (Haufler et al. 1985) and *Diplazium doederleinii* (Takamiya et al. 2001), and the angiosperm *Taraxacum officinale* (van der Hulst et al. 2000). In addition, although somaclonal mutation has been reported in some clonal organisms (e.g., King and Schaal 1990; Corradini et al. 2002), it has yet to be described in triploids. Documenting multiple origins as well as other sources of genetic diversity in triploid taxa is therefore of particular interest as a first step in evaluating the evolutionary potential of triploids.

Apios americana Medik., or groundnut, may represent another taxon where autotriploidy has evolved recurrently and would therefore be a good candidate to investigate the sources of genetic diversity in triploids. It is a perennial twining legume (Papilionoideae: Phaseoleae) that grows along riverbanks in eastern North America. This species has both triploid ($2n = 3x = 33$) and diploid ($2n = 2x = 22$) individuals roughly distributed in the north and the south of its range, respectively (Seabrook and Dionne 1976; Bruneau and Anderson 1988). Triploids are thought to be autopolyploids because of their morphological similarity with diploids (Seabrook and Dionne 1976; Bruneau 1986). Crossing experiments have shown that triploid individuals are completely sterile, whereas diploids are allogamous, though fruit production on these individuals is scarce (Bruneau and Anderson 1988). Groundnut takes advantage of an efficient asexual means of reproduction via tubers, which seem to be carried and distributed by water currents (Seabrook 1973). These tubers also were extensively used as food by Native American people and early American settlers (Duke 1983). The plant has been the object of studies to establish the feasibility of commercializing it as a new crop because of its high dry weight protein content (16.5 %; Walter et al. 1986), but this project has yet to give results (Reynolds et al. 1990). How-

ever, interest in this species is renewed since genistein, an anticarcinogenic compound, recently has been found in its edible tubers (Krishnan 1998). Triploidy in *A. americana* is suspected to have evolved multiple times on the basis of flower color (Seabrook 1973; Bruneau 1986), vegetative characteristics (Seabrook 1973), and preliminary isozyme studies (B. Connolly, unpubl. data). Nevertheless, multiple origins of polyploidy in *A. americana* have yet to be conclusively demonstrated.

The nuclear encoded H3-D histone gene is a good candidate for investigating these questions given that it possesses three variable introns and that it is in single copy in *Glycine* (Doyle et al. 1996), a close relative of *A. americana* in the papilionoid tribe Phaseoleae (Kajita et al. 2001). This locus has proved useful for showing multiple polyploid origins in *Glycine* (Doyle et al. 1999a; Doyle et al. 2002), and for resolving species relationships in the genus *Acacia* (Miller and Bayer 2000), another member of the Leguminosae (subfamily Mimosoideae). Our approach consists of estimating a gene genealogy via the construction of a haplotype network. Nuclear haplotype networks are particularly well suited for this type of study because they can potentially identify both diploid progenitors, document levels of heterozygosity in diploids and polyploids (no problem of null alleles), and discern between patterns of genetic diversity due to multiple origins of polyploidy and those due to mutation in the lineage following its formation. Similar approaches have been used to investigate recombination (Templeton et al. 1992), association between genotype and phenotype (Templeton et al. 1987), population structure (Excoffier and Smouse 1994), species boundaries (Shaw 1999; Templeton et al. 2000; Morando et al. 2003), modes of speciation (Barraclough and Vogler 2000), and hybridization (Templeton 2001; Doyle et al. 2002). Gene genealogy of the histone H3-D locus is used here specifically to assess multiple origins of triploidy in *A. americana*.

Materials and Methods

Sampling

We sampled 20 populations of *Apios americana* throughout its geographic range, but with particular emphasis in New England (Table 1) where triploidy is suspected to have arisen recurrently, and where both diploids and triploids are known to be present (Bruneau 1986). Usually only one individual per population was sampled although more individuals were evaluated in populations where both cytotypes were thought to be present. For brevity, a four-letter code was attributed to each population, in which the first two letters represent the state or province where the individual was collected, and the last two refer to the locality (Table 1). For populations containing more than one individual, a number was added to discern them. Two individuals of *A. priceana* B. L. Rob., the only other species of *Apios* in North America, were included to test whether the dataset is compatible with a hypothesis of complete lineage sorting among these species.

Ploidy Level Determination

To determine the ploidy levels, flow cytometry analyses were conducted on individuals for which fresh material was available. Tubers mainly were collected during the summer of 2000, and plants were grown in greenhouses. One gram of fresh leaves was cut on ice using scalpels in the extraction solution (0.44 M sucrose, 2.5% Ficoll 400, 25 mM tris-HCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 30% [v/v] glycerol, 8% PVP), replacing the solution three times for a total of 15 mL. The solution containing nuclei was then filtered successively using 75-, 45-, and 20-μm nylon filters. Nuclei were centrifuged at 200 × g for 15 min, washed in 10 mL PBS solution (2.68 mM KCl, 1.47 mM KH_2PO_4, 0.137 M NaCl, 15.56 mM $Na_2HPO_4 \cdot 7H_2O$, pH = 7.4), centrifuged again for 10 min at 200 × g, and resuspended in 2 mL of PBS solution. Rat hepathocytes (6.8 pg) were used as a standard, and were added in equal proportion to plant nuclei. This mixture was stained for one hour with 30 μL of propidium iodide (1 mg/mL) per mL of solution. Samples were analyzed with an FACScan cytometer (Becton Dickinson, Sunnyvale, CA); a total of 10,000 nuclei were processed for each sample.

The ploidy level of individuals for which fresh material was unavailable had to be evaluated via other methods. Chromosome counts were already available for some individuals from an earlier study (Bruneau 1986). When only herbarium specimens were available, ploidy levels were evaluated by pollen stainability using aniline blue in lactophenol (Hauser and Morrison 1964), which proved to be the easiest and most reliable method for discerning diploid and triploid individuals in *A. americana* (Bruneau 1986).

