

BRIEF COMMUNICATION

CHLOROPLAST DIVERSITY IN A PUTATIVE HYBRID SWARM OF  
*PONDEROSAE* (PINACEAE)<sup>1</sup>

BRYAN K. EPPERSON,<sup>2,5</sup> FRANK W. TELEWSKI,<sup>3</sup> AND ANN WILLYARD<sup>4</sup>

<sup>2</sup>Department of Forestry, Michigan State University, East Lansing, Michigan 48824 USA; <sup>3</sup>W. J. Beal Botanical Garden, Department of Plant Biology, Michigan State University, East Lansing, Michigan 48824 USA; and <sup>4</sup>Department of Biology, University of South Dakota, 414 E. Clark St., Vermillion, South Dakota 57069 USA

The *Ponderosae* subsection of the genus *Pinus* contains numerous taxa in disjunct mountain ranges of southern Arizona and New Mexico, differing for several leaf and cone traits, key among which is the number of leaf needles per fascicle. Trees with three needles are often found together with trees having five needles and mixed numbers. One taxonomic hypothesis is that there are swarms of hybrids between *P. ponderosa* and *P. arizonica*. A second hypothesis is that there are spatial mixtures of two separate taxa, five-needle *P. arizonica* and a “taxon X” containing three needle and mixed needle trees. We genotyped chloroplasts in one putative hybrid swarm on Mt. Lemmon using microsatellite markers and show that cpDNA is almost completely differentiated between two separate morphotypes corresponding to *P. arizonica* and “taxon X.” Little if any introgression has occurred on Mt. Lemmon, and the simplest explanation is that little or no effective hybridization has occurred. Further results indicate that not only is taxon X not of hybrid origin, it is more closely related to nonregional *Ponderosae* other than *P. ponderosa* and *P. arizonica*. The results further suggest that other putative hybrid swarms in the region are also spatial mixtures of distinct taxa.

**Key words:** chloroplast microsatellites; hybridization; introgression; Pinaceae; *Pinus ponderosa*; *Pinus arizonica*; *Ponderosae*; spatial genetic structure.

Populations of ponderosa pine in southern Arizona and New Mexico mountain ranges are unusual in that they frequently contain mixtures of morphotypes, which may include different species, subspecies, or conspecific morphological variants. All these types are placed in the *Pinus* subsection *Ponderosae* (Niebling and Conkle, 1990), and some of them can be artificially cross-fertilized (Conkle and Critchfield, 1988). Many forests have trees with intermediate morphology, with spatial distributions that suggest hybrid swarms. Hybrid swarms can have profound effects on the evolution of plant species, and hybridization and advanced introgression can alter population biology, for example, by allowing species to adapt to new climatic conditions (e.g., Grant, 1971). Among *Ponderosae* in the desert Southwest, trees differ for several highly heritable seed-cone and leaf-needle traits (Rehfeldt et al., 1996; Rehfeldt, 1999), including a key trait of number of needles per fascicle. Some *Ponderosae* populations have three needles, others have five, and others have mixtures of trees with three to four needles. Still other populations have mixtures of three-needle trees, five-needle trees and trees having intermediate mean numbers of needles. Populations with low needle numbers are purported to be most closely related to those in more northern regions, including northern Arizona and New Mexico, where ponderosa is generally considered a single taxon, *Pinus ponderosa* var. *scopulorum* Engelm., which has two to three needles. In contrast, the five-needle populations, a taxon variously regarded as a

separate species, *Pinus arizonica* Engelm., or variety, *Pinus ponderosa* var. *arizonica* (Engelm) Shaw (Kearney and Peebles, 1964), has a range that extends far south into the central mountains of Mexico.

