



Allozyme and microsatellite data reveal small clone size and high genetic diversity in aspen in the southern Cascade Mountains

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ABSTRACT

The most widely distributed tree in North America, quaking aspen (*Populus tremuloides*, Michx.), reproduces sexually via seed and clonally via suckers. The size of aspen clones varies geographically, generally smaller in the east and large in the arid Intermountain West. In order to describe clone size and genetic structure of aspen in the southern Cascade Mountains, 864 stems from six sites were assayed at 15 isozyme and 6 microsatellite loci. Although isozymes reveal significantly lower levels of allelic richness ($P < 0.001$) and expected heterozygosity ($P < 0.01$), differences in genet diversity (isozyme $G/N = 0.45$, microsatellite $G/N = 0.47$) and allele frequency variation (isozyme $F_{ST} = 0.02$, microsatellite $F_{ST} = 0.03$) were nonsignificant. While a majority of stands were monoclonal, such stands were small, and the number of clones per stand was positively correlated with stand size ($P < 0.0001$). High genetic diversity, low genetic differentiation, and a rapid decay of spatial genetic structure consistent with long distance gene flow during seedling recruitment indicate that sexual reproduction is a significant factor contributing to the genetic structure of these populations. These findings further resolve the geographic variation in clonal structure observed in aspen across North America, providing novel information for land management and conservation efforts.

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1. Introduction

Trembling aspen (*Populus tremuloides*, Michx., Salicaceae) is the most widely distributed tree in North America (Little, 1971), and is commonly known for its ability to propagate via suckering, or vegetative reproduction through roots (Greene et al., 2004). The repeated production of shoots by a single genetic individual produces stands composed of genetically identical stems connected at least initially via the underground root system (Barnes, 1966). By contrast, recruitment of seedlings is thought to be rare due to the narrow ecological requirements for seedling establishment (Jelinski and Cheliak, 1992; Romme et al., 1997), but when a large-scale disturbance occurs, seedling recruitment can produce large stands consisting of numerous genetic individuals (Tuskan et al., 1996). Given that this species is dioecious and has wind-dispersed seed (Every and Wiens, 1971), gene flow by both pollen and seeds may occur over large geographic distances (Jelinski and Cheliak, 1992).

Aspen stands have an important ecological role in the western U.S. Compared to surrounding conifer forests, aspen communities contain a greater diversity of butterflies (Chong et al., 2001) and birds (Rumble et al., 2001), and a greater species diversity and abundance of terrestrial snails (Karlin, 1961) and insects (Schimpf and MacMahon, 1985). Due to higher levels of plant and animal diversity within aspen communities compared to surrounding conifer forests, aspen communities have been considered oases for plant and animal diversity (Shepperd et al., 2006) and aspen have been considered a keystone species due to the importance of aspen in maintaining biodiversity on the landscape (Campbell and Bartos, 2001).

Aspen stands are in decline in Lassen National Forest (LNF) in the southern Cascade Mountains (Jones et al., 2005). Intensive sheep grazing in the late 19th century, ongoing cattle grazing, and fire suppression since the early 1900s have limited aspen regeneration. An inventory of 709 aspen stands on the LNF, of which 43 (6%) were completely dead, revealed that 528 (79%) of the remaining live stands were at a high risk of being replaced by conifers (District Records, ELRD, LNF). In addition, 641 stands (96%) contained a conifer component recommended for removal, and 357 stands (54%) were affected by excessive browsing. Stands were typically small, with a median size of 0.6 ha, isolated in thick conifer forests, and were commonly composed of a small number of sparsely spaced, decadent stems.

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Table 1

Location and size of aspen stands sampled from the Lassen National Forest in the southern Cascade Mountains.

| Site | Latitude (dd.ddd) | Longitude (dd.ddd) | Elevation (m) | Total no. samples | No. stands | Mean (s.d.) samples per stand | Mean stand size (acres) | Range of stand sizes (acres) |
|------------------------|-------------------|--------------------|---------------|-------------------|------------|-------------------------------|-------------------------|------------------------------|
| Harvey Mountain (HM) | 40.669 | –121.027 | 1700 | 126 | 20 | 6.4 (5.3) | 7.12 | 0.21–78.9 |
| Pine Creek Valley (PC) | 40.522 | –121.022 | 1750 | 135 | 38 | 3.5 (2.4) | 1.29 | 0.01–7.60 |
| Bogard Buttes (BB) | 40.551 | –121.115 | 1800 | 231 | 24 | 9.6 (14.0) | 20.7 | 0.03–233.3 |
| Prospect Peak (PP) | 40.597 | –121.289 | 1750 | 167 | 10 | 17.1 (30.3) | 41.0 | 0.17–324.1 |
| Peg Leg Mountain (PL) | 40.431 | –120.928 | 1650 | 89 | 18 | 4.9 (3.8) | 3.76 | 0.08–18.54 |
| Fredonyer Pass (FP) | 40.284 | –120.809 | 2000 | 116 | 15 | 8.3 (8.1) | 7.71 | 0.07–38.7 |
| Overall | | | | 864 | 125 | 6.9 (11.2) | 10.1 | 0.01–324.1 |

Due to its important ecological role (Campbell and Bartos, 2001; Shepperd et al., 2006), aspen is now the focus of intensive management activity, including efforts to thin conifers around stands, exclude browsing ungulates and to develop methods to stimulate suckering in existing stands (Jones et al., 2005). Understanding the distribution of aspen clones within and among stands, as well as the distribution of genetic variation among clones, is necessary to develop more effective and long-term management plans for this declining species.

Previous studies have concluded that the size of aspen stands varies across the species range. Aspen stands in eastern North America tend to be small, typically less than one to a few acres (Kemperman and Barnes, 1976; Wyman et al., 2003). In the arid west, by contrast, clones of aspen can become very large, consisting of thousands of stems covering tens or hundreds of acres of habitat (Barnes, 1966; Kemperman and Barnes, 1976; Mitton and Grant, 1996; DeWoody et al., 2008), although clones in some areas were found to be small in size, less than 10 acres (Yeh et al., 1995). Not every stand is necessarily monoclonal, however, and complex clonal boundaries with interdigitating stems have been reported in large stands (Mitton and Grant, 1980; Stevens et al., 1999; Namroud et al., 2005; Mock et al., 2008). Little work has been reported describing the clonal structure of aspen in the Pacific West of North America. A recent study of aspen in the central Sierra Nevada Mountains concluded that small stands tend to be monoclonal, with large stands composed of more than one clone (Hipkins and Kitzmiller, 2004).

While aspen clones may appear distinct morphologically, phenotypic variation is not always sufficient to accurately delineate clonal boundaries within stands (Wyman et al., 2003). Molecular genetic data can provide greater resolution of the number and distribution of aspen clones. Two types of genetic data most widely used in population genetic studies are isozymes and microsatellites (simple-sequence repeats, SSRs). Both types of analyses provide co-dominant data and both markers are accepted as selectively neutral for statistical purposes (van Oosterhout et al., 2004). Yet these two marker systems differ in rate and mechanism of mutation, both of which could affect the conclusions drawn from the data (Ellstrand and Roose, 1987; Arnaud-Haond et al., 2007). The lower levels of variation expected in isozymes may be insufficient to resolve individual clones. Yet faster-evolving microsatellite loci may display rates of somatic mutation sufficient to cause ramets (single stems) of a clone to appear to be genetically distinct, especially in a long-lived species (Mock et al., 2008). Combining data types may provide a more comprehensive picture of clonal delineation in *P. tremuloides*.