DNA Extraction, PCR Amplification, and Sequencing

Generally DNA was extracted following a modification of the Doyle and Doyle (1987) CTAB protocol. For some samples, especially from herbarium specimens but also from individuals for which we had limited quantities of tissue, DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Mississauga, Ontario) according to the manufacturer's recommendations.

The histone H3-D was amplified with two primers: H3D61, which is specific to the histone H3-D locus spanning the exon 1 and intron 1 boundary (Doyle et al. 1996; Doyle et al. 1999b), and KV-13 which is complementary to a highly conserved region of exon 4 (Kanazin et al. 1996; Fig. 1, Table 2). Polymerase chair reactions (PCR) in final volumes of 50 μL contained 1× PCR reaction buffer (Roche Diagnostics, Laval, Quebec; for a total $MgCl_2$ concentration of 1.5 mM), 1 mM of each primer, 200 μM of each dNTP, two units of Taq DNA polymerase, and between 30 and 100 ng of genomic DNA. Reaction conditions included an initial step of 7 min at 94°C, followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min elongation at 72°C, and finally, an extension step of 10 min at 72°C. Polymerase chain reaction products were purified using the QIAquick PCR purification kit (Qiagen), and then were cloned either with a TOPO TA cloning kit (Invitrogen, Burlington, Ontario) or in a U-vector (Qiagen) following the manufacturer recommendations. For the U-vector, chemically competent cells were prepared following Sambrook et al. (1989). To minimize the impact of possible PCR errors (Cronn et al. 2002), two PCR reactions were performed separately for each sample, and mixed prior to cloning.

TABLE 1. Locality, collectors, and ploidy levels of individuals included in this study of *Apios americana*. Individuals of both *Apios* species are sorted according to their code (first two letters represent the state or province, the second two represent the locality). The method used for assessing ploidy levels is indicated; the DNA content (2C value) is noted when flow cytometry was used. Haplotypes found in each individual are indicated, as well as the number of clones sequenced per individual. Haplotype sequences have been deposited in Genbank (accession nos. AY365194–AY365211).

Code	Collector[1]	Locality	Ploidy level	Method	Haplotypes[2]	No. of clones sequenced
Apios americana Medik.						
ALPK	Diamond 10500	Alabama, Pike	probably diploid	—	*j*	1
CTBP	Bruneau 136	Connecticut, Oneco	diploid	chromosome count $2n = 2x = 22$	*b, n*	3
CTMD	Joly 363	Connecticut, Madison	triploid	flow cytometry 2C = 4.37 pg	*b, e, n*	5
CTWR	Joly 366	Connecticut, Warren	triploid	flow cytometry 2C = 5.08 pg	*b, e, n*	0
FLDX	Godfrey 56032	Florida, Suwannee	diploid	pollen stainability	*k, o*	3
GAAT	Zomlefer and Giannasi s.n.	Georgia, Athens	diploid	presence of fruits	*i*	3
KYHL	Bruneau 251	Kentucky, Hematite Lake	diploid	chromosome count $2n = 2x = 22$	*a*	2
MABD	Joly 361	Massachusetts, Brimfield	triploid	flow cytometry 2C = 4.56 pg	*b, e, n*	0
MAEO	Joly 360	Massachusetts, East Otis	diploid	flow cytometry 2C = 3.05 pg	*g, n*	3
MAEV1	Joly 358	Massachusetts, Erving	diploid	flow cytometry 2C = 3.23 pg	*c, h*	5
MAEV3	Joly 376	Massachusetts, Erving	triploid	flow cytometry 2C = 4.59 pg	*b, e, n*	0
MSLK	Connolly s.n.	Mississippi, Abbeyville	diploid	flow cytometry 2C = 3.28 pg	*e, l*	4
NBBT	Bruneau s.n.	New Brunswick, Bouctouche	triploid	presence of three alleles (see Results)	*b, n*	9
NHAT	Joly 357	New Hampshire, Ashuelot	triploid	flow cytometry 2C = 4.84 pg	*b, e, n*	3
NYIT	Bruneau 972	New York, Ithaca	triploid	presence of three alleles	*d, f, l*	3
NYWT	Joly 368	New York, Waterloo	triploid	flow cytometry 2C = 4.66 pg	*d, f, l*	6
ONSL	Connolly s.n.	Ontario, Rock Port	triploid	chromosome count[3] $2n = 3x = 33$	*d, f, l*	2
PAEX	Joly 367	Pennsylvania, Exeter	triploid	flow cytometry 2C = 4.84 pg	*b, e, n*	5
QCDV	Joly 375	Quebec, Drumondville	triploid	flow cytometry 2C = 4.01 pg	*b, e, n*	4
QCIP	Joly 370	Quebec, Ile Perrot	triploid	flow cytometry 2C = 4.74 pg	*d, f, l*	2
QCSV	Joly 372	Quebec, Sabrevois	triploid	flow cytometry 2C = 4.96 pg	*b, e, n*	4
RIGV	Joly 362	Rhode Island, Greenville	triploid	flow cytometry 2C = 5.47 pg	*b, e, n*	6
VAFF	Wells s.n.	Virginia, Dyke Marsh	diploid	flow cytometry 2C = 3.38 pg	*c*	5
VTCP	Joly 351	Vermont, Chimney point	triploid	flow cytometry 2C = 5.14 pg	*b, e, n*	4
VTSB	Joly 352	Vermont, Stockbridge	triploid	flow cytometry 2C = 4.64 pg	*b, e, n*	0
WILG	Legit 2662	Wisconsin, La Grange	triploid	pollen stainability	*e, m*	3
Apios priceana B. L. Rob.						
APKY	Bruneau 257	Kentucky, Hematite Lake	diploid	Seabrook and Dionne 1976	*r*	1
APMS	McCook s.n.	Mississippi, Oxford	diploid	—	*p, q*	3

[1] Specimen vouchers for all accessions are deposited at the Herbier Marie-Victorin (MT) with the exception of ALPK which is deposited at the Carnegie Museum Section of Botany (CM).
[2] The haplotypes and relationships between them are depicted in Figure 4.
[3] The chromosome count was obtained from another individual of the same population.

