POPULATION GENETICS OF SHORTLEAF PINE (*Pinus echinata* Mill.): VARIATION, ISOENZYME LINKAGE AND REGENERATION EFFECTS ON DIVERSITY

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CHAPTER I

ISOENZYME VARIATION AND GENETIC STRUCTURE IN NATURAL POPULATIONS OF SHORTLEAF PINE (*Pinus echinata* Mill.)

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ABSTRACT

Seed from 126 trees from populations representing 15 geographic locations covering much of the natural range of shortleaf pine (*Pinus echinata* Mill.) were analyzed using 23 enzyme systems covering 39 loci to determine patterns of genetic variation and structure. Populations were polymorphic (p) at 87.2% of the loci, had 2.18 alleles (A) per locus and 2.35 alleles per polymorphic locus (A_p). Mean expected heterozygosity (H_e) was 0.194 and mean observed heterozygosity (H_o) was 0.174. Western populations had a higher p, higher A, similar A_p and a higher H_o and H_e than eastern populations, due in part to six private alleles (alleles seen only in one population) in the west but only one in the east. Genetic structure analysis revealed interpopulation genetic variation at 9% percent, meaning 91% of the genetic variation in shortleaf pine resides within populations. Interpopulation gene flow was 2.56, indicating two to three allele migrations per generation, which is relatively high and explains the low interpopulation genetic variation in the species. There was no apparent relationship between geographic distance among populations and their genetic distance. Shortleaf pine populations exist in naturally outcrossing random-mating populations and have a relatively large amount of natural variability. Western populations are more diverse than their eastern counterparts.

INTRODUCTION

Knowledge of the patterns of genetic variation within and among populations of a tree species and understanding its mating system will influence strategy for tree selection, breeding and seed production orchard design. Electrophoretic techniques, especially isoenzymes, have proven valuable in studying the mating systems and genetic structure of conifers. The presence of haploid (*n*) megagametophyte tissue in seeds greatly facilitate isoenzyme studies in conifers.

Perennial species with long life spans like conifers generally contain very high levels of genetic variation (Hamrick and Godt 1990; Mitton 1983). Conifers are one of the most genetically variable groups of species with higher mean heterozygosity (0.270) than monocots (0.165), dicots (0.113) or mammals (0.039) (Mitton 1983). In the past two decades, numerous species of conifers have been analyzed for their genetic variation, including *Pinus contorta* (Yeh *et al.* 1985; Yeh and Layton 1979), *Pinus ponderosa* (Yow *et al.* 1992; O'Malley *et al.* 1979), *Picea sitchensis* (Yeh and El-Kassaby 1980), *Pinus washoensis* (Niebling and Conkle 1990), *Pseudotsuga menziesii* (Yeh and O'Malley 1980; El-Kassaby and Sziklai 1982), *Pinus sylvestris* (Goncharenko *et al.* 1994), *Pinus sibirica* (Goncharenko *et al.* 1992) and *Pinus pumila* (Goncharenko *et al.* 1993). In these studies, interpopulation genetic variation ranged from 1.6 to 7.5 percent of the total genetic variation, and the percent of polymorphic loci ranged from 66 to 90. Pines, like other conifers, are highly variable.

Shortleaf pine (*Pinus echinata* Mill.) has the broadest geographic range of the southern pines (Figure 1). Considerable genetic variation is found in shortleaf pine, probably because it occurs at elevations from near sea level to 3,300 feet in the southern Appalachian Mountains, and because of its wide natural range (Tauer 1980; Dorman 1976). Shortleaf pine is used for construction lumber, plywood and paper, and accounts for more than 22 percent of the standing volume of the four major southern pines. Shortleaf pine would be expected to be highly variable at the isoenzyme level as suggested by studies of other pine with wide natural ranges.

In spite of its importance, to our knowledge there is only one report describing the mating system and pattern of genetic variation in natural populations of shortleaf pine. This recent study by Edwards and Hamrick (1995), based on 14 enzyme systems covering 22 loci in 18 populations, reported that the species had 91 percent polymorphic loci, an expected heterozygosity (H_e) of 0.115 and interpopulation genetic variation estimate (G_{ST}) of 0.026. The objective of our study was to describe isoenzymatic variation in shortleaf pine. We recognize the significant value of independent studies of the same species using a different sample of populations, individuals and isoenzyme loci. This paper presents the results of our isoenzymatic study of genetic variation in 15 natural populations of shortleaf pine using 23 enzyme systems covering 39 loci. Our considerably larger sample of loci makes the data complementary to the earlier report.

MATERIALS AND METHODS

This study is based on seeds collected from 126 shortleaf pine trees from 15 populations sampled from 11 states in the United States (Figure 1). Cones were collected during fall 1993 by Dr. Ron Schmidtling of the USDA Forest Service, Gulfport, MS from a shortleaf pine southwide seed source study (Wells 1973). Seeds were extracted, dried to approximately six percent moisture content and frozen at -20^{0}C for later use. Since only the megagametophytes were used, data represent the genetic structure of the trees' origin populations.

Seeds were thawed at room temperature for one hour and then immersed in water overnight prior to stratification. Water was drained and seeds were cold-stratified (4^{0}C) for sixty days, after which they were germinated on moist filter papers at room temperature. Ten megagametophytes were sampled from six to ten parent trees in each of the 15 populations. This sample size is sufficient to estimate maternal genotype and effective outcrossing rate (Yeh and Layton 1979). Megagametophytes were isolated, maintained on ice and ground in 0.14 mL Wendel and Parks (1982) extraction buffer.

The three electrophoresis gel systems used are described in Table 1. Gel preparation and loading followed Conkle *et al.* (1982) with the following modifications: 50 sec heating in a microwave oven after the boiling buffer is added to the starch suspension to avoid premature solidification and to

