RAPD markers reveal diversity within and among clonal and seedling stands of aspen in Yellowstone National Park, U.S.A.[1]

G.A. Tuskan, K.E. Francis, S.L. Russ, W.H. Romme, and M.G. Turner

Abstract: Fire in 1988 created a situation that allowed a rare aspen seedling recruitment event to occur within Yellowstone National Park. Through the use of (*i*) 194 randomly amplified polymorphic DNA (RAPD) markers from 14 select primers, (*ii*) measures of population diversity, and (*iii*) neighbor-joining analysis it was determined that the postfire aspen seedling population contains greater diversity within each of the four sampled stands than that found within all of the 10 sampled mature aspen stands that pre-existed the fire. Unlike previous studies, a large portion of the molecular variation in both the seedling and mature populations was partitioned among stands. Furthermore, variation was unexpectedly detected among ramets within each mature stand. The mature stands appear to be clonally derived, yet individual ramets within stands varied slightly and incrementally in their RAPD profile. These data suggest that somatic mutations may be occurring and accumulating in clonal aspen stands. A proposed scenario of stand establishment and development involving the accumulation of somatic mutations and elimination of genetically related seedlings arising from a rare founder event provides the theoretical basis for the observed differences among and within seedling and mature stands of aspen in Yellowstone National Park.

Résumé : En 1988, le feu a créé au Parc national de Yellowstone une rare situation permettant la régénération naturelle du peuplier par semences. L'utilisation de (*i*) 194 marqueurs d'amplification de régions anonymes polymorphes du génome (RAPD) obtenus à l'aide de 14 amorces choisies, (*ii*) de mesures de diversité de population et (*iii*) d'une analyse de « neighbor-joining » a permis de démontrer que la population de semis de tremble régénérés après feu démontrait une diversité génétique plus grande, au sein de chacun de quatre peuplements échantillonnés, que celle retrouvée au sein de chacun des 10 peuplements matures de peuplier échantillonnés et qui existaient avant le feu. Contrairement aux études précédentes, une proportion importante de la variation moléculaire retrouvée chez les populations de semis et les populations matures se retrouvait parmi les peuplements. De plus, une variation inattendue fut détectée parmi les ramets au sein de chacun des peuplements matures. Malgré le fait que les peuplements matures apparaissent être d'origine clonale, les patrons de RAPD des ramets individuels variaient légèrement et progressivement au sein des peuplements. Ces résultats suggèrent que des mutations somatiques peuvent se produire et s'accumuler au sein des peuplements clonaux de tremble. Les auteurs proposent un scénario d'établissement et de développement de peuplement impliquant une accumulation de mutations somatiques et l'élimination des semis génétiquement apparentés et découlant d'événements rares de fondation de populations. Ce scénario sert de toile de fond théorique pour expliquer les différences observées au sein et parmi les populations de semis et les populations matures de peuplier au Parc national de Yellowstone.

[Traduit par la Rédaction]

Introduction

Trembling aspen (*Populus tremuloides* Michx.) is unique among North American forest tree species in its distribution, reproductive biology, and reputed longevity. Trembling aspen has the widest distribution of any North American tree species, occurring from the Tropic of Capricorn to north of the Arctic Circle (Perala 1990). Its primary means of propagation is via root suckers, leading to the formation of large mature stands of younger ramets radiating outward from the stand's center (Barnes 1966). As a result of this clonal habit, a single aspen clone that occupies 40+ ha has been proposed to be the most massive organism on earth (Grant 1993). Stand establishment via seed propagation does occur, though successful seedling establishment requires a combination of exposed mineral soil, abundant moisture for several years after seed germination, and a lack of herbivory by ungulates (McDonough 1979). Many forest biologists believe that these combinations of factors have not occurred in the intermountain west of North American over the past 10 000 years (Cook 1983). The

Received November 1, 1995. Accepted June 14, 1996.

G.A. Tuskan.[2] Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, U.S.A.
K.E. Francis and S.L. Russ. Oak Ridge Institute for Science and Education, Oak Ridge, TN 37830, U.S.A.
W.H. Romme. Department of Biology, Fort Lewis College, Durango, CO 81301, U.S.A.
M.G. Turner. Department of Zoology, University of Wisconsin, Madison, WI 53706, U.S.A.

[1] Publication 4589, Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831.
[2] Author to whom all correspondence should be addressed.

lack of aspen seedling recruitment in the last 200 years has been documented in several regions of the United States (Jelinski and Cheliak 1992), leading to the speculation that many aspen clones may be thousands of years old (Barnes 1966; Libby and Ahuja 1993), though there is currently no means of directly verifying this estimate (Stettler and Ceulemans 1993). These unique aspects of aspen biology certainly influence intraspecific genetic diversity (Mitton and Grant 1996), phenotypic plasticity, and adaptation to environmental change.

In 1988, fire burned ca. 400 000 ha of land within Yellowstone National Park (Despain et al. 1989). The moist spring and summer of 1989, in combination with the exposed mineral soil resulting from the fires, allowed a rare seedling recruitment event to occur across burned areas inside the park (Romme et al. 1996). Prior to the fire, aspen in Yellowstone National Park was limited to lower elevation sites near the transition zone from coniferous forest to sagebrush grasslands (Despain 1991). Most prefire stands were located in the northern portion of the park, though small stands were also found near the western and southern entrances to the park. The newly established seedling stands have occurred not only within the prefire range of aspen, but also on the high, subalpine plateaus in areas previously dominated by lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.) (Kay 1993; Romme et al. 1996). These newly established seedling populations provide a unique opportunity to document changes in genetic diversity within and among seedling and mature stands of an otherwise clonally propagated species. In an attempt to provide this documentation, the objective of this study was to quantify the degree of genetic diversity within the newly established seedling population relative to the pre-existing mature stands of aspen in Yellowstone National Park.

Several authors have recently advocated the use of molecular markers, such as randomly amplified polymorphic DNA (RAPD), to study populations (Hadrys et al. 1992; Bachmann 1994). To be used reliably, artifactual variation in RAPD markers must be eliminated through the use of standardized protocols, quantified DNA template concentrations, consistent and accurate use of reaction components, and elimination of foreign template DNA (Munthali et al. 1992; Ellsworth et al. 1993). As two of several recommendations, Lynch and Milligan (1994) suggest that the number of sampled loci and the number of individuals per population be increased to minimize the bias in parameter estimates associated with the dominant nature of RAPD markers. Clark and Lanigan (1993) further advocate that both polymorphic and monomorphic bands be included in the determination of genetic diversity among populations and that segregation analysis be used to confirm allelism. Within these boundaries, RAPD markers have been suggested for use in studies of clonal populations, introgression and kinship (Bachmann 1994).