This study assayed aspen samples from six sites on the Lassen National Forest in the southern Cascade Mountains for isozyme and microsatellite variation in order to address three objectives. First, what is the distribution of clones within and among stands? Second, how is genetic variation partitioned within and among sites? Third, how may these findings be applied toward the management and conservation of this species?

2. Methods

2.1. Sample collection

Aspen from 125 stands within six study areas on the LNF were sampled for genetic analysis in summer 2003 (Table 1). A “stand” was defined as a patch of aspen separated from other aspen patches by a 30.5 m gap in which no living or dead aspen were detected. Narrower gaps were allowed to define stands when features such as streams, rock outcrops or roads were present that sharply defined the edge of the patch. At each site, the boundary of each stand of aspen was delineated using hand-held global positioning units. Within stands, stems were selected at random for genetic analysis, their position recorded, and leaves collected. A total of 864 stems were sampled.

2.2. Isozyme analysis

Two hole-punches of leaf tissue were submerged in 100 μ L Gottlieb's (1981) extraction buffer and frozen at -80°C for isozyme analysis. Starch gel electrophoresis resolved a total of 15 isozyme loci in three buffer systems (Conkle et al., 1982), using stain recipes adapted from Wendel and Weeden (1989). Three loci were resolved in a lithium borate electrode buffer-tris citrate gel buffer combination (system LB): malic enzyme (ME(7); EC 1.1.1.40) and phosphoglucose isomerase (PGI1 and PGI2; EC 5.3.1.9); five loci in a sodium borate electrode buffer-tris citrate gel buffer (system SB): aspartate aminotransferase (AAT1 and AAT2; EC 2.6.1.1), catalase (CAT; EC 1.11.1.6), and uridine diphosphoglucose pyrophosphorylase (UGPP1 and UGPP2; EC 2.7.7.9); and seven loci in a morpholine citrate electrode and gel buffer (system MC8): isocitrate dehydrogenase (IDH; 1.1.1.42), malate dehydrogenase (MDH1, MDH2, and MDH3; EC 1.1.1.37), phosphogluconate dehydrogenase (6PGD1 and 6PGD2; EC 1.1.1.44), and shikimic acid dehydrogenase (SKD; EC 1.1.1.25).

2.3. DNA analysis

Total genomic DNA was extracted from each sample using Qiagen[®] DNEasy-96 plant extraction kit, following the protocol for liquid nitrogen extraction. DNA quality and quantity were estimated using agarose gel electrophoresis.

Samples were analyzed for six microsatellite loci: ORNL-29 (Tuskan et al., 2004), PMGC-420, PMGC-433, PMGC-576, PMGC-649, and PMGC-2571 (International *Populus* Genome Consortium, http://www.ornl.gov/sci/ipgc/ssr_resource.htm). The forward primer for each locus was fluorescently labeled for automated analysis. Amplification reactions included approximately 1 ng template DNA, $1\times$ reaction buffer (provided with enzyme), 2.5 mM MgCl_2 , 1.25 mM each dNTP, 10.0 μM each primer, and 0.2 U HotStar-Taq (Qiagen[®]) in a 10 μL total reaction volume. Amplification took place using a touchdown program provided by S. DiFazio (personal communication): 95°C for 15:00 (mm:ss), four

cycles of 94 °C for 00:30, 55 °C for 00:30, 72 °C for 01:00, four cycles of 94 °C for 00:30, 52 °C for 00:30, 72 °C for 01:00, 25 cycles of 94 °C for 00:30, 50 °C for 00:30, 72 °C for 01:00, and a final extension of 72 °C for 15:00. The reaction conditions for PMGC-2571 were modified to anneal at 58 °C for the first four cycles. Amplification products were visualized on an ABI-3100 capillary system, and peaks scored using the peak label function in Genotyper (v3.7, Applied Biosystems, Inc.), with every sample verified by visual inspection. Twenty-seven samples (3%) were re-genotyped and the data were examined for quality assurance. Alleles were defined using the binning function in Allelogram v1.2 (Manaster, 2002).

2.4. Data analysis

Clones were delineated independently for isozyme and DNA data using the multilocus matches function in GENALEX v6 (Peakall and Smouse, 2005) and confirmed by visual inspection of raw data, with a mismatch of one allele tolerated between each pair. For both data types, the total number of unique genotypes (G), genet diversity (G/N , where N is the number of samples), mean and maximum number of stems sampled per clone, number of clones split among stands and sites, and the number of mismatches in clonal boundaries (e.g. one isozyme genotype corresponding to more than one microsatellite genotypes) were calculated for each data set. The null hypothesis of no correlation between stand size (in acres) and the number of unique genotypes per stand was tested using Pearson's product moment correlation coefficient as implemented by R v2.4.0 (R Development Core Team, 2006). In order to determine the probability that two genotypes would be identical by chance, the probability of identity, $Pr(I)$, was estimated over all loci for each data type using GENALEX v6 (Peakall and Smouse, 2005). This measure quantifies the probability of a specific multilocus genotype being randomly generated from the observed population, such that for alleles i and j with frequencies p_i and p_j , $Pr(I) = \sum p_i + \sum \sum (2p_i p_j)$, for all $i \neq j$ (Sherman, 2008).

In order to describe genetic variation and differentiation among sites of aspen, both data sets were reduced to a single sample per genotype per site. The microsatellite data set was tested for genotyping errors using MICRO-CHECKER V2.2.3 (van Oosterhout et al., 2004). When evidence of a null allele was found at a locus, Brookfield's (1996) method for the case of null-null homozygotes was used to estimate corrected allelic and genotypic frequencies for sites. Allele frequencies for isozyme loci and those microsatellite loci containing no null alleles were calculated using GENALEX v6 (Peakall and Smouse, 2005).

Six standard measures of genetic diversity were calculated for each data set: mean alleles per locus (A) and mean allelic richness (R_S) were determined using FSTAT V2.9.3.2 (Goudet, 1995, 2001), and percent polymorphic loci (P), observed heterozygosity (H_O), and expected heterozygosity (H_E) using GDA (Lewis and Zaykin, 2001). Differences in R_S , H_E , and G/N among data types were determined using the Wilcoxon Rank Sum test (equivalent to Mann-Whitney's U -test) as employed by R v2.4.0 (R Development Core Team, 2006).

Two analyses to describe the distribution of genetic variation among sites were conducted separately on the isozyme and microsatellite data sets. First, genetic differentiation among sites was estimated for isozyme genotypic data and the adjusted microsatellite allele frequency data using Weir and Cockerham's (1984) θ , with significance calculated over 1000 bootstraps using FSTAT V2.9.3.2 (Goudet, 1995, 2001). Second, spatial genetic structure was assessed within each site for both data types. Spatial classes were defined in 100 m intervals, with the largest class size ranging from 3200 m in PC to 6000 m in BB, HM, and PP. Following the method of Kalisz et al. (2001), kinship (f_{ij}) was estimated over all pairs of individuals within each spatial class, using first all

sampled stems and then one stem per genotype with spatial position defined as the midpoint of all stems sharing a genotype. For the microsatellite genotypic data, null alleles were coded as missing data, with different sample sizes among loci accounted for in the adjusted estimates of f_{ij} (Kalisz et al., 2001). As described in Kalisz et al. (2001), significance of observed kinship was tested by defining the confidence interval around the null hypothesis of no spatial genetic structure ($H_0: f_{ij} = 0$) by randomizing locations of individuals over 1000 replicates. Significant spatial genetic structure was identified as those estimates of f_{ij} occurring outside the 99% confidence interval around $H_0: f_{ij} = 0$.