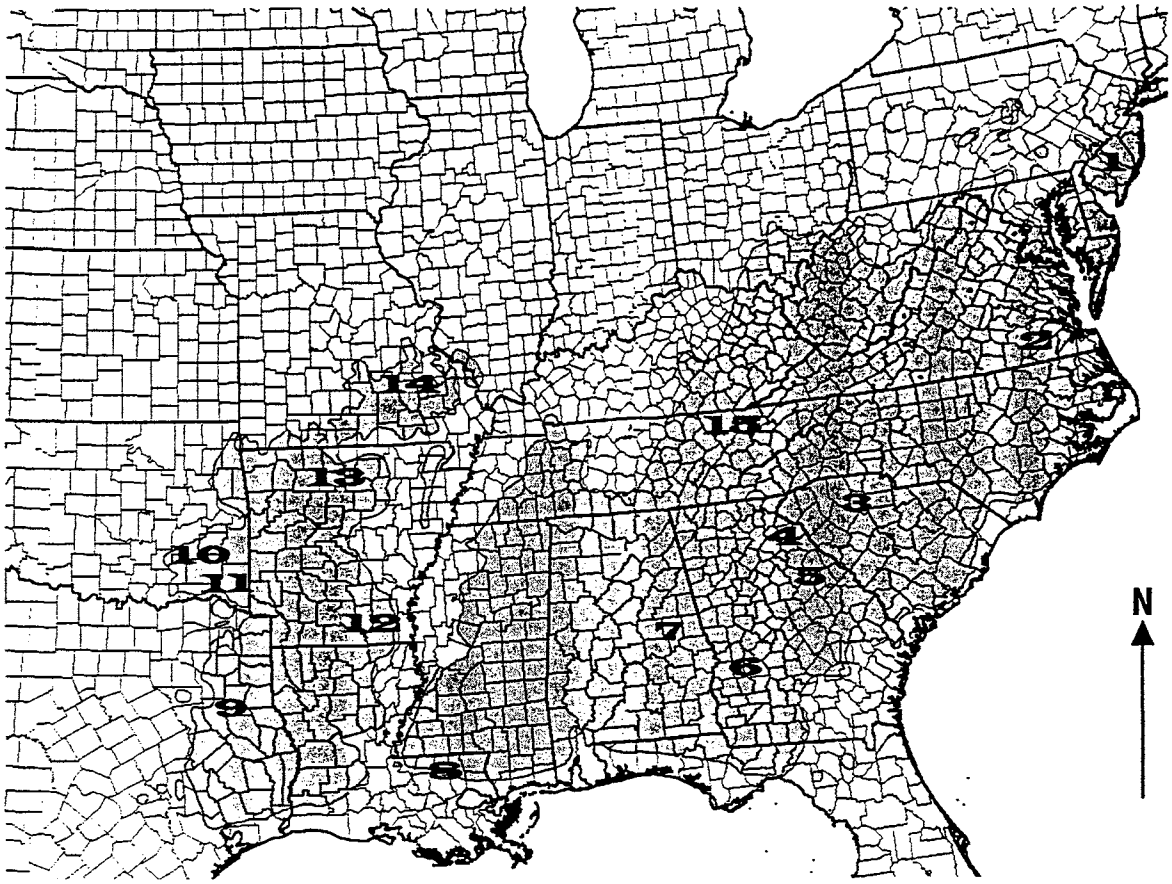

Figure 1. Natural range of shortleaf pine and location of the 15 seed source collections. 1: C453 - southern New Jersey; 2: C455 - southeastern Virginia; 3: C457 - western South Carolina; 4: C461 - northern Georgia, Clarke county; 5: C463 - northern Georgia, Putnam county; 6: C465 - southwestern Georgia; 7: C467 - east central Alabama; 8: C473 - southeastern Louisiana; 9: C475 - eastern Texas; 10: C477A - southeastern Oklahoma, Pushmataha county; 11: C477B - southeastern Oklahoma, McCurtain county; 12: C481 - southeastern Arkansas; 13: C483 - northern Arkansas; 14: C485 - south central Missouri; 15: C487 - northeastern Tennessee.

Table 1. Enzymes, their abbreviations (Abbr.), enzyme commission reference numbers (E.C.), buffer systems used for electrophoresis and number of loci consistantly scorable in each enzyme system

Enzyme	Abbr.	E.C. Number	Buffer System[a]	Scorable Loci
Aconitase	*Aco*	4.2.1.3	H	1
Acid phosphatase	*Acp*	3.1.3.2	H	2
Adenylate kinase	*Adk*	2.7.4.3	A	2
Alcohol dehydrogenase	*Adh*	1.1.1.1	H	2
Aldolase	*Ald*	4.1.2.13	H	2
Diaphorase	*Dia*	1.6.4.3	H	2
Fructose diphosphatase	*Fdp*	3.1.3.11	A	1
Fumarase	*Fum*	4.2.1.2	E	1
Glutamic dehydrogenase	*Gdh*	1.4.1.3	A	1
Glutamate-oxaloacetate transaminase	*Got*	2.6.1.1	A	2
Glucose-6-phosphate dehydrogenase	*G6pd*	1.1.1.49	H	2
Glycerate-2-dehydrogenase	*G2d*	1.1.1.29	A	1
Isocitric dehydrogenase	*Idh*	1.1.1.42	E	1
Malic dehydrogenase	*Mdh*	1.1.1.37	E	4
Malic enzyme	*Me*	1.1.1.40	E	1
Menadione reductase	*Mnr*	1.6.99.2	A	2
Peptidase	*Pep*	3.4.13.1	A	3
Phosphoglucose isomerase	*Pgi*	5.3.1.9	E	1
Phosphoglucomutase	*Pgm*	2.7.5.1	A	1
6-Phosphogluconate dehydrogenase	*6Pgd*	1.1.1.44	E	2
Sorbitol dehydrogenase	*Sdh*	1.1.1.14	A	1
Shikimate dehydrogenase	*Skdh*	1.1.1.25	H	2
Uridine diphosphoglucose pyrophosphorylase	*Ugpp*	2.7.7.9	E	2

[a] A: Electrode buffer - 28.6 mM Lithium hydroxide, 192 mM boric acid, pH 8.3; gel buffer - 45 mM Tris, 6.84 mM citric acid, 2.86 mM Lithium hydroxide, 19.2 mM boric acid, pH 8.3 (Conkle et al.1982).

E: Electrode buffer - 40 mM Citric acid, pH to 8.1 with N-(3-aminopropyl) morpholine; gel buffer - 20:1 dilution of electrode buffer (Strauss and Conkle 1986). H: Electrode buffer - 125mM Tris pH 7.0; gel buffer - 12.5mM Histidine-HCl, 0.35 mM EDTA, pH 7.0 (Cheliak and Pitel 1984).

strengthen gels; heating the vacuum flask on a hot plate while degassing; and using a spatula immediately after pouring to remove air bubbles. Each gel accommodated 50 wicks, 20 × 1.5 mm in size. Thirty-nine loci from 23 enzyme systems were resolved and consistently scorable in this study (Table 1). Recipes for staining enzymes are given in Table 2.

Allele frequencies for each locus were determined for each of the 15 populations studied. The most common allele at each locus was assigned an arbitrary value of 1.00 as described by Prakash *et al.* (1969). Alternate alleles were designated according to their relative migration with respect to the most common allele.

Existing literature suggests east-west genetic differences in shortleaf pine populations due to a drier western environment and isolation caused by the Mississippi river valley (Wells *et al.* 1977; Edwards and Hamrick 1995). Hence, the 15 populations sampled in this study were grouped into east (nine populations with 71 trees) and west (six populations with 55 trees) of the Mississippi river geographic regions. These groups were examined for within and between differences in genetic structure and compared to results of Edwards and Hamrick (1995). Allele frequency data were used to compute observed (H_o) and expected (H_e) heterozygosities (unbiased estimate, Levene 1949; Nei 1978). Genetic diversity for populations and regions was estimated by: percent polymorphic loci '*p*'; mean number of alleles per locus '*A*'; mean number of alleles per polymorphic locus 'A_p'; and H_o and H_e. A tree was

Table 2. Enzymes, references and modifications to stain recipes used in this shortleaf pine genetic variation study.