Materials and methods

Plant materials

Based on fire distribution and vegetation surveys conducted in 1989, the year after the fires, seedling and mature aspen populations within Yellowstone National Park were identified. Groups of individual stems were selected as clonal aggregates that (*i*) formed a discrete stand separated from the next nearest stand by a minimum of 100 m and (*ii*) shared a common leaf morphology (Kemperman 1977). The mature population was represented by stands of adult trees that pre-existed the fires at low elevations primarily on the northern range (Fig. 1). Four of these stands (two at Geode Creek and two at Frog Rock) had burned in 1988 and were re-establishing themselves through root suckers (Romme et al. 1996). The remaining six mature stands (two each at Lamar Valley and Specimen Ridge, and one each at MacMinn and Hebgen Lake) had not burned in 1988 (Table 1). In total, six geographic sites were represented by these mature stands. Four newly established seedling stands (one each at Madison River, Biscuit Basin, Fern Cascade, and Lewis Lake) were sampled from high-elevation sites on the subalpine plateau, each in a separate geographic area (Fig. 1). For both the seedling and mature populations, the sampled stands were mapped, the contours were delineated, and a transect was established through the longest axis of each stand. The transect was divided into 20 equal segments for the mature stands and 20–40 for the seedling stands. Individual stems were selected 90° to the right and then 90° to the left of the transect for each segment, for a total of 20 samples in the mature stands and 20–40 samples in the seedling stands. A single leaf was folded in half twice, and a microcentrifuge tube containing 1 mL of a 1× TE buffer (10 mM Tris·Cl, 1 mM EDTA) was used to punch four ca. 1 cm diameter disks from the leaf tissue from each sampled stem. The tubes containing the leaf discs were placed under dry ice and shipped the next day to the Oak Ridge National Laboratory (ORNL) for further sample preparation.

Fig. 1. Geographic locations of the seedling (●) and mature (■) aspen stands sampled within Yellowstone National Park, U.S.A.

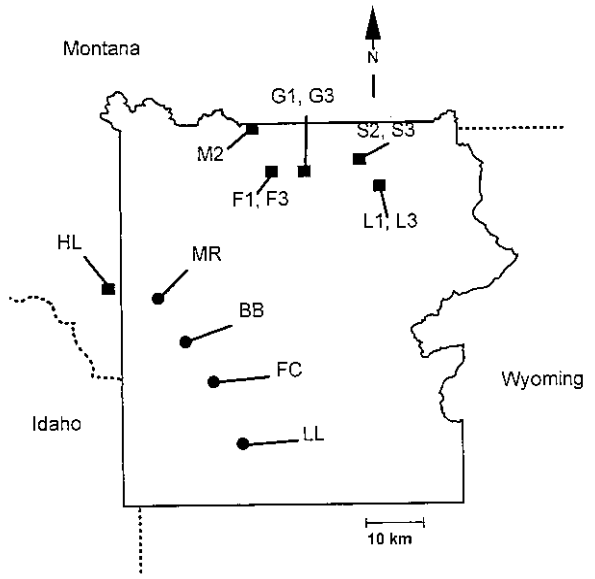


Table 1. Attributes of the Yellowstone National Park aspen populations sampled after the 1988 fires.

Population type and stand	Physiographic region	Estimated age (years)	Elev. (m)	Lat. (N)	Long. (W)
Seedling					
Biscuit Basin	Madison Plateau	3–4	2250	44°30′	110°50′
Madison River	Madison Plateau	3–4	2100	44°40′	111°00′
Lewis Lake	Pitchstone Plateau	3–4	2400	44°20′	110°40′
Fern Cascade	Madison Plateau	3–4	2350	44°25′	110°50′
Mature					
Hebgen Lake	Madison Plateau	Unknown	2050	44°40′	111°05′
Frog Rock 1	Blacktail Deer Plateau	105–120[a]	2100	44°55′	110°35′
Frog Rock 3	Blacktail Deer Plateau	105–120	2100	44°55′	110°35′
Lamar 1	Buffalo Plateau	110–120	1950	44°55′	110°20′
Lamar 3	Buffalo Plateau	110–120	1950	44°55′	110°20′
Specimen 2	Specimen Ridge	104–114	2050	44°50′	110°15′
Specimen 3	Specimen Ridge	104–114	2050	44°50′	110°15′
MacMinn	Blacktail Deer Plateau	105–115	1750	45°05′	110°40′
Geode 1	Blacktail Deer Plateau	105–120	2150	44°55′	110°30′
Geode 3	Blacktail Deer Plateau	105–120	2150	44°55′	110°30′

[a]Range of estimated ages for the largest ramets per stand per population, as measured prior to the 1988 Yellowstone fires.

In addition to the Yellowstone samples, a set of 23 full-sibling aspen progeny and their parents were obtained from the Aspen and Larch Genetics Cooperative in Grand Rapids, Minnesota. The maternal parent originated from northern Michigan, and the paternal parent originated from northern Ontario. Individual leaf samples were collected and handled in a manner identical with that described above. These genotypes and their amplified DNA products were used (*i*) as an outgroup in the neighbor-joining analysis of the Yellowstone population and (*ii*) in a segregation analysis (Clark and Lanigan 1993) to eliminate nonheritable bands from the Yellowstone data set (Riedy et al. 1992; Pellissier et al. 1992). That is, bands that were present in the progeny but were absent in the parents of the Lake States outgroup (i.e., 14 bands total) were eliminated from further analyses in the Yellowstone populations.

DNA extraction and amplification

Template DNA was initially extracted from four bulked leaf discs per sampled stem using an SDS extraction protocol. The isolated template DNA was then re-extracted because of suspected impurity, as indicated by the lack of polymerase chain reaction (PCR) amplification for all tested primers. The re-extraction protocol was based on a modified CTAB procedure (Doyle and Doyle 1987). Genomic DNA contained in 50 µL TE buffer in a 1.5-mL microcentrifuge tube was diluted with 200 µL of a 2× CTAB extraction buffer containing 1% (w/v) polyvinylpyrrolidone (PVP) and 50 µg/mL proteinase K. The DNA–buffer mixture was incubated at 60°C for 30 min, followed by an equal volume extraction with chloroform – isoamyl alcohol (24:1). The aqueous phase was transferred to a new microcentrifuge tube, RNase A (100 µg/ml) was added to the supernatant, and the entire volume was incubated at room temperature for 30 min. Genomic DNA was precipitated with a two-thirds volume of 0°C isopropanol. Following centrifugation at 8000 rpm for 8 min, the isopropanol was removed and the DNA pellet was rinsed twice with 75% (v/v) ethanol containing 10 mM ammonium acetate. The pellet was dried under vacuum and desiccant for 1 h and then suspended in 50 µL TE buffer, pH 8.0, for storage at 4°C. The DNA concentration was determined the following day for each sample using Hoefer TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, Calif.). A total of 280 individual DNA templates from the Yellowstone populations were used in the analysis.

