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Comparison of allozyme, RFLP, and RAPD markers for revealing genetic variation within and between trembling aspen and bigtooth aspen

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Abstract. We examined genetic variation in allozyme loci, nuclear DNA restriction fragment length polymorphisms (RFLPs), and random amplified polymorphic DNAs (RAPDs) in 130 trembling aspen (*Populus tremuloides*) and 105 bigtooth aspen (*P. grandidentata*) trees. In trembling aspen 10 out of 13 allozyme loci assayed (77%) were polymorphic (P), with 2.8 alleles per locus (A) and an expected heterozygosity (H_e) of 0.25. In contrast, bigtooth aspen had a much lower allozyme genetic variability (P = 29%; A = 1.4; H_e = 0.08). The two species could be distinguished by mutually exclusive alleles at *Idh-1*, and bigtooth aspen has what appears to be a duplicate 6PG locus not present in trembling aspen. We used 138 random aspen genomic probes to reveal RFLPs in *Hin*dIII digests of aspen DNA. The majority of the probes were from sequences of low copy number. RFLP results were consistent with those of the allozyme analyses, with trembling aspen displaying higher genetic variation than bigtooth aspen (P = 71%, A = 2.7, and H_e = 0.25 for trembling aspen; P = 65%, A = 1.8, and H_e = 0.13 for bigtooth aspen). The two species could be distinguished by RFLPs revealed by 21 probes (15% of total probes assayed). RAPD patterns in both species were studied using four arbitrary decamer primers that revealed a total of 61 different amplified DNA fragments in trembling aspen and 56 in bigtooth aspen. Assuming a Hardy-Weinberg equilibrium, estimates of P = 100%, A = 2, and H_e = 0.30 in trembling aspen and P = 88%, A = 1.9, and H_e = 0.31 in bigtooth aspen were obtained from the RAPD data. Five amplified DNA fragments were species diagnostic. All individuals within both species, except for 2 that likely belong to the same clone, could be distinguished by comparing their RAPD patterns. These results indicate that (1) RFLPs and allozymes reveal comparable patterns of genetic variation in the two species, (2) trembling aspen is more genetically variable than bigtooth aspen at both the allozyme and DNA levels, (3) one can generate more polymorphic and species-specific loci with DNA markers than with allozymes in aspen, and (4) RAPDs provide a very powerful tool for "fingerprinting" aspen individuals.

Key words: *Populus tremuloides* – *Populus grandidentata* – Isozyme – Restriction fragment length polymorphism – Random amplified polymorphic DNA

Introduction

The assessment of genetic variation is a major concern of plant breeders and population geneticists. This is important at several levels. First, the ability to reliably distinguish members of different species is critical in controlling the material entering a breeding program and in population genetic analyses. Second, the ability to reliably identify or "fingerprint" different genotypes is important in breeding programs that rely on clonal propagation in testing and/or production and in population genetic analyses of naturally clonal species. Finally, an estimate of the amount of variation within a species is useful for predicting potential genetic gain in a breeding program and in testing population genetic hypotheses. Traditionally, morphological and phenological characteristics have been used for these purposes. Since such characteristics are often controlled by multiple genes and subject to varying degrees of environmental modification

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and interaction, differences between clones or closely related species are not always absolute. Natural interspecific hybridization and introgression can make this problem even more difficult. Many of these traits are also difficult to analyze because they do not have the simple genetic control assumed by many population genetic models.

Allozyme markers have provided a valuable tool for studies of genetic variation in natural populations (Adams 1983). Allozyme markers have also been used to unambiguously identify species and hybrids (e.g., Adams 1983; Ayala 1983; Rajora 1990) and to delineate clones (Cheliak and Pitel 1984), providing many advantages over morphological methods. While allozyme analysis is relatively fast and inexpensive, in some cases species-diagnostic alleles cannot be found, and in most species there are insufficient numbers of allozyme markers available for examining large portions of the genome.

Restriction fragment length polymorphisms (RFLPs) provide a very large number of genetic markers for detecting and analyzing genetic diversity in plants (Burr et al. 1983; Helentjaris et al. 1985), and for distinguishing different species and individuals within species (Clegg 1989). RFLPs have been successfully used for these purposes in a number of crop plants (e.g., Beckmann and Soller 1986; Chase et al. 1991; McGrath and Quiros 1992; Messmer et al. 1991; Neuhausen 1992; Zhang et al. 1992).