Enzymes	Recipe used [a]	Additions and modifications to stain recipes [b]
Aco	1	cis-Aconitic acid -10 mL; NBT replaces MTT.
Acp	1	Acetate buffer, pH 5.0 - 25 mL; alpha-naphthyl acid phosphate - 25 mg; fast garnet GBC salt - 40 mg.
Adk	1	Tris-Hcl buffer - 25 mL; NADP / MTT / PMS / $MgCl_2$ - 2 mL; NAD not used.
Adh	2	0.2M tris-Hcl buffer - 25 mL; PMS - 1 mL.
Ald	1	Tris-Hcl buffer - 25 mL; fructose-1,6-diphosphate - 150 mg; arsenic acid - 40 mg; glyceraldehyde-3-phosphate dehydrogenase - 150 Units.
Dia	1	Tris-Hcl buffer - 25 mL; 2,6-dichlorophenol indophenol - 0.3 mg; NADH - 15 mg.
Fdp	1	Tris-Hcl buffer - 20 mL; G6PDH - 150 Units; NAD / MTT / PMS - 2 mL.
Fum	1	NAD / MTT / PMS - 4 mL.
Gdh	1	Tris-Hcl buffer - 25 mL; L-Glutamic acid - 1 g.
Got	2	Phosphate buffer - 30 mL.
G6pd	2	0.2M tris-Hcl buffer - 25mL; D-glucose-6-phosphate - 100 mg; MTT replaces NBT.
G2d	1	Tris-Hcl buffer - 25mL; NAD / MTT / PMS - 3 mL.
Idh	1	Tris-Hcl buffer - 25mL; NADP / $Mgcl_2$ / NBT / PMS - 2 mL.
Mdh	1	Tris-Hcl buffer / DL-malic acid - 25mL; NBT used.
Me	1	A electrophoresis buffer / DL-malic acid - 12 mL; NBT replaces MTT.
Mnr	2	Menadione - 25 mg.
Pep	1	L-leu-L-tyr / L-val-L-leu / L-leu-L-ala - 10 mg; dimethyl formamide - 5 mL.
Pgi	2	0.2M tris-Hcl buffer - 25 mL; D-fructose-6-phosphate - 15 mg; G6PDH - 30 Units.
Pgm	1	Tris-Hcl buffer - 25 mL; glucose-1-phosphate - 70 mg; G6PDH - 30 Units; glucose-1,6-diphosphate - 0.3 mg; NADP / NBT replaces NAD / MTT.
6pgd	2	0.2M tris-Hcl buffer - 25 mL; 6-phosphogluconic acid - 10mg; $Mgcl_2$ / PMS - 1mL.
Sdh	1	Tris-Hcl buffer - 25 mL; sorbitol - 1g.
Skdh	2	0.2M Tris-Hcl buffer - 25 mL; Shikimic acid - 40 mg; $Mgcl_2$ / PMS - 1 mL.
Ugpp	3	No modification.

[a] 1: O' Malley *et al.*, 1980; 2: Conkle *et al.*, 1982; 3: Strauss and Conkle, 1986.

[b]: Some of these modifications are based on Ernst (unpublished), Dept. For., Univ. Neb., Lincoln, NE; If quantity of a chemical is given, it indicates quantity used different from the original recipe.

considered polymorphic at a locus if more than one allele was present at that locus irrespective of frequencies.

Wright's F statistics (Wright 1965; 1969; 1978; Nei 1977) were used to describe genetic structure in shortleaf pine. F statistics measure differentiation among populations by examining deviations of heterozygote and homozygote frequencies from expected under Hardy-Weinberg equilibrium. F_{IT} quantifies the deviation in genotypic frequencies from a hypothetical population that mates at random with no genetic structure, F_{ST} measures differentiation among populations, and F_{IS} represents the level of deviation of heterozygote frequencies within populations from expected under Hardy-Weinberg equilibrium.

Contingency χ^2 tests for heterogeneity of allele frequencies among populations (Workman and Niswander 1970) were performed using the formula:

[1] $$\chi^2 = 2N \left(\sum_{j=1}^{k} \sigma^2_{pj} / \hat{p}_j \right)$$

where N denotes the number of individuals sampled in all populations, $\hat{p}_j$ and σ^2_{pj} denote the weighted mean and variance of frequencies of the jth allele across all populations, and k is the number of alleles at a locus.

Nei's (1972) genetic distance coefficients (D_n) among all possible pairs of populations were estimated. Nei's genetic distance is based on the identity of genes between populations, $D_n = -\log_e I$, where I is the normalized identity of genes between two populations. D_n measures the cumulative allele differences

per locus. A phenogram was constructed to visualize the results. The D_n values were clustered using the unweighted-pair-group-method algorithm (UPGMA) (Sneath and Sokal 1973). F_{IT}, F_{IS}, F_{ST}, D_n and UPGMA cluster analysis were calculated using the BIOSYS-1 computer program (Swofford and Selander 1981).

An estimate of the level of gene flow was obtained from F_{ST} by solving

[2] $$N_e m = (1/F_{ST} - 1) \div 4$$

where $N_e m$ is gene flow (Wright 1931), N_e is effective population size and m is the proportion of migrants exchanged per generation. Since N_e and m are usually not known, gene flow is often reported as $N_e m$, which estimates the number of migrants per generation.

RESULTS AND DISCUSSION

Genetic diversity

Twenty-three enzyme systems assayed in shortleaf pine identified 85 electrophoretic variants in 39 loci (Figure 2). Thirty four of the 39 loci assayed exhibited polymorphism (87.2 %), which is in close agreement with the 91 percent reported by Edwards and Hamrick (1995). These data show considerable natural variation in shortleaf pine, higher than observed in *Pinus contorta* Dougl. (65.8%, Yang and Yeh 1993), *Pinus pumila* (Pall.) Regel. (77.7%, Goncharenko *et al.* 1993), *Pinus ponderosa* Dougl. ex. Laws. (81%, Yow *et al.* 1992), *Robinia pseudoacacia* (70.8%, Surles *et al.* 1989), all plant species (51%, Edwards and Hamrick 1995), all woody plants (65%, Hamrick *et al.* 1992), and all conifers (67.7%, Hamrick 1989), but slightly lower than *Pinus sylvestris* L. (90.5 %, Goncharenko *et al.* 1994) and *Pinus taeda* L. (90.0%, Edwards and Hamrick 1995). Seven of the 85 electrophoretic variants found were seen in only one population and are considered private alleles (Slatkin 1985). Six of the seven private alleles (*Mnr-2, Fdp, Sdh, Adk-1, G6pd-1 and 6pgd-2*) were in the western region and the other (*Ald-1*) in the eastern. It is of note that three (*Mnr-2, Adk-1* and *Sdh*) of the six private alleles in the western populations occurred in the northern Arkansas population (C483). The *Mdh-4* allele was present only in one eastern and one western population, and the *Ald-2* allele only in three eastern populations. The occurrence of private alleles in the populations studied may be an artifact of sampling.

