Persistent Nuclear Ribosomal DNA Sequence Polymorphism in the *Amelanchier* Agamic Complex (Rosaceae)

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Individual plants of several *Amelanchier* taxa contain many polymorphic nucleotide sites in the internal transcribed spacers (ITS) of nuclear ribosomal DNA (nrDNA). This polymorphism is unusual because it is not recent in origin and thus has resisted homogenization by concerted evolution. *Amelanchier* ITS sequence polymorphism is hypothesized to be the result of gene flow between two major North American clades resolved by phylogenetic analysis of ITS sequences. Western North American species plus *A. humilis* and *A. sanguinea* of eastern North America form one clade (A), and the remaining eastern North American *Amelanchier* make up clade B. Five eastern North American taxa are polymorphic at many of the nucleotide sites where clades A and B have diverged and are thought to be of hybrid origin, with *A. humilis* or *A. sanguinea* as one parent and various members of clade B as the other parent. Morphological evidence suggests that *A. humilis* is one of the parents of one of the polymorphic taxa, a microspecies that we refer to informally as *A.* "erecta." Sequences of 21 cloned copies of the ITS1–5.8S gene–ITS2 region from one *A.* "erecta" individual are identical to *A. humilis* sequence or to the clade B consensus sequence, or they are apparent recombinants of *A. humilis* and clade B ITS repeats. *Amelanchier* "erecta" and another polymorphic taxon are suspected to be relatively old because both grow several hundred kilometers beyond the range of one of their parents. ITS sequence polymorphisms have apparently persisted in these two taxa perhaps because of polyploidy and/or agamospermy (asexual seed production), which are prevalent in the genus.

Introduction

Phylogenetic studies based on nrDNA ITS sequences have provided novel insights into plant evolution and hybridization (Baldwin et al. 1995; Sang, Crawford, and Stuessy 1995; Wendel, Schnabel, and Seelanan 1995*a*; Buckler and Holtsford 1996*a*, 1996*b*). NrDNA is phylogenetically useful in part because of sequence homogeneity among repeats within the same species (Hillis and Dixon 1991; Baldwin et al. 1995). This homogeneity is attributed to concerted evolution, a process that leads to greater similarity among members of a repeated family within a species than among species (Dover 1982; Arnheim 1983).

We used ITS sequences for phylogenetic inference in *Amelanchier,* small trees and shrubs of the North Temperate Zone that are commonly called shadbushes or serviceberries (Jones 1946). We uncovered extensive ITS sequence polymorphism within individuals of several eastern North American *Amelanchier* taxa. This finding contrasts with homogenization among repeats within individuals of most examined plants (Baldwin et al. 1995). Within-individual nrDNA polymorphisms may occur when concerted evolution is not fast enough to homogenize repeats in the face of high rates of mutation and/or recent interspecific hybridization. Concerted evolution may also be disrupted by loss of sexual recombination or location of nrDNA loci on nonhomologous chromosomes.

Eastern North American *Amelanchier* forms an agamic complex, an array of phenotypically similar entities (microspecies) that appear to have been created by hybridization and perpetuated by agamospermy (Grant 1981, pp. 434–461). Hybridization occurs between most *Amelanchier* taxa, creating hybrid swarms and possibly spawning new taxa (Fernald 1950, pp. 760–767; Cruise 1964; Landry 1975; Weber and Campbell 1989; Campbell and Wright 1996). Agamospermy has been documented in six of the seven taxa that have been studied (see Campbell and Wright 1996) and is facultative; meiosis is mostly bypassed in the formation of seeds but some are produced sexually. All *Amelanchier* agamosperms for which a chromosome number is known are tetraploid.

In this report we postulate that hybridization created much of the ITS sequence polymorphism in *Amelanchier* individuals and that agamospermy and/or polyploidy may have been responsible for the apparent retardation of concerted evolution. Our primary focus here is on the origin and maintenance of this polymorphism, especially in the undescribed taxon *Amelanchier* "erecta".

Abbreviations: ITS, internal transcribed spacer; nrDNA, nuclear ribosomal DNA.

Key words: *Amelanchier,* agamospermy, hybridization, nuclear rDNA internal transcribed spacer (ITS), phylogeny reconstruction, polymorphism.

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Materials and Methods

Plant Samples

We used single-individual samples of 26 accessions of *Amelanchier,* including 19 taxa from eastern North America (taxa 1–19, table 1), five from western North America (taxa 20–24), and the Asian *A. asiatica* (taxon 25). Our sample contains three apparently undescribed species (taxa 5, 6, and 17, table 1) that we refer to informally as *A.* "dentata," *A.* "erecta," and *A.* "seroti-

Table 1
Taxa of *Amelanchier*[a] and Two Outgroups Used in this Study, Their Reproductive Status, and Their Origin

Taxon	Reproductive Status[b]	Origin[c]	Accession
1. *A. arborea* (Michx. f.) Fern.	C[d]	Maine	Campbell, 91-1
2. *A. bartramiana* (Tausch) Roemer	I, S, D	Maine	Campbell, B3
3. *A. bartramiana* × "dentata"	H	Quebec	Campbell, 91-38
4. *A. canadensis* (L.) Medicus	C, A, T	Maine	Campbell, 91-51
5. *A.* "dentata"	H	Quebec	Campbell, 91-39
6a. *A.* "erecta"	C, A, H, T	Maine	Campbell, BP1
6b. *A.* "erecta"	H	Maine	Campbell, 95-3
7. *A.* "erecta" × *laevis*	A, H	Maine	Campbell, 95-1
8. *A. fernaldii* Wieg.	H	Quebec	Campbell, 91-46
9. *A. humilis* Wieg.	D/T	Vermont	Campbell, 95-10
10. *A. intermedia* Spach	H, D/R	Maine	Campbell, DB25
11. *A. laevis* Wieg.	C, A, T	Maine	Campbell, L11
12. *A. lucida* Fern.	U	Nova Scotia	Dibble, 3473
13. *A. nantucketensis* Bick.	C, A, T	Massachusetts	Dibble, 2907
14. *A.* × *neglecta* (Eggelst.) Eggelst.	C, A, H, T	Maine	Campbell, H30
15. *A. quinti-martii* Louis-Marie	H	New Brunswick	Dibble, 3515
16. *A. sanguinea* (Pursh) DC.	C[d]	Vermont	Campbell, 95-13
17. *A.* "serotina"	H	Maine	Campbell, 91-28
18. *A. stolonifera* Wieg.	R	Maine	Campbell, DB78
19. *A. wiegandii* Nicls.	H, T	Quebec	Campbell, 91-40
20. *A. alnifolia* (Nutt.) Nutt.	T	WNA[e]	AA,[f] 1167-74
21. *A. cusickii* Fern.	U	WNA	AA, 1753-81
22. *A. florida* Lindl.	T	WNA	AA, 820-76
23. *A. pumila* Nutt.	U	Colorado	AA, 1321-81
24. *A. utahensis* Koehne	U	Colorado	Campbell, 91-48
25. *A. asiatica* (Sieb. & Zuc.) Endl.	D	South Korea	AA, 510-87
26. *Malacomeles denticulata* (Kunth) Engler	U	Mexico	T17M-96s[g]
27. *Peraphyllum ramosissimum* Nutt.	D	Colorado	Campbell, 91-49

[a] Following species circumscription in Phipps et al. (1990), except numbers 5, 6, 12, 13, 15, and 17.