The presence of trees with mixed numbers of needles per fascicle (and mean numbers not near 3.0 or 5.0) in the region has contributed to taxonomic confusion and frequent revision of low vs. high needle number *Ponderosae* morphotypes in the region, but most authors have considered them to be hybrids between *scopulorum* and *arizonica* (e.g., Peloquin, 1984). A quite different interpretation was made by Rehfeldt (Rehfeldt et al., 1996; Rehfeldt, 1999), who found some genetic evidence for a “taxon X,” a previously unrecognized taxon with some individual trees having primarily three-needle fascicles and other trees having many threes but also frequent fascicles with higher numbers (four and five) of needles. Thus what appear to be hybrids on some mountains may instead be part of a single taxon X.

One particular population, containing a mix of pure five-needle trees, pure three-needle trees, and trees with intermediate average numbers (Epperson et al., 2001), on the south-facing slope of Mt. Lemmon, is known to exhibit a very strong cline, one of the strongest ever observed for a genetically based trait in any species (Epperson et al., 2003). Pure or near-pure three-needle trees grow at the summit of Mt. Lemmon, and pure five-needle trees (*P. arizonica*) less than 1 km downslope, at 300 m lower elevation, where it is considerably warmer. Trees with intermediate numbers of needles are highly concentrated in the center of a study transect, a pattern that appears to be caused by hybridization (Epperson et al., 2001). When this population was analyzed using allozymes, almost no spatial differentiation was observed along the transect. However, no differentiation was found between the two study outgroups (Epperson et al., 2003); hence, the allozyme distributions did not allow conclusions

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<sup>5</sup> Author for correspondence (e-mail: epperson@msu.edu), fax: 517-432-1143

regarding hybridization, although they did reduce the number of possible histories of the taxa. One remaining possibility is simply that taxa have not been separated long enough for mutation and genetic drift to have differentiated them for allozyme loci, even though they are genetically differentiated for needle and cone traits. It is possible that very little hybridization occurs, and the spatial differentiation of the needle trait is maintained largely through interspecific differences in fitness along the transect. The other remaining possibility is that the taxa were differentiated for allozymes but more recent hybridization has been so frequent that very advanced introgression has already occurred.

If there has been insufficient time since separation for the accumulation of mutations for allozymes, then the use of molecular markers that mutate at higher rates should allow discrimination between the hypotheses discussed. Genetic differentiation between isolated taxa is determined by rates of genetic drift and mutation, and hence would occur on a faster time scale. In this article, we report the results of assays for chloroplast microsatellites from the Mt. Lemmon and outgroup populations. If advanced introgression were indicated, then the results would support the concept that intermediate trees have adapted to intermediate elevation conditions, and the most common taxonomic treatment of *Ponderosae* is correct. If not, then the cline on Mt. Lemmon is due to interspecific competition, and other taxonomic treatments are required.

## MATERIALS AND METHODS

Study trees included: 105 randomly sampled along a belt transect on the south-facing slope of Mt. Lemmon; 15 three-needle outgroup trees, five from each of three distances, ca. 0.60, 0.85, and 1.03 km, respectively, north from the top of the transect and on the opposite, north-facing slope of Mt. Lemmon (considered to be the most likely location in the Santa Catalina Mountains of any three-needle pine population that might be "uncontaminated" by introgression); and 15 trees in an outgroup of the five-needle type in Bear Canyon, located ca. 10.75 km southeast from the transect, in the Santa Catalina Mountains, Pima County, Arizona, as described in detail in Epperson et al. (2003). Leaf bud and needle tissues were immediately placed on ice, and materials for DNA extraction were placed in a freezer by the end of each day. These trees had been assayed for hundreds of needle counts, as was described earlier (Epperson et al., 2001).