There are some differences between allozyme and RFLP methods for the detection of genetic variation, with the fundamental difference being that they detect genetic variation at different levels (Clegg 1989). Allozymes are gene products, while RFLPs reveal differences directly at the level of DNA in both coding and noncoding regions. Correlations between levels of allozyme and DNA polymorphisms have been reported (Chase et al. 1991; Messmer et al. 1991), but discrepancies between the two levels of polymorphisms have also been found (see Clegg 1989 for review).

Recently, another class of molecular marker, random amplified polymorphic DNAs (RAPDs), has been developed, in which DNA is amplified by the polymerase chain reaction (PCR) using arbitrary short (ca. 10 nucleotides) primers (Williams et al. 1990). Different sequences may be amplified in different organisms, depending on the nucleotide sequence of the primer used and on the source of the template DNA, resulting in a genome-specific "DNA fingerprint". The high variability of DNA fingerprints described in humans, animals, and plants, allows the identification of different individual genotypes and species (Halward et al. 1991; Klein-Lankhorst et al. 1991; Quiros et al. 1991). RAPD assays require less time than RFLP assays, but there is some loss of information because RAPD markers are dominant rather than codominant.

Allozymes, RFLPs, and RAPDs each have advantages and disadvantages as markers for assessing genetic variation. However, relatively little effort has been devoted to applying all three types of markers to the same set of individuals. Such a study would provide experimental data to verify the relative merits of the different methods and would indicate whether measures of variability obtained by one method are similar to those obtained by another method. Hence, we decided to compare these markers for assessing genetic variation in two aspen species.

Trembling aspen (*Populus tremuloides* Michx.) is the most widely distributed tree species in North America (Perala 1990). In northeastern North America, its range overlaps with a related species, bigtooth aspen (*P. grandidentata* Michx.) (Laidly 1990). Natural hybridization between the two species, due to overlap in flowering times, has been reported (Andrejak and Barnes 1969; Barnes 1961, Einspahr and Joranson 1960; Henry and Barnes 1977; McComb and Hanson 1954; Pauley 1956). Aspens are among the most important commercial hardwoods in the United States, particularly in the Great Lakes region, because of their rapid growth rate and high quality of wood (Spencer et al. 1990).

Previous studies of trembling aspen employing molecular markers include geographic surveys of allozyme variation (Cheliak and Dancik 1982; Hyun et al. 1987; Jelinski and Cheliak 1992; Lund et al. 1992; Mitton and Grant 1980), the use of allozymes to delineate clones (Cheliak and Pitel 1984), and the use of a bacteriophage M 13 repeat probe to identify minisatellite DNA sequences for "fingerprinting" individuals and clones (Rogstad et al. 1991). No studies on molecular genetic variation in bigtooth aspen have been reported. The objectives of our study were (1) to evaluate levels of genetic variability in trembling aspen and bigtooth aspen at both the protein and DNA levels with allozyme, RFLP, and RAPD markers, (2) to determine the potentials of these markers for reliably distinguishing the two species, and (3) to examine the utility of RAPD markers for "fingerprinting" individual genotypes.

Materials and methods

One hundred and thirty trembling aspen and 105 bigtooth aspen trees were sampled from Michigan, Minnesota, and Wisconsin. Care was taken to select trees that were morphologically clearly members of one of the two species and did not appear to be hybrid derivatives. Vegetative buds were collected from 40 trembling aspen and 20 bigtooth aspen trees in the summers of 1989 and 1990 to extract enzymes for gel electrophoresis. In July 1991, leaf tissues were collected from these 60 trees and the other 175 aspen individuals for both allozyme and DNA analyses. Rapidly expanding leaves one or two nodes from the shoot tip were collected for good activities of enzymes and high yields of DNA.