[b] A = agamospermous (from Campbell and Wright 1996); C = self-compatible (producing fruit after self-pollination); H = putative hybrid taxon; I = self-incompatible (not producing fruit after self-pollination); D = diploid, R = triploid, T = tetraploid (chromosome count in Campbell and Wright 1996 or references in Campbell, Greene, and Dickinson 1991); U = no data available about reproductive status.

[c] State (United States), province (Canada), or country (taxa 25 and 26).

[d] Unpublished data from pollinator exclusion bags.

[e] Precise geographic location unknown beyond western North America.

[f] Arnold Arboretum.

[g] Supplied by Yucca Do Nursery, Waller, Tex.

na." The first two undescribed taxa are morphologically similar to but consistently differ from *A. sanguinea* and *A. humilis* (Campbell and Wright 1996), respectively.

Our sample includes six taxa that have been considered to be hybrids (taxa 3, 7, 10, 14, 15, and 19, table 1). The origins of three putative hybrids—*A. bartramiana* × "dentata" (taxon 3, table 1), *A.* "erecta" × *laevis* (taxon 7, table 1; Campbell and Wright 1996), and *A.* × *neglecta* (taxon 14, table 1; *A. bartramiana* × *laevis*; Weber and Campbell 1989)—seem clear because all three occur with, and are morphologically intermediate between, the parents. Evidence for a hybrid origin is less compelling for *A. intermedia* (taxon 10, table 1; *A. canadensis* × *laevis*) and *A. wiegandii* (taxon 19, table 1; *A. arborea* × *sanguinea*; Landry 1975). The parentage of *A. quinti-martii* (taxon 15, table 1) is somewhat controversial; there is agreement that A. *bartramiana* is one parent, but both *A. arborea* (Lalonde 1957) and *A. humilis* (Louis-Marie 1960) have been proposed as the other parent.

For outgroups, we used single-individual samples of *Malacomeles* and *Peraphyllum,* which are morphologically tied to *Amelanchier* (Jones 1946, p. 14) and which ITS sequence data showed to be sister taxa to *Amelanchier* (Campbell et al. 1995). Voucher specimens of all samples are in the University of Maine herbarium.

Total genomic DNA was isolated from leaves using the 2 × CTAB procedure of Doyle and Doyle (1987). Most DNAs were further purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients (Sambrook, Fritsch, and Maniatis 1989).

PCR and DNA Sequencing

Polymerase chain reaction (PCR) amplification, direct sequencing of both ITS1 and 2 and 69 nucleotides at the 3′ end of the 5.8S gene in genomic DNA, and identification of the boundaries of the ITS regions and coding sequences follow Campbell et al. (1995). All ITS sequences were aligned manually. Sequences for several taxa were obtained using an ABI 373 automated sequencer (Applied Biosystems, Inc., Foster City, Calif.). Resulting chromatograms were manually edited using the software Sequence Navigator 1.0 (Applied Biosystems, Inc., Foster City, Calif.). Manual, cycle, and automated sequencing of genomic DNA of one individual of *A.* "erecta" (accession Campbell BP1, table 1) all yielded identical sequences. Automated sequences of two individuals of *A.* "erecta" from the same site (6a

and b, table 1) are identical. Sequencing was performed in both directions for all genomic DNAs. Pairwise sequence divergence was calculated in PAUP 3.1.1 (Swofford 1993) as mean distance between sequences. In the DNA format, sequence polymorphisms are treated as equivalent to any base of that polymorphism. For example, the distance from a nucleotide site with an A/C polymorphism to a site with A or to a site with C is 0. Sequences used in this study are available in the GenBank Libraries (accession numbers U16193 for *Malacomeles,* U16197 for *Peraphyllum,* U151591 for *A. bartramiana,* and U71156–U71179 for the remaining *Amelanchier* in table 1).

To further characterize intragenomic variation we sequenced 21 clones from a single *A.* "erecta" individual. PCR-amplified DNA of the ITS1–5.8S–ITS2 region from *A.* "erecta" (taxon 6a, table 1) was ligated into the pCRII cloning vector in the TA cloning kit according to instructions of the manufacturer (Invitrogen Co., San Diego, Calif.), and the resulting recombinant plasmids were used to transform competent cells provided with the kit. The transformation mixture was incubated in SOC medium at 37°C with agitation and plated on LB agar with ampicillin (50 μg/ml) and X-Gal (25 μg). White colonies were selected for growth, and plasmid DNA was isolated according to an alkaline lysis miniprep protocol (Sambrook, Fritsch, and Maniatis 1989). The ITS1–5.8S–ITS2 region was digested from the plasmid with *Eco*RI, gel-isolated, and PCR-amplified. ITS1 and ITS2 were sequenced separately and in one direction by dsDNA cycle sequencing following instructions of the manufacturer (Gibco BRL, Gaithersburg, Md.).

We analyzed the association between ITS sequence polymorphism and agamospermy, polyploidy, hybridization, and the phylogenetic information of nucleotide sites. We made the following comparisons of levels of polymorphism: (1) in *A. bartramiana,* the only studied taxon in which there is evidence of predominant sexuality, with those in taxa in which agamospermy has been documented (see table 1); (2) in diploids and polyploids (see table 1); (3) in accessions that are clearly hybrids (taxa 3, 7, and 14, table 1) with those in accessions that have been hypothesized to be of hybrid origin (taxa 10, 15, and 19, table 1) and accessions that have not previously been hypothesized to be of recent hybrid origin (all remaining *Amelanchier* in the data set); and (4) at autapomorphic, phylogenetically informative, and variable but neither autapomorphic nor informative nucleotide sites (i.e., with one nucleotide type and at least one polymorphism involving that nucleotide type).