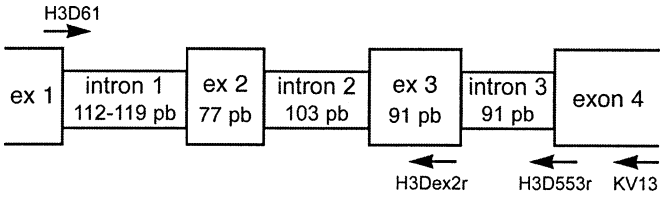

FIG. 1. H3-D histone locus in *Apios americana*, showing intron and exon lengths and primer positions. Primer sequences are given in Table 2.

Sequencing was conducted using the primers H3D61 and H3D553r, the latter also being specific to the single copy H3-D histone, spanning the junction between intron 3 and exon 4 (Doyle et al. 1996; Doyle et al. 1999b; Fig.1, Table 2). Cycle sequencing was performed using the BigDye terminator chemistry (Applied Biosystems, Foster City, CA), and sequenced products were run on an ABI 310 automated sequencer (Applied Biosystems). Sequences were verified and edited using Sequencher (ver. 4.1, GeneCodes, Inc., Ann Arbor, MI). Several clones were sequenced per individual (from three to nine; Table 1) attempting to sample every allele, but sequencing had to be limited to the available clones for some individuals. Sequences were aligned by eye, and indels were coded using the simple gap coding method of Simmons and Ochoterena (2000).

Single-Strand Conformational Polymorphism Analysis

The cloning procedure does not always guarantee that all alleles from one individual will be found unless large numbers of clones are sequenced. To evaluate whether the lack of alleles in particular individuals is due to an artifact of the cloning procedure or whether it is biologically significant, individuals included in this study were genotyped using single-strand conformational polymorphism (SSCP; Hongyo et al. 1993). This method assumes that DNA strands with distinct sequences have different secondary conformations and will migrate at different rates on a nondenaturing gel. Because SSCP analyses work better with fragments of 400 bp or less (Hayashi 1992), a new primer, H3Dex2r, was designed (Fig.1, Table 2). This primer is situated at the 3′ end of exon 3 and thus discards information situated in the third intron, but very few mutations are located in this region and these never were important in discerning haplotypes. Polymerase chain reaction amplification conditions for the SSCP analysis were performed as described above. The PCR product was mixed with an equal volume of denaturing loading buffer (deionized formamide and 0.01% xylene cyanol). Samples were denatured for 4 min at 95°C, then placed on ice until gel loading. The samples were run in a 6% polyacrylamide gel (37:1 acrylamide to methylbisacrylamide) with 1× TBE buffer and 4% urea on a vertical electrophoresis system at constant power (17,5 W) and temperature (4°C) for 16 h. Different concentrations of urea (0%, 2%, and 4%) in the gel were tested, but 4% urea was used because it gave the best resolution overall. Fragments were detected using silver staining (Bassam et al. 1991).

Silver staining reveals both complementary DNA strands, which given their distinct nucleotide composition should migrate at different rates on the gel. Accordingly, we would expect homozygous diploid individuals to have two bands, heterozygous diploids to have four bands, and triploids with three alleles to have six bands. However, patterns become more complicated, because one or both strands may have one or several secondary conformations (Sheffield et al. 1993). For this reason, the SSCP analysis was used only as a genotyping method and not to draw conclusions regarding shared alleles among individuals. Nevertheless, the identity of most bands on the SSCP gel was confirmed by sequencing. Bands were removed from the gel, placed in water overnight, and then amplified and sequenced as previously described.

Identification of Polymerase Errors and Recombinants

Before proceeding with the analysis, we removed sequences that included polymerase errors or that were the result of PCR recombination. The SSCP method was used to identify possible polymerase errors that can occur in the PCR reaction because it allows individuals with the same genotype to be identified. Point mutations detected in individuals with the same genotype must necessarily be caused by polymerase. Sequencing of SSCP bands also helped to identify PCR errors. As a further means of identifying polymerase errors, direct sequencing (without cloning) also was performed for all individuals using primers H3D61, H3Dex2r, and H3D553r, all of which were necessary because of length variation between alleles.

Recombinant sequences have been found to be frequent in PCR reactions (Bradley and Hillis 1997; Judo et al. 1998; Cronn et al. 2002; Doyle et al. 2002). PCR recombination can occur when the DNA polymerase makes a pause or disengages from the template before elongation is complete. If this incomplete fragment anneals to a nonidentical template (an allele in the case of a single copy nuclear gene) in a subsequent cycle, a chimeric sequence will be produced. A PCR recombinant can be identified when a sequence obtained from the cloning procedure is made up of two regions from different alleles. However, it may be difficult to identify a recombinant consisting of several alternating small parts of the original alleles or when one or both ''real'' alleles are not recovered by the cloning procedure. Another means of

TABLE 2. Sequences for the primers used to study the H3-D locus in *Apios americana*.

Primer	Sequence (5′–3′)[1]	Reference
H3D61	GCTTGCAACCAAGgttygttt	Doyle et al. 1996
H3Dex2r	GTCCTGGGCAATTTCMCGC	this study
H3D553r	TGGAAACGCAGATCAGTctg	Doyle et al. 1996
KV-13	AGCTGGATGTCCTTGGGCAT	Kanazin et al. 1996

[1] Lowercase letters represent intron nucleotides.

identifying recombinants is by parsimony analysis. As long as recombination events are relatively rare, the few recombinant haplotypes can be identified in a parsimony analysis by their abnormally high levels of homoplasy. These homoplasies also will tend to be physically clustered in the same DNA region. Recombination should be invoked only if a single recombination event can resolve two or more homoplasies (Aquadro et al. 1986). In our analysis, only sequences that clearly could be identified as PCR recombinants were removed because similar sequences also can be produced in vivo via crossing-over or gene conversion.