Bud tissues proved to be somewhat better than needle tissues for DNA extraction in a procedure that we have routinely applied to *Pinus resinosa* (Walter and Epperson, 2005). High quality DNA was successfully extracted from 97 transect trees, 14 north slope (OH) trees, and 15 Bear Canyon (BC) trees. Four chloroplast microsatellites that we have routinely used in studies of *P. resinosa* (Walter and Epperson, 2005) were assayed. The primers were developed by Vendramin and colleagues (1996), and our designations for them with the original PT numbers in parenthesis are: *cp3* (PT15169), *cp5* (PT30204), *cp12* (PT71936), and *cp14* (PT87268). Details of the PCR and genotyping followed

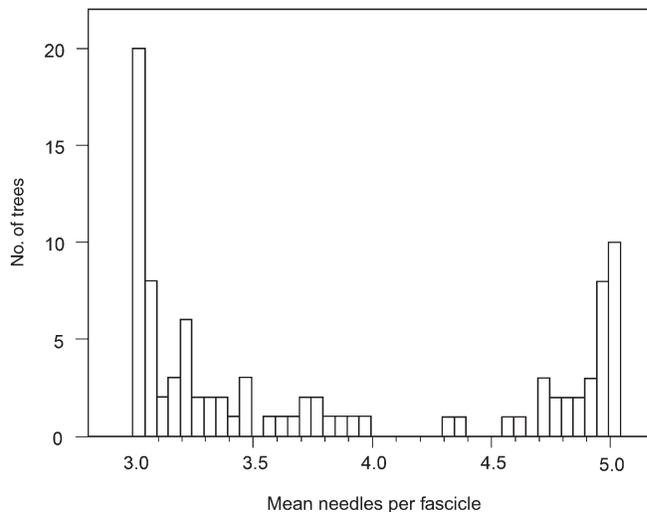


Fig. 1. Histogram of mean needle counts for individual trees.

precisely those reported in Walter and Epperson (2005). Four-locus genotypes were obtained for all but three trees, each of which would not amplify for one or more loci.

Measures of genetic diversity within and among groups of trees were calculated using Lewis and Zaykin's (2001) GDA program (version 1.0 d16c), including the actual number of alleles, "effective heterozygosity" or diversity, Weir's  $\theta$  estimate (Weir, 1996), and Nei's (1978) measures of genetic identity. These measures were calculated after partitioning the transect population into two groups, "5's" and "3's," following Ploquin's (1984) definition of a five-needle type, by including only the 32 trees (Fig. 1) that had mean needle values  $\geq 4.6$ . The remaining 64 trees we initially labeled "3's." Thus there are four groups of study trees: the transect "3's," transect "5's," north slope (OH), and Bear Canyon (BC).

To further understand taxon X type trees, we assayed three trees for three nucleotide sequences that were recently used in a large phylogenetic study that surveyed multiple accessions of all 17 species in subsection *Ponderosae* (Willyard et al., in press). Two trees had nearly all three-needle fascicles (mean number of needles 3.026 and 3.030), and a third had mixed numbers of needles (mean 3.706). The chloroplast *trnG* intron and *LEA-like* and *WD-40* nuclear loci were PCR-amplified and sequenced; GenBank (FJ234429–FJ234437). The three sequences were added to a *trnG* haplotypic network of all *Ponderosae* species (Willyard et al., in press). A strict consensus tree was built for each nuclear locus over the most parsimonious trees from ten iterations of parsimony ratchet (Nixon, 2005), and these trees were tested for consistency with the original Bayesian and parsimony analyses (Willyard et al., in press).

## RESULTS AND DISCUSSION

In the entire set of sampled trees, four alleles were found for *cp3* and *cp12*, five for *cp14*, and three for *cp5*. The three smaller

TABLE 1. Genetic diversity  $H_e$  for chloroplast microsatellites (*cp3*, *cp5*, *cp12* and *cp14*) and Weir's (1996)  $\theta$  among four groups<sup>a</sup> of *Ponderosae* (Pinaceae).

Group	N	$H_e$				Total
		<i>cp3</i>	<i>cp5</i>	<i>cp12</i>	<i>cp14</i>	
"3's"	62	0.28	0.64	0.45	0.59	0.49
"5's"	32	0.66	0.17	0.23	0.06	0.28
OH	14	0.25	0.65	0.51	0.62	0.51
BC	15	0.46	0.40	0.25	0.43	0.39
Total	123	0.49	0.57	0.55	0.55	0.42
$\theta$		0.24	0.18	0.41	0.27	0.28

<sup>a</sup> See Materials and Methods for definitions of groups.