All trees were examined for their allozyme patterns at ten enzyme systems encoded by 14 loci: aconitase (ACO, E.C. 4.2.1.3), glutamate oxaloacetate transaminase (GOT, E.C. 2.6.1.1), isocitrate dehydrogenase (IDH, E.C. 1.1.1.42), leucine aminopeptidase (LAP, E.C. 3.4.11.1), menadione reductase (MNR, E.C. 1.6.99.2), peroxidase (PER, E.C. 1.11.1.7), phosphoglucomutase (PGM, E.C. 2.7.5.1), 6-phosphogluconate dehydrogenase (6PG, E.C. 1.1.1.44), phosphoglucose isomerase (PGI, E.C. 5.3.1.9), and superoxide dismutase (SOD, E.C. 1.15.1.1). Laboratory methods and the inheritance of these loci have been previously described (Liu and Furnier 1993).

All 235 aspen trees were assayed for their RFLP patterns by the methods of Liu and Furnier (1993). All RFLPs were revealed by digestion of DNA with *Hin*dIII. We used *Pst*I-restricted random genomic DNA sequences of trembling aspen as the source of probes. The methylation-sensitive restriction enzyme *Pst*I was used to digest DNA because this type of library is enriched for low-copy-number sequences. Genomic DNA probes can potentially reveal more variation than cDNA probes since these sequences may include noncoding DNA regions that tend to accumulate mutations more rapidly than coding regions (Devey et al. 1991; Zamir and Tanksley 1988). A total of 143 aspen genomic probes were hybridized with Southern blots of aspen DNA. Only 5 probes (3.5%) were discarded because of weak signal intensity. Due to the relatively large numbers of trees and genomic probes, a subset of 28 of the probes was hybridized with DNA from all trees, and the entire set of 138 usable probes was hybridized with DNA from 15–50 trees of each species. Poor enzyme digestion and low DNA yields for some trees reduced the sample population for the subset of 28 probes to approximately 80–90 individuals from each species.

Allelic structures of the aspen RFLPs have been previously investigated by inheritance and linkage analyses (Liu and Furnier 1993). A probe may reveal several monomorphic bands on a Southern blot, in which case it is impossible to determine precisely how many monomorphic loci are involved. A conservative method was used to estimate the number of such loci. For each probe, we compared the size of the probe and the restriction fragments generated. Fragments that could be spanned by the probe were considered to belong to the same monomorphic locus for that probe.

For the RAPD assays, PCR was performed according to the protocol of Williams et al. (1990), except that an incubation of 7 min at 72° was added at the final stage. Four primers, each 10 nucleotides long (5′–3′: F-01, ACGGATCCTG; F-02, GAGGATCCCT; F-03, CCTGATCACC; F-05, CCGAATTCCC), were used to direct amplification in a Coy Tempcycler (model 50). Amplified DNA fragments were resolved electrophoretically on 1.4% agarose gels, followed by staining with ethidium bromide. Amplified products of the two species were run side by side on gels to make clear interspecific comparisons of the RAPD patterns. One negative control that included all the components of the PCR reaction except the template DNA was made for each amplification to monitor any possible contamination. Amplification of DNA was repeated once, and only those fragments amplified reproducibly were included in the analysis. A locus was counted if a band could be unambiguously scored. Since RAPD markers are dominant, a locus was considered to be polymorphic if the presence and absence of the band were observed and monomorphic if the band was present among all individuals. Two alleles were assigned for a polymorphic locus and one allele for a monomorphic locus.

Three measures of genetic variation were estimated for each species based on all of the allozyme, RFLP, and RAPD loci assayed. These were the percentage of loci polymorphic (P; a locus was considered polymorphic if more than one allele was detected), average number of alleles per locus (A), and average heterozygosity. We estimated both observed heterozygosity (H_o) based on a direct count, and expected heterozygosity (H_e) based on the unbiased estimate of Nei (1978). Estimates A and H_e were also made based only on the polymorphic loci (A_p, H_{ep}). Observed heterozygosity could not be determined for RAPD data because heterozygotes could not be distinguished from dominant homozygotes. Expected heterozygosity, however, could be estimated. If a Hardy-Weinberg equilibrium is assumed the frequency of a recessive allele can be estimated as the square root of the frequency of individuals lacking the band (the frequency of recessive homozygous genotypes). This allele frequency was then used to estimate H_e. To verify the assumption of this method, all polymorphic allozyme and RFLP loci were tested within each species for deviations from the Hardy-Weinberg equilibrium using a χ^2 goodness-of-fit test. Alleles with low frequencies were bulked with the next more frequent allele if the expected number of individuals in a class was less than one (Snedecor and Cochran 1967). In some cases, even bulking did not yield the desired minimum class sizes, resulting in a test not being accepted as valid. All tests of significance were conducted at the $\alpha = 0.05$ level. Since there were multiple tests with the species, we set α for each individual test (α_i) at $\alpha_i = 1-(1-0.05)^{1/k}$, where k is the number of tests. This assures an overall type I error rate of 0.05 (Hocking 1985).