Network Construction and Phylogenetic Analysis

The gene genealogy of the H3-D locus was estimated via a haplotype network constructed using statistical parsimony (Templeton et al. 1992). This algorithm was preferred over traditional phylogenetic reconstruction methods (i.e., cladistics) because such techniques often make assumptions that are not valid at the intraspecific level (Posada and Crandall 2001). It is based on the parsimony criterion because although maximum parsimony is known to have drawbacks when times of divergence are long (Felsenstein 1978), it tends to be the method of choice when evolutionary times are shorts (Felsenstein 1981; Sober 1988). Haplotype networks are constructed only when parsimony has a probability of at least 0.95 of being true as determined by coalescent theory. When parsimony cannot be justified at this probability level, both parsimonious and nonparsimonious connections are allowed between haplotypes until their cumulative probability exceeds 0.95, thereby fixing an upper limit to parsimony beyond which haplotype relationships are not considered. Hence, the resulting 95% plausible set of alternative networks is not necessarily just a set of alternative maximum parsimony networks, but also can include nonparsimonious alternatives. Statistical parsimony therefore has the advantage of not requiring further validation methods, which may not give accurate support when variation is limited, as in intraspecific studies (Crandall 1994). The method is implemented by the TCS (ver. 1.13) software (Clement et al. 2000) available at http://InBio.byu.edu/Faculty/kac/crandall_lab/tcs.htm.

Because the divergence between haplotypes of *A. americana* and *A. priceana* was too high to be connected at the 95% limit using statistical parsimony (see Haplotype Network in Results), a branch-and-bound analysis was conducted using PAUP* (Swofford 1998) with haplotypes from both species. This served to root the *A. americana* haplotype tree, but also to see whether the histone data is consistent with the hypothesis of complete lineage sorting between *A. priceana* and *A. americana*.

RESULTS

Ploidy Level Determination

Flow cytometry analyses revealed two distinct groups of individuals based on the nuclear DNA content. Four individuals considered to be diploids possess an average of 3.24 pg (SD = 0.14) of DNA per nucleus, and 13 presumed triploids have 4.84 pg (SD = 0.29) of DNA per nucleus on average (Table 1). Diploids possess two-thirds the DNA content of triploids, as expected. Although no individuals were evaluated for both chromosome counts and DNA amount, a few individuals that were evaluated for DNA content in this study (CTWR, QCIP, and VTCP) also have chromosome counts for other individuals from the same population (Bruneau 1986) and the same ploidy level was obtained by both methods. Pollen from diploids stained with aniline blue in lactophenol were uniform in size and stained at a very high proportion (near 100%) whereas pollen from triploids stained poorly and showed a great variability in size and shape.

Results indicate that triploid individuals are located in the northern part of the species range, whereas diploids occur in the southern portion of the range (Fig. 2). The two cytotypes overlap slightly in distribution, the northernmost diploid sampled being found in northern Massachusetts (MAEV), and the southernmost triploid population sampled was from Pennsylvania (PAEX). Only one population (MAEV) was suspected to contain both diploids and triploids based on the production of fruits on only certain individuals, and cytometry analysis confirmed that both ploidy levels were present in the population (Table 1).

Sequencing

The number of clones sequenced per individual is indicated in Table 1. The H3-D region between exons 1 and 4 in *A. americana* has a length ranging from 405 to 411 bp. Sequence divergence within *A. americana* ranges from 0.00 to 3.86%, but when *A. priceana* is included, sequence divergence reaches 5.30%. Mutations are distributed equally among introns 1 (eight mutations), 2 (10), and 3 (seven), whereas exons 2 and 3 bear one and 10 mutations, respectively. The pattern is different for gaps, however, since they are only located in introns (introns 1, 2, and 3 have one, two, and one gaps, respectively). Sequencing revealed a hypervariable region in the first intron of the H3-D histone consisting of long strings of thymines and a few adenosines. This region was not included in the analyses because of a lack of confidence in homology assessment (alignment) at the nucleotide level (Kelchner 2000). The final matrix used for the network construction is available upon request from SJ.

Single-Strand Conformational Polymorphism Analysis

For all alleles of the histone H3-D locus, both complementary strands have secondary conformations on the SSCP gel, as shown by the presence of duplicated band patterns for all individuals analyzed (an upper and a lower pattern; Fig. 3). The sequencing of bands further confirmed that the bands in the lower and upper bands were homologous.

Single-strand conformational polymorphism analyses showed that all diploids sampled had different genotypes (Fig. 3). Six diploid individuals showed evidence of heterozygosity by harboring more than two bands: ALPK, CTBP, FLDX, MAEO, MAEV1, and MSLK. The remaining three diploids showed only two bands, as expected if a single allele were present. All triploids appear heterozygous, having more than two bands, although the exact number of bands is difficult to assess beyond doubt because of lack of resolution between alleles, some of which appear to have similar migration rates. Four distinct genotypes were found among trip-