3. Results

3.1. Clonal diversity

852 stems were genotyped for isozyme data, 864 stems for microsatellite data, and 851 stems genotyped for both markers. A fraction of genotypes in both data sets (12.9% of isozyme genotypes and 7.9% of microsatellite genotypes) displayed more than two alleles at up to three loci (data not presented). Triploidy has been described in trembling aspen (Every and Wiens, 1971), but no sample displayed patterns indicative of polyploidy in both isozyme and microsatellite loci. Four microsatellite loci demonstrated an excess of homozygotes consistent with null alleles (ORNL-29, PMGC-420, PMGC-576, and PMGC-649). Mean error rates were 0.031 mistypes per reaction (range among loci 0.0–0.074), and 0.015 mistypes per allele (range over loci 0.0–0.037). Although error due to stuttering or large-allele dropout was possible, results of tests were marginally significant, and replicates indicate neither phenomenon significantly affected data quality.

Isozymes resolved 387 genotypes, microsatellites resolved 406 genotypes, and the combined data identified 409 unique genotypes, indicating a low level of inconsistency between the two marker types (Table 2). Mismatches of clonal boundaries resulted in 19 isozyme genotypes containing more than one microsatellite genotype, and 5 microsatellite genotypes containing more than one isozyme genotype. The probability of identity for isozyme genotypes ($Pr(I)_I = 1.3 \times 10^{-5}$) was greater than that for microsatellite genotypes ($Pr(I)_D = 4.0 \times 10^{-7}$), but both values indicate the data sets provide sufficient power to distinguish individual clones. The presence of null alleles in the microsatellite data is expected to decrease the power to identify clones, in particular to distinguish between samples heterozygous for a null allele and true homozygotes. However, the probability of identity estimates for the two marker types indicate that even with null alleles, the microsatellite data provide greater power than the isozyme data for clone delineation.

Table 2

Isozyme and microsatellite data reveal concordant levels of clone diversity in aspen from the southern Cascade Mountains.

| | Isozyme | Microsatellite | Combined |
|--|-----------|----------------|-----------|
| No. of samples (N) | 852 | 864 | 851 |
| No. or genotypes (G) | 387 | 406 | 409 |
| Genet diversity (G/N) | 0.45 | 0.47 | 0.48 |
| Mean (s.d.) stems per genotype | 2.2 (2.1) | 2.1 (1.9) | 2.1 (1.9) |
| Max stems per genotype | 19 | 14 | 14 |
| No. monoclonal stands | 69 | 66 | 66 |
| No. genotypes in >1 stand | 26 | 24 | 23 |
| No. genotypes in >1 site | 2 | 0 | 0 |
| No. mismatches in clonal boundaries ^a | 19 | 5 | NA |

Clonal membership and diversity were estimated separately for 15 isozyme loci, six microsatellite loci, and the combined data set.

^a Number of genets corresponding to more than one genotype in the alternate data set.

Differences in genet diversity (G/N) were nonsignificant between data types ($P > 0.05$): 0.45 for isozymes, 0.47 for microsatellites, and 0.48 for the combined data (Table 2). The congruence between data types in measures of genet diversity indicate the microsatellite data likely provide sufficient power for clonal delineation, despite the presence of null alleles. However, the clones identified with these six microsatellite loci might be considered a conservative measure, and the addition of loci lacking nulls may further resolve clonal relationships.

The pattern of distribution of genets among stands was consistent at the six sampling sites. The mean number of stems sampled per genotype, maximum number of stems sampled for a genotype, and number of genotypes split among stands were identical for microsatellite and combined data, but slightly higher for the isozyme data (Table 2). The modal number of stems per genotype was equal to 1.0 for all three data types, indicating a left skew to the number of ramets sampled per clone. A moderate proportion of stems (205, 24%) contained a unique genotype at both isozyme and microsatellite data, indicating that there was a single ramet sampled of that clone.

The number of monoclonal stands was consistent among data sets: 69 (56%) for isozyme data, 66 (53%) for microsatellite and combined data. Monoclonal stands were small (mean 1.13 acres, median 0.57 acres, range 0.01–7.6 acres). Correlation between stand size and the number of genotypes was equal and highly significant for all data types ($r^2 = 0.95$, $P < 0.0001$; Fig. 1).

A total of 23 clones (based on the combined data set) occurred in more than one stand (Table 2). Adjacent stands that share a clone were likely once connected and have become fragmented as the aspen reproduce via suckering. If so, these results provide evidence that 46 stands arose via fragmentation. Disregarding one clone that was interrupted by a road (potentially fragmented due to anthropogenic causes), of the 22 putatively fragmented clones, 20 were broken into two stands, and two were separated into three stands. In order to estimate the linear extent of aspen coverage lost due to this fragmentation, we calculated the total distance between stands which were previously connected to be approximately 2565 m. For comparison, the current length of the 46 stands delineated in the survey, measured across their long axis, totalled

8514 m. Thus, the gap distance of 2565 m indicates that these stands have lost 30.1% of their linear extent.

3.2. Genetic variation

While the complete data set was used for clonal identification, in order to create fully diploid multilocus genotypes for analyses of genetic variation and differentiation, the allele with the highest frequency in the complete data set was dropped from each individual locus displaying a polyploidy banding pattern. This method did not change estimates of differentiation or inbreeding when compared to the approach where the major allele was retained (data not presented), likely due to the small proportion of genotypes involved. Further, this approach minimizes the potential that two distinct genotypes would appear identical in diploid form. Tests of Hardy–Weinberg equilibrium were conducted solely on the isozyme data, as the presence of null alleles makes the microsatellite data inappropriate for such interpretation.

Isozymes contain fewer alleles per locus ($A = 3.67$), lower allelic richness ($R_S = 2.61$), and lower levels of heterozygosity, both observed ($H_O = 0.332$) and expected ($H_E = 0.345$) than microsatellites ($A = 16.2$, $R_S = 9.06$, $H_O = 0.515$, $H_E = 0.740$, Table 3). Results of Wilcoxon rank sum tests over per-locus estimates indicate allelic richness ($P < 0.001$) and heterozygosity ($P = 0.002$) differed significantly among data types. Levels of allelic richness (which accounts for different sample sizes) and heterozygosity were consistent among sites within each data type.

3.3. Genetic differentiation

Low but significant genetic differentiation was observed among sites for both isozymes ($\theta = 0.022$; 95% C.I. 0.012–0.035) and microsatellites ($\theta = 0.029$; 95% C.I. 0.020–0.037), indicating gene flow in aspen is not unrestricted across the landscape.