Results

A total of 14 allozyme loci with 38 alleles were inferred from the ten enzyme systems surveyed in both trembling aspen and bigtooth aspen. Trembling aspen was considerably more variable than bigtooth aspen (Table 1). Two factors may account for the differences in genetic variabilities observed between the two species. One is the number of alleles per locus, with trembling aspen displaying twice as many alleles per locus as bigtooth aspen. The second factor is the large difference in numbers of polymorphic loci detected in trembling aspen (10 out of 13 loci) and bigtooth aspen (4 out of 14 loci). If monomorphic loci are excluded from consideration, the interspecific difference in expected heterozygosities is much lower (H_{ep}, Table 1). Observed genotype frequencies deviated from those expected under Hardy-Weinberg equilibrium at 3 (*Aco-1*, *Per-1*, and *Pgi-2*) of the 10 polymorphic loci in trembling aspen and none of the 4 polymorphic loci in bigtooth aspen.

In all bigtooth aspen individuals, we observed a 6PG band ($R_f = 0.33$) migrating slightly faster than the *6Pg-2* ($R_f = 0.30$) allozyme. This faster band was not present in any of the trembling aspen individuals. We suspected that this was another 6PG locus in bigtooth aspen rather than just another allele of *6Pg-2*. To confirm that these individuals were not all heterozygotes, we examined the allozyme pattern in 20 interspecific hybrids from each of two trembling aspen × bigtooth aspen full-sib families. As expected, no segregation was observed, and both of the bigtooth aspen bands were present in every hybrid progeny. This does not, however, rule out the alternative possibility that post-translational modification mecha-

Table 1. Genetic variation detected by allozyme, RFLP, and RAPD markers in trembling and bigtooth aspens. Measures are percentage of loci polymorphic (P), mean number of alleles per locus (A), mean number of alleles per locus based only on polymorphic loci (A_p), observed heterozygosity (H_o), expected heterozygosity (H_e), and expected heterozygosity based only on polymorphic loci (H_{ep}). Standard errors are in parentheses

Species	Marker	Trees	Loci	P	A	A_p	H_o	H_e	H_{ep}
Trembling	Allozyme	118	13	77	2.8 (1.7)	3.3 (1.6)	0.19 (0.05)	0.25 (0.06)	0.32 (0.06)
	RFLP	91	41	71	2.7 (1.5)	3.3 (1.3)	0.21 (0.03)	0.25 (0.04)	0.35 (0.04)
	RAPD	102	61	100	2.0 (0.0)	2.0 (0.0)		0.30 (0.03)	0.30 (0.03)
Bigtooth	Allozyme	96	14	29	1.4 (0.9)	2.5 (1.0)	0.07 (0.04)	0.08 (0.04)	0.28 (0.11)
	RFLP	75	37	65	1.8 (0.8)	2.3 (0.7)	0.12 (0.02)	0.13 (0.02)	0.19 (0.03)
	RAPD	95	56	87	1.9 (0.3)	2.0 (0.0)		0.31 (0.10)	0.35 (0.08)

Table 2. Numbers of aspen RFLP loci revealed by genomic DNA probes

Species	Probes surveyed	Polymorphic probes	Number of probes revealing			
			1 locus	2 loci	3 loci	≥4 loci
Trembling	138	117 (85%)	72 (52%)	33 (24%)	18 (13%)	15 (11%)
Bigtooth	138	104 (75%)	73 (53%)	33 (24%)	18 (13%)	14 (10%)

nisms generate a two-banded allele that is present in bigtooth aspen but not in trembling aspen.

In addition to the species-diagnostic putative 6PG gene duplication, trembling aspen and bigtooth aspen could be distinguished by mutually exclusive alleles at *Idh-1*. Trembling aspen was polymorphic for two *Idh-1* alleles, whereas bigtooth aspen was monomorphic for a third allele at this locus. One other locus (*Aco-2*) was almost species diagnostic with bigtooth aspen fixed for allele 1, which was present in trembling aspen at a very low frequency (0.02).