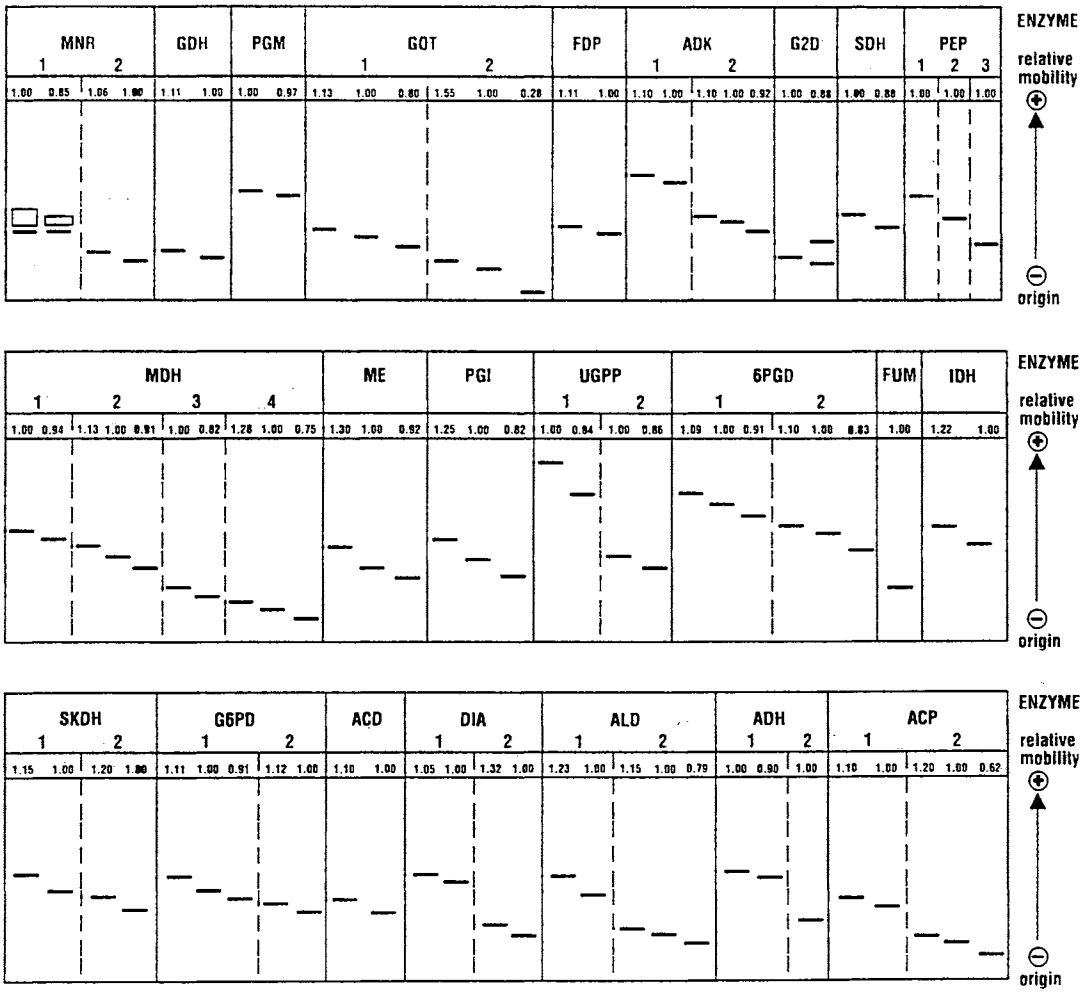

Figure 2. Relative mobility and designation of all electrophoretic variants at the 39 loci studied in shortleaf pine.

Genetic diversity measures for the 15 populations of shortleaf pine sampled show a mean percent of polymorphic loci within populations much lower (53.9 %) than for the species as a whole (Table 3). The same trend was noted for mean number of alleles per locus and mean number of alleles per polymorphic locus. Similar trends were reported by Edwards and Hamrick (1995), although our values are slightly lower due to differences in enzymes scored and populations sampled. Lower values for genetic diversity measures within populations compared to the whole species may be attributed in part to the seven private alleles, and the *Mdh-4* and *Ald-2* loci discussed above. However, the differences seen between this study and that of Edwards and Hamrick (1995) may be due to sampling.

H_o for the species was 0.174, which was lower than the expected value of 0.194 (Table 3). The same trend was seen for eastern (H_o= 0.164, H_e = 0.186) and western (H_o= 0.189, H_e = 0.205) populations. When considered over all populations or as two regions, the species shows some deviation from Hardy-Weinberg expectations. Some deviation is expected in a species whose natural range extends over thousands of miles, and is probably due to violations of the assumptions under which Hardy-Weinberg expectations are made, such as random mating and the absence of selection and migration. The assumption of random mating cannot be completely fulfilled due to the wide range of shortleaf pine. Studies in almost all pine species report a significant level of migration. Lower H_o values can also be caused by inbreeding. In spite of these obvious

Table 3. Summary of genetic diversity of shortleaf pine for all populations and by region. The data is based on 39 different loci.

Region	Polymorphic loci 'p'	Mean number of alleles per locus 'A' (se)	Mean number of alleles per polymorphic locus 'Ap'	H_o (se)	H_e (se)[1]
East					
Southern New Jersey	58.97	1.59 (0.08)	2.00	0.217 (0.034)	0.226 (0.034)
Southeastern Virginia	48.72	1.54 (0.10)	2.11	0.126 (0.027)	0.170 (0.032)
Western South Carolina	56.41	1.67 (0.11)	2.18	0.153 (0.030)	0.183 (0.033)
Northern Georgia (Clarke County)	53.85	1.54 (0.08)	2.00	0.185 (0.039)	0.219 (0.037)
Northern Georgia (Putnam County)	48.72	1.54 (0.10)	2.11	0.179 (0.041)	0.174 (0.034)
Southwestern Georgia	53.85	1.62 (0.10)	2.14	0.185 (0.039)	0.197 (0.035)
East-central Alabama	38.46	1.41 (0.09)	2.07	0.150 (0.041)	0.174 (0.037)
Northeastern Tennessee	51.28	1.54 (0.09)	2.05	0.137 (0.027)	0.161 (0.031)
Southeastern Lousiana	46.15	1.51 (0.10)	2.11	0.143 (0.034)	0.169 (0.035)
Mean	50.71	1.55	2.09	**0.164**	0.186
Within East Region	**76.92**	**2.03**	**2.33**		**0.186**
West					
Eastern Texas	41.03	1.49 (0.10)	2.19	0.170 (0.039)	0.163 (0.036)
Southeastern Oklahoma (Pushmataha county)	66.67	1.77 (0.10)	2.15	0.205 (0.034)	0.226 (0.035)
Southeastern Oklahoma (McCurtain county)	53.85	1.56 (0.09)	2.05	0.144 (0.033)	0.153 (0.029)
Southeastern Arkansas	61.54	1.80 (0.12)	2.29	0.220 (0.038)	0.221 (0.034)
Northern Arkansas	69.23	1.77 (0.09)	2.11	0.201 (0.034)	0.205 (0.032)
South-central Misssouri	58.97	1.74 (0.11)	2.26	0.191 (0.038)	0.207 (0.035)
Mean	58.55	1.69	2.18	**0.189**	0.196
Within West Region	**84.62**	**2.13**	**2.33**		**0.205**
Overall mean	53.85	1.61	2.12	**0.174**	0.190
Within Species	**87.18**	**2.18**	**2.35**		**0.194**