TABLE 2. Nei's identity measure among groups<sup>a</sup> of *Ponderosae*.

Group	"3's"	"5's"	OH	BC
"3's"	—	0.60	0.97	0.61
"5's"	0.46	—	0.63	0.97
OH	0.50	0.32	—	0.66
BC	0.37	0.90	0.30	—

<sup>a</sup> See Materials and Methods for definitions of groups.

Notes: Values above diagonal are based on allele frequencies, those below on haplotype frequencies.

groups, transect "5's," north slope (OH), and Bear Canyon (BC) had similar numbers of alleles per locus, averaging 2.75, 2.50, and 2.50, respectively, whereas the larger sample of transect "3's" had somewhat larger numbers, averaging 3.5. For all trees combined, the effective heterozygosity or diversity was very similar among loci, ranging from 0.49 to 0.57 (Table 1). There were large differences among the four groups in their amounts of diversity, and the averages over loci were greater for the transect "3's" (0.49) and OH group (0.51) than for the transect "5's" (0.28) and BC (0.39). The pure five-needle pines have less diversity than the "3's," and the relatively large value for BC (especially for *cp14*) is due to the fact that BC was not all pure fives (Epperson et al., 2003). The amount of differentiation among groups was large and statistically significant, and the overall estimate of Weir's  $\theta$  was 0.28. Values ranged from 0.18 to 0.41 over loci (Table 1). The overall value of  $\theta$  is 0.36 between transect "3's" and transect "5's." Values of Nei's identity were much higher for "3's"-OH (0.97) and "5's"-BC (0.97) than for any other pair of groups (Table 2), and UPGMA (not shown) also clustered these two pairs.

A large number (27) of haplotypes, combinations of alleles at the four loci, were found in the entire sample. Among all four groups, by far the most (nine) private haplotypes (5, 11, 19, 21, 22, 23, 25, 26, and 27) were found in the transect "3's," fewer (six) were found among the transect "5's" (haplotypes 13, 14, 16, 17, 18, and 20), the OH group (haplotypes 8, 15, and 24) and the BC trees (haplotype 9). Only eight haplotypes (1, 2, 3, 4, 6, 7, 9, 10, and 12) were found in more than one group. Values of  $\theta$  averaged 0.07 among all four groups (Table 3), slightly larger (0.08) between the transect "3's" and "5's."

Most strikingly, among the 22 haplotypes found in the transect, 12 (nos. 2, 5–7, 11, 19, 21–23, 25–27) were private for the "3's" and 7 (nos. 10, 13, 14, 16–18, 20) for the "5's" (Table 4). The only haplotypes found in both groups of trees were nos. 1, 3, and 4. Haplotype 1 is the most common of all haplotypes (total frequency ca. 0.21), and it was equally frequent in both the transect "3's" and "5's," as well as in OH and BC. Based on inspection of the array of observed haplotypes (Table 4), it appears very likely that haplotype 1 is either the ancestral type or

TABLE 3. Haplotype diversities and Weir's (1996)  $\theta$  among groups<sup>a</sup> of *Ponderosae*.

Group	<i>N</i>	<i>H<sub>e</sub></i>
"3's"	62	0.88
"5's"	32	0.79
OH	14	0.51
BC	15	0.82
Mean		0.85
$\theta$		0.07

<sup>a</sup> See Materials and Methods for definitions of groups.

TABLE 4. Definition of observed haplotypes in terms of nominal sizes of amplified fragments for the four chloroplast simple sequence repeats (cpSSRs).