The majority of the 138 genomic DNA probes revealed RFLPs, with approximately 50% of the probes revealing single loci and a few generating large numbers of loci (Table 2). These results suggest that the methylation-sensitive restriction enzyme *Pst*I was effective in generating libraries with low-copy sequences in aspen. The subset of 28 probes used on most of the trees revealed 41 loci in trembling aspen and 37 loci in bigtooth aspen. Observed genotype frequencies deviated from those expected under Hardy-Weinberg equilibrium at 5 of the 29 polymorphic RFLP loci in trembling aspen and none of the 24 polymorphic loci in bigtooth aspen.

The results of RFLP analyses agree with those from the allozyme analyses, with trembling aspen displaying more alleles per RFLP locus and higher values of H_o and H_e than bigtooth aspen (Table 1). Even when only polymorphic loci were considered (H_{ep}), trembling aspen was almost twice as heterozygous as bigtooth aspen. We compared estimates of genetic variation obtained for the complete sample of trees with the above 28 probes to results obtained from a large number of probes. The remaining 110 probes were hybridized to *Hin*dIII digests of 15–50 aspen DNA samples. The total number of RFLP loci obtained in trembling aspen from this survey was 184, with 131 (71%) being polymorphic. For bigtooth aspen, 181 loci were observed, with 108 (60%) being polymorphic. These results agree well with those based on the subset of 28 probes used for the larger sample of trees (Table 1). Of the 138 probes 21 (15%) revealed species-diagnostic markers. These probes generated a total of 49 restriction fragments in trembling aspen and 26 restriction fragments in bigtooth aspen that were mutually exclusive in the two species.

The RAPD assays revealed a large amount of variation (Tables 1 and 3, Fig. 1), with the four primers amplifying a total of 61 repeatable DNA fragments in trembling aspen and 56 in bigtooth aspen. The amplified products ranged in size from 0.33 kb to 2.75 kb. Estimates of genetic variability based on the RAPD data were similar in the two species, although trembling aspen had more polymorphic loci (Table 1). A total of 5 amplified fragments were found to distinguish the two aspen species, with the 5 fragments present in all of the individuals of bigtooth aspen examined but absent from trembling aspen (Table 3, Fig. 1).

Primer F-05 generated the highest number of fragments among the primers used and had the greatest capacity for discriminating individual trees within the species. For trembling aspen, the 110 individuals analyzed with this primer all had unique RAPD genotypes (Table 3, Fig. 1). For bigtooth aspen, primer F-05 revealed 87 unique genotypes among the 97 trees assayed (Fig. 1), and the addition of data from one other primer uniquely identified all but 2 individuals. These 2 individuals, which are shown in the last two bigtooth aspen lanes in Fig. 1,