[1] Unbiased estimate based on conditional expectations (Levene, 1949; Nei, 1978).

violations, the deviation from expected is only ten percent, which indicates that the populations mate nearly at random over vast areas, and thus no reason for concern over skewed allele frequency distributions. Western populations showed a higher percentage of polymorphic loci, higher mean number of alleles per locus, same mean number of alleles per polymorphic locus and a higher H_o and H_e than their eastern counterparts (Table 3). These differences may be due to the six private alleles and one rare allele showing polymorphism in the west, while only one private and one rare allele are present in the east, as discussed above. The source of and selective advantage/disadvantage, if any, of these private alleles is of interest. Why are they more numerous in western populations? If the occurrence of these private and rare alleles is not an artifact of sampling, their source may be a lower frequency of disturbance in western populations over their evolutionary history, and/or a higher frequency of interspecific hybridization. Whatever the reason, these data show a relatively-large amount of natural variability in shortleaf pine, and the western populations are more diverse than their eastern counterparts.

A considerably higher level of heterozygosity was found at the *Idh* locus in the western populations ($H_0 = 0.167$ or 16.7%) than in eastern populations ($H_0 =$ 0.044 or 4.4%) due to a higher-than-average frequency of the faster allele in the western populations. This observation is in agreement with Edwards and Hamrick (1995), although their values were lower ($H_0 = 0.0458$ and 0.0109 respectively). Contingency χ^2 test for heterogeneity of allele frequencies among populations at the *Idh* locus was not significant ($\alpha = 0.05$, $p = 0.1199$) when

tested for all populations, but was significant ($\alpha = 0.05$, $p = 0.0223$) when tested as two regions, which confirms a significant difference in *Idh* allele frequencies between the two regions. According to Huneycutt and Askew (1989), polymorphism at the *Idh* locus is due to hybridization with loblolly pine, suggesting 16.7 percent of the trees in the western populations we sampled are of hybrid descent. These data raise questions regarding the adequacy of the *Idh* locus in identifying hybrids. If the *Idh* locus is a true indicator of hybrids between loblolly and shortleaf pine, these results suggest that 16 percent of the trees in these stands are hybrids or their descendants, which seems improbable. The value of *Idh* as an indicator of hybrids is also in doubt because four of the eight populations in our study, and some of the western populations of Edwards and Hamrick (1995) with polymorphic *Idh* loci are outside the present natural range of loblolly pine.

Genetic structure

Wright's fixation index estimate (F_{ST}) for shortleaf pine was 0.089 (Table 4). In other words, 91 percent of the genetic variation in shortleaf pine resides within populations and 9 percent among populations, indicating that these populations are genetically quite similar. As two regions, the F_{ST} value was much lower (0.006), suggesting that more than 99 percent of the total variation is found within regions. The higher F_{ST} estimate of this study compared to 0.026 by Edwards and Hamrick (1995) is in part due to the six private alleles, only three of which were studied by Edwards and Hamrick. G_{ST} or F_{ST} values obtained for other pine species average about 0.08 (Guries and Ledig 1982; Furnier and Adams 1986; Moran *et al.* 1988), except for *Pinus pumila* (F_{ST} = 0.037) and *Pinus sibirica* (F_{ST} = 0.020) (Goncharenko *et al.* 1992). Hence, we conclude that the mating pattern and genetic structure in shortleaf pine is quite similar to that found in other pines.

The F_{IS} values for the 15 populations averaged 0.018 (Table 4). The positive average value suggests a 1.8 percent deficiency in heterozygotes within shortleaf pine populations, a very small deficiency. F_{IT} values averaged 0.105 over all populations, which indicates a 10.5 percent heterozygote deficiency. These data agree with the deviation between H_o and H_e values. When analyzed as two regions, a ten percent heterozygote deficiency was seen both within regions and for the entire species. A ten-fold increase in the F_{IS} value when F_{IT}

Table 4. Wright's fixation indices for shortleaf pine for all populations and by region with probability of chi-square distribution for heterogeneity of allele frequencies among populations.

Locus	As 15 Populations			As two regions			Contingency
	F_{IS}	F_{IT}	F_{ST}	F_{IS}	F_{IT}	F_{ST}	Chi-square
Mnr-1	-0.065	-0.001	0.060	0.000	0.002	0.002	ns
Mnr-2	-0.167	-0.010	0.135	-0.036	-0.018	0.018	***
Gdh	-0.169	-0.067	0.088	-0.075	-0.067	0.007	*
Pgm	0.116	0.190	0.084	0.208	0.209	0.001	*
Got-1	0.074	0.142	0.073	0.134	0.135	0.001	*
Got-2	0.117	0.248	0.148	0.234	0.242	0.010	***
Fdp	-0.077	-0.005	0.067	-0.012	-0.006	0.006	ns
Adk-1	-0.200	-0.011	0.157	-0.014	-0.007	0.007	***
Adk-2	-0.169	-0.049	0.103	-0.061	-0.054	0.007	***
G2d	-0.052	0.048	0.095	0.015	0.016	0.000	**
Sdh	-0.111	-0.007	0.094	-0.019	-0.009	0.009	**
Mdh-1	-0.091	-0.015	0.070	-0.024	-0.018	0.006	ns
Mdh-2	-0.099	-0.037	0.056	-0.042	-0.039	0.003	ns
Mdh-3	-0.169	-0.044	0.107	-0.055	-0.050	0.005	**
Mdh-4	-0.062	-0.018	0.041	-0.020	-0.019	0.000	ns
Me	0.169	0.247	0.094	0.157	0.174	0.020	***
Pgi	-0.148	-0.041	0.093	-0.034	-0.024	0.009	*
Ugpp-1	0.122	0.178	0.064	0.146	0.151	0.006	ns
Ugpp-2	-0.266	-0.097	0.133	-0.114	-0.114	0.000	***
6pgd-1	-0.295	-0.206	0.068	-0.224	-0.213	0.009	ns
6pgd-2	-0.128	-0.011	0.104	0.021	0.029	0.008	***
Idh	-0.145	-0.049	0.084	-0.076	-0.055	0.020	ns
Skdh-1	0.338	0.432	0.141	0.395	0.396	0.001	***
Skdh-2	-0.136	-0.044	0.081	-0.065	-0.050	0.014	ns
G6pd-1	0.496	0.561	0.130	0.545	0.555	0.024	**
G6pd-2	0.419	0.470	0.089	0.349	0.349	0.000	*
Aco	0.243	0.293	0.066	0.279	0.280	0.002	ns
Dia-1	0.160	0.223	0.075	0.253	0.257	0.005	ns
Dia-2	-0.070	-0.013	0.053	-0.018	-0.015	0.003	ns
Ald-1	-0.111	-0.007	0.094	-0.008	-0.004	0.004	**
Ald-2	-0.152	-0.045	0.093	-0.052	-0.039	0.013	**
Adh-1	-0.053	0.004	0.055	0.046	0.055	0.010	ns
Acp-1	0.328	0.363	0.051	0.328	0.328	0.000	ns
Acp-2	0.041	0.114	0.076	0.109	0.112	0.004	*
Mean	0.018	0.105	0.089	0.091	0.097	0.006	

NOTE: ***, significant at $P < 0.01$; **, significant at $P < 0.05$;*, significant at $P < 0.10$; ns, not significant.

remained unchanged supports the earlier inference that the heterozygote deficiency for the entire species is due to the violation of some Hardy-Weinberg assumptions. Higher F_{IS} values for the two regions can also be due to differences in the level of disturbance and/or the level of inter-specific hybridization. Hardy-Weinberg assumptions are met when populations are studied in smaller units, and in such a case the genotypic proportions fulfill the expectations. Hence, by comparing the F_{IS} and F_{IT} estimates with the H_o and H_e values, we conclude that in spite of shortleaf pine's wide geographic distribution, its among population genetic structure is quite similar.