Measures of kinship were similar for isozyme and microsatellite data. Significant fine-scale genetic structure was observed when all stems were included in the data set, with estimates of f_{ij} exceeding the 99% CI around $H_O:f_{ij} = 0$ for distance classes less than 1000 m in

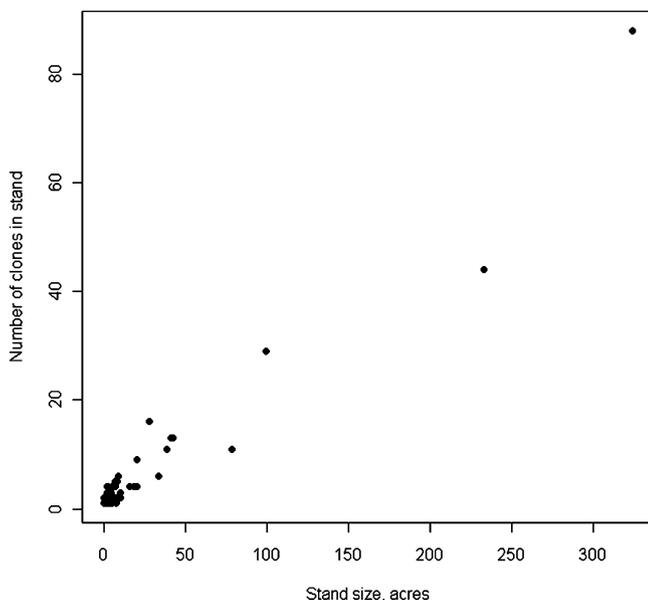


Fig. 1. The number of aspen clones per stand increases with stand size in the southern Cascade Mountains ($r^2 = 0.95$, $P < 0.001$). Clones were identified from the combined data for 15 isozyme and six microsatellite loci; data from the individual data sets produced congruent correlations.

Table 3

Genetic diversity statistics for six collections of aspen from the southern Cascade Mountains.

| Data/Site | N^a | P^b | A^c | R_S^d | H_O^e | H_E^f |
|-----------------------|-------|-------|-------|---------|---------|---------|
| Isozyme | | | | | | |
| HM | 50 | 73.3 | 2.73 | 2.56 | 0.359 | 0.333 |
| PC | 43 | 80.0 | 2.93 | 2.71 | 0.360 | 0.344 |
| BB | 106 | 73.3 | 2.80 | 2.44 | 0.325 | 0.322 |
| PP | 109 | 80.0 | 3.00 | 2.47 | 0.338 | 0.328 |
| PL | 25 | 80.0 | 2.67 | 2.65 | 0.350 | 0.333 |
| FP | 51 | 66.7 | 2.40 | 2.21 | 0.336 | 0.332 |
| Overall | 383 | 75.6 | 3.67 | 2.61 | 0.332 | 0.345 |
| Microsatellite | | | | | | |
| HM | 50 | 100.0 | 10.7 | 9.02 | 0.519 | 0.719 |
| PC | 41 | 100.0 | 9.5 | 8.51 | 0.478 | 0.739 |
| BB | 117 | 100.0 | 12.2 | 8.76 | 0.557 | 0.752 |
| PP | 113 | 100.0 | 10.8 | 8.03 | 0.522 | 0.772 |
| PL | 24 | 100.0 | 7.5 | 7.44 | 0.474 | 0.735 |
| FP | 52 | 100.0 | 9.0 | 7.60 | 0.545 | 0.728 |
| Overall | 395 | 100.0 | 16.2 | 9.06 | 0.515 | 0.740 |

Fifteen isozyme loci and six microsatellite loci were assessed for each sample. Site labels follow Table 1. The discrepancy between observed and expected heterozygosity can be attributed to null alleles.

^a Mean number of unique genotypes assessed for diversity statistics.

^b Percent polymorphic loci.

^c Mean alleles per locus.

^d Mean allelic richness.

^e Observed heterozygosity.

^f Expected heterozygosity.

BB and PP, and less than 500 m all other sites. Analyses of one stem per genotype detected spatial genetic structure in the smallest distance classes (<200 m) in BB and PP (BB depicted in Fig. 2A, PP equivalent), but not in the remaining sites (PC presented in Fig. 2B, others equivalent). Estimates of kinship were significantly smaller than zero for distance classes greater than 1000 m in all sites for both data types and data sets (Fig. 2), indicating lower levels of relatedness than expected on average, potentially due to the obligatory outcrossing mating system of this dioecious species or the rare and episodic nature of recruitment from seed. Correspondence between isozyme and microsatellite data in measures of genet diversity, genetic differentiation, and spatial autocorrelation indicate the null alleles likely do not bias interpretation of the distribution of genetic variation in these samples.

4. Discussion

4.1. Clonal distribution and concordance of data types

Stands of aspen in the southern Cascade Mountains contain large amounts of clonal and genetic diversity as assayed by isozyme and microsatellite markers. Single clones of aspen do not appear to reach the large size expected in western North America.

Rather, stands composed of single clones are small compared to reports in the Intermountain West (less than 8 acres in area compared to tens or hundreds of acres, Mitton and Grant, 1996; DeWoody et al., 2008; Mock et al., 2008), with larger stands being composed of more than one genetic individual (clonal diversity significantly correlated with stand size). The small size of most clones is consistent with the moderate level of genet diversity (Table 2). Previous studies delineating clones of aspen report a wide range of values of genet diversity, potentially due to the metric's sensitivity to sampling design (Arnaud-Haond et al., 2007) as well as biological causes. Values range from 0.26 in Ontario, Canada (Cheliak and Pitel, 1984) to 0.92 in Alberta, Canada (Jelinski and Cheliak, 1992). The only previous study of aspen in the Pacific West reported a genet diversity of 0.30 in the Sierra Nevada Mountains (Hipkins and Kitzmiller, 2004). Evidence is mounting that aspen genets are smaller than previously reported. Studies designed to assess population-level diversity but sampling multiple stems of a putative clone have reported greater levels of genet diversity than predicted by morphological characteristics (Tuskan et al., 1996; Wyman et al., 2003; Mock et al., 2008). The only study to date to exhaustively sample plots of *P. tremuloides* reported high levels of genet diversity ($G/N = 0.63$) and a large proportion of single-ramet clones (48% of sampled stems, Namroud et al., 2005).