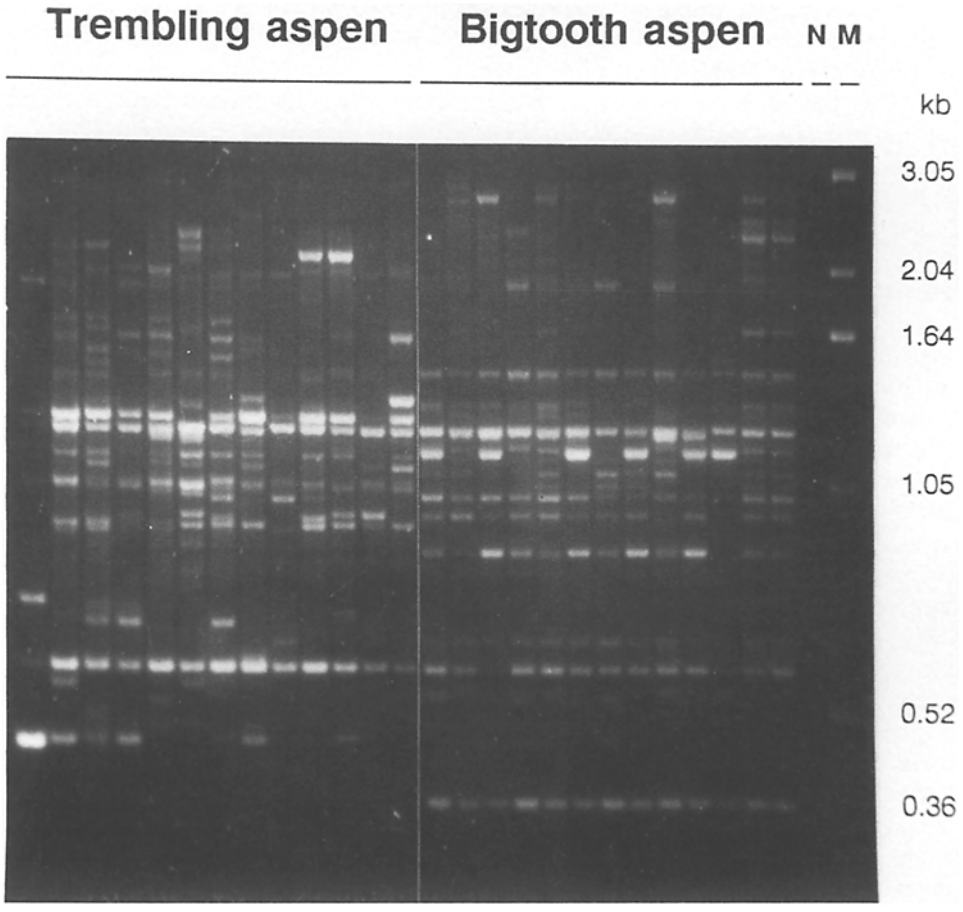

Fig. 1. RAPD patterns generated in trembling and bigtooth aspens by primer F-05. The 0.36-kb DNA fragment in bigtooth aspen is absent from trembling aspen. *N* Negative control lane, *M* DNA molecular weight standard lane

Table 3. Trembling and bigtooth aspen RAPD assays

Primer	Species	Amplified fragments[a]	Polymorphic fragments	Trees	Unique genotypes	Species-diagnostic fragments (size)
F-01	Trembling	12	12 (100%)	97	71 (73%)	
	Bigtooth	12	8 (67%)	95	33 (35%)	2 (950 bp, 940 bp)
F-02	Trembling	14	14 (100%)	96	77 (80%)	
	Bigtooth	11	10 (91%)	91	68 (76%)	1 (500 bp)
F-03	Trembling	15	15 (100%)	103	83 (81%)	
	Bigtooth	14	13 (93%)	95	68 (72%)	1 (610 bp)
F-05	Trembling	20	20 (100%)	110	110 (100%)	
	Bigtooth	19	18 (95%)	97	87 (91%)	1 (360 bp)

[a] Amplified fragments that were not repeatable were excluded

were adjacent trees and had identical RAPD genotypes for all four primers, suggesting that they were members of the same clone. The other three primers had lower capacities for fingerprinting because they generated fewer bands than primer F-05. The extreme case was primer F-01, which generated only 33 different genotypes among the 95 bigtooth aspen trees.

Discussion

Since allozyme variation may only reflect differences in protein-coding genes, the question is often posed of whether protein polymorphism is a good predictor of overall levels of genetic diversity (Clegg 1989). In this study, allozymes and RFLPs generally yield comparable

estimates of alleles per locus and heterozygosity within each species, with a higher value of percentage of loci polymorphic for the RFLPs in bigtooth aspen (Table 1). Genetic variation was greater for RFLPs than for allozymes in previous studies of inbred maize (Messmer et al. 1991) and *Brassica campestris* (McGrath and Quiros 1992). This result might be expected since RFLPs and RAPDs can detect variation in both coding and non-coding regions.

RAPDs consistently revealed greater variation than allozymes and RFLPs in both aspen species, with the difference being particularly striking in bigtooth aspen. However, several factors must be considered in using RAPD markers for assessing genetic variation in diploid outcrossing organisms. The first factor is the dominant nature of RAPD markers. If a locus is polymorphic, with the recessive allele present at low frequency, almost all copies of that allele will be carried in heterozygotes and will go undetected by the RAPD assay, resulting in that locus being scored as monomorphic. This will lead to an underestimate of P, A, and H_e. In our data, RAPD markers actually yielded higher estiamtes of variation, with no monomorphic loci in trembling aspen and few in bigtooth aspen.

