GENETIC VARIATION IN SHORTLEAF PINE, *PINUS ECHINATA* MILL. (PINACEAE)

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ABSTRACT

Shortleaf pine, *Pinus echinata* Mill., is the most widely distributed yellow pine of the southeastern United States. Allozyme diversity at 22 loci was determined for 18 populations of shortleaf pine sampled from throughout its geographic range. Compared to plant species possessing similar life history traits, shortleaf pine had more polymorphic loci (P = 91%) and a higher mean number of alleles per locus (A = 2.77) but less expected heterozygosity (H_e = 0.115). This result was due to the presence of several polymorphic loci with skewed allele frequencies, a pattern typical of many conifers. The G_{ST} value of shortleaf pine (0.026) was somewhat lower than G_{ST} values for other pine species with continuous geographic ranges. Differences in allele frequencies between eastern and western sections of its range were slight. However, significantly more hybridization between shortleaf pine and loblolly pine, *P. taeda* L., was found in populations west of the Mississippi River (4.6% West vs. 1.1% East).

Key words: *Pinus echinata*, *Pinus taeda*, genetic diversity, allozymes, hybridization

INTRODUCTION

Plant species are not only defined taxonomically by their characteristics but their traits can also influence their genetic composition. Ecological factors and life history traits such as regional distribution, life span, habit, geographic range, mating system, and pollen and seed dispersal mechanisms all influence genetic diversity and structure in plant species. In general, long-lived, woody species have more allozyme variation than other types of plants (HAMRICK *et al.* 1979, 1992; HAMRICK & GODT 1989). Furthermore, plant species with a boreal-temperate distribution, a continuous and regional geographic range, and with wind-dispersed pollen and seeds have most of their genetic variation within their populations (LOVELESS & HAMRICK 1984; HAMRICK & GODT 1989). Such pollen and seed dispersal mechanisms promote gene flow; increasing genetic diversity within populations and minimizing among population variation.

Shortleaf pine is a yellow pine belonging to Subsection *Australes* Loud. and is native to 22 states, making it the most widely distributed of the southeastern United States pines (Figure 1) (CRITCHFIELD & LITTLE 1966; LITTLE 1971; LAWSON 1990). Its distribution is naturally subdivided into western and eastern regions, since shortleaf pine does not compete well with the hardwoods that dominate the Mississippi River flood plain (LOWERY 1986). Paleoecological data indicate that the Mississippi River flood plain has separated these two regions at least since the end of the last glacial epoch and there has been speculation that present day populations were founded by individuals from separate glacial refugia (DELCOURT *et al.* 1983).

Shortleaf pine grows sympatrically with loblolly pine throughout most of its range, but locally the two species are often found in different habitats (MCCUNE 1988). Artificial crosses between shortleaf pine and loblolly pine produce viable, hybrid offspring which are intermediate to the parental species for some, but not all, morphological traits (DUFFIELD 1952; CRITCHFIELD 1963; KENG & LITTLE 1961; LITTLE & RIGHTER 1965). In addition to such traits, loblolly pine, shortleaf pine, and their hybrids can be distinguished by their genotypes at an isocitrate dehydrogenase locus (IDH) (HUNEYCUTT & ASKEW 1988).

In this paper we estimate levels of genetic diversity for shortleaf pine and determine how genetic diversity is distributed within and among populations and geographic regions. We also use the IDH locus to identify the frequency of natural hybrids between shortleaf pine and loblolly pine within populations and to determine whether there are regional differences in the frequency of hybridization.

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MATERIALS AND METHODS

Study sites

Eighteen naturally occurring shortleaf pine populations were sampled from 10 southeastern states, ranging from Virginia (VA) to Oklahoma (OK) (Figure 1). Where possible, branch samples from 48 individuals of varying ages were collected from each population. Populations TAR, PMO, WTN, and CMI had 43, 44, 36, and 47 individuals, respectively. Branch material was placed in a plastic bag and kept cool to avoid protein denaturation. Upon return to the lab, samples were refrigerated at 5 °C until enzyme extraction. Enzyme extraction was accomplished with a mortar and pestle. Needle and bud materials were covered with liquid nitrogen and a pinch of sea sand was added to facilitate the creation of a fine powder. A phosphate- polyvinyl-pyrrolidone buffer was then added to the extract to stabilize the proteins (MITTON *et al.* 1979). The crude extract was absorbed onto filter paper wicks which were placed in microtest plates for storage at –70°C.