DNA characterization relied on a PCR procedure for generating RAPD markers (Welsh and McClelland 1990; Williams et al. 1990). The RAPD reactions were conducted in a Perkin–Elmer 9600 thermocycler (Perkin–Elmer Corp., Norwalk, Conn.) with a 35-cycle protocol involving 1 min at 94°C denaturation, 1 min at 36°C annealing, and 2 min at 72°C polymerization steps within each cycle, followed by a 5-min final polymerization step. Each reaction contained 50 mM KCl, 10 mM Tris–HCl (pH 8.0), 2.5 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 2.0 ng bovine serum albumin, 0.5 units *Taq* polymerase, 200 µM each dNTP, 10 ng primer, and 1.0 ng DNA template, to a final volume of 10 µl. A control reaction containing all PCR components except the DNA template was run with each set of reactions to verify the lack of foreign DNA. Amplified PCR products (i.e., RAPD markers) were resolved using a 1.5% (w/v) agarose 0.5× TBE gel containing 0.1 µg/mL ethidium bromide electrophoresed under constant voltage (60 V) for 2.5 h. A 100-bp DNA standard (BRL, Grand Island, N.Y.) was used to assign molecular weights to individual RAPD bands. Photographs of each gel were taken under a UV light source (254 nm) and used to score bands for data analysis. A total of 380 decamer primers (Operon Technologies Inc., Alameda, Calif. and University of British Columbia, Vancouver, B.C.) were screened based on band resolution and band number per primer using four separate intrapopulational bulked DNA samples. Of the 380 screened primers, 103 showed little or no amplification. Twenty-eight of the screened primers produced discrete, discernible, multiple polymorphic bands. Fourteen of these 28 primers were selected for use in this study (a list of tested and selected primers, as well as the molecular weights of the analyzed markers may be obtained from the author). Randomly selected sets of DNA templates were periodically used as replicates to verify RAPD banding patterns across genotypes and across primers.

Table 2. Descriptive variables based on RAPD markers for seedling and mature populations of aspen from Yellowstone National Park.

Population type or stand	No. of samples	No. of unique genotypes	Avg. no. of bands per individual	% polymorphic loci
Seedling				
Biscuit Basin	19	19	81±2.0	59.0
Madison River	39	39	84±1.0	68.7
Lewis Lake	16	16	79±2.0	51.8
Fern Cascade	20	20	81±2.0	63.6
Mature				
Hebgen Lake	20	19	89±0.5	18.5
Frog Rock 1	17	13	88±0.2	4.1
Frog Rock 3	20	17	91±0.4	7.2
Lamar 1	18	18	96±1.4	27.7
Lamar 3	20	20	93±1.3	29.2
Specimen 2	16	16	82±0.5	16.9
Specimen 3	18	16	84±1.0	19.0
MacMinn	19	18	81±0.5	26.2
Geode 1	20	13	97±0.6	10.8
Geode 3	18	14	93±0.8	23.6

Data collection and analysis

All gel photographs were independently scored by two technicians for the presence or absence of RAPD bands across all primers and genotypes. The two derived matrices were compared, and inconsistencies were reconciled before the final binary matrix was created. Within this matrix each sampled stem was represented by a vector of 1 (for band presence) and 0 (for band absence). This matrix was input into the computer program RAPDPLOT (Kambhampati et al. 1992) to calculate a similarity index for each pairwise combination of genotypes based on Nei and Li's (1985) formula:

$$S = \frac{2N_{AB}}{N_A + N_B}$$

where N_{AB} is the number of bands that individual A and B have in common, N_A is the total number of bands for individual A, and N_B is the total number of bands for individual B. The binary matrix was also used to obtain estimates of Wright's F_{ST} statistic through the use of the RAPDFST program (Kambhampati et al. 1992), as modified by Dr. Steve Bao, Oak Ridge National Laboratory, to allow the analyses of larger matrices.

Neighbor-joining analysis was performed using the similarity matrix created by RAPDPLOT as an input file in the NEIGHBOR program of PHYLIP 3.5c (Felsenstein 1993). NEIGHBOR utilizes the neighbor-joining function of Saitou and Nei (1987) to construct successive linkages without the assumption of a molecular clock. Individuals were input in random order; the Lake States population was used as an outgroup. Altering the input order of the samples did not change the overall topography of the phenogram. The phenograms were plotted within and among populations using the DRAWTREE program of PHYLIP (Felsenstein 1993).

Results

A total of 194 RAPD markers from 14 selected primers were scored and analyzed in this study. Of these markers, 31 were monomorphic across all populations (Fig. 2).

Fig. 2. RAPD profiles created through Polymerase Chain Reaction amplification using Operon AB9 primer applied to individual samples collected within the (A) Geode 3 (mature) and the (B) Madison River (seedling) aspen stands from Yellowstone National Park. Distinctions among the two stands can be seen in terms of total variability across molecular markers within each stand. Arrows designate differences in RAPD profiles within and among stands at molecular markers 650 and 1150 base pairs (bp). Note the marker at 380 bp is monomorphic in both stands; 31 markers were monomorphic across all sampled populations. The outermost lane on each gel represents a 100-bp DNA standard.

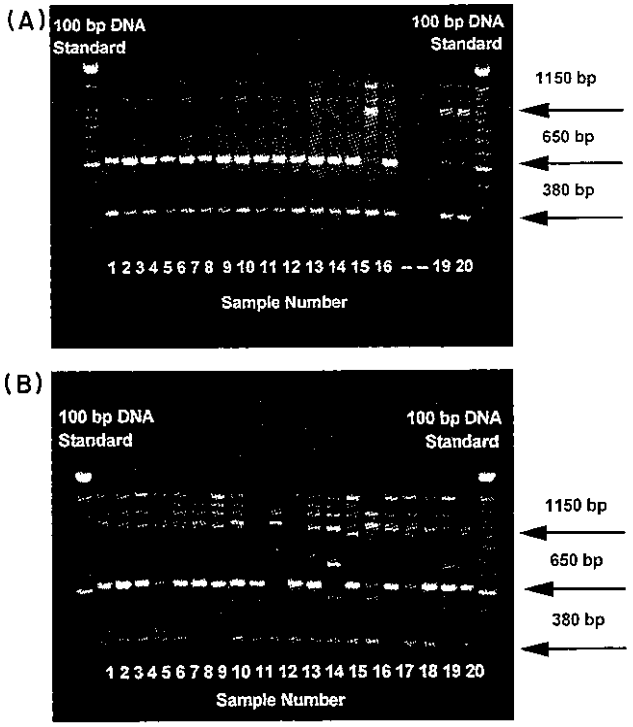


Table 3. Average similarity index for pairwise comparisons within (on the diagonal) and among (below the diagonal) seedling and mature populations of aspen from Yellowstone National Park.