One-tailed probability of contingency χ^2 tests for heterogeneity of allele frequencies among populations (Table 4) were significant for 14 of the 34 polymorphic loci ($\alpha = 0.05$). Thus, the allele frequencies of these 14 loci are significantly different in at least one of the populations, and they are largely responsible for the 9 percent genetic variation seen among populations. All private alleles except *Fdp* (which had a very low frequency of the alternate allele) showed significant allele frequency differences among populations using the χ^2 test.

Interpopulation gene flow, N_em was estimated to be 2.56, meaning approximately two to three allele migrations occur per generation. This relatively-high migration rate suggests that populations of shortleaf pine are genetically linked by constant gene flow through a network of connecting stands. It is impossible to estimate the absolute amount of gene flow among populations, because migration is dependent on the effective number of migrants, not just

migration rate (Wright 1931), and because effective population size in natural populations is difficult to obtain (Allendorf and Phelps 1981). However, one migration every two generations ($N_e m$ = 0.5) is generally enough to annul any genetic difference resulting from drift (Wright 1931). Hence, $N_e m$ values greater than one may be considered a sufficient level of migration among populations to prevent differentiation. In this study, there is a relatively high rate of migrants between populations per generation, consequently the among-population genetic differences are small. However, differentiation among populations can still occur due to directional, adaptive selection.

Cluster analysis using UPGMA with arithmetic averages gave a cophenetic correlation coefficient of (Sneath and Sokal 1973) r_{cs} = 0.71 on the matrix of Nei's genetic distances. The cophenetic correlation coefficient is a measure of the agreement between the values in the phenogram, obtained by UPGMA clustering, to the original genetic distance coefficients (D_n) of Nei (1972). A phenogram of the 15 shortleaf pine populations with Nei's genetic distance coefficients for every possible pair is presented in Figure 3. The mean genetic distance was very low (0.022), indicating that an average difference of about two percent in structural genes exists between any two of the populations. The D_n value was lowest between southeastern Virginia and western South Carolina and between Northern Georgia-Putnam county and southwestern Georgia (0.009). The 0.009 value means that less than one allelic substitution per 100

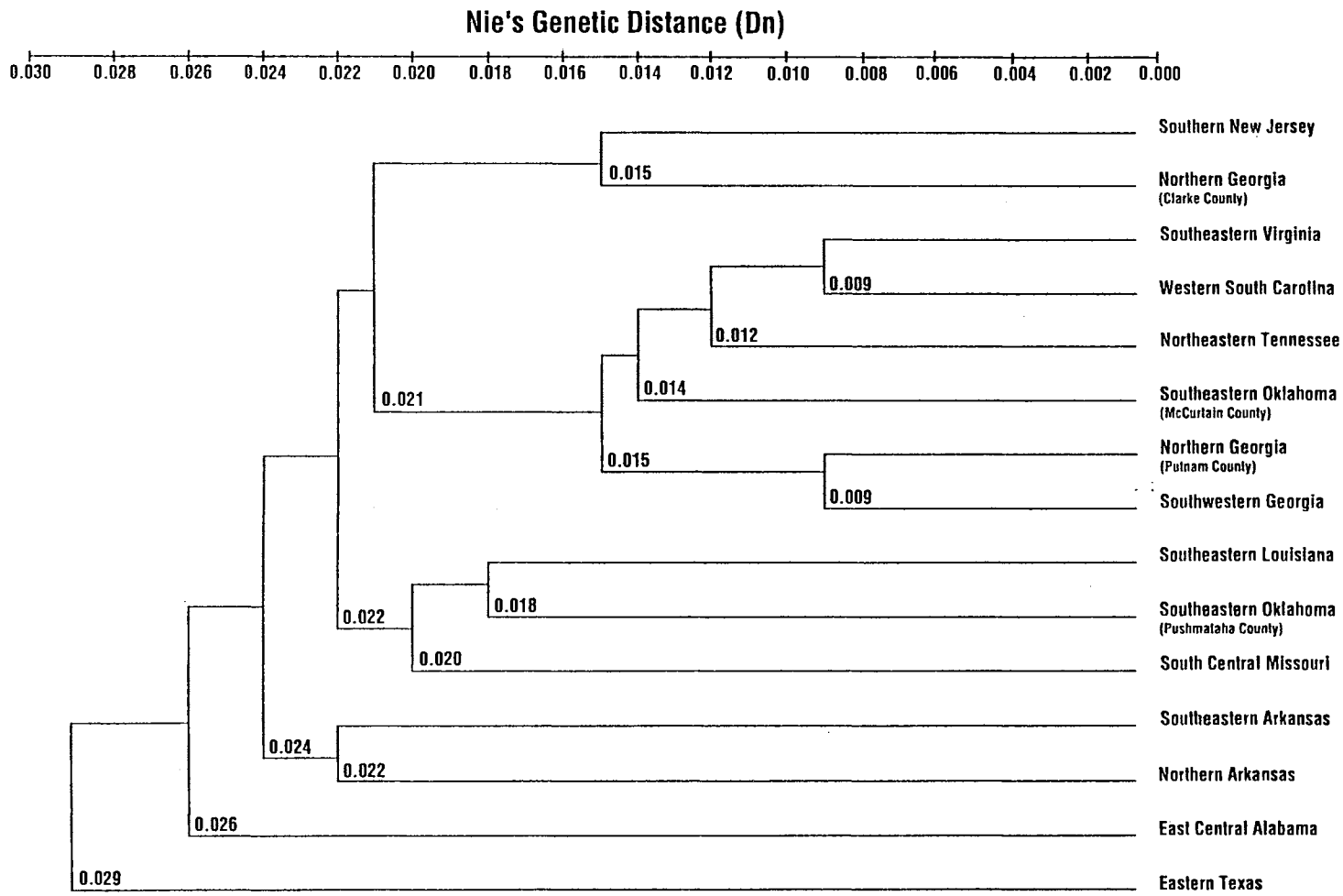

Figure 3. Phenogram of shortleaf pine populations based on Nei's genetic distance coefficient (D_n) showing genetic differentiation levels.

loci occurred between the genomes of these populations. The eastern Texas source showed the highest genetic difference from the rest of the populations. The southeastern Virginia source is more genetically similar to the western South Carolina source than to south New Jersey; north Georgia-Putnam and southwestern Georgia to southeastern Virginia than to northern Georgia-Clarke; and southeastern Oklahoma-Pushmataha county to southeastern Louisiana than to southeastern Oklahoma-McCurtain county. Also, the eastern Texas source maintains the same level of genetic difference with the Oklahoma sources as it does to south New Jersey. These results show the absence of a meaningful correlation between geographic distance among populations and their genetic distance.