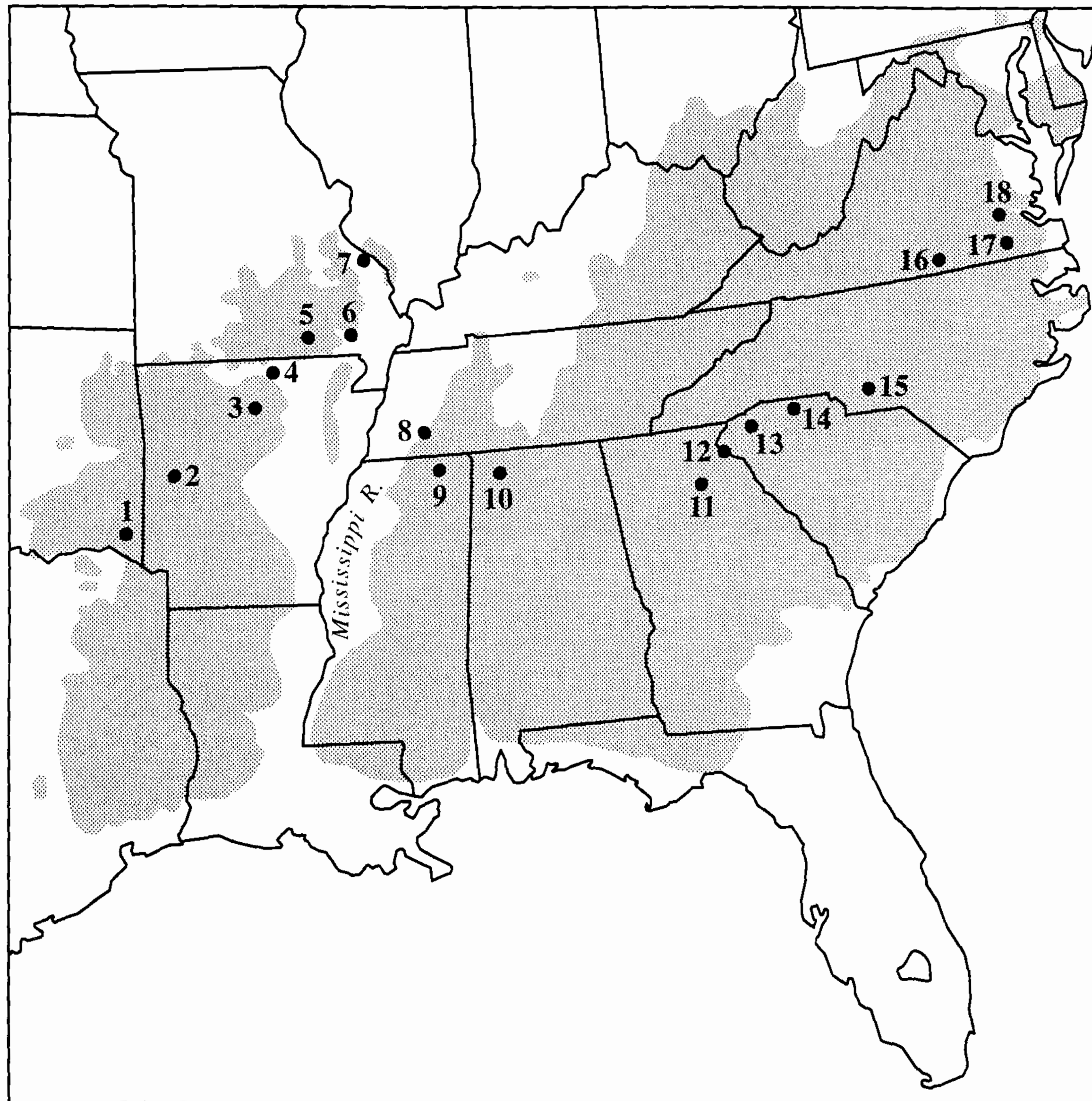

Figure 1 Range map of shortleaf pine and populations sampled: (1) BOK – Bokhoma City, Oklahoma; (2) WAR – Waldron, Arkansas; (3) TAR – Tilly, Arkansas; (4) NAR – Norfolk, Arkansas; (5) WSMO – Willow Springs, Missouri; (6) GMO – Greenville, Missouri; (7) PMO – Perrysville, Missouri; (8) WTN – Williston, Tennessee; (9) CMI – Corinth, Mississippi; (10) CAL – Cherokee, Alabama; (11) TMGA (Thompson Mills, Georgia; (12) TGA – Toccoa, Georgia; (13) WSC – Walhalla, South Carolina; (14) ISC – Inman, South Carolina; (15) CNC – Concord, North Carolina; (16) TVA – Turberville, Virginia; (17) SVA – South Hill, Virginia; (18) GVA – Green Bay, Virginia

Table 1 Electrode and gel buffer systems and electrophoretic conditions used to resolve twenty-two putative loci in shortleaf pine. Buffer systems are as described by Soltis *et al* (1983) with the exceptions of 1 and 2 (both modifications of buffer system 6) and 4 (a modification of buffer system 8)

System	Electrode buffer	Gel buffer	Enzyme systems	Initial setting
1	0.4 M NaOH 0.3 M boric acid pH 8.6	0.015 M Tris 0.004 M citric acid pH 7.6	ADH, MNR, PER PGI, PGM, TPI	200 V 3 hrs.
2	0.05 M NaOH 0.27 M boric acid pH 8.0	0.100 M Tris 0.016 M citric acid pH 8.45	AAT	200 V 5 hrs.
3	0.40 M citric acid, trisodium 1.0 M HCl to pH 7.0	0.005 M Histidine HCl 1.0 M NaOH to pH 7.0	FDP, IDH, MDH SKDH, 6–PGD	55 mA 5.5 hrs.
4	0.388 M LiOH 0.263 M boric acid pH 8.0	0.004 M LiOH 0.029 M boric acid 0.033 M Tris 0.006 M citric acid pH 7.6	DIA, FE	40 mA 4.5 hrs.