Population type and stand	Biscuit Basin	Madison River	Lewis Lake	Fern Cascade	Hebgen Lake	Frog Rock 1	Frog Rock 3	Lamar 1	Lamar 3	Specimen 2	Specimen 3	MacMinn	Geode 1	Geode 3
Seedling														
Biscuit Basin	773													
Madison River	672	745												
Lewis Lake	664	663	766											
Fern Cascade	685	707	670	735										
Mature														
Hebgen Lake	671	693	669	679	950									
Frog Rock 1	682	645	611	639	690	984								
Frog Rock 3	674	715	653	693	705	724	968							
Lamar 1	685	691	655	690	687	721	745	920						
Lamar 3	663	678	617	687	653	679	742	724	890					
Specimen 2	708	694	637	699	727	675	732	713	698	926				
Specimen 3	691	693	637	689	695	695	715	692	725	758	948			
MacMinn	631	670	637	655	654	668	692	719	691	673	656	923		
Geode 1	664	668	644	659	707	632	711	734	704	652	636	705	975	
Geode 3	667	689	687	657	673	634	692	725	695	656	698	659	733	927
Lake States outgroup	569	565	544	559	604	572	576	636	594	580	592	582	650	636

There were 74 markers that were unique to the Yellowstone populations, and thus absent in the Lake States outgroup. Another 15 markers were unique to the Lake States outgroup. The mature stands typically had higher numbers of markers per individual than did the seedling stands, 89 ± 2 versus 81 ± 1, respectively, while the seedling stands had higher average percent polymorphic loci per stand, 18.3 ± 2.7% versus 60.8 ± 3.6%, respectively (Table 2). In the extremes, 4% of the loci were polymorphic in the Frog Rock 1 stand and 69% in the Madison River stand. The number of unique genotypes per stand was identical with the number of samples per stand in the seedling population, whereas the number of unique genotypes per mature stand varied from 13 genotypes per 20 samples to 20 genotypes per 20 samples (Table 2). Ramets sharing identical genotypes occurred within 7 of the 10 mature stands.

The similarity index within a seedling stand averaged 0.755 ± 0.009, indicating that pairwise combinations of individuals within such a stand contain ca. 76% of the same markers (Table 3). The average similarity index within a mature stand was considerably higher than that for the seedling population, 0.941 ± 0.009. Average similarity index among seedling stands was 0.677 ± 0.003 (Table 3), indicating that pairwise combinations of seedlings from alternate stands were less similar to each other than were combinations of seedlings from within a stand. The average similarity index among mature stands was only slightly higher than that for the seedling population, indicating that pairwise combinations of individuals from alternate mature stands were no more or less similar to each other than were pairwise combinations of individuals from alternate seedling stands, even though the seedling stands were established in a single event. The average similarity index for pairwise combinations of individuals from seedling and mature stands was 0.669 ± 0.004, but ranged from 0.611 to 0.715. The average similarity index for pairwise combinations of individuals from the Yellowstone stands and the Lake States outgroup was 0.590 ± 0.008.

The probability that two individuals from within a single stand share all markers by chance alone within the seedling population ranged from 1.31×10^{-20} to 3.94×10^{-24}, based on the formula (Weising et al. 1994)

$$I = [S^2 + (1 - S)^2]^{n/S}$$

where I is the probability of two individuals sharing all markers, S is the average similarity index, and n is the average number of markers per individual.

The probability of two individuals from within a mature stand sharing all markers by chance ranged from 5.72×10^{-2} to 1.29×10^{-10} (Table 2). In the Geode 3 stand there is a 1 in 2 000 000 likelihood that chance alone accounts for the occurrence of five identical samples. Thus, these values suggest that the individuals within a mature stand that share all markers must be identical by descent, i.e., clonal.

The results of the neighbor-joining analysis confirmed and demonstrated that individuals within seedling stands were highly variable; nevertheless, each seedling stand formed a discrete cluster, separate from the other seedling stands (Fig. 3). An exception to this occurred in the seedling stand from Madison River, where one minor and two major clusters within the stand were differentiated. The neighbor-joining analysis also indicated that the seedling stands were more similar to each other than they were to the mature population. Relative to the mature population, the seedling stands were most similar to a cluster containing the Hebgen Lake and Specimen stands.

There was no relationship between geographic proximity among the mature stands and estimates of similarity

as indicated by the neighbor-joining analysis. For example, the two Specimen stands were more similar to the Hebgen Lake stand than they were to the two Lamar stands, despite the fact that Specimen and Lamar are geographically closer (Fig. 1). This lack of relationship between geographic proximity and average similarity value may indicate independent, temporally separate establishment dates for each mature stand.

As expected, individuals within the mature stands were not as variable as individuals within the seedling stands, though generally each individual within a mature stand had a unique multiband RAPD phenotype. The variation within disjunct mature stands appears to originate from two distinct sources, as indicated by (*i*) large distance between clusters of individuals within a stand and (*ii*) short, incremental distance among the majority of the individuals within a stand (Fig. 3). An example of all types of individuals present within a mature stand is found in Geode 3, where individuals 5, 10, 11, 13, and 14 are identical, individuals 1, 2, 3, 4, 6, 7, 8, 9, 12, and 16 form an incrementally joined cluster, and individuals 15, 19, and 20 form a second, sharply segregated cluster (Fig. 4).

The Lake States outgroup formed as a discrete cluster segregated by a considerable distance from the Yellowstone populations (Fig. 3). Within the Lake States outgroup individual seedlings were more similar to each other than were individuals within any of the Yellowstone seedling stands, as indicated by the difference in average branch length within the two groups of seedlings, 0.019 ± 0.002 versus 0.046 ± 0.001, respectively. By comparison, the mature stands from Yellowstone were far less variable than either of the seedling populations. Of the Yellowstone populations, the two mature stands from Geode were more similar to the Lake States outgroup than were other mature stands; the Yellowstone seedling stands were the least similar to the Lake States outgroup.

Discussion

Results from this study indicate that (*i*) the postfire aspen seedling population within Yellowstone National Park is highly genetically variable and (*ii*) the prefire mature population is clonal in nature, but is variable among and within stands.

Several other studies have characterized genetic variability within and among aspen populations in other regions of North America (Cheliak and Dancik 1982; Hyun et al. 1987; Jelinski and Cheliak 1992; Lund et al. 1992; Liu and Furnier 1993; Yeh et al. 1995). Though the methods used to characterize populations with isozymes and RAPD markers are not completely analogous, comparisons can be made for several of the derived metrics. Estimates of percent polymorphic loci on a per-population basis ranged from 67% (Hyun et al. 1987) to 100% (Lund et al. 1992), with the estimate across all previous isozyme studies averaging 85%. Based on RAPD markers, Yeh et al. (1995) and Liu and Furnier (1993) reported an average percent polymorphic loci across populations of 90% and 100%, respectively. In the current study, the percent polymorphic RAPD markers per stand ranged from 52% to 69% for the seedling population. The lower values for seedling population reported in this study appear to be the result of the number and type of markers used. That is, the largest number of loci previously studied was 15 isozyme markers by Hyun et al. (1987) and 61 RAPD markers by Liu and Furnier (1993), compared with 194 RAPD markers examined in this study. Also, it is not clear from discussions in Liu and Furnier (1993) or Yeh et al. (1995) that all monomorphic markers were included in their analyses. Moreover, RAPD markers are dominant, and thus the estimate of polymorphic loci in the current study may be biased downward.