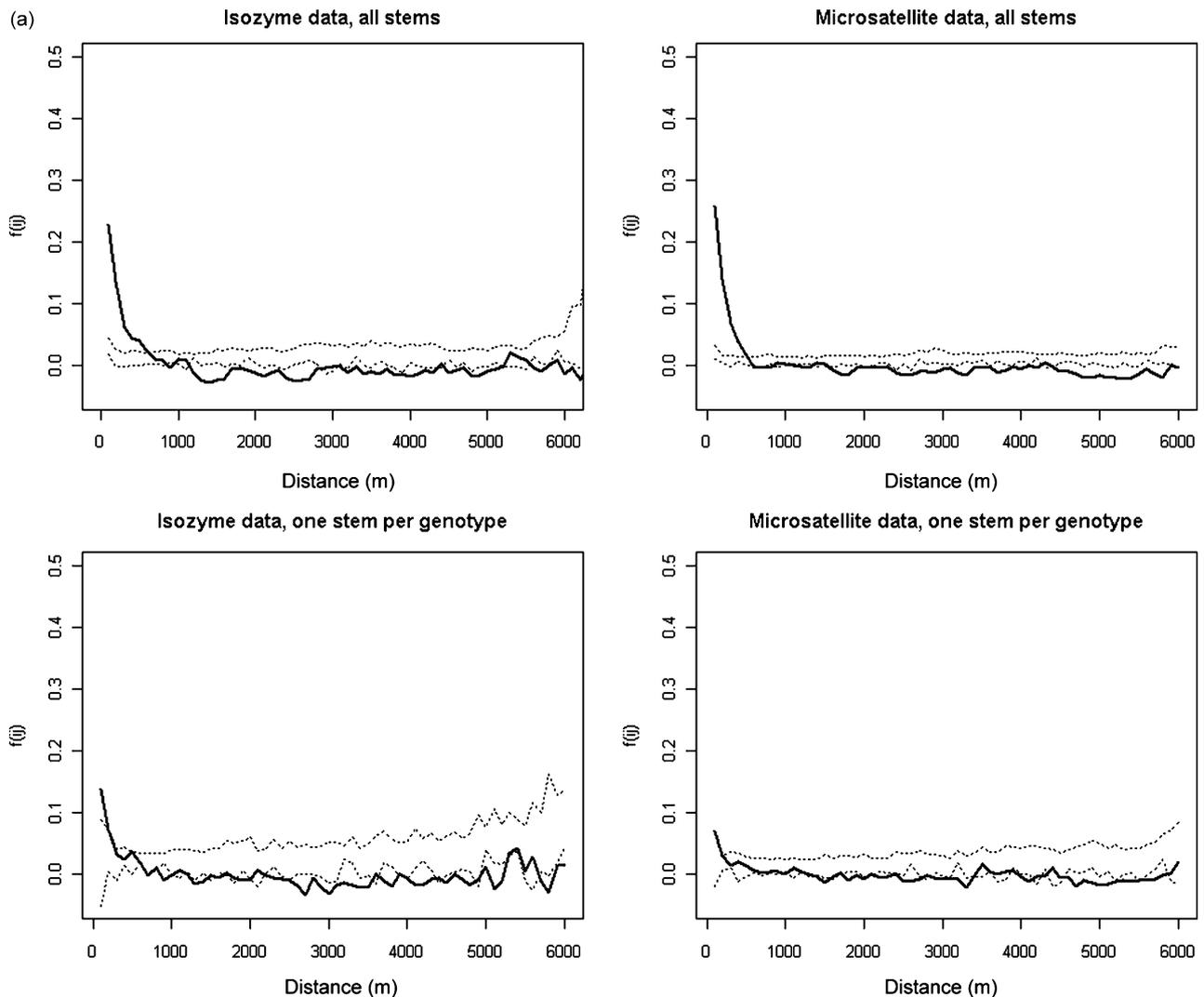


Fig. 2. Spatial genetic structure decays over short distances in collections of aspen from the southern Cascade Mountains. When a single stem per genotype was analyzed, significant structure was observed only in the smallest distance classes and only at two of the six sites (BB and PP). Solid lines indicate estimates of kinship (f_{ij}) over all pairs of samples in a distance class. Dashed lines represent the 99% C.I. around the $H_0: f_{ij} = 0$. Part A depicts results for site BB; Part B for site PC.

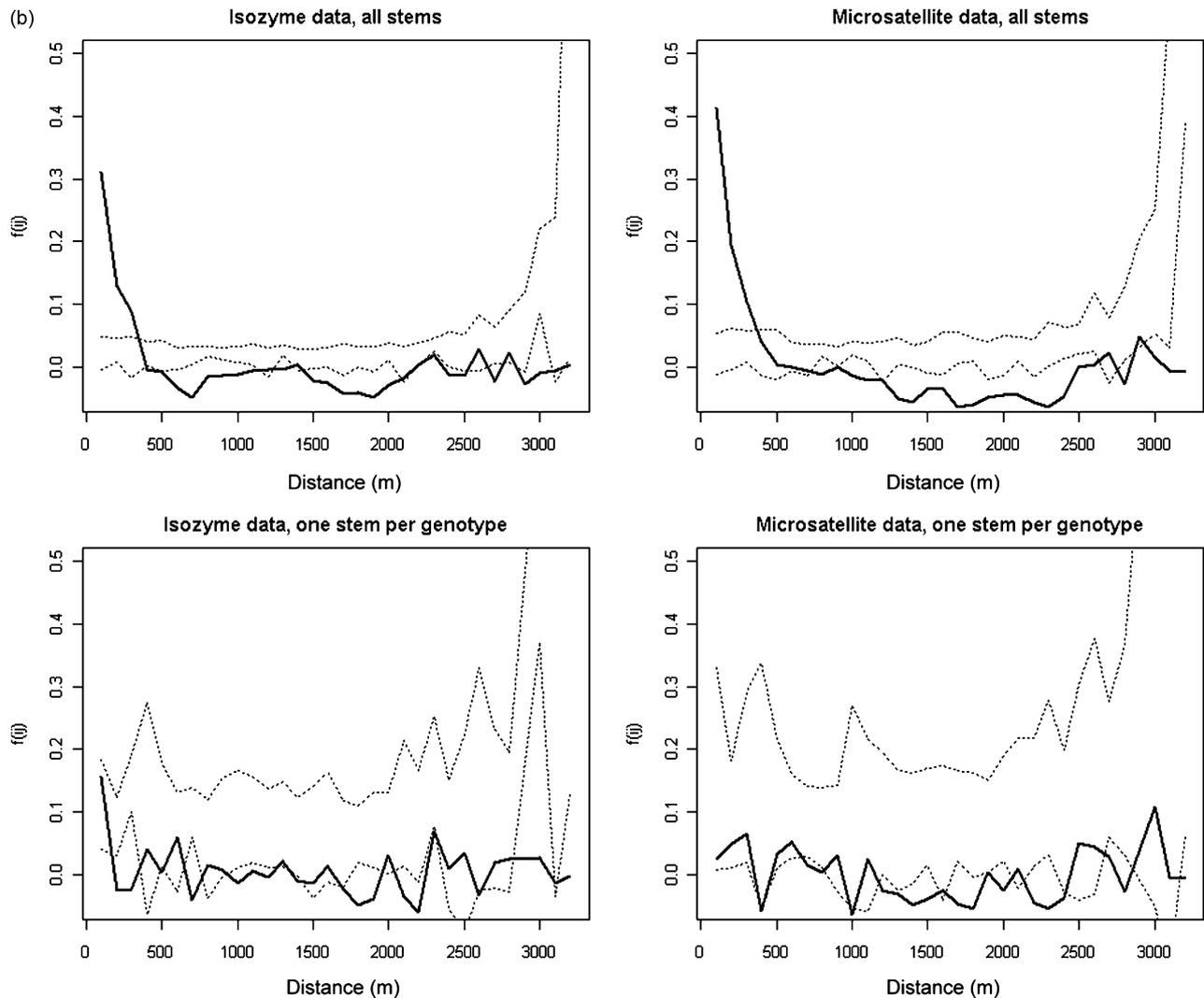


Fig. 2. (Continued).

The results of this study support observations and inventory work on the LNF that indicate aspen stands are in decline, as they are elsewhere in California (Di Orio et al., 2005) and in the western U.S. (Kay, 1997; Bartos, 2001). Assessing the 45 stands that putatively arose through fragmentation, as identified by the occurrence of one clone in adjacent stands, we estimate these once contiguous stands have lost 30.1% of their linear extent. This information indicating decline of the extent of aspen on the LNF are consistent with Forest inventory data that 43 of 709 inventoried aspen stands were completely dead, and 532 of 666 living stands (79.3%) were at a high risk of being lost.