Allozyme Analysis

Samples were run on 10% starch gels using four gel electrode buffer systems (Table 1). The 14 enzyme systems stained (22 putative loci resolved) were alcohol dehydrogenase (*Adh*), aspartate aminotransferase (*Aat–1*, *Aat–2*), diaphorase (*Dia–2*, *Dia–3*), fluorescent esterase (*Fe–1*, *Fe–2*), fructose-1,6-di-phosphatase (*Fdp*), isocitrate dehydrogenase (*Idh*), malate dehydrogenase (*Mdh*), menadione reductase (*Mnr*), peroxidase (*Per–1*, *Per–2*), phosphoglucoisomerase (*Pgi–1*, *Pgi–2*), phosphoglucomutase (*Pgm–1*, *Pgm–2*), 6-phosphogluconate dehydrogenase (*6-Pgd–1*, *6-Pgd–2*), shikimic dehydrogenase (*Skdh*), and triose-phosphate isomerase (*Tpi–1*, *Tpi–2*).

Data Analysis

Statistics of genetic diversity were calculated at the population, region, and species levels. Two regions, east and west, were based on the location of the population relative to the Mississippi flood plain. Populations BOK, WAR, TAR, NAR, WSMO, GMO, and PMO were pooled to represent the west, while the remaining populations made up the east (Figure 1). Standard measures of genetic variation used at all levels included percent polymorphic loci (P; a locus was considered polymorphic if it contained more than one allele regardless of the frequency of that allele), mean number of alleles per locus (A), mean number of alleles per polymorphic locus (A_p), observed heterozygosity (H_o), and expected heterozygosity ($H_e = 1 - \Sigma\, p_i^2$; also referred to as genetic diversity) (Table 2).