A second important factor is the assumption that each fragment represents 1 RAPD locus with only two alleles, which correspnds to the presence and absence of the fragment observed on the gel. This probably is not always the case, since deletion and insertion events that occur in the region between the primers will result in an amplified fragment migrating to a different position, causing it to be scored as a separate locus rather than an additional allele at the first locus. This would lead to an overestimate of P, an underestimate of A, and its effect on H_e would depend on the allele frequencies. Another artifact of the RAPD scoring procedure can clearly inflate estimates of variability. For codominant markers, one can observe loci for which any given species is monomorphic for any given allele. For the dominant RAPD markers, the only monomorphic loci observed are those that are monomorphic for the dominant allele (presence of a band). Portions of the genome that are monomorphic for the recessive allele (absence of a band) are not observed. This inability to observe loci monomorphic for one of the two alleles will result in an overestimate of P, A, and H_e. One might correct for this by counting the number of loci monomorphic for the dominant allele and doubling that to obtain an estimate of the number of monomorphic loci. This correction assumes that all loci scored as monomorphic are indeed monomorphic (see discussion above) and that the probability of a locus being monomorphic is independent of whether the fixed allele is dominant or recessive. When this procedure is applied to our data, $P=78\%$, $A=1.8$, and $H_e=0.28$ for bigtooth aspen, still clearly higher than the estimates obtained for allozyme and RFLP data. Since $P=100\%$, none of the estimates change for trembling aspen.

Another factor is the assumption of Hardy-Weinberg equilibrium made for estimating H_e from RAPD data. Analyses of our allozyme and RFLP data showed that genotype frequencies at 73% of the polymorphic loci in trembling aspen and 92% in bigtooth aspen did not differ significantly from those expected under Hardy-Weinberg equilibrium. All but 1 of these deviations was due to a deficiency of heterozygotes, as would be expected when combining individuals from a broad geographic area (Wahlund effect). The relatively low number of loci showing deviations suggests that the assumption of Hardy-Weinberg equilibrium made in estimating H_e from our RAPD data is probably reasonable, but it is always possible that allozyme and RFLP loci many not be representative of the RAPD loci.

Our observation of higher levels of RAPD variation than allozyme and RFLP variation may simply be due to different capacities of the different markers to detect genetic variability, but because of the factors discussed above, RAPD markers will probably be less reliable than allozymes and RFLPs for the estimation of population genetic parameters in populations of outcrossing diploids, such as the aspens.

Surveys of allozyme variation in trembling aspen have been previously reported from Alberta (Cheliak and Dancik 1982; Jelinski and Cheliak 1992), Ontario (Hyun et al. 1987), and Minnesota (Lund et al. 1992). Results of our allozyme survey of trembling aspen in the Great Lakes region are in good agreement with those of Hyun et al. (1987) and Lund et al. (1992). All three studies reported approximately 80% of the loci to be polymorphic and an observed frequency of heterozygotes of approximately 0.2. Levels of variation reported by Cheliak and Dancik (1982) and Jelinski and Cheliak (1992) were higher, with P being 92% and 89%, and H_o being 0.42 and 0.32, respectively, for their analyses. Jelinski and Cheliak (1992) suggested that the higher diversity observed in the western part of the species' range may be due to drier weather, which hinders seedling establishment, resulting in the primary mode of reproduction being asexual by root suckers (Barnes 1975; Kemperman and Barnes 1976; McDonough 1985). Under some circumstances, asexual propagation increases the amount of variation by allowing the accumulation of mutations (Cheliak and Dancik 1982; Muller 1964). Alternatively, the different amounts of allozyme variation observed in the Alberta and Great Lakes area studies may simply result from different sampling schemes and differences in the loci assayed.

It is apparent from this study that trembling aspen maintains considerably higher levels of allozyme and RFLP variation than bigtooth aspen. One possible expla-

nation is that the wider range of environments in which trembling aspen grows has led to an accumulation of greater levels of genetic diversity. Our data do not allow us to distinguish this from the alternative that trembling aspen originally had a greater level of genetic diversity that allowed it to colonize a broader range of environments. It is possible that bigtooth aspen, with its range confined to eastern North America, experienced a mild population bottleneck during the Pleistocene glaciation, leaving contemporary bigtooth aspen to be derived from a smaller number of refugial populations than contemporary trembling aspen. Measures of allozyme variability in bigtooth aspen, including percentage of loci polymorphic (29%), alleles per locus (1.4), and expected heterozygosity (0.08) are also lower than those values of 65%, 2.2, and 0.18, respectively, reported as the means of 110 long-lived woody perennials (Hamrick and Godt 1989).