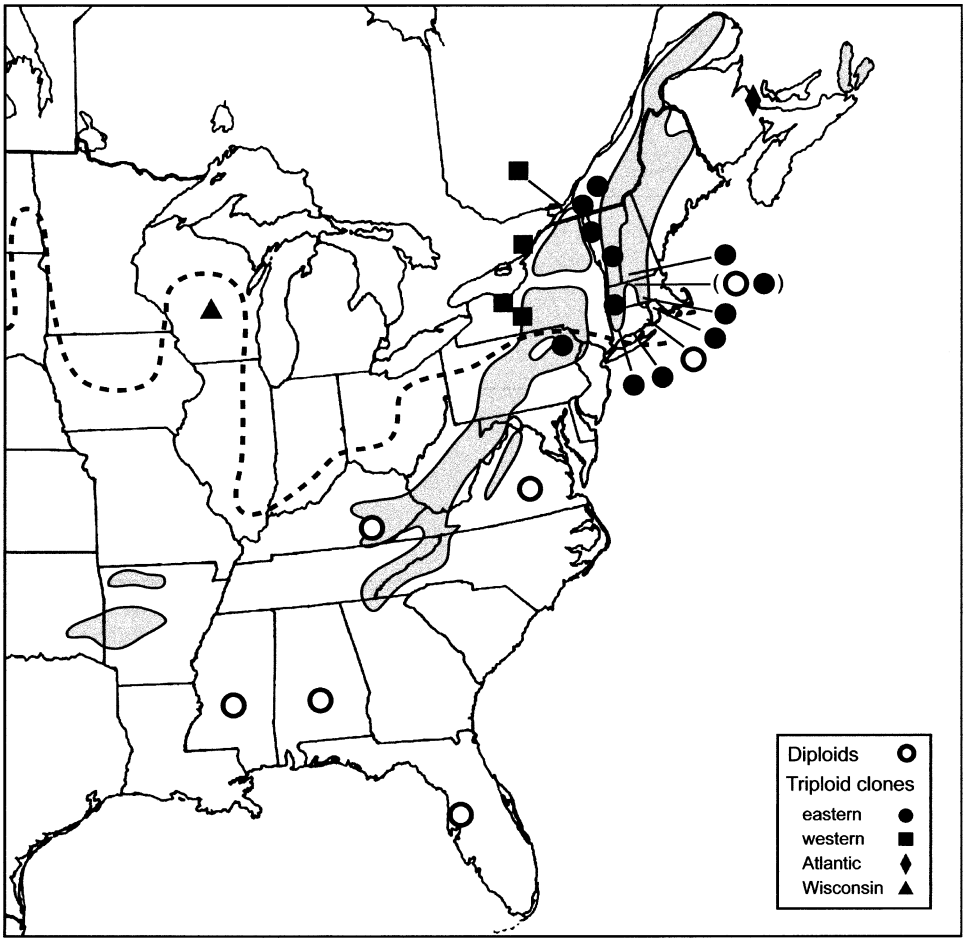

FIG. 2. Geographic location of the individuals of *Apios americana* sampled in this study. The broken line indicates the maximum limit of the Wisconsin glaciation 18,000 years ago. Open circles represent diploids, and triploids are represented by filled circles, squares, triangles, and diamonds, with the different forms identifying the different clones. The mountains are represented by a shaded area.

loids, depicting four distinct triploid clones. The first identified individuals CTMD, CTWR, MABF, MAEV3, NHAT, PAEX, QCDV, QCSV, RIGV, VTCP, and VTSB as belonging to a single clone (eastern clone, see Haplotype Network section; Fig. 3). Triploid individuals NYIT, NYWT, ONSL, and QCIP also have identical H3-D genotypes and represent a second clone (western clone; Fig. 3). The third and fourth genotypes were each found in a single individual: NBBT (Atlantic clone) and WILG (Wisconsin clone; Fig. 3). Thus the SSCP was useful for confirming the genetic identity of certain individuals, but also to complete our sequence dataset where the cloning procedure failed to recover every allele of each individual.

Polymerase Errors and PCR Recombinants

Several polymerase errors and PCR recombinants were detected in the dataset. Twenty-five sequences contained PCR errors and 14 were recombinant making a total of 34 sequences that had to be removed from the initial 85 (three sequences were recombinants in addition to having PCR errors, and two sequences possessed two PCR errors). The proportion of PCR recombinants in the whole dataset is 16.5%, which falls within the range found in a previous empirical study (Cronn et al. 2002). The proportion of polymerase errors per sequence is 27.1%. Assuming that each sequence is 411 bp in length, we obtained a polymerase error per nucleotide ratio of 6.58×10^{-4}. This is slightly higher than other reported estimates for Taq polymerases (1.1×10^{-4}, Tindall and Kundel 1988; 2.0×10^{-5}, Lundberg et al. 1991).

Haplotype Network

The H3-D region between exons 1 and 4 produced a network free of uncertainty consisting of 15 *A. americana* haplotypes and three *A. priceana* haplotypes (Fig. 4). For ease of discussion, a letter (*a*–*r*) has been assigned to each haplotype. Sequences for these haplotypes have been deposited in Genbank (accession nos. AY365194–AY365211). The limit of parsimony calculated by the algorithm (Templeton et al. 1992) is eight steps. Consequently, the sequences of *A. priceana* were too distant from those of *A. americana* to be connected to the network using statistical parsimony. Standard phylogenetic analysis was therefore used to connect the two subnetworks (Fig. 4). The branch-and-bound analysis gave 160 most-parsimonious trees of 55 steps (CI = 0.909, RI = 0.926; results not shown), where the two species form

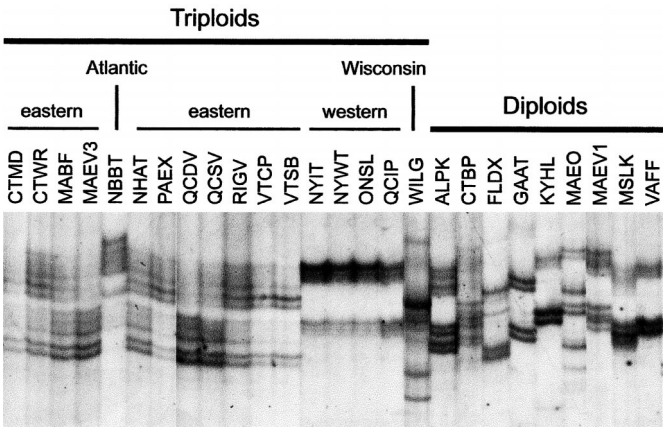

FIG. 3. Single-strand conformational polymorphism analysis gel of the histone H3-D alleles for diploid and triploid *Apios americana* individuals. All migrating strands possess a second stable conformer, as shown by the duplication of the band patterns. Sequencing of bands confirmed that the upper and the lower patterns were homologous. Interpretation of the gel focuses on only one of these duplicated patterns. Six diploids (ALPK, CTBP, FLDX, MAEO, MAEV1, MSLK) appear heterozygous due to the presence of more than two bands, whereas three other diploids (GAAT, KYHL, VAFF) are more likely homozygous, harboring two bands. Triploids appear heterozygous, each with more than two bands present. Four genotypes were found among triploids; these four clones are identified.

two distinct clades separated by 10 nonhomoplasious mutations.