For each polymorphic locus in each population, deviations from Hardy Weinberg expectations were examined by calculating Wright's fixation index and using χ^2 to test for significant deviations from the expected value of $F = 0$ (WRIGHT 1922; LI & HORVITZ 1953). F_{IS} values were also calculated (Table 3; NEI 1973). Among population variation was quantified three ways. First, χ^2 tests were used to test for significant allele frequency heterogeneity among populations (WORKMAN & NISWANDER 1970). Next, Nei's genetic identities were calculated for pairwise comparisons of divergence between populations (NEI 1972, 1977). And finally, total genetic diversity at polymorphic loci (H_T) was partitioned into a within population component (H_S) and an among population component (D_{ST}) so that

$$H_T = H_S + D_{ST}$$

(Table 3). Among population variation was compared to total genetic variation to give $G_{ST} = D_{ST} / H_T$ (Table 3). The G_{ST} values were calculated for each polymorphic locus and then averaged over all loci. Among

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Table 2 Summary of genetic diversity within eighteen populations of shortleaf pine based on twenty–two putative loci. Numbers in parentheses refer to locations indicated on Figure 1

Population	P	A_p	A	H_o (sd)	H_e (sd)
West					
BOK (*1*)	54.6	2.58	1.86	0.108 (0.010)	0.119 (0.036)
WAR (*2*)	59.1	2.46	1.86	0.096 (0.009)	0.115 (0.036)
TAR (*3*)	59.1	2.62	1.95	0.129 (0.011)	0.129 (0.036)
NAR (*4*)	54.6	2.25	1.68	0.094 (0.009)	0.104 (0.031)
WSMO (*5*)	54.6	2.75	1.95	0.129 (0.011)	0.134 (0.036)
GMO (*6*)	59.1	2.69	2.00	0.125 (0.011)	0.118 (0.035)
PMO (*7*)	63.6	2.57	2.00	0.133 (0.011)	0.124 (0.036)
Mean	**57.8**	**2.56**	**1.90**	**0.116**	**0.120**
Within West	**72.7**	**2.88**	**2.36**		**0.120**
East					
WTN (*8*)	50.0	2.82	1.90	0.131 (0.013)	0.130 (0.040)
CMI (*9*)	59.1	2.54	1.91	0.118 (0.010)	0.127 (0.037)
CAL (*10*)	57.1	2.58	1.90	0.099 (0.011)	0.098 (0.033)
TMGA (*11*)	57.1	2.83	2.05	0.102 (0.009)	0.103 (0.030)
TGA (*12*)	45.5	2.80	1.82	0.110 (0.010)	0.109 (0.038)
WSC (*13*)	45.5	2.90	1.86	0.098 (0.010)	0.105 (0.032)
ISC (*14*)	45.5	2.60	1.73	0.085 (0.009)	0.091 (0.033)
CNC (*15*)	50.0	2.64	1.82	0.099 (0.009)	0.113 (0.033)
TVA (*16*)	50.0	2.64	1.82	0.089 (0.009)	0.102 (0.033)
SVA (*17*)	45.5	2.70	1.77	0.090 (0.009)	0.107 (0.033)
GVA (*18*)	40.9	2.78	1.73	0.099 (0.009)	0.101 (0.034)
Mean	**49.6**	**2.71**	**1.85**	**0.102**	**0.108**
Within East	**77.3**	**2.94**	**2.50**		**0.110**
Mean	**52.8**	**2.65**	**1.87**	**0.107**	**0.113**
Within species	**90.9**	**2.95**	**2.77**		**0.115**

region variation was calculated in the same way.