Haplotype	<i>cp3</i>	<i>cp5</i>	<i>cp12</i>	<i>cp14</i>	No.
1	121	137	144	161	26
2	121	<b>138</b>	<b>145</b>	<b>163</b>	5
3	<b>122</b>	137	144	161	17
4	<b>122</b>	<b>138</b>	144	161	6
5	121	<b>138</b>	<b>145</b>	<b>162</b>	8
6	121	<b>139</b>	<b>145</b>	161	10
7	121	<b>139</b>	<b>145</b>	<b>162</b>	9
8	121	<b>138</b>	<b>145</b>	161	2
9	<b>122</b>	<b>138</b>	<b>146</b>	161	1
10	<b>122</b>	137	144	<b>160</b>	4
11	121	137	<b>145</b>	<b>162</b>	13
12	121	<b>138</b>	144	161	3
13	<b>123</b>	137	<b>145</b>	161	1
14	<b>123</b>	137	<b>143</b>	161	1
15	<b>122</b>	<b>138</b>	144	<b>162</b>	2
16	<b>120</b>	137	144	161	2
17	121	137	<b>143</b>	161	1
18	<b>122</b>	137	<b>143</b>	161	1
19	<b>122</b>	137	<b>145</b>	161	1
20	<b>123</b>	137	144	161	2
21	<b>122</b>	137	144	<b>162</b>	1
22	<b>122</b>	<b>138</b>	<b>145</b>	<b>162</b>	1
23	121	<b>139</b>	<b>146</b>	<b>162</b>	2
24	121	137	<b>145</b>	<b>163</b>	1
25	<b>120</b>	<b>138</b>	<b>145</b>	<b>163</b>	1
26	<b>122</b>	<b>139</b>	<b>143</b>	<b>163</b>	1
27	121	137	<b>145</b>	<b>164</b>	1

Notes: Alleles differing from haplotype 1 are in boldface. Also shown is the number of trees.

the one closest to the ancestral type. Haplotype 3 is very similar to 1, differing only by an allele (122) that is 1 bp larger at *cp3*, and it is the second most frequent overall (ca. 0.14). Haplotype 4 differs from 1 by that same allele, plus an allele (138) that is 1 bp larger at *cp5*, and it has fairly low frequency (total frequency ca. 0.05). A median network (Bandelt et al., 1995) indicated extensive homoplasy (not shown), which is very often the case in conifer data sets of chloroplast microsatellites (Gugerli et al., 2001; Ribeiro et al., 2002; Cuenca et al., 2003). Hence, statistical phylogenetic analyses cannot be used to fully determine the ancestral relationships among the haplotypes observed in these trees. The pattern of Nei identities (Table 2) for haplotypes is similar to that for allele frequencies, although as would be expected the values are smaller (since mutations at any one of the four loci causes a different haplotype).

The spatial distributions of 94 chloroplast genotypes in the transect area were characterized by spatial autocorrelation statistics, specifically unweighted Moran's *I* statistics (Epperson, 2003), using the PASSAGE program of Rosenberg (2002). Each allele of each locus was analyzed separately, after transformation into values 1 or 0 for the presence or absence of that allele in the (haploid) genotype. Alleles that were present in five or fewer trees were considered as not sufficiently informative and thus not analyzed. Analyses of nine spatially informative alleles (alleles 121 and 122 of *cp3*; 137, 138, and 139 of *cp5*; 144 and 145 of *cp12*; and 161 and 162 of *cp14*) indicated that eight of them had statistically significant correlograms (allele 138 of *cp5* was the exception) at the 5% level. Mean correlograms per locus are shown in Fig. 2, and they indicate a high degree of autocorrelation at short distances, with increasingly