Phylogenetic Analyses

Phylogenetic relationships were reconstructed using parsimony as implemented in PAUP. We performed heuristic searches, with 10 replications of RANDOM addition of taxa and TBR (tree bisection-reconnection) branch swapping on the full data set and branch-and-bound searches on data sets with 11 taxa. Bootstrapping, used as an index of support for individual clades, was implemented in PAUP using heuristic searches of 100 CLOSEST taxon-addition sequences. We employed decay indices for another perspective on the robustness of individual clades. Decay indices were computed by heuristic searches, with CLOSEST taxon-addition sequences, for trees one or more steps longer than the most parsimonious trees, with each set of trees of one length summarized by semistrict consensus.

We removed putative hybrids (taxa 3, 5–8, 10, 14, 15, 17, and 19, table 1) from some phylogenetic analyses. We switched the data format in PAUP from DNA to symbols, so that polymorphisms would be recognized as distinct character states, and then entered possible *Amelanchier* hybrids into the analysis individually and in groups to examine their location relative to the parents and their impact on tree topology. We included some ITS clones of *A.* "erecta" as separate "taxa."

Results

ITS Sequence Polymorphism

Polymorphism for nucleotide states at a site, spread more or less uniformly over ITS1 and ITS2, is a conspicuous feature of many *Amelanchier* ITS sequences (fig. 1 and table 2). In direct sequences of genomic DNA, roughly equal band intensity of two or more nucleotide states suggests superimposition of two or more repeat types in approximately equimolar proportions. One hundred seventy-three total polymorphisms comprise 1.4% of all the sites for all 25 taxa of *Amelanchier.* Polymorphisms occur at 62 variable sites (fig. 1), 14 of which are autapomorphic, 19 phylogenetically informative, and 29 variable but neither autapomorphic nor phylogenetically informative. Eleven polymorphisms make up 2.8% of all nucleotides for the 25 *Amelanchier* taxa at the autapomorphic sites; 103 polymorphisms account for 21.7% of all nucleotides at phylogenetically informative sites; and 59 polymorphisms make up 8.6% of all nucleotides at the variable but neither autapomorphic nor phylogenetically informative sites.

Polymorphism is especially concentrated at the 17 sites distinguishing the majority of clades A and B (see section below on ITS phylogeny of *Amelanchier*). The frequency of polymorphism is 21.9% at these sites, but only 0.6% at phylogenetically uninformative sites (table 2). Polymorphism at these potentially informative sites is lower in clade A taxa (mean of 3.3%) than in clade B taxa (mean of 30.4%), where it equals or exceeds 25% in seven taxa. The percentage of polymorphic sites across these 17 nucleotide positions ranges from 0 (e.g., *A. bartramiana, A. canadensis,* A. *laevis,* and *A. stolonifera*) to 100 in *A.* "dentata" and *A.* "erecta" (table 2). When one or more of these 17 is not polymorphic in eastern North American taxa, it is the clade B repeat type nucleotide that consistently appears in direct sequences of genomic DNA (fig. 1).

Polymorphism is not clearly associated with agamospermy or polyploidy. ITS sequences of tetraploid agamosperms *A. laevis, A. canadensis,* and *A. nantuckетensis* (table 1) are not more polymorphic than those of sexual, diploid *A. bartramiana* (table 2). The impact of hybridization on polymorphism depends on the sequence divergence of the parents. When parents have

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                                                  I*vIvvvvvvv**Iv*vI*vvvI*vIIvvIIvvvvIv*v*vvII*vvI***I*IvIII*vvI
                                                                                                  11111111111111223333234444444455555555555
                                                  11112344556678888999901112477899999001233340445689903456667778
                                                  23691235371460124345974799334734568287901240191908965313792691
1. arborea                                        CAAAGCCGGCCTTCCTTCYCCCACCTTGGCTGCGCGACAGTTTCCCCRGAATTTGTTCTTRC
2. bartramiana                                    CAAAGCYGGCCTTCCTTCYCCCACCTCGGCTGCGCGACAGTTTCACCGGAATTTGTTCTTGC
3. bartramiana X "dentata"                        CAARRCYGGCCTTCCTTYYCCCACCTCGGMTGCGCKACAGTTKYMCCRGAAYTYGYYYTTGS
4. canadensis                                     CAAAGCCGGCCTTYCTTCCCCCACCTTGGCTGYGCGACAGTTTCCYYGGAATTTGTTCTTGC
5. "dentata"                                      MAARRCCGGCCTTCCTTYCMCCRCCYYGGMYGCGCKACAGTTKYCCCRGAAYTYGYYYTTGS
6. "erecta"                                       MAARRCCGGCCTTCCTTYCMCCRCCYHGGMYGCGCKACAGTYKYCCCRGAAYTYGYYYTTGS
7. "erecta" X laevis                              MAAARYCGKCCTTCCTTYCCCCACCTTRRMTGCGCKACAGTTTCCCCRGAATTTGTTCTTGC
8. fernaldii                                      CAAAGCCGGCCYTCCTTCCCCCACCTYGGCTGCGCKACAGTTTCCCCRGAATTTGTTCTTGC
9. humilis                                        AAAGGCCKGCMTTCCTTTYCCCGCCCAGGACGCGCTACAGTTGTCCCAGAACTCKCCTTTGG
10. intermedia                                    CAAAGCCGGCCTTCCTTCCCCCACCTTRRCTGCGCGACAGYTTCCCCGGAATTTGTTCTTGC
11. laevis                                        CAAAGYCGKCCTTCCTTCCCCCACCTTRRCTGCGCGACAGTTTCCCCGGAATTTGTTCTTGC
12. lucida                                        CAAAGCCGGCCTTYCTTCCCCCACCTTGGCTGYGSGACAGTYTCCCCGGAATTTGTTCTTGC
13. nantucketensis                                CAAAGCCGGCCTTTCTTTCCCCCACCTTGGCTGYGCGACAGTTTCCCCGGAATTTGTTCTTGC
14. X neglecta                                    CAAAGYYGKCCTTCCTTCYCCCACCTYRRCTGCGCGACAGTTTCMCCGGAATTTGTTCTTGC
15. quinti-martii                                 CAARRCCGGCCTTCCTTYCCCCRCCTYGGMTGCGCGACAGTTTCMCCRGAATTTGTTCTTGS
16. sanguinea                                     AAAGGCCKGCCTTCCTTTCCCCGCCCCGGACGCGCTACAGTTGTCCCAGAACTCGCCTTTGG
17. "serotina"                                    CAARRCCGGCCTTYCTTYCMCCACCTTGGCTGYGCKACAGTTKYCCCRGAAYTYGYYYTTGS
18. stolonifera                                   CAAAGCCGGCCTTTCTTCCCCCACCTTGGCTGCGCGACAGTTTCCCCGAAATTTGTTCTTGC
19. wiegandii                                     MAARRCCGGCCTTCCTTYCMCCRCCYYGGMTGCGCGACAGTTTYCCCRGAAYTYGYYYTTGS
20. alnifolia                                     AARGGCCGGSCTTCCTTTCMCCGCCCMGGACRCRCTACAGTTGTCCCAGAACTCGCCTTTGG
21. cusickii                                      AAAGRCCGGCCTTCCTTTCCCCGCCCCGGACGCGCTACAGTTGTCCCAGAAYTCGCCTTTGG
22. florida                                       AAAGRCCGGCCTTCCYTTCCCCGCCCCGGRCGCGCTATAGTTGTCCCAGAAYTCGCCTTTGG
23. pumila                                        AAARRCCGGCCTCCYTYTCCCCGCCCCGGACGCGCTACAGTTGTCCCAGAATTCGCCTTTGG
24. utahensis                                     AAAAGCCGGCCTTCCTTTCCCCGCCCCGGACGCGCTACMATTGTCCCAGAATTCGCYCTYGG
25. asiatica                                      CGAAGCCGGCCCTCCCTCTYYRGAYCCGGCTGCGCGRCARTTTCCCCGGGGTCTGTTCCTGC
26. MALACOMELES                                   CAAAGCTGGCCTTCTTTTCCCTGCCTCGACTGCGCGACAGTTACCCCAAAATTTGTTCTGTA
27. PERAPHYLLUM                                   CAAAGCTGGCCTTCTTTTCCCTACCTCGGCTGCGCGACAGTTACCCCAGAATTTGTCCGCTT
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FIG. 1.—Variable nucleotide sites for nrDNA ITS1, the 69 bases at the 3′ end of the 5.8S gene, and ITS2 for 25 *Amelanchier* taxa. *Malacomeles* and *Peraphyllum* also vary at other sites. Order of taxa follows that of table 1. Sequence symbols: A, C, G, T = dATP, dCTP, dGTP, dTTP; H = A, C, or T; K = G or T; M = A or C; R = A or G; S = C or G; Y = C or T. Site numbering follows the ITS DNA sequence in figure 2. Symbols above the sites indicate that the site is autapomorphic (*), phylogenetically informative ("I"), or variable but neither autapomorphic nor phylogenetically informative ("v").