Two indirect methods were employed to estimate gene flow. The first was based on the average frequency of "rare" alleles (BARTON & SLATKIN 1986). An allele was considered "rare" if found in only one population (region) (SLATKIN 1985). The second method used WRIGHT's (1931) formula:

$$Nm(W) = \frac{(1 - F_{ST})}{4F_{ST}}$$

Where N is the effective population size of the recipient population and m is the rate of gene flow. $Nm(W)$ estimates the number of migrants per generation. Here, F_{ST} was considered equivalent to G_{ST} (NEI 1977).

Lastly, IDH genotypes were used to determine the percent hybridization occurring in each population. All heterozygous individuals containing an allele from each parental species were considered to be hybrids.

RESULTS

Genetic Diversity

Twenty of the 22 loci resolved (91%) were polymorphic in at least one population. Throughout the species, the 20 polymorphic loci averaged 2.95 alleles. *Mnr* and *Fe–1* were the only monomorphic loci, and when averaged with the other twenty loci gave a value of 2.77 alleles per locus. Expected heterozygosity at the species level was rather low (0.115) due to several loci exhibiting very skewed allele frequencies. Fifty three percent of the loci were polymorphic within populations (Table 2). The number of alleles per polymorphic locus within populations was 2.65, slightly less than at the species level. Across all loci, the average number of alleles within populations (1.87) was much less than that for the species as a whole. The mean expected heterozygosity within populations (0.113) was close to that for the species .

Table 3 Statistics of genetic variation for twenty polymorphic loci in shortleaf pine (NEI 1973, 1977; WRIGHT 1922)

Locus (alleles)	Genetic diversity			Genetic structure			
				As 18 populations		As 2 regions	
	H_T	H_S	D_{ST}	F_{IS}	G_{ST}	F_{IS}	G_{ST}
Aat–1 (4)	0.261	0.254	0.007	0.024	0.026	–0.002	0.004
Aat–2 (4)	0.334	0.322	0.012	0.022	0.037	0.053	0.007
Adh (2)	0.011	0.011	0.000	–0.040	0.033	–0.015	0.009
Dia–2 (3)	0.048	0.047	0.001	–0.036	0.014	–0.022	0.001
Dia–3 (2)	0.001	0.001	0.000	–0.009	0.008	–0.001	0.000
Fe–2 (2)	0.111	0.110	0.002	–0.035	0.015	–0.021	0.001
Fdp (2)	0.015	0.015	0.001	0.304	0.034	0.324	0.006
Idh (3)	0.050	0.049	0.001	–0.048	0.021	–0.037	0.011
Mdh (3)	0.022	0.022	0.000	–0.026	0.017	–0.010	0.001
Per–1 (2)	0.019	0.018	0.000	–0.026	0.015	–0.013	0.004
Per–2 (2)	0.496	0.470	0.026	0.127	0.053	0.167	0.007
6-Pgd–1 (5)	0.475	0.461	0.014	0.106	0.030	0.129	0.003
6-Pgd–2 (5)	0.234	0.226	0.007	–0.019	0.031	0.012	0.001
Pgi–1 (2)	0.007	0.007	0.000	1.000	0.059	1.000	0.002
Pgi–2 (4)	0.212	0.205	0.007	0.046	0.033	0.073	0.003
Pgm–1 (2)	0.008	0.008	0.000	–0.018	0.014	–0.005	0.001
Pgm–2 (2)	0.003	0.003	0.000	–0.016	0.015	–0.004	0.003
Skdh (5)	0.213	0.208	0.006	0.045	0.027	0.069	0.002
Tpi–1 (3)	0.002	0.002	0.000	–0.011	0.010	–0.001	0.001
Tpi–2 (2)	0.002	0.002	0.000	–0.022	0.020	–0.002	0.001
Mean	0.126	0.122	0.004	0.068	0.026	0.085	0.003

Overall levels of genetic variation in the eastern and western regions were very similar (Table 2). The eastern region had 77% polymorphic loci, 2.50 alleles per locus, and 2.94 alleles per polymorphic locus. The western region had 73% polymorphic loci, 2.36 alleles per locus, and 2.88 alleles per polymorphic locus. Expected heterozygosity for the east was 0.110 and for the west was 0.120.