Although theory predicts that isozymes will resolve lower levels of diversity than microsatellites, and thus provide lower power to identify individual clones (Ellstrand and Roose, 1987), the number and delineation of clones identified across 124 stands were remarkably similar in the two data sets (Table 2). Three possible mechanisms could result in the observed disagreements. First, somatic mutation could produce multiple genotypes in one marker system but not the other. This is more likely the case for the 19 cases where stems matched at isozyme but not microsatellite loci (Arnaud-Haond et al., 2007). Mutation at isozyme loci, while rare, is possible in long-lived, vegetatively reproducing species, and have been observed in Great Basin bristlecone pine (Lee et al., 2002). Second, the probability of identity may be sufficiently high that two clones may have matching genotypes by chance alone.

Increasing the number of loci would distinguish between these possibilities. Third, mistypes due to triploidy or null alleles at microsatellite loci may have contributed to the number of mismatches.

4.2. Distribution of genetic variation

Studies of aspen diversity report high levels of genetic variation, indicating that aspen is not only one of the most widely distributed hardwood species but also one of the most genetically diverse (Cheliak and Dancik, 1982; Lund et al., 1992; Jelinski and Cheliak, 1992; Yeh et al., 1995; Stevens et al., 1999; Wyman et al., 2003). Although both marker types provided sufficient power to identify clones in the southern Cascade Mountains, isozyme and microsatellite markers reveal significantly different levels of allelic diversity and expected heterozygosity (Table 3). The differences are consistent with evolutionary theory, such that isozymes are slower evolving and display fewer alleles per locus than microsatellites. In addition, these results are consistent with previous studies comparing marker types (Larsson et al., 2007).

The moderate levels of genetic differentiation observed among sites ($\theta_{ISO} = 0.02$; $\theta_{DNA} = 0.03$) are congruent between data types in this study, and with other isozyme (Jelinski and Cheliak, 1992) and microsatellite (Wyman et al., 2003; Namroud et al., 2005) studies of aspen. This level of differentiation is inconsistent, however, with

a previous study of aspen in the Pacific West which found higher levels of allele frequency variation in the central Sierra Nevada ($F_{ST} = 0.39$, Hipkins and Kitzmiller, 2004). This discrepancy may be due to biological differences between the two regions. For example, genetic similarity between genotypes was found to be associated with elevation in the Sierra Nevada, which were sampled across a larger elevational gradient (1550–2350 m; Hipkins and Kitzmiller, 2004) than the sites reported here (1650–2000 m; Table 1). Other ecological differences between the sites, such as soil composition (California Geological Survey, 2002), may affect demographic processes and thus the genetic differentiation observed between populations. Additional investigation into the association between ecological factors and genetic structure is warranted.

The rapid decay of spatial genetic structure among clones reveals two processes to be critical to the distribution of genets. First, the limited fine-scale genetic structure indicates that gene flow regularly occurs over large distances at these sites. Second, the lack of kinship is consistent with genets arising from sexual reproduction and not somatic mutation within old, established clones. Tests of a single stem per clone indicate kinship coefficients are significant for distances <200 m at sites BB and PP, but are nonsignificant at the remaining sites (Fig. 2). When assaying kinship in an outcrossing plant species, spatial genetic structure is determined by the distance of pollen dispersal to the maternal plant, and the range of seed dispersal away from the maternal plant (Kalisz et al., 2001). In the absence of inbreeding, significant fine-scale spatial genetic structure has been shown to result from limited dispersal of siblings around maternal plants (Hamrick and Nason, 1996; Kalisz et al., 2001). Given the lack of inbreeding in this dioecious species (isozyme data reveal $F_{IS} = -0.033$), the significant kinship observed among neighboring clones may be due to limited seed dispersal when those genets were established. We know of one previous study describing similar patterns of spatial genetic structure in stands of *P. tremuloides* in Quebec (Namroud et al., 2005).

Previous empirical (Tuskan et al., 1996) and simulation (Namroud et al., 2006) studies have suggested three processes interact to maintain the high levels of genetic variation and clonal diversity observed in aspen stands. First, large-scale disturbance events recruit large numbers of genetically diverse individuals. Second, as seedlings mature, interclonal competition results in clone death, with available space becoming occupied with suckers from the established clones (Tuskan et al., 1996; Stevens et al., 1999). Third, old, established clones are expected to accumulate somatic mutations (Tuskan et al., 1996; Wyman et al., 2003). As somatic mutations accumulate in clones and stands decline and fragment, the remaining stems may appear to be distinct clones, increasing the overall variation observed at a location. Together, large-scale seed recruitment, interclonal competition, and somatic mutation may be considered the canonical model of aspen regeneration.

This canonical model is likely sufficient to explain the high levels of variation observed in aspen from the southern Cascade Mountains, but not necessarily the small clone size. Large stands consistently contained multiple genets, and likely arose from substantial disturbance events (Fig. 1). The lack of fine-scale genetic structure among clones at most sites and the low level of differentiation among sites are consistent with long distance pollen and seed dispersal at stand establishment (Table 3, Fig. 2). However, the concordance of clonal boundaries in isozyme and microsatellite data, markers displaying markedly different rates of mutation, indicate that most intrastand variation is likely due to small clone size rather than somatic mutation in old clones, although putative somatic mutations were observed.

Misidentification of stands as distinct genets which arose through senescence and fragmentation may have consequences when designing management strategies. In some cases microsatellite data may inflate measures of genotypic diversity due to somatic mutations, whereby isozyme data would identify those clones sharing a single origin. Does a certain level of genetic differences classify two stems as unique individuals, or is it the process of recruitment that defines a clone? One goal of conservation biology is to maintain the processes, not simply the objects, of ecosystems (Bowen, 1999). In this sense, the fact that a stand arose through repeated suckering may be of greater interest to managers than the number of genetic differences between individual ramets at highly variable (microsatellite) loci, in which case classifying two stems as distinct clones based on somatic mutations should be avoided. Although the potential exists for somatic mutation to change the selective pressures on a single ramet (e.g. palatability), rates of mutation are expected to be greater in microsatellite loci than coding regions of the genome, and are thus expected to overestimate functional differences between ramets.

Given the lack of either vegetative or sexual reproduction in most stands of aspen in LNF over the past century, the genetic structure observed in extant stands is a snapshot of past recruitment. The genetic structure of future stands may vary depending on the scale of the disturbance events leading to regeneration, and the potential for seedling recruitment. Long-term and fine-scale demographic studies are necessary to test the relative contribution of each process to the maintenance of genet diversity in aspen.

4.3. Implications for conservation and management

Aspen clones in the southern Cascade Mountains do not reach the large size reported in the Intermountain West (Rocky Mountains and Colorado Plateau). These results, when combined with data from the central Sierra Nevada Mountains (Hipkins and Kitzmiller, 2004), further resolve geographic variation in clone size reported for aspen. In Eastern North America, both clone size and stand size tends to be small, while in the Intermountain West, both clone size and stand size can be very large. In the Pacific West, however, clone size is small while stand size is variable. Small stands are often monoclinal and large stands are composed of many genetic individuals. The most effective and appropriate management plans will account for this geographic variation in clonal structure.

Most stands of aspen in LNF are small in size and composed of mature and decadent stems and typically recruitment or regeneration is sparse or unsuccessful (Jones et al., 2005). The patchy and isolated nature of the small, monoclinal stands is not consistent with the large-scale disturbance and subsequent intraclonal competition described by the canonical model of aspen regeneration. If the small size of aspen stands is a result of natural ecological and biological processes, small-scale disturbances likely contribute to the recruitment of new individuals more frequently than expected in the canonical model. Alternatively, livestock grazing and subsequent encroachment by conifers into what were originally large stands may have produced the patchy distribution of these stands.