equivalent ITS sequences, polymorphism is low; *A. intermedia,* for example, has only three polymorphisms (fig. 1), all at phylogenetically uninformative sites, for a total of 0.6% polymorphism. In contrast, putative hybrids between members of clades A and B, such as *A.* "dentata" and *A.* "erecta," have 4% or more total polymorphism.

Table 2
Percent Within-Individual Polymorphism at ITS-Region[a] Sites that Are Phylogenetically Uninformative and that Are Phylogenetically Informative for Clades A and B in *Amelanchier*

Taxa	Percent Within-Individual Polymorphism		
	Uninformative Sites[b]	Clade A/B Sites[c]	Total[d]
25 *Amelanchier* taxa	0.6	21.9	1.4
Clade A taxa	0.5	3.3	0.7
Clade B taxa	0.6	30.4	1.7
A. bartramiana	0.4	0	0.4
A. bartramiana × "dentata"	0.6	70.6	3.2
A. canadensis	0.6	0	0.8
A. "dentata"	0.4	100	4.0
A. "erecta"	0.6	100	4.2
A. "erecta" × *laevis*	1.0	29.4	2.0
A. fernaldii	0.2	17.6	0.8
A. laevis	0.8	0	0.8
A. quinti-martii	0.4	41.2	1.8
A. "serotina"	0.6	64.7	3.2
A. stolonifera	0	0	0
A. wiegandii	0.4	82.3	3.4
A. × *neglecta*	1.4	5.9	1.6

[a] ITS1, 69 nucleotides at the 3′ end of the 5.8S gene, and ITS2.

[b] There are 475 phylogenetically uninformative sites; these include 29 sites where one or more taxon is polymorphic and 14 autapomorphic sites.

[c] There are 17 phylogenetically informative sites separating the majority of taxa in clades A and B.

[d] There are 494 total sites in the data set.

ITS Sequence Analyses

GC content in ITS1 and ITS2 averages 68% in *Amelanchier,* which is toward the high end of the range recorded for plants (Baldwin et al. 1995). Because alignment of *Amelanchier* sequences is straightforward, only one complete sequence is shown (fig. 2). Within *Amelanchier,* alignment requires introducing five indels: two single-base deletions in *A. alnifolia* (after sites 56 and 488, fig. 2) and, in *A. asiatica,* a single-base deletion (after site 49), a three-base insertion (after site 52), and a single-base insertion (at site 413). Alignment of the two outgroup genera with *Amelanchier* required no indels for *Malacomeles* and a single-base deletion (after site 488) and a single-base insertion (after site 545; fig. 2) in *Peraphyllum.* ITS1 is 212 bp in *Amelanchier* (211 bp in *A. alnifolia,* 214 bp in *A. asiatica*), *Malacomeles,* and *Peraphyllum.* ITS2 is 213 bp for all taxa except *A. alnifolia* (212 bp) and *A. asiatica* (214 bp).

ITS sequences of clade A taxa (see section below on ITS phylogeny of *Amelanchier*) diverge from one another from 0% (*A. alnifolia* and *A. humilis*; *A. alnifolia, A. cusickii,* and *A sanguinea*) to 0.6% (*A. florida* and *A. utahensis*). Clade B taxa sequences diverge from one another from 0% (*A. arborea, A. canadensis, A. laevis,* and *A. lucida*) to 0.8% (*A. bartramiana* and *A. stolonifera*). Ten eastern North American putative hybrids (taxa 3, 5–8, 10, 14, 15, 17, and 19, table 1) are equivalent to one or both parents in ITS sequences. Sequence divergence within *Amelanchier* reaches a maximum of 5.0% between *A. asiatica* and *A. alnifolia, A.*

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ITS 1
TCGAACCTGCAMAGCAGARCRACCC   GAGAACCAGTTTCAACGCCGGGGGT
                                                .100
CGG---CGGGCCTTCGGGCTCGGCG   TCCCCCTGTCCCGGGAGYCMGCTCC