Three mutations are homoplasious within *A. americana* (α, β, and γ; Fig. 4). Two of them (α, β) occur on the same branches and are clustered physically in the gene region (position 369 for one mutation, and the deletion of nucleotides 372 to 374 in the other), indicating a potential recombination event. Because the haplotypes involved (*e* and *k*) are not present in the same individuals, this recombination event would have to have occurred in vivo. Because it is difficult to identify which haplotype arose from recombination, these were left in the network. This decision does not change any conclusions made here.

A total of 11 haplotypes were found in *A. americana* diploids and seven in triploids, with some (*e, l, n*) shared by both ploidy levels (Fig. 4). The network shows that five of the nine diploids (CTBP, FLDX, MAEO, MAEV1, and MSLK) are heterozygous for the histone H3-D. Four triploid clones can be identified based on their distinct sets of haplotypes. The largest triploid clone (12 populations sampled) possesses haplotypes *b, e,* and *n,* and occupies a territory restricted to the eastern side of the Appalachian Mountains ranging from Pennsylvania to Quebec (eastern clone; Figs. 2, 4). The second largest clone (four populations sampled) is characterized by the presence of haplotypes *d, f,* and *l,* and is located west of the Appalachian Mountains from New York state to southwestern Quebec (western clone; Figs. 2, 4). The third clone identified by the network is represented by a single individual from Wisconsin that possesses haplotypes *e* and *m* (Wisconsin clone; Figs. 2, 4). A fourth clone, the Atlantic clone, is also represented by a single individual and possesses the haplotypes *b* and *n* (Atlantic clone; Figs. 2, 4) and differs from the eastern clone only in lacking the *e* allele. All triploid individuals are heterozygous for the H3-D data, although not all at the same level. Only two alleles were found in the Wisconsin and Atlantic clones, whereas the eastern and western clones both have three different alleles at the histone H3-D locus according to the network.

DISCUSSION

Multiple Origins of Autotriploidy

Both the network analyses and the SSCP revealed four different triploid clones. The network indicates that the eastern, western, and Wisconsin clones possess distinct sets of haplotypes. Because these haplotypes are each separated by several steps on the network, it is unlikely that one clone evolved by mutation from another one. More importantly, none of these triploid haplotypes is directly derived from another triploid haplotype, supporting the hypothesis of multiple independent origins. The eastern and Wisconsin triploid clones share one allele, but the other alleles found in these clones are different and not closely related suggesting that they must have evolved independently. Evidence from the network for a fourth triploid origin of the Atlantic clone is less clear because it differs from the eastern clone only by the absence of haplotype *e.* However, the SSCP analysis clearly shows that it possesses a different genotype, suggesting that the Atlantic clone must possess a third allele that was not sampled by the cloning procedure, even though nine clones were sequenced for this individual. Band isolation on the SSCP gel also failed to give a good candidate for the missing allele. The absence of this allele makes it uncertain whether this clone represents a distinct triploid origin or whether it evolved by mutation from the eastern clone (the missing allele could have diverged from allele *e* of the eastern clone). Regardless, our study presents clear evidence for at least three distinct origins of autotriploidy in *A. americana*. This represents a conservative evaluation since it is likely that a more intensive sampling regime would reveal further triploid clones and more independent origins, particularly in western and Atlantic parts of the range, where our sampling is limited.

Concordant with previous studies, the histone H3-D in *A. americana* also appears to be in single copy since no diploid and triploid was found with more than two and three alleles, respectively. The strong morphological similarity between diploid and triploid of *A. americana* (they are undistinguishable), as well as the present histone data, support the hypothesis that *A. americana* triploids originated through autopolyploidy. This is supported by both the haplotype network and the branch-and-bound analyses that show that *A. americana* haplotypes belong to a clade distinct from that of *A. priceana*. However, because no other species were included in the study and because phylogenetic relationships within the genus *Apios* are uncertain, complete lineage sorting cannot be confirmed, nor can we ascertain that no other species are involved in the formation of triploid *A. americana*; but the results do not contradict either of these hypotheses. All other species of the genus are located in Asia lending further support to an autopolyploid rather than allopolyploid origin of *A. americana* triploids.