Estimates of the proportion of the total variation associated with differences among geographic populations also varied among previous studies, with F_{ST} values ranging from 0.003 (Lund et al. 1992) to 0.068 (Hyun et al. 1987) across all isozyme loci. Likewise, Yeh et al. (1995) reported that only 2.6% of the total variation was attributed to the among-population component in an analysis of molecular variance. Such estimates indicate that there is very little subdivision among populations. In the current study, F_{ST} estimates for the seedling population averaged 0.311 (Table 3), indicating that 31% of the total variation could be attributed to differences among seedling stands. Across all loci and all Yellowstone populations, F_{ST} equaled 0.561, suggesting that subdivision among the aspen stands is greater than previously reported. In support of this position, RAPD priming sites are generally considered randomly distributed throughout the genome and thus do not typically represent functional sequences (Williams et al. 1990). Hence, RAPD markers tend to be neutral and may be more indicative of variation among and within populations than are isozymes. If so, data from this study supports Chong et al.'s (1994) conclusion that geographic isolation is important in aspen. It should be noted that using the frequency of the null genotype to estimate allele frequency overestimates F_{ST} statistics, regardless of compensations made in loci number and sample size (Lynch and Milligan 1994). Thus, the above comparisons of isozyme data to RAPD data should be viewed with cautions.

In comparison to the Yellowstone seedling population, the mature stands had a lower percentage of polymorphic loci (Table 2). This difference was anticipated as a result of the clonal reproductive habit of aspen (Barnes 1966); however, the amount of variation detected within each mature stand was unexpected. The mature stands occasionally contained as many unique genotypes as sampled stems. This cryptic variation was not expressed at the morphological level, where stands were segregated and sampled based on leaf and stem morphology, as well as spatial isolation. From the neighbor-joining analysis, it appears that the variation within stands results from (*i*) interdigitation of similar but distinct progenitor genotypes and more commonly from (*ii*) small incremental differences among individual samples. In the latter case, RAPD markers may be revealing sequentially accumulated somatic mutations in lateral meristems of a single genetic origin. In support of this hypothesis, Fig. 2A shows a group of three individuals from the Geode 3 stand, samples 15, 19, and 20, which differ from all other individuals within the stand, but which share variant markers at 650, 1100, and 1150 base pairs. Such uniformity within this group, and within the remaining individuals within the stand, suggests that the variation

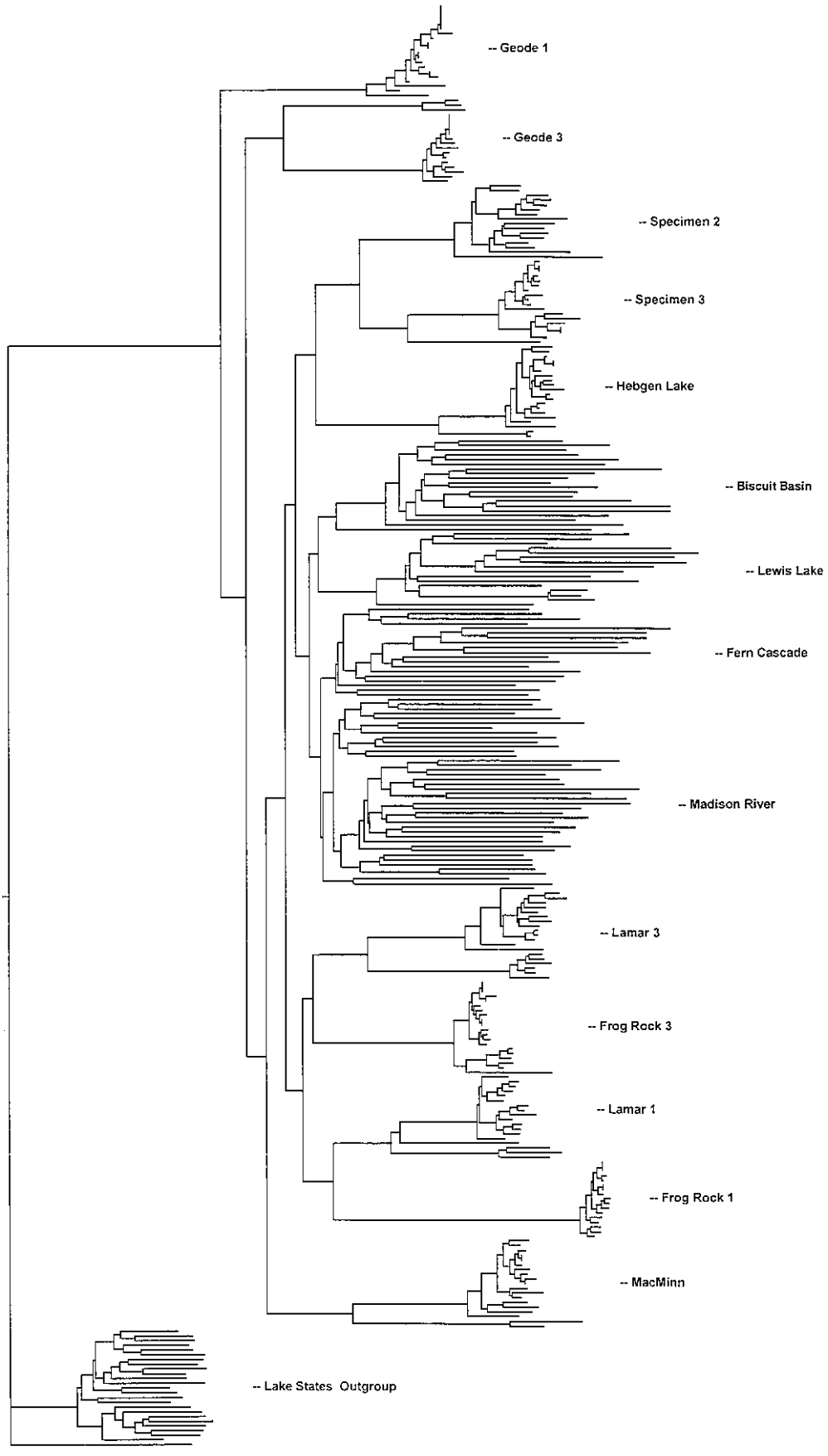

-- Geode 1
-- Geode 3
-- Specimen 2
-- Specimen 3
-- Hebgen Lake
-- Biscuit Basin
-- Lewis Lake
-- Fern Cascade
-- Madison River
-- Lamar 3
-- Frog Rock 3
-- Lamar 1
-- Frog Rock 1
-- MacMinn
-- Lake States Outgroup