CGGGCGCACAAACRAACACCGGCGC   GTGYTGCGCCAAGGAACHCGAACGA
                                                .200
AAGAGCGCGCTCCCGCCGCCCCGGA   AACGGTGCGCGMGCGGGYGCGTCGT
              5.8S
CKTCTTCAATATGTCAAAACGACTC   TCGGCAACGGATATCTCGGCTCTCG
                                                .300
CATCGATGAAGAACGTAGCGAAATG   CGATACTTGGTGTGAATTGCAGAAT

CCCGTGAACCATCGAGTCTTTGAAC   GCAAGTYGCGCCCAAGCCKTTAGGC
                        ITS 2                   400
CGAGGGCACGCCTGCCTGGGCGTCA   CACGCCGTTGTCCCCCCGCGCCTCY

CTCGGGAGCGTC-GGGGGGCGGAGG   ATGGCCTCCCGTGCGCCACCCCGCG
                                                .500
CGGTTGGCACAAATGCCGRGTCCCC   GGCGGCGAACGCCACGACAATCGGT

GGTTGYCAAACCTCGGTTGCCTGTT   GTGCGCTTTCGCCGCGCYCC-GGGC

GGCTCGCGACGAYCGCYGYTCTGCT   TCGGCSGAGCTTTCAACG
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FIG. 2.—Sequence of the *Amelanchier* "erecta" ITS1–5.8S–ITS2 region of nrDNA from directly sequenced genomic DNA. Sites are numbered from 1 at the 5′ end of ITS1, to the 3′ end of ITS1 (site 215), to the end of the 5.8S gene (site 378), and to the end of ITS2 (site 593). The 21 polymorphic sites (12, 19, 21, 93, 95, 114, 129, 143, 187, 193, 202, 332, 344, 400, 469, 506, 543, 563, 567, 569, and 581) are underlined, and the 17 sites at which clades A and B differ are covered by a line. Sequence symbols are as in figure 1; "–" = gap (for alignment of *A. asiatica* at sites 54–56 and 413 and *Peraphyllum* at site 546).

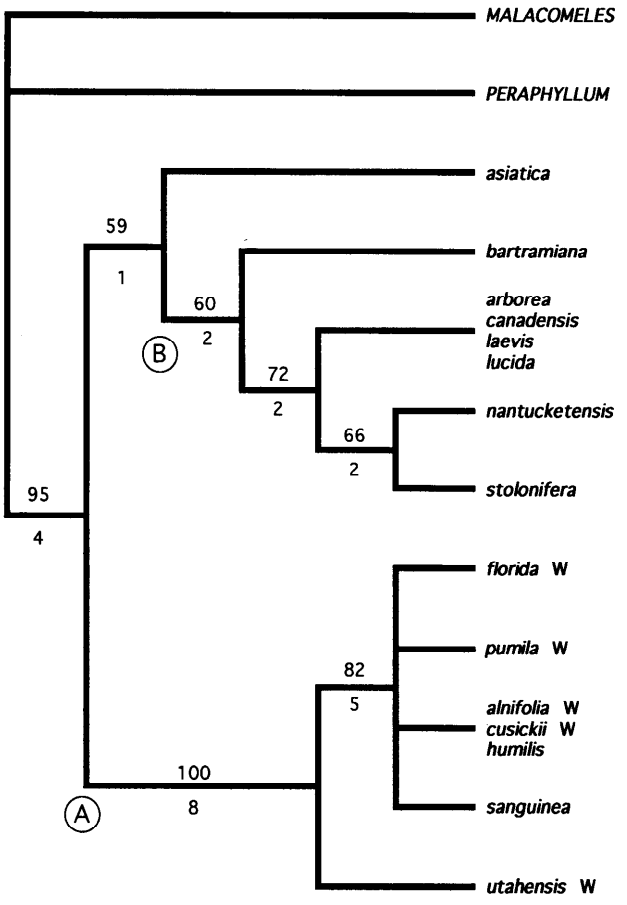

FIG. 3.—Strict consensus of three most parsimonious trees of 59 steps derived from Fitch parsimony analysis (branch-and-bound search) in PAUP 3.1.1 of ITS1, 69 bases at the 3′ end of the 5.8S gene, and ITS2 nrDNA sequences (see fig. 1) for *Amelanchier* species and outgroups *Malacomeles* and *Peraphyllum*. *Amelanchier* taxa not shown here (taxa 3, 5–8, 10, 14, 15, 17, and 19, table 1) are putative hybrids. Species in the *arborea-canadensis-laevis-lucida* group and the *alnifolia-cusickii-humilis* group have equivalent sequences. *Amelanchier* species followed by a "W" grow in western North America. Numbers above branches indicate bootstrap % values for clades found in both strict consensus and bootstrap majority-rule trees. Numbers below branches are decay index values. Clades A and B are indicated by circled letters at the base of the clade. Consistency index = 0.875, and retention index = 0.932.

humilis, and *A. sanguinea.* Sequences of *Peraphyllum* and *Malacomeles* differ from one another by 3.2% and from those in *Amelanchier* by 1.8% (*Peraphyllum* and *A. bartramiana* × "dentata") to 6.0% (*Malacomeles* and *A. humilis*).

ITS Phylogeny of *Amelanchier*

Branch-and-bound searches of taxa that are not obviously of hybrid origin (taxa 1, 2, 4, 9, 11–13, 16, 18, and 20–27, table 1) yield three maximally parsimonious trees of 59 steps (strict consensus shown in fig. 3). Salient features of these trees are: (1) *Amelanchier* is monophyletic, (2) five western North American taxa plus *A. humilis* and *A. sanguinea* form the well-supported clade A, (3) the remaining eastern North American taxa form the weakly supported clade B, (4) relationships within clades A and B are weakly supported or unresolved, and (5) *A. asiatica* is the most divergent species in the genus, with nine autapomorphies (fig. 1). All taxa of clade A differ from all taxa of clade B by 15 substitutions. There is one additional substitution associated with the basal split within each clade. In clade A the additional substitution is a transition at site 569 (fig. 1) on the branch connecting *A. utahensis* and the remainder of the clade (fig. 3). In clade B the additional substitution is a transition at site 143 on the branch connecting *A. bartramiana* and the remainder of the clade. Thus, six of the seven taxa in clade A differ from six of the seven taxa in clade B at 17 sites, including 12 transitions and 5 transversions (table 3).

When the data format in PAUP is switched from DNA to symbols so that polymorphisms are recognized as distinct character states, *A.* × *neglecta* attaches to the tree near *A. laevis* (not shown in fig. 3), in accord with the prediction that hybrids will attach at the base of the clade that includes the most derived parent (McDade 1992). Addition of this hybrid does not radically alter tree topology. Inclusion of other putative hybrids in phylogenetic analysis as well as apparent recombinant *A.* "erecta" clones, however, leaves relationships in the genus more unresolved. However, clade A and the sister group relationship of *A. nantucketensis* and *A. stolonifera* remain after inclusion of hybrids.