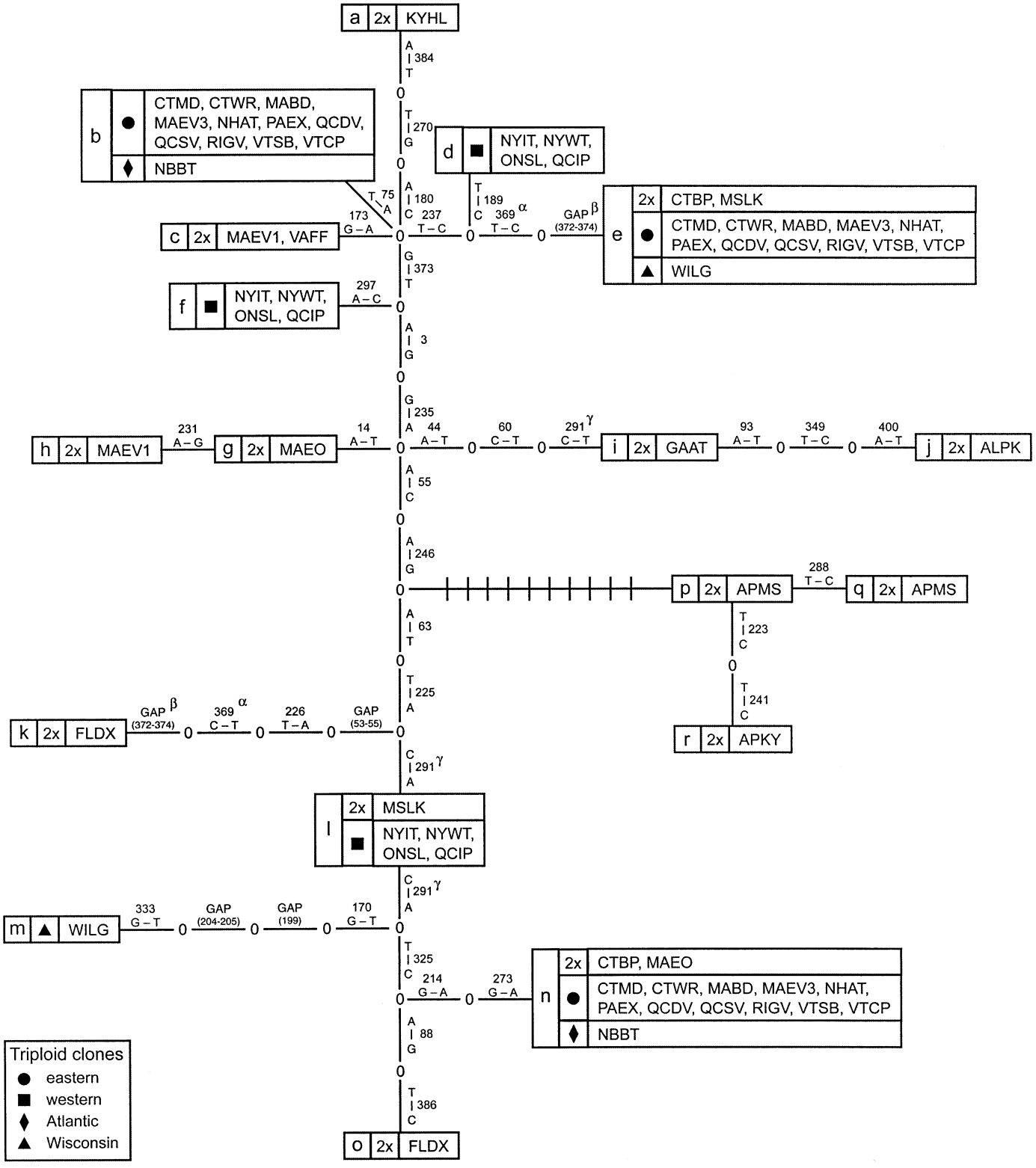

FIG. 4. Haplotype network of *Apios americana* and *A. priceana* accessions obtained with the single-copy histone H3-D locus via statistical parsimony analysis. Haplotypes (*a–r*) for each individual are indicated, as are the ploidy levels (2×) and the triploid clones (filled forms). Zeros along the network branches represent missing haplotypes. Branches represent mutations; the position and nature of the mutations are indicated. The relative positions of mutations between nodes (i.e., along a given branch) are arbitrary. Greek letters indicate homoplasious mutations on the network. The limit of parsimony calculated by the algorithm is eight steps. Therefore, the relationship between haplotypes of *A. americana* (*a–o*) and *A. priceana* (*p–r*) was evaluated using standard phylogenetic analysis. The two subnetworks are separated by 10 nonhomoplasious mutations shown as bars along the branch to indicate that it has not been reconstructed via the same method as for the rest of the network.

Sources of Genetic Diversity

Because autotriploid individuals of *A. americana* are generally sterile (Seabrook and Dionne 1976; Bruneau and Anderson 1988), they cannot rely on segregation and recombination to combine beneficial traits in a single genome to increase their fitness. The only possibility for creating new genotypes in such lineages is either by mutation or via multiple origins. Although somatic mutations have been reported in certain clonal plants (e.g., King and Schaal 1990; Corradini et al. 2002), our analyses suggest that they may not be important in *A. americana*. It is possible that the Atlantic clone (NBBT) originated from the eastern clone by somatic mutation, but there are otherwise no examples of mutation in the H3-D locus following the formation of a triploid clone. In contrast, multiple origins of triploidy clearly enhance the global genetic diversity in autotriploid individuals of *A. americana*. This variability increases the likelihood of forming successful triploid genotypes that could adapt to new environments, and gives the opportunity for autotriploids to be better fit than their diploid progenitors in these new habitats (Mooney and Johnson 1965). This also should be true for other plant species in which multiple origins of autotriploidy have been documented (Haufler et al. 1985; van der Hulst et al. 2000; Takamiya et al. 2001). Indeed, our study showing multiple origins of autotriploidy, as well as others suggesting an important role of triploidy in tetraploid formation (Harlan and deWet 1975; deWet 1980; Haufler et al. 1985; Ramsey and Schemske 1998), suggest that triploidy may play an important role in plant evolution, given the preponderance of polyploidy in flowering plants in general (Stebbins 1950; Grant 1981; Masterson 1994; Otto and Whitton 2000). The frequency of formation of triploidy in *A. americana*, as for other species for which multiple autotriploid origins have been described, also indicates that autopolyploidy and triploidy may be present in many unsuspected plant species.

Diploid Progenitors

Nuclear haplotype networks have the advantage over competing methods (e.g., isozyme data, morphological analysis, chloroplast DNA) that in addition to evaluating the number of recurrent origins of polyploids, they can be used to determine their progenitors (Doyle et al. 2002). In natural populations, it is likely that the individuals that gave rise to the triploids no longer exist today. Nevertheless, shared alleles between cytotypes may be indicative of the geographical origin of the triploid clone. In our analysis, diploids and triploids sometimes share identical alleles. The allele shared between cytotypes sometimes has a restricted distribution, as for haplotype *n*, which is found in the eastern and Atlantic triploid clones and which also is present in diploids from Massachusetts (MAEO) and Connecticut (CTBP). In contrast, others have a widespread distribution, such as haplotype *e*, which is present in the eastern and Wisconsin triploid clone and in diploids from Connecticut (CTBP) and Mississippi (MSLK). This latter individual (MSLK) also possesses an allele (*l*) in common with the western triploid clone. Determining whether range expansion or gene flow is the cause of the wide geographic distribution of these alleles in *A. americana* would require intensive population sampling, which is beyond the scope of this study. Nonetheless, the presence of shared alleles between the two cytotypes (alleles that differ among the triploid clones sampled) supports the hypothesis that triploids are of distinct origins. It does not, however, suggest precisely from which diploid population these triploid clones evolved.