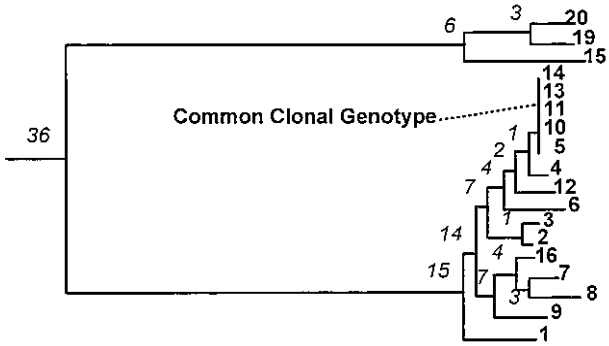
Fig. 3. Phenogram generated using a neighbor-joining algorithm and demonstrating the relationship between seedling and mature aspen populations from Yellowstone National Park. The relationship between alternate branches within a single stand is as follows, from the upper most branch to the lower most branch, respectively: Geode 1: 1, 3, 4, 7, 8, 9, 5, 10, 14, 13, 6, 12, 16, 2, 19, 17, 18, 15, 20, 11; Geode 2: 20, 19, 15, 14, 13, 11, 10, 5, 4, 12, 6, 3, 2, 16, 7, 8, 9, 1; Specimen 2: 15, 16, 17, 11, 4, 2, 13, 3, 8, 5, 1, 6, 7, 9, 10, 14; Specimen 3: 4, 8, 7, 6, 17, 5, 18, 16, 5, 15, 12, 20, 2, 13, 14, 11, 9, 12; Hebgen Lake: 16, 13, 18, 19, 20, 15, 14, 7, 6, 4, 5, 3, 1, 8, 10, 17, 11, 9, 12; Biscuit Basin: 25, 23, 32, 31, 33, 36, 27, 37, 29, 24, 26, 34, 36, 39, 35, 22, 30, 21, 40, 24; Lewis Lake: 11, 4, 15, 2, 10, 8, 9, 6, 3, 7, 5, 17, 12, 13, 14, 1; Fern Cascade: 10, 17, 13, 12, 11, 9, 16, 15, 19, 20, 5, 7, 2, 6, 4, 1, 14, 3, 13; Madison River: 34, 32, 31, 27, 35, 28, 40, 37, 30, 25, 8, 29, 5, 18, 1, 3, 12, 6, 9, 2, 11, 8, 7, 10, 13, 14, 20, 19, 16, 4, 15, 17, 21, 26, 23, 22, 39, 38, 33; Lamar 3: 15, 6, 5, 20, 9, 11, 12, 13, 14, 16, 19, 17, 10, 15, 7, 1, 2, 3, 4, 5; Frog Rock 3: 20, 3, 13, 10, 11, 18, 4, 19, 17, 16, 5, 14, 1, 15, 7, 6, 2, 8, 9; Lamar 1: 12, 14, 9, 10, 11, 17, 12, 4, 5, 7, 20, 18, 13, 15, 16, 3, 1, 5, 2; Frog Rock 1: 5, 12, 13, 4, 7, 2, 3, 1, 16, 9, 8, 10, 18, 14, 15, 11, 19; MacMinn: 3, 5, 7, 14, 15, 16, 18, 13, 5, 17, 4, 1, 2, 10, 11, 9, 6, 20, 19; and Lake States Outgroup: 16, 17, 10, 20, 23, 1, 21, 19, 15, 14, 18, 12, 11, 22, 5, 4, 2, 13, 6, P1, 7, 3, 9, 5, P2. Numeric designations within a stand represent the linear sampling of individual stems within a single stand of trees.

contained in these individuals represents the clonal propagation and interdigitation of two genotypes. Figure 4 depicts neighbor-joining phenogram for the Geode 3 stand based on all 194 markers. The analysis separates the three individuals noted above from all other individuals. These individuals vary in nine markers among themselves and in 36 markers from the remaining individuals in the stand. Within the remaining individuals, the common genotype, samples 5, 10, 11, 13, and 14, varies from the next most similar individual, sample 4, by one marker. This cluster then varies from the next most similar individual, sample 12, by two additional markers, and so on. The sequential accumulation of such markers, the spatial association of alternate markers within the sampling transect, and the central location of the common clonal genotype suggest that all sampled stems originated from two genotypes that subsequentially accumulated somatic mutations over vegetative generations. These relationships are not seen in the Lake States seedling outgroup or Yellowstone seedling population. Similar evidence of sequentially accumulated somatic mutations within a stand occurs in all other mature stands (Fig. 3). Similarly, Liu and Furnier (1993) and Yeh et al. (1995) each postulate that somatic mutations may be affecting genetic diversity detected in aspen in their respective studies.

Comparisons of the average number of bands per population also support the occurrence and accumulation of somatic mutations in the mature population. That is, all point mutations within the template DNA for a given primer sequence will be expressed as the same null allele. Alternatively, each point mutation in template DNA at a potential priming site, creating homology to a given primer, will be expressed as additional, separate markers. Thus, over time, somatic mutations in a clonally propagated organism should result in an increase in the average number of bands per genotype, as is seen in the mature stands in Yellowstone.

Several authors have suggested that somatic mutations provide clonal organisms with a mechanism for enhanced survival and fitness (Cook 1983; Gill and Halverson 1984; Herms and Mattson 1992), particularly in long-lived organisms that may occupy variable environments (Manning and Dickson 1986; Ellstrand and Roose 1987). The occurrence of accumulated somatic mutations has never been directly demonstrated in aspen, let alone the ecological or

Fig. 4. Neighbor-joining phenogram for the Geode 3 stand. Bold numbers at the ends of each branch represent sequentially sampled stems; italicized numbers at the nodes represent the number of polymorphic markers that account for the differentiation among samples.



evolutionary significance of such variation in this or other organisms. Nonetheless, several reports provide indirect evidence for such events. Whitham and Slobodchikoff (1981) and Gill and Halverson (1984) both demonstrated differential feeding by insects on adjacent branches within single plants. In the case of Gill and Halverson (1984), this pattern of herbivory was repeated over successive years, suggesting a genetic basis for such a feeding pattern. Using restriction site variation in rDNA, King and Schaal (1990) were able to demonstrate that mutations did occur in successive generation of the apomictic species *Taraxacum officinale* Weber. Somatic mutations were expressed as the loss of an *Eco*RI restriction site in offspring of 2 of the 31 examined lineages. Likewise, Jeffreys et al. (1988) reported mutations in hypervariable minisatellite DNA in humans. Alternatively, Rogstad et al. (1991) suggested that somatic mutations have not occurred in the aspen populations they characterized using M13 probes. They reported no variation within ca. 12 markers revealed among the 10 tested ramets of a single clone. The inability of Rogstad et al. (1991) to detect somatic mutations should be expected given the limited number of individuals and limited number of markers examined in their study. Results from the present study, based upon a larger number of (*i*) examined markers, (*ii*) sampled individuals, and (*iii*) cell divisions (Klekowski 1988;

Klekowski and Godfrey 1989) potentially represented in each mature stand, bolster the argument for the occurrence and accumulation of somatic mutations in aspen.