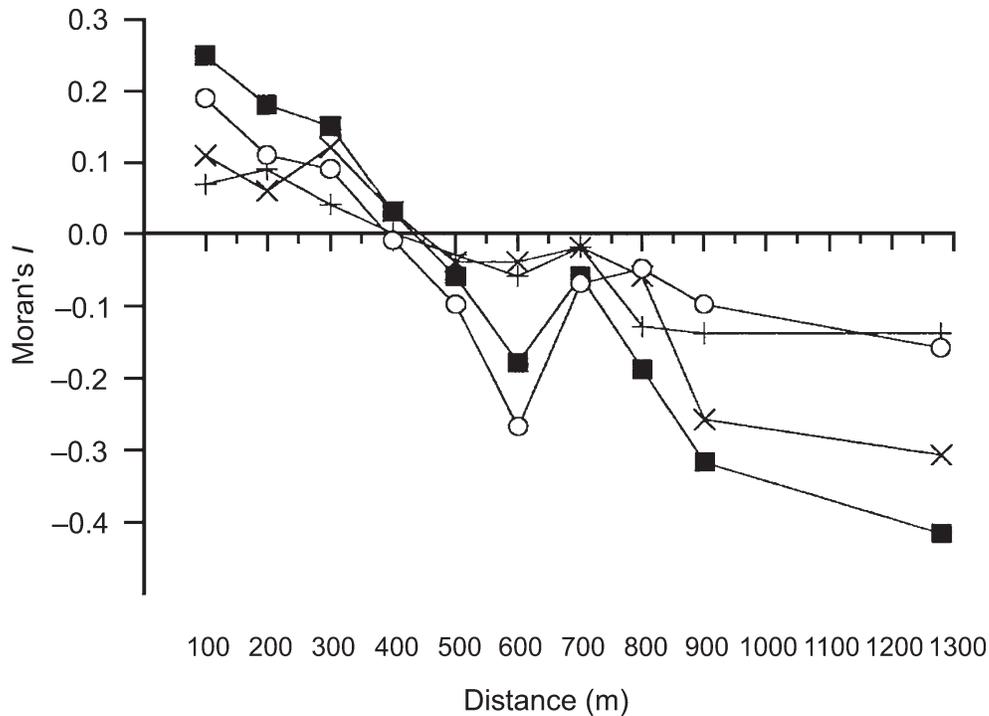


Fig. 2. Correlograms of mean (unweighted)  $I$ -statistics for each of the four chloroplast loci for the entire transect on Mt. Lemmon:  $cp3$  (X),  $cp5$  (+),  $cp12$  (■), and  $cp14$  (○).

large negative values at larger distances, the characteristic of a cline (Epperson, 2003).

In a separate spatial analysis of only the 64 three-needle transect pines, one of nine spatially informative alleles exhibited a significant correlogram, and only two of 72 coefficients (8 distance classes  $\times$  9 alleles) were nominally significant at the 5% level. There is very little autocorrelation (Fig. 3). For the 32 five-needle pines,  $cp5$ ,  $cp12$  and  $cp14$  had one allele in great majority, with only a few trees having variants. However,  $cp3$  had enough variation that in principle there could be spatial information for two alleles. Neither allele had any significant  $I$ -statistics or significant correlograms. The average  $I$ -statistics for the four distance classes are:  $-0.07$  (0–100 m);  $-0.02$  (100–200 m);  $0.01$  (200–300 m); and  $-0.04$  (300–527 m). There is very little if any spatial structuring of alleles within the five-needle transect trees.

Unlike allozymes chloroplast microsatellites (cpSSRs) highly differentiate two types of trees on Mt. Lemmon and surrounding areas, one a near pure five-needle taxon and the other a group of trees with needle numbers ranging from purely three to mixes having averages of more than 4.0 needles per fascicle (Epperson et al., 2001). Because the latter group is completely consistent with Rehfeldt's (1999) "taxon X" (discussed later), we will henceforth refer to it as such, and the five-needle pines will be referred to as *Pinus arizonica*. It is clear that there are two groups that have almost completely different chloroplast genotypes. Based on cpSSR allele frequencies, the average value of  $\theta$  (0.36) between transect *P. arizonica* vs. taxon X was much greater than that (0.08) observed for allozymes (Epperson et al., 2003).