Trembling and bigtooth aspens can usually be distinguished by morphological and phenological traits (Barnes 1969), but the large amount of intraspecific variability in these traits and the ability of the two species to hybridize can make the task of reliably determining an individual's identity difficult at times. Identifying the origin of a genotype is particularly difficult when F_1 hybrids have backcrossed to either of the parent species (Farmer and Barnes 1978). In our study, 2 allozyme markers, RFLPs revealed by 21 probes, and 5 RAPD fragments have been demonstrated to be species specific and can thus be used to reliably distinguish the two aspen species and their hybrid derivatives. Having a large number of markers permits us to detect not just F_1 interspecific hybrids but also advanced generation hybrid derivatives. This will be useful in more accurately determining the extent and role of introgression in the evolution of these two species. The large number of species-diagnostic markers would also be useful in a backcross breeding program designed to transfer resistance to *Hypoxylon* canker from bigtooth aspen to trembling aspen. In this capacity, species-diagnostic markers are particularly useful if their distribution in the genome is known (Edwards 1992; Hospital et al. 1992). Linkage studies show that among the RFLP loci revealed by the 21 species-diagnostic probes, 6 loci revealed by 3 probes are linked, with *G316-1, G316-2, G316-3*, and *G65-1* in one group and *G65-2* and *G207* in another (Liu and Furnier 1993). Of the remaining 18 probes, 5 revealed RFLPs that did not appear to be linked, and 13 did not produce RFLPs segregating in the family used for linkage mapping. Neither of the species-diagnostic allozyme markers were linked to other species-diagnostic markers (Liu and Furnier 1993). These results indicate that our species-diagnostic markers are dispersed in the genome. Linkage analyses have not been performed on the RAPD markers. The proportions of markers that were species diagnostic were 14% (2/14 loci), 15% (21/138 probes), and 6% (5/86 fragments with different sizes) for allozymes, RFLPs, and RAPDs, respectively. The similarity in these figures suggests that the advantage of RFLPs and RAPDs over allozymes lies primarily in the formers' abilities to generate a much larger number of markers.

Morphological and phenological traits have also long been used to distinguish members of different aspen clones (Barnes 1969), but some useful phenological traits can only be measured at certain times of the year, and other traits, such as leaf morphology, require very detailed analyses due to environmentally induced intraclonal variation. Allozyme markers avoid the problem of environmental variation and have been useful in delineating aspen clones (Cheliak and Pitel 1984), but the power of this technique is limited by the relatively low number of available allozyme markers. Although RFLP technology using an M 13 repeat probe that yields DNA "fingerprints" has been effective in distinguishing aspen individuals (Rogstad et al. 1991), it is more labor intensive and requires greater amounts of DNA than the RAPD method. Our study demonstrates that RAPD markers are very useful for discriminating individual genotypes in aspens. The ability of a single primer to generate fragments distinguishing all genotypes in a sample of 110 different trembling aspen clones (Table 3, primer F-05) illustrates the immense power of this method. The usefulness of RAPD markers for fingerprinting has recently been exploited in several other plant species (Klein-Lankhorst et al. 1991; Quiros et al. 1991; Wilde et al. 1992).

Our study shows that similar levels of genetic variation are revealed by allozyme and RFLP data. Both types of markers reveal that trembling aspen is genetically more variable than bigtooth aspen. RAPD markers reveal high levels of variation in both species, but this may be partly due to the method of designating loci and the assumptions required to obtain expected heterozygosity estimates, factors that suggest caution in the use of RAPD markers for estimating levels of variation. Our data also demonstrate that RFLPs and RAPDs can provide a large number of polymorphic markers in aspens that will be useful both for distinguishing species and individuals with species. This suggests a high potential for RFLPs and RAPDs to be useful as markers to assist aspen breeding programs and basic genetic research.

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