Finally, the full sibling versus half-sibling versus non-sibling relationship among individuals within the Yellowstone seedling population may be inferred from the neighbor-joining analysis. Given that the average branch length within the Lake States outgroup is half that of the Yellowstone seedling population, and that the Lake States outgroup is composed of full siblings from a single cross, it is likely that each stand within the Yellowstone seedling population represents a group of related individuals, half-siblings for $q < 1/3$, where q is the frequency of the null allele. That is, Yellowstone seedlings from individual stands are more similar to each other than they are to individuals from other stands, yet they coalesce over a greater distance than do the full-sibling outgroup. Each sampled seedling stand may represent a single seed cast from an open-pollinated mother tree, with the exception of the Madison River stand, which may have arisen through the contributions of two or three mother trees. Hebgen Lake is the first mature stand to cluster with the seedling population (Fig. 3). It is likely then that the parental source for the sampled seedling population is located west of the park boundary, which is also the direction of the prevailing wind. There are additional stands of mature aspen within 2 to 3 km of the Hebgen Lake stand that were not sampled. The proximity of the Madison River seedling stand to the potential parental source may also explain the occurrence of multiple putative mother-tree origins in Madison River not found in the other seedling stands.

In an overall unified scenario of stand establishment and development in aspen, we propose that a disturbance (e.g., fire), in concert with seed availability and subsequent favorable climate, facilitates seed germination and seedling establishment. Over time, seedlings are eliminated via natural selection within a spatially localized homogeneous environment, leading to the survival of morphologically similar genotypes. Simultaneously, somatic mutations accumulate in the surviving genotype as millions of cell divisions occur over time. The interdigitation among morphologically similar clones occurs over time as natural selection eliminates related genotypes established in a single, rare seedling establishment event. The neighbor-joining analysis supports this scenario in that the degree of similarity (i.e., branch length) between any two major clusters within a single mature stand is comparable to that detected among individuals within a single seedling stand (Fig. 3 or 4). Furthermore, interdigitation in aspen has been reported previously (Mitton and Grant 1980; Cheliak and Pitel 1984; Rogstad et al. 1991). In all cases, the general morphology of the clones was indistinguishable, suggesting a degree of relatedness among the interdigitated genotypes. If this hypothesized scenario is correct, then the number of surviving progenitor genotypes (i.e., the number of major clusters within a mature stand) and the number of derivative genotypes (i.e., the number of small incremental subdivisions within a major cluster) could provide a relative estimate of stand age. Based on this premise, data from this study would suggest that Frog Rock 1 and Geode 1 are the oldest mature stands (i.e., each having a single incremental cluster), Lamar 1, Lamar 3, Frog Rock 3, Hebgen Lake, Specimen 2, Specimen 3, and Geode 3 are intermediate, and MacMinn is the youngest mature stand.

The results from the current study raise several questions. The ecological significance, i.e., impacts at the somatic level, and the evolutionary significance, i.e., impacts at the reproductive level, of accumulated somatic mutations in aspen have not been fully investigated or defined. It is possible that somatic mutation (*i*) provides genetic variation upon which natural selection associated with rapidly evolving pest populations or fluctuating climatic conditions acts to eliminate certain genotypes and thus (*ii*) contributes to the long-term survival of individual clones. If so, then questions related to the merits of this adaptive strategy need to be placed within the context of global climate change models (Romme and Turner 1991; Franklin et al. 1992), which predict species range distribution changes associated with calculated temperature and moisture changes. Prior to this effort, the proposed scenario of aspen stand establishment needs to be tested in other populations along with the use of maternally inherited cytoplasmic markers to confirm the relationship among individuals in seedling stands. And finally, processes that lead to monoclonal stands need to be documented. That is, how do seedling stands that contain dozens of genotypes within several dozen square metres develop into mature stands that contain one or two progenitor genotypes that occupy dozens of hectares? Are most of the seedling genotypes eliminated early in stand development through random processes such as ungulate browsing (Jelinski and Fisher 1991) or are seedling genotypes progressively eliminated over time via directional selection associated with climate and (or) the physical environment?

Acknowledgments

The authors thank Mike and Cyndi O'Hara for assistance with sample collection, Bailian Li and the Aspen and Larch Genetic Cooperative for supplying the full-sibling aspen progeny, Karen Segert and Migdalia Plaza for laboratory assistance during DNA extractions, Joseph Tuskan for assembling the graphics files, and Lee Gunter, Toby Bradshaw, Steve Strauss, and Dave O'Malley for the their insights and suggestions on an earlier version of this manuscript. Research was partially supported by National Science Foundation grants BSR-9016281 and BSR-9018381 through the Ecosystems Studies Program and by the U.S. Department of Energy, under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.

References

Bachmann, K. 1994. Molecular markers in plant ecology. Tansley review 63. New Phytol. **126**: 403–418.

Barnes, B.V. 1966. The clonal growth habit of American aspens. Ecology, **47**:439–447.

Cheliak, W.M., and Dancik, B.P. 1982. Genic diversity of natural populations of a clone forming tree *Populus tremuloides*. Can. J. Genet. Cytol. **24**: 611–616.

Cheliak, W.M., and Pitel, J.A. 1984. Electrophoretic identification of clones in trembling aspen. Can. J. For. Res. **14**: 740–743.

Chong, D.K.X, Yang, R.-C., and Yeh, F.C. 1994. Nucleotide divergence between populations of trembling aspen estimated with RAPDs. Curr. Genet. **36**: 374–376.

Clark, A.G., and Lanigan, C.M.S. 1993. Prospects for estimating nucleotide divergence with RAPD's. Mol. Biol. Evol. **10**: 1096–1111.

Cook, R.E. 1983. Clonal plant populations. Am. Sci. **71**: 244–253.

Despain, D.G. 1991. Yellowstone vegetation. Consequences of environment and history in natural setting. Roberts Rinehart Publishers, Boulder, Colo.

Despain, D.G., Rodman, A., Schullery, P., and Shovic, H. 1989. Burned area survey of Yellowstone National Park: the fires of 1988. Internal Report, Geographic Information Systems Laboratory, Yellowstone National Park.

Doyle, J.J., and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. **19**: 11–15.

Ellstrand, N.C., and Roose, M.L. 1987. Patterns of genotypic diversity in clonal plant species. Am. J. Bot. **74**: 123–131.

Ellsworth, D.L., Rittenhouse, K.D., and Honeycutt, R.L. 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. BioTechniques, **14**: 214–217.

Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. [Produced and distributed by the author.] Department of Genetics, University of Washington, Seattle.

Franklin, J.F., Swanson, F.J., Harmon, M.E., Perry, D.A., Spies, T.A., Dale, V.H., McKee, A., Ferrell, W.K., Means, J.E., Gregory, S.V., Lattin, J.D., Schowalter, T.S., and Larsen, D. 1992. Effects of global climatic change on forests in northwestern North America. *In* Global warming and biological diversity. *Edited by* R.T. Peter and T.E. Lovejoy. Yale University Press, New Haven, Conn.

Gill, D.E., and Halverson, T.G. 1984. Fitness variation among branches within trees. *In* Evolutionary Ecology: 23rd Symposium of the British Ecological Society. *Edited by* B. Shorrocks. Blackwell Scientific, Oxford, London. pp. 105–116.