The differences are even stronger in terms of haplotypes. As described earlier, among the 22 four-locus cpSSR haplotypes found among transect trees, most (19) were found in only one

or the other group. Further, although homoplasy is common and precluded a complete haplotype phylogeny, haplotype 1 is likely ancestral to, or at least older than, all or most other haplotypes. Haplotype 1 and the other two (3 and 4) presently shared may well constitute an ancestral polymorphism preceding separation of taxon X and *P. arizonica*. Moreover, assuming for the moment haplotype 1 to be ancestral, further inspection of the array of haplotypes (Table 4) is consistent with major clade differences between taxon X and *P. arizonica*. Remarkably, there are two apparent major clades of seven haplotypes and five haplotypes, respectively, each marked by different double-mutations (plus additional mutations) from haplotype 1. Of the 64 genotyped taxon X type trees in the transect sample, 49 (>75%) have one of these 12 haplotypes, none of which were present in *P. arizonica*.

It should be noted that although it may be considered arbitrary to have initially divided the transect trees into two groups in which 4.6 or greater is treated as "5's" and less than 4.6 "3's" (taxon X), other authors have used the same cutoff (Peloquin, 1984). Moreover, there are very few trees in our samples that are near the cutoff (Fig. 1). Only three transect trees had mean numbers of needles from 4.0 to 4.6, and only two from 4.6 to 4.7. Once the two groups are formed, nearly all haplotypes are contained in only one or the other group. Once the division is made, it becomes clear that *P. arizonica* is quite distinct from the other trees.

For the entire transect, the spatial distribution of cpSSR alleles has high levels of autocorrelation for short distances and increasingly large negative correlations at large distances, the latter being characteristic of clines. The structure is highly correlated with that observed for mean needle number per tree (Epperson et al., 2001). Both needle number and chloroplast alleles exhibit among the strongest clinal patterns reported in the lit-

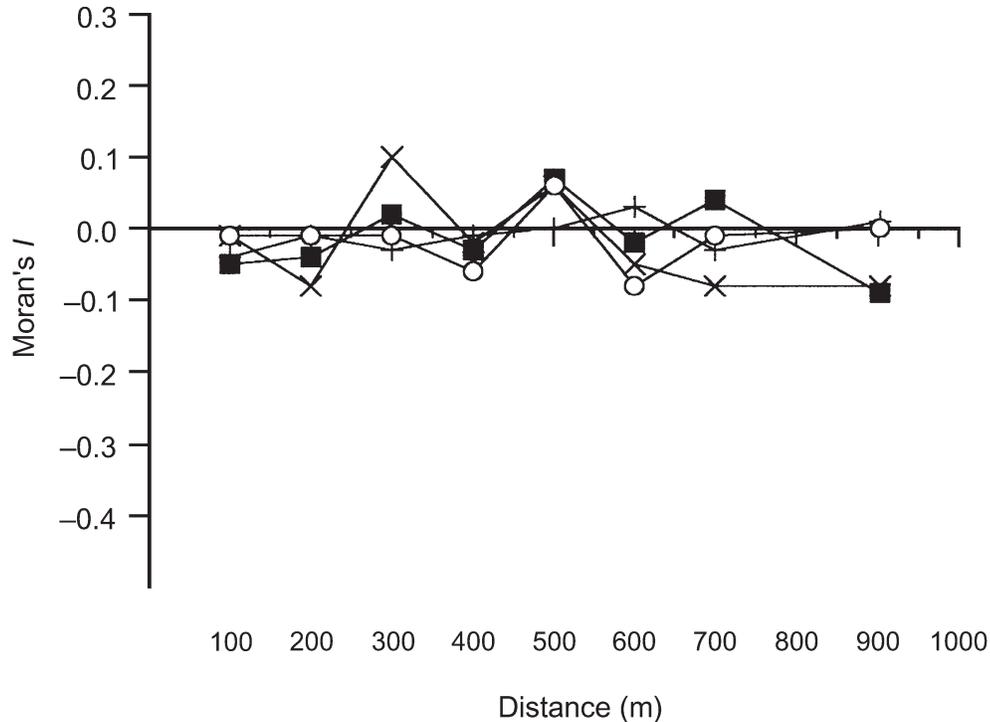


Fig. 3. Correlograms of mean (unweighted)  $I$ -statistics for each of the four chloroplast loci for only three-needle transect trees. Symbols follow those for Fig. 2.