ITS Sequences of *A.* "erecta" Clones

Amelanchier "erecta" clones have the nucleotide composition of the majority of clade A taxa (clones 7, 8, and 19, table 3) or clade B taxa (clones 1 and 15). Clones 2, 3, 5, 10, 12, and 21 each contain one or two nucleotides that are exceptions to an otherwise uniformly clade A or B repeat type. Clones 4, 9–14, 16–18, 20, and 22 are apparent recombinants of clade A and B re-

Table 3
Nucleotides of the ITS1–5.8S–ITS2 Region of 21 Clones from One Individual of *Amelanchier* "erecta" at Nucleotide Positions[a] Where Clades A (Nucleotides Underlined) and B (Nucleotides Not Underlined) Differ

	Nucleotide Position																	
	ITS1									|5.8S|		ITS2					
Clone(s)	12	19	93	114	129	143	187	193	202	344	400	469	543	563	567	569	581
7, 19 . . .	A	G	T	G	C	A	A	C	T	G	T	A	C	C	C	T	G
8	A	G	T	G	C	C	A	C	T	G	T	A	C	C	C	T	G
3	A	G	C	G	C	A	A	C	T	G	T	A	C	C	C	T	G
21	A	G	T	G	C	A	A	C	T	G	T	G	C	C	C	T	G
10	C	A	T	G	C	A	A	C	T	G	T	A	C	C	C	T	G
12	C	A	T	G	C	C	A	C	T	G	T	A	C	C	C	T	G
17	C	A	C	A	T	C	G	C	T	G	T	A	C	C	C	T	G
9	A	G	C	A	T	T	C	T	G	G	T	A	C	C	C	T	G
4	C	A	C	A	T	T	C	T	G	G	T	A	C	C	C	T	G
14	C	A	C	A	?[b]	T	C	T	G	G	T	A	C	C	C	T	G
18	C	A	C	A	T	T	?[b]	T	G	G	C	A	C	C	C	T	G
13	T[c]	A	C	A	T	T	C	T	G	T	C	A	C	C	C	T	G
11, 22 . .	A	G	T	G	C	A	A	C	T	T	C	G	T	T	T	C	C
16	A	G	C	A	T	T	C	T	G	G	T	G	T	T	T	C	C
20	C	A	C	A	T	T	C	T	G	G	T	A	T	T	T	C	C
2	C	A	C	A	T	T	C	T	T	T	C	A	T	T	T	C	C
5	C	A	C	A	T	T	C	T	G	G	C	G	T	T	T	C	C
1, 15 . . .	C	A	C	A	T	T	C	T	G	T	C	G	T	T	T	C	C

[a] See figure 2 for base composition and location of these positions in complete sequence of the *Amelanchier* "erecta" genomic ITS1–5.8S–ITS2 region.
[b] Uncertain nucleotide.
[c] Apparent nonrevertant mutation, showing a nucleotide not present in clade A or B.

peats. Clone 4, for example, has the nucleotide composition of clade B at the first nine sites (12 to 202) and then conforms to clade A for the final eight sites (344 to 581). Clone 10 possesses the clade B for nucleotide sites 12 and 19 and has the clade A repeat type for the remainder of the region. Clone 9 is like clade A for the first two marker nucleotides, then switches to clade B nucleotides through site 202, and reverts to the clade A repeat type for the remainder of the region. Some of the apparent recombinations might be due to PCR jumping early in PCR amplification, although this is not likely because no footprint A or T autapomorphies occur at the putative break points (Pääbo, Irwin, and Wilson 1990). Nucleotides at some positions, such as 12 and 19 in clones 9, 10, and 12, could be the result of reverse mutation or represent very local double recombinations or conversion. Two apparent transitions occur at sites not shown in table 3.

The largest section of the ITS1–5.8S–ITS2 region in which there is no nucleotide marker distinguishing clades A and B starts 13 nucleotide positions before the 3′ end of ITS1 (site 202) and extends 129 nucleotide positions into the 5.8S gene (site 344, table 3). This region contains eight possible recombination events (clones 4, 9, 11, 14, 16, 18, 20, and 22), more than any other section between polymorphic sites.

ITS Sequences of Putative Hybrids

Amelanchier × *neglecta* shows the complete additivity of parental genomes expected in an F_1 hybrid. It is polymorphic at the transition (site 143, fig. 1) and transversion (site 441) distinguishing the parents—*A. bartramiana* and *A. laevis*—and at the six sites where one of the parents is polymorphic. Other putative hybrids diverge from this pattern.

Amelanchier bartramiana × "dentata" is polymorphic for parental nucleotides at the one site (441, fig. 1) where the parents differ, at the two polymorphic sites found in *A. bartramiana,* and at 14 of the 20 polymorphic sites of *A.* "dentata." Thirteen of these polymorphisms are at sites differentiating clades A and B. At the other four clade A/clade B sites this putative hybrid shows the nucleotide of *A. bartramiana. Amelanchier* "erecta" × *laevis* has all four polymorphisms of *A. laevis* but only six of the 21 polymorphisms of *A.* "erecta." The *A. laevis* nucleotide appears in *A.* "erecta" × *laevis* at the other 15 sites where *A.* "erecta" is polymorphic. This loss of *A.* "dentata" and *A.* "erecta" polymorphisms may be due to segregation within their hybrid genomes.

ITS sequences of the putative parents of *A. intermedia—A. canadensis* and *A. laevis*—are equivalent, apart from possessing different polymorphisms involving a common state. None of the four *A. canadensis* polymorphisms and only two of the four *A. laevis* polymorphisms appear in our sample of *A. intermedia.* The *A. wiegandii* ITS is polymorphic at one of the four sites where its putative parents—*A. arborea* and *A. sanguinea*—are polymorphic and at 15 of the 17 sites differentiating the parents. It shows the predominant clade B nucleotide at the other two sites.

ITS sequences point to *A. bartramiana* and *A. humilis* as parents of *A. quinti-martii* because its genome

combines the nucleotides of these two species at a site where *A. bartramiana* is autapomorphic (site 441, fig. 1) and at seven other sites where the two species differ. At the other 10 sites where the clade B ITS repeat differs from that of *A. humilis, A. quinti-martii* shows clade B nucleotides.