CONCLUSION

Our study is in close agreement with Edwards and Hamrick (1995) in the following aspects: shortleaf pine is polymorphic at about 87 percent of its loci, genetic diversity measures were lower within populations than for the species as a whole, and western populations had higher genetic diversity measures than eastern populations, perhaps due to the higher number of private alleles seen in the western populations. However, the studies differ in that we found H_o to be 10% lower than H_e compared to the 7% difference of Edwards and Hamrick (1995), probably due to differences in enzyme scored and populations sampled. We found a higher level of heterozygosity at the *Idh* locus in western populations than eastern populations, higher than Edwards and Hamrick (1995). Such high values have compelled us to question the adequacy of the *Idh* locus as a way of identifying hybrids, as was suggested by Huneycutt and Askew (1989). Further, the higher frequency of private alleles in our study may be the reason for our higher F_{ST} estimate of 0.089, and consequently a lower N_em value of 2.56 than Edwards and Hamrick (0.026 and 9.95 respectively). However, studies conducted in other pines show that a F_{ST} value of 0.08 is characteristic of pines and a N_em value above 0.5 is sufficient to prevent genetic drift.

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CHAPTER II

SEGREGATION AND LINKAGE RELATIONSHIPS OF ISOENZYMES IN SHORTLEAF PINE (*Pinus echinata* Mill.)

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ABSTRACT

Segregation and linkage relationships were analyzed between 28 isoenzyme loci in 10 natural stands representing much of the natural range of *Pinus echinata* Mill. (shortleaf pine). A total of 203 possible two-locus combinations were tested. Three linkage groups were revealed in this study at a linkLOD of 4.0. The first linkage group (A) consisted of *Pgi* and *Adh-1*; *Gdh, Idh, Skdh-2, G6pd-2* and *Aco* were mapped to the second linkage group (B); the third group (C) had two loci: *Mdh-2* and *Mdh-3*. A moderate linkage between *Mnr-2* and *Dia-2* and weak linkages between *Mnr-1* and *Dia-1*, and *Got-2* and *6pgd-2* were also detected. The significance of these results in shortleaf pine is discussed and compared to linkage maps previously reported in other conifers including pines.

INTRODUCTION

Since their introduction into population genetics by Hunter and Markert in 1957, the use of isoenzymes as genetic markers in forest trees has led to the accumulation of information on levels of genetic variation in a wide variety of species, including conifers. In conifers, isozymes have been used extensively for estimating mating system parameters (Burczyk et al. 1996; Prat and Caquelard 1995; Innes and Ringius 1990; Neale and Adams 1985; Cheliak et al. 1985; Yazdani et al. 1985), phylogenetic studies (Goncharenko et al. 1995a; Conkle et al. 1988; Millar et al. 1988), pollen migration studies (Burczyk et al. 1996; Harju and Nikkanen 1996; Smith and Adams 1983), hybrid zone studies (Copes and Beckwith 1977), provenance research (Bergmann and Ruetz 1991; Falkenhagen 1985), biosystematics (Millar et al. 1988; Strauss et al. 1992), investigating genetic diversity among and within populations (El-Kassaby and Ritland 1996; Bergmann and Hattemer 1995; Goncharenko et al. 1995b, 1994, 1993; Roberds and Conkle 1984; Raja et al. 1997; Surles et al. 1989; O'Malley et al. 1979) and more recently to examine whether evolutionary processes formed the basis for quantitative genetic variation seen in morphological traits (Yang et al. 1996). Unlike morphological traits in conifers, isozymes exhibit simple Mendelian inheritance and codominant expression.

Rarely found in other diploid organisms, the haploid nature of the megagametophyte tissue in conifer seeds offers a great advantage in genetic segregation studies. Each megagametophyte represents a single meiotic event

in the parent plant, which enables the detection of heterozygous and homozygous parents at any given locus by analyzing a number of seeds from each parent. An observed variation is said to be genetic if the megagametophytes from a heterozygous tree show 1:1 segregation for the locus of interest. Similarly, the segregation of markers in megagametophytes from a heterozygous tree with a 1:1:1:1 ratio at two loci forms evidence for joint independent segregation, or an absence of linkage (Bartels 1971).

When genes on the same chromosome fail to segregate independently they are said to be linked, which is a well-known genetic phenomenon (Boyle and Morgenstern 1985). Therefore, the establishment of inheritance of isozymes and linkage relationship among isozyme loci is crucial to the utilization of enzyme systems (Rudin 1976). Information derived from linkages of marker loci with quantitative characters can be a powerful tool in tree breeding (Tauer et al. 1992; Newton et al. 1991; King et al. 1990; Beckmann and Soller 1983). Interpretation of multi-locus population data also requires information about linkage (Epperson and Allard 1987).

Shortleaf pine (*Pinus echinata* Mill.), an important tree species of the southeast United States, has the broadest geographic range of the southern pines, ranging from New York to Texas and from southern Ohio to northern Florida. One of the four major southern pines, shortleaf pine makes up more than 22 percent of the standing volume of all southern pines and is widely used for construction lumber, plywood, pulp and paper. Several studies have been reported on linkage analysis of isozymes in pines (Strauss and Conkle 1986;

Guries et al. 1978; Szmidt and Muona 1989; Adams and Joly 1980; Conkle 1981; Eckert et al. 1981), but none so far in shortleaf pine. In this paper, we report on segregation and linkage of 28 isoenzyme loci in 10 natural stands representing a broad sample across the natural range of shortleaf pine in the United States.