erature (Epperson, 2003). This also means that there are very strong (spatial) cross-correlations between cpSSR alleles, mean number of needles per fascicle (namely  $>4.6$  vs.  $<4.6$ ) and elevation. In stark contrast, the spatial distribution of genotypes within taxon X (i.e., after excluding *P. arizonica*) is fully consistent with that of a freely interbreeding population, based on distances of seed and pollen dispersal of pines (Epperson et al., 2001). Whereas within taxon X, there is a concentration of trees with relatively high needle counts toward the bottom of the transect, nearest to the *P. arizonica* trees (Epperson et al., 2001), no such spatial differentiation is seen among chloroplast genotypes. In other words, there is a cline of needle numbers but not chloroplast genotypes within taxon X transect trees. Hence there is no correspondence of haplotype with needle number within taxon X along the transect. There is also almost no spatial structure within the *P. arizonica* population. Hence, the data also indicate that within each separate population there is free interbreeding. In addition, these results suggest that the cline in needle traits along the entire transect is caused by differential growth and/or survival across two distinct taxa, rather than adaptation of genotypes within a single species.

The sequence data showed, perhaps surprisingly, that all three taxon X samples share a chloroplast lineage with a group of *Ponderosae* taxa with current geographic ranges predominantly limited to Mexico and Central America. Based on *trnG* haplotypes, both *P. ponderosa* and *P. arizonica* var. *arizonica* chloroplast lineages are distinct from this "Mexican-plus-taxon X" haplotype. In the *LEA-like* and *WD-40* gene trees, the three taxon X accessions all resolve with clades that include most of the same Mexican and Central American taxa as with the chloroplast haplotype, and again these clades generally do not contain either *P. ponderosa* or *P. arizonica*. Of three taxon X samples in

two nuclear gene trees, the one exception was one sample joining a gene tree clade that contained both *P. arizonica* and *P. ponderosa* var. *scopulorum*, and in this case, the clade also contained some Mexican taxa. Willyard et al. (in press) noted that low phylogenetic resolution and somewhat different inferences from each gene tree could be explained either by the retention of ancestral polymorphism or by ancient hybridization events early in the formation of the entire *Ponderosae* subsection.

In total, the results clearly show that there has not been massive homogenization of the chloroplast genome on Mt. Lemmon. Indeed, no introgression was detected. The results strongly support the existence of taxon X, and they further suggest that many if not all of the variable-needle populations in southern Arizona and New Mexico are spatial mixtures of distinct taxa rather than hybrid swarms. Further, it appears that five-needle populations on Mt. Lemmon are taxonomically distinct and genetically separate from three-needle or intermediate-needle populations. The simplest explanation is that there has not been substantial hybridization, and this could be due to little or no effective hybridization each generation. It is worth pointing out that the results do not necessarily completely prove that hybridization never takes place. For example, it is a theoretical possibility that the same results could occur if contact between *P. arizonica* and taxon X is very recent, pollinations occur only from taxon X to *P. arizonica* (not vice versa), and hybrid pollinations are fairly infrequent and occur primarily on those trees of *P. arizonica* that are spatially nearest to taxon X trees. The same scenario could also mean that some of the mixed needle trees in the center of the transect are early generation hybrids. However, we have no evidence that any of these conditions are in operation.

The sequence data not only provided no evidence that taxon X is an intermediary of *P. ponderosa* and *P. arizonica*, consis-

tent with the cpSSR evidence that taxon X is not the result of massive hybridization, they indicate that taxon X is more closely related to a group of Mexican and Central American *Ponderosae* than it is to either *P. ponderosa* or *P. arizonica*. It should be noted that only one of the Mexican sister species, *P. engelmannii*, has a range approaching Mt. Lemmon, with the nearest population being ca. 80 km away. However, *P. engelmannii* has a number of distinctive leaf and cone characteristics not shared with taxon X trees, and there is no indication that taxon X trees are more closely related to *P. engelmannii* than to others in the Mexican clade.

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