Grant, M.C. 1993. The trembling giant. Discover, **14**: 83–89.

Hadrys, H., Black, M., and Schierwater, B. 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol. Ecol. **1**: 55–63.

Herms, D.A., and Mattson, W.J. 1992. The dilemma of plants: to grow or defend. Q. Rev. Biol. **67**: 283–335.

Hyun, J.O., Rajora, O.P., and Zsuffa, L. 1987. Genetic variation in trembling aspen in Ontario based on isozyme studies. Can. J. For. Res. **17**: 1134–1138.

Jeffreys, A.J., Royle, N.V., Wilson, V., and Wong, Z. 1988. Spontaneous mutation rates to new length alleles at tandem-repetitive hypervariable loci in human DNA. Nature (London), **332**: 278–281.

Jelinski, D.E., and Cheliak, W.M. 1992. Genetic diversity and spatial subdivision of *Populus tremuloides* in a heterogeneous landscape. Am. J. Bot. **79**: 728–736.

Jelinski, D.E., and Fisher, L.J. 1991. Spatial variability in the nutrient composition of *Populus tremuloides*: clone-to-clone differences and implications for cervids. Oecologia, **88**: 116–124.

Kambhampati, S., Black, W.C., and Rai, K.S. 1992. RAPD-PCR for identification and differentiation of mosquito species and populations: techniques and statistical analysis. J. Med. Entomol. **29**: 939–945.

Kay, C.E. 1993. Aspen seedlings in recently burned areas of Grand Teton and Yellowstone National Parks. Northwest Sci. **67**: 94–104.

Kemperman, J.A. 1977. Aspen clones: development, variability and identification. Ontario Ministry of Natural Resources, Toronto. For. Res. Inf. Pap. 101.

King, L.M., and Schaal, B.A. 1990. Genotypic variation within asexual lineages of *Taraxacum officinale*. Proc. Natl. Acad. Sci. U.S.A. **87**: 998–1002.

Klekowski, E.J. 1988. Genetic load and its cause in long-lived plants. Trees, **1**: 195–203.

Klekowski, E.J., and Godfrey, P.J. 1989. Ageing and mutation in plants. Nature (London), **340**: 389–391.

Libby, W.J., and Ahuja, M.R. 1993. The genetics of clones. *In* Clonal forestry I: genetic and biotechnology. *Edited by* M.R. Ahuja and W.J. Libby. Springer-Verlag, Berlin. pp. 5–13.

Liu, Z., and Furnier, G.R. 1993. Comparison of allozyme, RFLP, and RAPD markers for revealing genetic variation within and between trembling aspen and bigtooth aspen. Theor. Appl. Genet. **87**: 97–105.

Lund, S.T., Furnier, G.R., and Mohn, C.A. 1992. Isozyme variation in quaking aspen in Lake States. Can. J. For. Res. **22**: 521–524.

Lynch, M., and Milligan, B.G. 1994. Analysis of population genetic structure with RAPD markers. Mol. Ecol. **3**: 91–99.

Manning, J.T., and Dickson, D.P.E. 1986. Asexual reproduction, polyploidy and optimal mutation rates. J. Theor. Biol. **118**: 485–489.

McDonough, W.T. 1979. Quaking aspen—seed germination and early seedling growth. USDA For. Serv. Res. Pap. INT-234.

Mitton, J.B., and Grant, M.C. 1980. Observations on the ecology and evolution of quaking aspen, *Populus tremuloides*, in the Colorado Front Range. Am. J. Bot. **67**: 202–209.

Mitton, J.B., and Grant, M.C. 1996. Genetic variation and natural history of quaking aspen. BioScience, **46**: 25–31.

Munthali, M., Ford-Lloyd, B.V., and Newbury, H.J. 1992. The amplification of polymorphic DNA for fingerprinting plants. PCR Methods Appl. **1**: 274–276.

Nei, M., and Li, W.H. 1985. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. **76**: 5269–5273.

Pellissier, M.S., Haymes, K.M., and Williams, S.M. 1992. Parentage analysis using RAPD PCR. Nucleic Acids Res. **20**: 5493.

Perala, D.A. 1990. *Populus tremuloides* Michx. quaking aspen. *In* Silvics of North America. Vol. 2. Hardwoods. *Edited by* R.M. Burns and G.H. Honkala. U.S. Dep. Agric. Agric. Handb. 654. pp. 555–569.

Riedy, M.F., Hamilton, W.J., and Aquadro, C.F. 1992. Excess of non-parental bands in offspring from known primate pedigrees assayed using RAPD PCR. Nucleic Acids Res. **20**: 918.

Rogstad, S.H., Nybom, H., and Schaal, B.A. 1991. The tetrapod "DNA fingerprinting" M13 repeat probe reveals genetic diversity and clonal growth in quaking aspen (*Populus tremuloides*, Salicaceae). Plant Syst. Evol. **175**: 115–123.

Romme, W.H. 1982. Fire and landscape diversity in subalpine forests of Yellowstone National Park. Ecol. Monogr. **52**: 199–221.

Romme, W.H., and Turner, M.G. 1991. Implications of global climate change for biogeographic patterns in the greater Yellowstone ecosystem. Conserv. Biol. **5**: 373–386.

Romme, W.H., Turner, M.G., Wallace, L.L., and Walker, J. 1995. Aspen, elk, and fire in northern Yellowstone National Park. Ecology, **76**: 2097–2106.

Romme, W.H., Turner, M.G., Gardner, R.H., Hargrove, W.W., Tuskan, G.A., Despain, D.G., and Renkin, R. 1996. A rare episode of sexual reproduction in aspen (*Populus tremuloides* Michx.) following the 1988 Yellowstone fires. Nat. Areas J. In press.

Saitou, N., and Nei, M. 1987. The neighbor joining method: a new Method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**: 406–425.

Stettler, R.F., and Ceulemans, R.J. 1993. Clonal materials as a focus for genetic and physiological research in forest trees. *In* Clonal forestry I: genetic and biotechnology. *Edited by* M.R. Ahuja and W.J. Libby. Springer-Verlag. Berlin. pp. 68–86.

Weising, K., Nybom, H., Wolff, K., and Meyer, W. 1994. DNA fingerprinting in plants and fungi. CRC Press, London.

Welsh, J., and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. **18**: 7213–7218.

Whitham, T.G., and Slobodchikoff, C.N. 1981. Evolution by individuals, plant–herbivore interactions, and mosaics of genetic variability: the adaptive significance of somatic mutations in plants. Oecologia, **49**: 287–292.

Williams, J.G.K., Kubelik, A.R., Livak, K.L., Rafalski, J.A., and Tingey, S.C. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. **18**: 6531–6535.

Yeh, F.C., Chong, D.K.X., and Yang, R.C. 1995. RAPD variation within and among natural populations of trembling aspen (*Populus tremuloides* Michx.) from Alberta. J. Hered. **86**: 454–460.