Genetic Structure

For the most part, genotype frequencies conformed to Hardy Weinberg expectations. Eight of the 208 χ^2 tests showed significant deviations. Based on chance alone we would expect to see approximately ten significant deviations. The mean F_{IS} value over all loci was 0.068, further indicating that there is little deviation from random mating within populations and suggesting the absence of significant genetic structure (Wahlund effect) within the populations (Table 3).

Heterogeneity χ^2 tests for allele frequency differences among populations were significant for 14 of the 20 polymorphic loci. Values of G_{ST} ranged from 0.010 (*Tpi–1*) to 0.059 (*Pgi–1*) with a mean G_{ST} of 0.026, indicating that most of the genetic diversity (97.4%) occurred within populations (Table 3). Nei's genetic identities (*I*) were high for all pairwise comparisons (mean = 0.995). The lowest genetic identity was between BOK and TMGA (0.972) and the highest was between ISC and GVA (0.999). There was no statistically significant correlation between the geographic distance among populations and their genetic identities ($r = -0.086$, $p = 0.294$). Gene flow among populations was high with *Nm* due to the Slatkin method (*Nm(S)*) equal to 6.47 with 9 private alleles and to the Wright method (*Nm(W)*) equal to 9.95.

Loci with significant differences ($p < 0.05$) in allele frequencies between regions included *Aat–1*, *Aat–2*, *Adh*, *Fdp*, *Idh*, *Per–1*, *Per–2*, *Pgi–2*, *Skdh*, and *6–Pgd–1*. The proportions of total genetic diversity due to differences among regions (G_{ST}) were quite low ranging from 0.000 (*Dia–3*) to 0.011 (*Idh*, Table 3). The mean G_{ST} value among the two regions was 0.004

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Table 4 Shortleaf pine populations and their percent hybridization as measured using the IDH locus

Region			
Western		Eastern	
Popualtions	% hybrids	Populations	% hybrids
BOK	5.21	WTN	0.00
WAR	2.08	CMI	3.19
TAR	3.49	CAL	1.11
NAR	7.29	TMGA	4.55
WSMO	6.25	TGA	0.00
GMO	4.35	WSC	0.00
PMO	3.41	ISC	1.04
Mean	**4.58**	CNC	0.00
sd	**1.80**	TVA	2.08
		SVA	0.00
		GVA	0.00
		Mean	**1.09**
		sd	**1.57**

Table 5 Comparison of genetic variation for shortleaf pine with average values of genetic variation for all plants, for all woody plants, for all gymnosperms, and for loblolly pine (HAMRICK, unpublished data; HAMRICK *et al.* 1992)

Categories	Sample size *N*	Within species			Within populations			Among populations
		P	A	H_e	P	A	H_e	G_{ST}
All species	655	51.3	1.97	0.150	34.6	1.52	0.113	0.228
Woody plants	191	65.0	2.22	0.177	49.3	1.76	0.148	0.084
Gymnosperms	89	71.1	2.38	0.169	53.4	1.83	0.151	0.073
Loblolly pine	–	90.0	2.89	0.221	72.7	1.93	0.218	0.078
Shortleaf pine	–	91.1	2.77	0.115	52.8	1.87	0.113	0.026

indicating that almost all of the genetic diversity within the species (99.6%) exists within regions. Thus, approximately 16.7% of the variation among populations is due to differentiation among regions. Genetic identity between the regions was 0.999.

All seven western populations contained hybrids between loblolly pine and shortleaf pine, as indicated by the IDH locus (Table 4). In this region, the fewest were found in WAR (2.1%) and the most were found in NAR (7.3%). Less than half of the eastern populations contained hybrids. Overall, the western region had a significantly higher percentage of hybrids than the eastern region (4.6% vs. 1.1%, $p < 0.0005$).