Amelanchier "dentata," *A.* "erecta," and *A.* "serotina" individuals are all highly polymorphic at nucleotide sites distinguishing clades A and B repeats. *Amelanchier fernaldii* is polymorphic at only three of these sites. These four taxa plus *A. quinti-martii* and *A. wiegandii* therefore are possibly of hybrid origin, with *A. humilis* or *A. sanguinea* as one parent and another eastern North American *Amelanchier* as the other parent.

Hybridization may be responsible for some polymorphism at autapomorphic and at variable but neither autapomorphic nor phylogenetically informative sites. Five polymorphisms at autapomorphic sites 94 and 441 (fig. 1) may be attributable to hybridization involving *A. bartramiana.* At six variable but neither autapomorphic nor informative sites (positions 21, 32, 43, 53, 173, and 174, fig. 1), 14 polymorphisms that occur in four putative hybrids (taxa 3, 7, 10, and 14, table 1) are also present in one of the parents.

Discussion

ITS Polymorphism and Hybridization

In *Amelanchier,* polymorphism at phylogenetically informative ITS sites could simply be the result of high mutation rates at these positions, with mutations accumulating in asexual *Amelanchier,* as in the nrDNA of obligately asexual *Taraxacum officinale* (King and Schaal 1990). But one would then expect a random association of point mutation/polymorphism between species and not the highly nonrandom association observed in *Amelanchier.* Polymorphism could also be ancestral, with nonpolymorphic taxa the product of lineage sorting. This would require the accumulation of 17 linked polymorphisms within a genome, an unlikely scenario given that there is some sexuality in facultative agamosperms. The origin of polymorphism through gene flow between divergent lineages is more plausible and consistent with the high incidence of hybridization in the genus.

If *A.* "dentata" and *A.* "erecta" are hybrids of plants from clade A and plants from clade B, then *A. bartramiana* × "dentata" and *A.* "erecta" × *laevis* are backcrosses to clade B plants. Such backcrossing is corroborated by the observation that our samples of *A. bartramiana* × "dentata" and *A.* "erecta" × *laevis* show the clade B parental ITS repeat nucleotide at the clade A/clade B informative sites where they are not polymorphic (4 sites in *A. bartramiana* × "dentata" and 12 in *A.* "erecta" × *laevis*). Apparent fixation of some polymorphisms to clade B nucleotides in *A. fernaldii, A. quinti-martii, A.* "serotina," and *A. wiegandii* suggest a similar history of backcrossing. These may also have lost some polymorphism through concerted evolution, mutation, preferential PCR amplification of one repeat type, or further hybridization. Putative erosion of polymorphism would appear to be well advanced in *A. fernaldii,* which is polymorphic at only three of the 17 sites distinguishing clade A and B repeat types.

The five most polymorphic eastern North American taxa in our sample—*A.* "dentata," *A.* "erecta," *A. quinti-martii, A.* "serotina," and *A. wiegandii*—may be closely interrelated. *Amelanchier humilis* and *A. sanguinea* have been considered conspecific (Landry 1975) and one of the parents of *A. quinti-martii* and *A. wiegandii* (Lalonde 1957; Landry 1975). The ancestry of *A.* "serotina" is not readily apparent, but ITS polymorphism at 11 of the 17 nucleotide sites distinguishing clades A and B ITS repeats suggests that its history is like that of *A.* "dentata" and *A.* "erecta." It is possible that polymorphisms in these five taxa arose during a small number of original hybridizations and persisted through diversification.

Concerted Evolution, ITS Polymorphism, Polyploidy, and Agamospermy

Variability within multigene families depends upon number of gene copies; rates of mutation, speciation, and concerted evolution; number and chromosomal location of loci; and proportion of sexual and asexual reproduction. Mechanisms of DNA turnover vary in their rate, bias, and size of DNA on which they are effective, depending on chromosome and species (Dover et al. 1993). Concerted evolution is generally highly effective in the nrDNA family, the most broadly studied multigene family (Hillis and Dixon 1991), with rates of about 10^{-2} to 10^{-4} turnover events per kilobase per generation (Dover 1989).

Extensive polymorphism within species is unusual in ITS sequences of angiosperms (Baldwin et al. 1995) and other eukaryotes (Wesson, Porter, and Collins 1992; Vogler and DeSalle 1994). Within-individual polymorphic nrDNA may occur in transition stages of concerted evolution (Strachan, Webb, and Dover 1985); when mutation rate exceeds the rate of concerted evolution, as in length variants in the intergenic spacer (e.g., Appels and Honeycutt 1986; Rogers and Bendich 1987; Schaal, Leverich, and Nicto-Soleto 1987; Jorgensen and Cluster 1988; Crease and Lynch 1991; Bobola, Smith, and Klein 1992; Linares, Bowen, and Dover 1994); as a result of interspecific hybridization (e.g., Sites and Davis 1989; Arnold, Bennett, and Zimmer 1990; Delseny et al. 1990; Rieseberg, Carter, and Zona 1990; Crease and Lynch 1991; Rieseberg 1991; Soltis and Soltis 1991; Kim and Jansen 1994; Sang, Crawford, and Stuessy 1995); when pseudogenes evolve (Buckler and Holtsford 1996*b*); or when location of nrDNA loci on nonhomologous chromosomes potentially disrupts concerted evolution (Appels and Honeycutt 1986; Polans, Weeden, and Thompson 1986; Seperak, Slatkin, and Arnheim 1988; Karvonen and Savolainen 1993; Suh et al. 1993; Jellen, Phillips, and Hines 1994; Vogler and DeSalle 1994).

Reports of extensive, within-plant ITS polymorphism, other than as the product of recent interspecific hybridization (see above), include Winteraceae (Suh et al. 1993), peonies (Sang, Crawford, and Stuessy 1995), conifers (Bobola, Smith, and Klein 1992; Karvonen and

Savolainen 1993), *Zea* (Buckler and Holtsford 1996*a*, 1996*b*), and *Amelanchier*. Sequence divergence between two clones each from five species in three Winteraceae genera was 0–1.4% for ITS1 and ITS2. On the other hand, sequence divergence ranged from 4.7% to 7.0% between two clones each from four species in three other genera. Suh et al. (1993, p. 1054) suggested that the "high polyploid state may provide an opportunity for different arrays of nrDNA to evolve independently."

Five species of *Paeonia* show an additive pattern of nrDNA ITS repeat types that was hypothesized to be the result of hybridization (Sang, Crawford, and Stuessy 1995). The geographic distributions of the putative parents of some of these species—which include three tetraploids, one diploid, and one whose chromosome number is unknown—are distant from these species. For example, both parents of a species of the Mediterranean region grow in eastern Asia. Nine species show partial homogenization of ITS repeats. A long generation time was suggested as a mechanism that might retard concerted evolution in *Paeonia*.