The success of autopolyploids also has been thought to rely in part on their mode of formation and consequently, on the nature of their progenitors. For example, Stebbins (1980) suggested that only autopolyploids formed via unreduced gametes from genetically distinct individuals, rather than from either unreduced gametes from a single individual or from somatic chromosomal doubling, have evolutionary potential. Given that all triploids investigated in this study are heterozygous and that all have three distinct alleles with the exception of the Wisconsin clone (and this also could be attributed to an unsampled allele), the histone data support Stebbins' hypothesis that these viable triploids were formed via unreduced gametes between genetically distinct individuals. This may be another way by which triploid individuals can increase their evolutionary potential. Since they must reproduce vegetatively, heterozygosity present at the time of formation is ''fixed'' in a lineage. Therefore, in a way similar to that of fixed heterozygosity at homeologous loci in allopolyploids and that of the increased genetic diversity of autotetraploid taxa due to tetrasomic inheritance, autotriploids may take advantage of the heterosis effect resulting from their potential fixed heterozygosity. Haufler et al. (1985), in an investigation of *Cystopteris protrusa*, revealed that all sampled triploid clones were heterozygous for all allozymes tested. Similarly, not only are all triploid *A. americana* heterozygous for the H3-D loci, but almost all triploid clones possess three distinct alleles. Such levels of heterozygosity could certainly provide an advantage to triploid *A. americana* over diploid individuals. Two-thirds of the diploid *A. americana* sampled in this study also show evidence of heterozygosity as determined from both SSCP and network analyses. Overall, high levels of heterozygosity in diploids and triploids support the notion that *A. americana* is an outcrossing species, possibly with a partial self-incompatibility system as suggested by Bruneau and Anderson (1988).

Cytogeography and Phylogeography

Flow cytometry analyses were clearly useful in discerning diploid and triploid individuals, and provide a good alternative to chromosome counts, which are not easily obtained for this species (Bruneau 1986). The results reported here on ploidy levels of different individuals of *A. americana* are concordant with previous cytogeographic studies of this species (Seabrook and Dionne 1976; Bruneau 1986). Even though the two cytotypes are sympatric for a small portion of the range of this species, from Massachusetts to Pennsylvania, only one mixed cytotype population was found. The only other known population containing both cytotypes was located in Connecticut (Bruneau 1986), but no longer exists due to urban development (S. Joly, pers. obs. 2000).

The absence of tetraploids in *A. americana* is peculiar, but is in agreement with previous cytological studies for this species (Seabrook and Dionne 1976; Bruneau 1986). Al-

though further investigation could eventually reveal tetraploid individuals, their absence to date is striking especially given that triploids often are seen as ''bridges'' in the formation of tetraploids (Harlan and deWet 1975; deWet 1980; Ramsey and Schemske 1998). Following this model, triploids are more likely to form unreduced gametes because of their meiotic instability, which increases the probability of tetraploid formation. The paucity of mixed cytotype populations could reduce the probability of tetraploid formation since backcrossing of triploid to diploid no longer is a possibility (Ramsey and Schemske 1998). The allogamous nature of *A. americana* also reduces the probability of tetraploid formation because the triploid bridge has been shown to be less important in allogamous compared to autogamous species (Ramsey and Schemske 1998). A confounding factor is also the late flowering of *A. americana*, which may reduce the probability for viable seed production, especially in the northern part of the range where even diploid individuals may not set fruits, and where triploids predominantly are found (Bruneau and Anderson 1988).

Triploid individuals of *A. americana* are mainly located within regions formerly covered by ice during the Wisconsinan glaciation, which had its maximum expansion 18,000 years ago, whereas diploid populations are almost completely excluded from this area. Similar cytogeographic patterns have been reported in other polyploid species (Stebbins 1950; Favarger 1967; Ehrendorfer 1980; Soltis 1984). Selection hypotheses would imply that this cytogeographic pattern is either the result of an adaptation by polyploids to harsh habitats (Lewis 1980; Levin 1983) or the consequence of a better colonizing ability of polyploids (Stebbins 1950; Ehrendorfer 1980). The latter hypothesis involves glaciation as an important factor in structuring cytotype distribution, but glaciation need not be implicated. In fact, a latitudinal distribution of cytotypes is a pattern common to many but not all polyploids (Lewis 1980), with the polyploids being located either north or south of their diploid progenitors. In contrast, a neutralistic hypothesis would favor colonization by triploids following glaciation due simply to their proximity to open habitats. Determining whether selection or postglaciation colonization played a role in structuring cytotypes in *A. americana* requires intensive ecological investigations, which are not available for *A. americana*.

Our results, principally the distribution of the western and eastern triploid clones, clearly show that the triploid clones are clustered geographically (Fig. 3). The distribution of the triploid clones also may give some insight on possible phylogeographic patterns in eastern North America. For example, the segregation of the western and eastern triploid clones on either side of the Appalachian Mountains suggests that no genetic exchange occurred over this geographical barrier and that colonization after the retreat of the ice following the last glaciation may have followed separate migration routes on either side of these mountains. The barrier to gene flow caused by the Appalachian Mountains has other biological consequences since the western and eastern triploid clones of *A. americana* have been reported to differ in morphological characteristics. The western triploid clone has dark-colored flowers and strong shoot pubescence, whereas the eastern clone has light-colored flowers and weak shoot pubescence (Seabrook 1973; Bruneau 1986). Although flower color may vary under different growing conditions (S. Joly, pers. obs. 2001), shoot pubescence seems reliable as an indicator for differentiating triploid clones.

In conclusion, results show that triploid *A. americana* are distributed in more northern latitudes than diploids, a pattern that could be explained either by differential selection between cytotypes or by postglaciation colonization. At least three distinct origins of autotriploidy are reported for *A. americana* based on a nuclear haplotype network and on SSCP analysis of the histone H3-D gene, and our results suggest high levels of heterozygosity for both diploids and triploids. Multiple origins of triploidy and high levels of heterozygosity suggest that, in contrast to the traditional concept of autotriploidy as maladaptive, autotriploid taxa should be seen as genetically variable, with latitude for adapting to new or variable environments. This study also demonstrates the potential of nuclear haplotype networks applied to evolutionary studies at the species level such as autopolyploidy, and gives another taxonomic level for which the histone H3-D gene can be useful.

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