Documentation of genets in more than one stand is of particular interest to management as an indicator of the reduction in spatial extent and fragmentation of aspen stands. This knowledge can better inform aspen restoration plans on the LNF by supporting the need for restoration treatments to prevent further disarticulation of stands and loss of ecological services and products that aspen stands provide, such as biodiversity, including conditions necessary for a suite of obligate understory plant assemblages, forage, wildlife habitat, and conservation of riparian soil moisture

(Winternitz, 1980; Harper et al., 1981; Gifford et al., 1984; DeByle, 1985; Mueggler, 1985; Chong et al., 2001). Restoration treatments aimed at restoring the presence of aspen may also be prescribed in the gaps now separating once-contiguous stands.

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Appendix A

See Tables A.1 and A.2.

Table A.1

Isozyme allele frequencies observed in six collections of aspen from the southern Cascade Mountains.

| Locus, Allele | HM | PC | BB | PP | PL | FP |
|---------------|-------|-------|-------|-------|-------|-------|
| <i>6pgd1</i> | | | | | | |
| 32 | 0.010 | 0.012 | | | 0.020 | |
| 34 | 0.530 | 0.605 | 0.519 | 0.423 | 0.700 | 0.569 |
| 36 | 0.410 | 0.372 | 0.407 | 0.468 | 0.240 | 0.431 |
| 38 | 0.050 | 0.012 | 0.075 | 0.108 | 0.040 | |
| <i>6pgd2</i> | | | | | | |
| 10 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 | 0.500 |
| 17 | 0.500 | 0.500 | 0.500 | 0.500 | 0.440 | 0.500 |
| 20 | | | | | 0.040 | |
| 21 | | | | | 0.020 | |
| <i>AAT1</i> | | | | | | |
| 39 | 0.040 | 0.155 | 0.198 | 0.298 | 0.140 | 0.235 |
| 41 | 0.260 | 0.274 | 0.267 | 0.120 | 0.320 | 0.275 |
| 44 | 0.690 | 0.500 | 0.505 | 0.577 | 0.500 | 0.490 |
| 48 | 0.010 | 0.060 | 0.030 | 0.005 | 0.040 | |
| 52 | | 0.012 | | | | |
| <i>AAT2</i> | | | | | | |
| 39 | 0.040 | 0.071 | 0.015 | 0.010 | | 0.020 |
| 41 | 0.120 | 0.083 | 0.010 | 0.024 | | 0.030 |
| 44 | 0.350 | 0.405 | 0.554 | 0.614 | 0.580 | 0.550 |
| 48 | 0.340 | 0.214 | 0.289 | 0.281 | 0.280 | 0.280 |
| 52 | 0.120 | 0.214 | 0.123 | 0.071 | 0.120 | 0.110 |
| 54 | 0.030 | 0.012 | | | 0.020 | 0.010 |
| 50 | | | 0.010 | | | |
| <i>CAT</i> | | | | | | |
| 25 | 0.978 | 0.952 | 0.977 | 0.995 | 0.960 | 1.000 |
| 28 | 0.022 | 0.048 | 0.023 | 0.005 | 0.040 | |
| <i>IDH</i> | | | | | | |
| 30 | | | 0.118 | 0.005 | 0.040 | 0.157 |
| 32 | 0.280 | 0.314 | 0.363 | 0.221 | 0.480 | 0.471 |
| 34 | 0.720 | 0.686 | 0.519 | 0.775 | 0.480 | 0.373 |
| <i>MDH1</i> | | | | | | |
| 22 | | | | 0.018 | | |
| 27 | 1.000 | 1.000 | 1.000 | 0.982 | 1.000 | 1.000 |
| <i>MDH2</i> | | | | | | |
| 20 | | 0.012 | | 0.068 | 0.020 | |
| 22 | 0.030 | | 0.005 | 0.009 | | |
| 23.5 | | 0.047 | | | | |
| 24.5 | 0.970 | 0.942 | 0.995 | 0.914 | 0.980 | 1.000 |
| Null | | | | 0.009 | | |
| <i>MDH3</i> | | | | | | |
| 11 | 0.460 | 0.523 | 0.439 | 0.414 | 0.400 | 0.343 |
| 15 | 0.060 | 0.047 | 0.099 | 0.270 | 0.120 | 0.176 |

Table A.1 (Continued)

| Locus, Allele | HM | PC | BB | PP | PL | FP |
|---------------|-------|-------|-------|-------|-------|-------|
| 19 | 0.450 | 0.349 | 0.420 | 0.275 | 0.460 | 0.471 |
| 21 | | 0.012 | | | | |
| Null | 0.030 | 0.070 | 0.042 | 0.041 | 0.020 | 0.010 |
| <i>ME(7)</i> | | | | | | |
| 17 | 1.000 | 1.000 | 0.981 | 0.977 | 1.000 | 0.990 |
| 18.5 | | | 0.019 | 0.023 | | 0.010 |
| <i>PGI1</i> | | | | | | |
| 43 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| <i>PGI2</i> | | | | | | |
| 12 | 0.530 | 0.593 | 0.579 | 0.468 | 0.600 | 0.529 |
| 18 | 0.070 | 0.081 | 0.042 | 0.050 | 0.060 | 0.010 |
| 20 | 0.030 | 0.035 | 0.005 | 0.014 | 0.040 | |
| 22 | 0.330 | 0.267 | 0.350 | 0.441 | 0.300 | 0.461 |
| 24 | 0.040 | 0.023 | 0.023 | 0.023 | | |
| 28 | | | | 0.005 | | |
| <i>SKD</i> | | | | | | |
| 13 | 0.210 | 0.023 | 0.019 | 0.024 | | 0.050 |
| 15 | 0.150 | 0.256 | 0.154 | 0.217 | 0.208 | 0.200 |
| 17 | 0.630 | 0.721 | 0.827 | 0.759 | 0.792 | 0.750 |
| 21 | 0.010 | | | | | |
| <i>UGPP1</i> | | | | | | |
| 60 | 1.000 | 0.977 | 1.000 | 0.995 | 0.940 | 0.980 |
| 61 | | 0.023 | | 0.005 | 0.060 | 0.020 |
| <i>UGPP2</i> | | | | | | |
| 45 | 0.580 | 0.616 | 0.743 | 0.638 | 0.680 | 0.539 |
| 50 | 0.420 | 0.384 | 0.248 | 0.362 | 0.320 | 0.461 |
| Null | | | 0.009 | | | |

Allele indicates the distance the band migrated from origin in mm.