ITS restriction-fragment-length polymorphism within individuals of spruces (Bobola, Smith, and Klein 1992) and Scots pine (Karvonen and Savolainen 1993) was attributed to the large number of nucleolar organizing regions (NORs) in conifers. There are eight NORs per haploid genome in Scots pine, for example.

With the exception of one species of *Paeonia* and *Zea* pseudogenes (Buckler and Holtsford 1996*a*, 1996*b*), all within-individual plant ITS polymorphisms discussed above are associated with polyploidy or multiple NORs. Concerted evolution may not be effective in polyploids, especially allopolyploids, because they are likely to bear nrDNA loci on nonhomologous chromosomes. Turnover does occur among nonhomologous nrDNA loci (e.g., Krystal et al. 1981; Dvořák 1990; Wendel, Schnabel, and Seelanan 1995*a*), but it may be considerably slower than turnover within and between homologous chromosomes (Saghai-Maroof et al. 1984; Appels and Honeycutt 1986; Polans, Weeden, and Thompson 1986; Jellen, Phillips, and Hines 1994; Linares, Bowen, and Dover 1994; but see Dubcovsky and Dvořák 1995). The number of NORs in *Amelanchier* is not known, although acetocarmine squashes of early meiotic prophase in microsporocytes of *A.* "erecta" show only one large NOR (unpublished data).

If polyploidy can retard nrDNA concerted evolution, then allopolyploids would be expected to contain more polymorphism than diploids either because of genetic heterogeneity created by hybridization or because of divergence of chromosomally distinct NORs over time. Many polyploids for which ITS sequence data are available (e.g., Baldwin 1992; Hsiao et al. 1994; Sun et al. 1994; Baldwin and Robichaux 1995; Wendel, Schnabel, and Seelanan 1995*a*), however, do not show the extensive nucleotide site polymorphism present in *Amelanchier*. Given the high incidence of hybridization in *Amelanchier* and disomic isozyme inheritance in *A. laevis* (R. D. Overath, personal communication), allopolyploidy is the likely condition of our tetraploid samples of *A. canadensis, A. laevis,* and *A. nantucketensis*. That these allopolyploid shadbushes are not more polymorphic in ITS sequences than diploid *A. bartramiana* (table 2) may be because they evolved within the eastern North American lineage in which sequence divergence is low.

Similarly, agamospermy is not always associated with extensive, within-individual ITS sequence polymorphism; agamospermous *A. canadensis, A. laevis,* and *A. nantucketensis* show levels of polymorphism similar to sexual *A. bartramiana* (table 2). Agamospermous taxa formed by hybridization between taxa with divergent ITS sequences would be extensively polymorphic, as we infer for *A.* "erecta" and other eastern North American *Amelanchier*. DNA turnover via gene conversion and unequal crossing over is possible through mitosis but at a considerably lower rate than in meiosis (Jinks-Robertson and Petes 1993). Agamospermy may then be responsible for the apparent failure of concerted evolution to remove polymorphism that has persisted during separation of our sample of *A.* "erecta" in central Maine from *A. humilis,* which does not grow in New England east of western Vermont. This separation may have been from dispersal by *A.* "erecta" approximately 400 km east of the limit of distribution of *A. humilis* in western Vermont or from the retreat of *A. humilis* from Maine. Our sample of *A.* "dentata" is several hundred kilometers away from the geographic range of one of its putative parents, *A. sanguinea*. We do not have direct evidence about reproductive mode in *A.* "dentata." Agamospermy is indirectly implicated because (1) *A. sanguinea* is self-compatible (table 1); (2) self-compatibility is strongly linked to polyploidy and agamospermy in *Amelanchier* and relatives (Campbell, Greene, and Dickinson 1991); and (3) *Amelanchier* hybrids with at least one agamospermous parent are agamospermous (Weber and Campbell 1989; Campbell and Wright 1996).

Potential for concerted evolution is apparently less in facultatively agamospermous *Amelanchier* than in some vertebrates. Concerted evolution operates effectively in triploid, parthenogenetic lizards (Hillis et al. 1991). Gene conversion is strongly implicated; thus, heteroduplex formation must occur during the specialized cell divisions leading to the formation of ova in parthenogenetic vertebrates. Endomitosis creates a hexaploid cell that proceeds through two conventional meiotic divisions. The significant bias documented by Hillis et al. (1991) would accelerate concerted evolution (Dover 1982), perhaps compensating for the constraint on DNA turnover imposed by asexuality. Agamospermy in *Amelanchier,* in contrast, precludes meiosis completely; the megasporocyte or its immediate derivatives degenerate and nearby cells develop mitotically into chromosomally unreduced megagametophytes (Campbell and Wright 1996). Avoidance of meiosis limits the formation of heteroduplex molecules required for efficient molecular turnover and concerted evolution. Nevertheless the diversity of sequences among *A.* "erecta" clones indicates that recombination or gene conversion has occurred in this facultative agamosperm.

Twelve *A.* "erecta" clones (numbers 4, 9–14, 16–18, 20, and 22, table 3) are possibly recombinants of

clade A and B repeat types. Chimeric nrDNA repeats were also reported by Sites and Davis (1989) and Wendel, Schnabel, and Seelanan (1995*b*). These apparent recombinants may thus represent transition stages in the homogenization of the ITS region (see Strachan, Webb, and Dover 1985). Backcrossing might lead to apparent loss of a minority repeat type in direct sequences of genomic DNA, thus simulating homogenization via DNA turnover.

Conclusions

ITS sequences resolve two major clades in North American *Amelanchier*. Individuals of several *Amelanchier* taxa are polymorphic at ITS nucleotide sites where these clades have diverged, and most of these taxa are hypothesized to be hybrids between the clades. Geographic separation of two highly polymorphic taxa, *A.* "dentata" and *A.* "erecta," from their putative clade A parents suggests that some time has passed since their hybrid origin and that concerted evolution has not been effective in homogenizing the ITS region. Both agamospermy and polyploidy could retard concerted evolution, agamospermy by eliminating sexual recombination and polyploidy by separating nrDNA arrays at different loci. Polyploidy and within-individual ITS sequence polymorphism are associated in several plant groups, but this is the first report of an association between agamospermy and persistent ITS sequence polymorphism. Sequences of ITS clones from an individual of *A.* "erecta" are either identical (or nearly so) to those of clade A or B ITS repeats, or they are apparent recombinants of clade A and B repeats. Recombination is consistent with the observation that agamospermy in *Amelanchier* is facultative and with the possibility that the ITS region is in a transition stage of concerted evolution.

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