DISCUSSION

Compared to the average values for all plant species, for all woody species, and for all gymnosperms, shortleaf pine has a higher proportion of polymorphic loci (P = 91%) and alleles per locus (A = 2.77) but lower expected heterozygosity (H_e = 0.115) (Table 5, HAMRICK *et al.* 1992). A priori, we would expect many of shortleaf pine's loci to have a common allele and several low frequency alleles, a pattern typical of many conifer species (HAMRICK *et al.* 1992, 1994). Twelve of the twenty polymorphic loci have H_T values less than 0.10, indicating that the common allele at these loci has a frequency of 0.95 or higher (Table 3). Such skewed allele frequencies are responsible for the low mean H_e value observed. While several factors can have a significant influence on genetic diversity within woody plant species, we know of nothing in the biology or evolutionary history of shortleaf pine that might explain this observation (HAMRICK *et al.* 1992). *P. taeda* (loblolly pine), which has a similar distribution and

recent biogeographical history, has similar levels of polymorphism (P = 90%) and alleles per polymorphic locus (A_p = 2.89) but has significantly higher mean heterozygosity (H_e = 0.221), indicating that fewer of its loci have highly skewed allele frequencies (Table 5; HAMRICK unpublished data).

Since most woody plants are outcrossing, they often have less genetic differentiation among populations than nonOwoody plant species (Table 5). Partitioning genetic diversity among woody plant populations is, therefore, primarily influenced by their effective population size and geographic distribution (HAMRICK *et al.* 1992). More specifically, pine species that occur in widely distributed, isolated populations have more among population genetic differentiation (i.e. higher G_{ST} values) presumably as a result of limited gene flow. Examples of pine species with disjunct ranges are bishop pine (*P. muricata*, G_{ST} = 0.220, MILLAR 1988), aleppo pine (*P. halepensis*, G_{ST} = 0.300, SCHILLER *et al.* 1986), and torrey pine (*P. torreyana*, G_{ST} = 1.00, LEDIG & CONKLE 1983). In contrast, species with more continuous geographic distributions should have more gene flow among populations and their populations should be less differentiated genetically. Pines with this sort of geographic distribution include jack pine (*P. banksiana*, G_{ST} = 0.030, DANCIK & YEH 1983), lodgepole pine (*P. contorta*, G_{ST} = 0.036, WHEELER & GURIES 1982), ponderosa pine (*P. ponderosa*, G_{ST} = 0.015, HAMRICK *et al.* 1989), and pitch pine (*P. rigida*, G_{ST} = 0.023, GURIES & LEDIG 1982).

Throughout much of its range shortleaf pine is continuously distributed and, therefore should have relatively little genetic heterogeneity among its populations. Our results support this prediction (G_{ST} = 0.026). In addition, genetic identities between shortleaf pine populations are quite high (0.972 to 0.999) as are estimates of gene flow ($Nm(S)$ = 6.47 and $Nm(W)$ = 9.95). Gene flow rates greater than 4.0 migrants per generation are sufficient to prevent population differentiation due to genetic drift (WRIGHT 1931).

Given their presumed evolutionary history, we had expected more heterogeneity in allele frequencies between the two geographic regions. This is obviously not the case since less than 17% of the total variation among populations is due to region of origin. Interestingly, loblolly pine, which is thought to have a similar paleoecological history but has more genetic diversity among populations (G_{ST} = 0.078), has a similar level of genetic diversity among these two regions (23% of the total among population diversity) (HAMRICK unpublished data).

Despite their genetic similarity, shortleaf pine from the eastern and western regions differ in one important aspect; the seven western populations had significantly more hybridization between shortleaf pine and loblolly pine than the eleven eastern populations. Loblolly pine usually sheds its pollen earlier than shortleaf pine; however, flowering time can differ extensively, both temporally and spatially (DORMAN & BARBER 1956). The flowering times of shortleaf pine and loblolly pine are associated with latitude (i.e. trees in lower latitudes flower earlier than trees in higher latitudes) (DORMAN & BARBER 1956). Apparently, the dry, warm climate, such as that found in the west and lower latitudes of the shortleaf pine distribution, creates more phenological overlap between the two species and, therefore, more opportunities to hybridize.

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