Table A.2

Microsatellite allele frequencies observed in six collections of aspen from the southern Cascade Mountains.

| Locus, Allele | HM | PC | BB | PP | PL | FP |
|-----------------|--------|-------|-------|-------|-------|-------|
| <i>ORNL-29</i> | | | | | | |
| 233 | 0.260 | 0.188 | 0.254 | 0.202 | 0.250 | 0.094 |
| 235 | 0.063 | 0.040 | 0.022 | 0.057 | | 0.027 |
| 237 | 0.120 | 0.027 | 0.087 | 0.061 | 0.080 | 0.127 |
| 239 | 0.032 | 0.034 | | 0.003 | 0.080 | 0.020 |
| 241 | | | 0.006 | | | |
| 243 | 0.038 | 0.020 | 0.053 | 0.131 | 0.180 | 0.241 |
| 245 | 0.057 | 0.148 | 0.108 | 0.144 | 0.200 | 0.087 |
| 247 | | | 0.016 | 0.010 | | |
| 249 | | | 0.003 | | | |
| 251 | | | | | | 0.007 |
| 255 | 0.025 | 0.013 | 0.065 | 0.010 | | |
| 257 | | | 0.003 | | | |
| 263 | | 0.007 | 0.019 | 0.020 | | 0.013 |
| 265 | 0.013 | | 0.037 | 0.077 | | 0.007 |
| 267 | | 0.007 | 0.006 | 0.017 | | 0.007 |
| 269 | | | | 0.02 | | |
| 271 | | | 0.003 | | | |
| Null | 0.392 | 0.516 | 0.319 | 0.241 | 0.200 | 0.372 |
| <i>PMGC-420</i> | | | | | | |
| 77 | | | | 0.013 | | |
| 79 | 0.0340 | 0.037 | 0.061 | 0.027 | 0.090 | 0.074 |
| 81 | 0.076 | 0.131 | 0.121 | 0.211 | 0.12 | 0.103 |
| 83 | 0.747 | 0.643 | 0.658 | 0.489 | 0.620 | 0.605 |
| 87 | 0.009 | | 0.007 | 0.030 | | |
| 89 | | 0.009 | | | | |
| Null | 0.135 | 0.180 | 0.154 | 0.230 | 0.170 | 0.218 |
| <i>PMGC-576</i> | | | | | | |
| 100 | 0.019 | | | 0.007 | | |
| 124 | | | | | | 0.009 |
| 146 | 0.019 | 0.034 | 0.050 | 0.007 | 0.030 | |
| 148 | 0.009 | | | | | |
| 150 | | | | 0.007 | | |
| 152 | 0.009 | 0.042 | 0.066 | 0.110 | 0.050 | 0.017 |
| 160 | 0.019 | | | 0.018 | 0.020 | 0.059 |
| 162 | 0.028 | 0.017 | | | | 0.017 |
| 164 | 0.102 | 0.059 | 0.062 | 0.117 | 0.020 | 0.152 |

Table A.2 (Continued)

| Locus, Allele | HM | PC | BB | PP | PL | FP |
|---------------|-------|-------|-------|-------|-------|-------|
| 166 | 0.427 | 0.195 | 0.254 | 0.242 | 0.27 | 0.381 |
| 168 | 0.009 | 0.051 | 0.062 | | 0.020 | 0.009 |
| 171 | 0.028 | 0.034 | 0.039 | 0.139 | 0.05 | 0.034 |
| 173 | | | 0.039 | | | |
| 178 | | 0.017 | 0.008 | | 0.020 | |
| 180 | 0.019 | 0.051 | 0.042 | | 0.02 | |
| 182 | | | 0.004 | 0.004 | 0.020 | |
| 188 | 0.186 | 0.204 | 0.270 | 0.146 | 0.310 | 0.220 |
| 190 | 0.028 | 0.009 | 0.012 | 0.004 | | |
| 192 | | | 0.004 | | | |
| 194 | 0.009 | | | | | |
| 200 | 0.009 | | | | | |
| 202 | 0.019 | | | 0.004 | | |
| 204 | 0.009 | | 0.008 | | | |
| 206 | | | | 0.007 | | |
| Null | 0.054 | 0.287 | 0.083 | 0.190 | 0.180 | 0.103 |
| PMGC-649 | | | | | | |
| 77 | | | 0.007 | | | |
| 85 | | | 0.003 | | | |
| 89 | | | 0.003 | | | |
| 91 | 0.036 | 0.017 | 0.043 | 0.043 | 0.060 | 0.137 |
| 93 | 0.050 | 0.009 | 0.007 | 0.009 | 0.010 | 0.048 |
| 97 | 0.014 | 0.009 | | 0.003 | | |
| 99 | 0.022 | 0.087 | 0.072 | 0.072 | 0.14 | 0.193 |
| 101 | | | | 0.017 | | |
| 103 | | | 0.003 | | | |
| 105 | | | 0.016 | 0.003 | | |
| 109 | | | 0.013 | 0.006 | | 0.008 |
| 111 | | | 0.003 | 0.003 | | |
| 113 | 0.201 | 0.260 | 0.242 | 0.029 | 0.230 | 0.177 |
| 115 | 0.093 | 0.087 | 0.085 | 0.040 | 0.010 | 0.008 |
| 117 | 0.093 | 0.121 | 0.134 | 0.283 | 0.140 | 0.169 |
| 120 | 0.108 | 0.043 | 0.049 | 0.035 | 0.040 | 0.024 |
| 122 | 0.007 | 0.043 | 0.036 | 0.040 | | 0.016 |
| 125 | 0.014 | 0.009 | 0.039 | 0.020 | 0.030 | 0.024 |
| 128 | 0.007 | 0.017 | | | | 0.008 |
| 130 | 0.014 | 0.009 | 0.007 | | | 0.008 |
| 133 | | | | | | 0.016 |
| 135 | | | 0.003 | | | |
| Null | 0.341 | 0.289 | 0.235 | 0.397 | 0.340 | 0.164 |
| PMGC-433 | | | | | | |
| 178 | | | 0.004 | 0.031 | | |
| 186 | 0.250 | 0.081 | 0.102 | 0.250 | 0.125 | 0.039 |
| 188 | 0.050 | 0.163 | 0.157 | 0.197 | 0.104 | 0.069 |
| 190 | 0.140 | 0.081 | 0.174 | 0.145 | 0.083 | 0.078 |
| 192 | 0.230 | 0.302 | 0.288 | 0.096 | 0.375 | 0.196 |
| 194 | 0.070 | 0.035 | 0.042 | 0.039 | 0.063 | 0.304 |
| 196 | 0.070 | 0.140 | 0.076 | 0.197 | 0.188 | 0.108 |
| 198 | 0.120 | 0.093 | 0.085 | 0.035 | 0.042 | 0.088 |
| 202 | 0.030 | | 0.013 | | | 0.118 |
| 208 | 0.040 | 0.105 | 0.059 | 0.009 | 0.021 | |
| PMGC-2571 | | | | | | |
| 82 | 0.157 | 0.093 | 0.080 | 0.070 | 0.208 | 0.049 |
| 84 | 0.059 | 0.128 | 0.050 | 0.018 | 0.104 | 0.029 |
| 86 | 0.069 | 0.047 | 0.097 | | 0.021 | 0.039 |
| 88 | 0.265 | 0.244 | 0.265 | 0.386 | 0.229 | 0.353 |
| 92 | 0.029 | 0.012 | 0.004 | | | |
| 94 | 0.029 | 0.035 | 0.008 | | | |
| 96 | 0.020 | | 0.008 | | | |
| 100 | 0.049 | 0.012 | 0.084 | 0.118 | 0.042 | 0.049 |
| 102 | 0.029 | 0.070 | 0.021 | 0.026 | 0.063 | 0.010 |
| 104 | 0.029 | 0.035 | 0.017 | | 0.104 | 0.078 |
| 106 | 0.147 | 0.256 | 0.261 | 0.250 | 0.208 | 0.225 |
| 108 | 0.020 | 0.023 | | 0.004 | | |
| 110 | | | | 0.079 | 0.021 | 0.020 |
| 112 | 0.098 | 0.047 | 0.105 | 0.044 | | 0.147 |
| 114 | | | | 0.004 | | |

Allele indicates size in bp. Adjusted frequencies are provided for loci having null alleles.

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