MATERIALS AND METHODS

Plant material, electrophoresis and enzyme detection procedures

As part of a population genetics study of shortleaf pine (Raja et al. 1997) wind-pollinated seeds from 126 trees belonging to fifteen populations were collected in the Fall of 1993 by Dr. Ron Schmidtling of the USDA Forest Service Southern Research Station, Gulfport, MI from their shortleaf pine southwide seed source study plantations (Wells 1973). From the large number of single-tree collections of shortleaf pine, 13 trees which were found to be heterozygous at six or more isozyme loci were selected for the linkage analysis. Ten of the above mentioned populations were represented. Selection of the most heterozygous individuals from a much larger population helps in maximizing the number of pairwise comparisons possible, and enables adequate interconnectedness or 'bridges' between the selected trees for heterozygous loci, which is necessary for building an elaborate and reliable linkage analysis incorporating comparisons between pairs of loci heterozygous among trees (Stam 1993). Seed source codes, their geographic locations, seed extraction and storage procedures, sample preparation, starch gel electrophoresis and isoenzyme detection procedures followed protocols described by Raja et al. (1997). The 10 populations represented in this study were western South Carolina (C457), northern Georgia, Putnam county (C463), southwestern Georgia (C465), east central Alabama (C467), eastern Texas (C475), southeastern Oklahoma, Pushmataha county (C477A), southeastern Oklahoma, McCurtain county (C477B), southeastern Arkansas (C481), northern Arkansas

(C483) and south central Missouri (C485). Thirty-four loci belonging to 20 enzyme systems were resolved and consistently scorable in this study. The enzyme systems were aconitase (*Aco*, EC 4.2.1.3, 1 locus), acid phosphatase (*Acp*, EC 3.1.3.2, 2 loci), adenylate kinase (*Adk*, EC 2.7.4.3, 2 loci), alcohol dehydrogenase (*Adh*, EC 1.1.1.1, 2 loci), aldolase (*Ald*, EC 4.1.2.13, 2 loci), diaphorase (*Dia*, EC 1.6.4.3, 2 loci), glutamic dehydrogenase (*Gdh*, EC 1.4.1.3, 1 locus), glutamate-oxaloacetate transaminase (*Got*, EC 2.6.1.1, 2 loci), glucose-6-phosphate dehydrogenase (*G6pd*, EC 1.1.1.49, 2 loci), glycerate-2-dehydrogenase (*G2d*, EC 1.1.1.29, 1 locus), isocitric dehydrogenase (*Idh*, EC 1.1.1.42, 1 locus), malic dehydrogenase (*Mdh*, EC 1.1.1.37, 4 loci), malic enzyme (*Me*, EC 1.1.1.40, 1 locus), menadione reductase (*Mnr*, EC 1.6.99.2, 2 loci), phosphoglucose isomerase (*Pgi*, EC 5.3.1.9, 1 locus), phosphoglucomutase (*Pgm*, EC 2.7.5.1, 1 locus), 6-phosphogluconate dehydrogenase (*6Pgd*, EC 1.1.1.44, 2 loci), sorbitol dehydrogenase (*Sdh*, EC 1.1.1.14, 1 locus), shikimate dehydrogenase (*Skdh*, EC 1.1.1.25, 2 loci) and uridine diphosphoglucose pyrophosphorylase (*Ugpp*, EC 2.7.7.9, 2 loci). Sixty megagametophytes were analyzed per parent to ensure reliable results, since it has been reported that satisfactory estimates of inheritance and linkage can be made from twelve or more megagametophytes (Chaisurisri and El-Kassaby 1993; O'Malley et al. 1979; Boyle and Morgenstern 1985). Data shown in this study represent the parent trees, since only the megagametophytes were used.

Linkage Analysis

Segregation of all the loci scored was tested for goodness-of-fit to the expected 1:1 Mendelian segregation ratio using a Chi Square (χ^2) goodness-of-fit test. Analysis of linkage between loci that passed the χ^2 test was performed using JoinMap version 1.4 (Stam 1993). This program analyzes segregation data from multiple parents of unknown pedigree and combines them into one map. Segregation data of the sixty megagametophytes analyzed, at each heterozygous locus for each parent, was entered into the computer as backcross data (H × A). A minimum LOD (logarithm of the odds) score for linkage (linkLOD) of 4.0 was used to identify linkage groups using two-point linkage analysis. The most likely order of loci within groups was determined using multi-point analysis with a minimum linkLOD of 4.0 and a minimum LOD for mapping (mapLOD) score of 0.5. Internal consistency in the data set was tested by constructing another map with a linkLOD of 4.0 and mapLOD of 1.0 and comparing it with the earlier map for significant differences in the ordering of markers. The Kosambi (1944) mapping function was used to determine the centiMorgan (cM) distance between markers.

RESULTS

Segregation analysis

The 23 enzyme systems tested revealed 34 isozyme loci of which 28 segregated in at least one of the 13 shortleaf pine trees studied. *Mnr-2*, *Got-1*, *Mdh-1*, *Mdh-2*, *Mdh-3*, *Skdh-1*, *Dia-1* and *Dia-2* segregated in one parent; *Pgm* and *Mdh-4* segregated in two parents and the remaining 18 in three or more parents (Table 1). All the loci with the exception of *Ugpp-1* exhibited a 1:1 Mendelian segregation ratio at $\alpha = 0.05$ ($P = 0.0068$) using χ^2 analysis (Table 1). The distribution of heterozygous loci across the 13 parent trees is also presented in Table 1 to demonstrate the interconnectedness for heterozygous loci among the trees analyzed. All the trees analyzed were adequately interconnected to enable a reliable linkage analysis.

Linkage analysis

Two-point linkage analysis of all testable two-locus combinations (203 combinations) revealed three linkage groups involving nine loci and 18 unlinked loci at a linkLOD of 4.0. *Ugpp-1* was excluded from analysis since it failed the χ^2 test for 1:1 segregation. The eighteen unlinked loci were *Mnr-1*, *Mnr-2*, *Got-1*, *Got-2*, *Ald-2*, *G2d*, *Mdh-1*, *Mdh-4*, *Me*, *Ugpp-2*, *6pgd-1*, *6pgd-2*, *Acp-1*, *Acp-2*, *Skdh-1*, *Pgm*, *Dia-1* and *Dia-2*. Recombination fractions (R = recombination percentage $\div$ 100), the associated standard errors (S_R) and actual LOD scores

Table 1. Distribution of heterozygous isozyme loci (x) in the 13 shortleaf pine trees studied with segregation chi-square test for 1:1 ratio.

Isozyme	Parent Trees													No. of trees	Allele	Allele	Segregation Chi-square	*P*
Locus	1	2	3	4	5	6	7	8	9	10	11	12	13	segregating	A	B	for 1:1 ratio	
6pgd-1					x	x	x	x	x	x		x	x	8	194	205	0.3033	0.5819
6pgd-2		x	x			x		x	x		x		x	7	218	185	2.7022	0.1002
Aco				x			x	x			x			4	79	82	0.0559	0.8131
Acp-1			x							x		x	x	4	111	109	0.0182	0.8927
Acp-2			x	x		x					x			4	113	110	0.0404	0.8408
Adh-1	x	x		x						x			x	5	138	132	0.1333	0.7150
Ald-2	x				x	x								3	74	89	1.3804	0.2400
Dia-1												x		1	25	35	1.6667	0.1967
Dia-2							x							1	27	20	1.0426	0.3072
G2d		x	x		x		x	x	x				x	7	190	202	0.3673	0.5445
G6pd-2				x			x				x			3	64	66	0.0308	0.8608
Gdh				x			x				x			3	76	91	1.3473	0.2458
Got-1	x													1	22	36	3.3793	0.0660
Got-2	x		x	x	x	x	x	x	x	x	x	x		11	302	335	1.7096	0.1910
Idh				x			x	x			x			4	108	115	0.2197	0.6392
Mdh-1											x			1	31	29	0.0667	0.7962
Mdh-2				x										1	23	35	2.4828	0.1151
Mdh-3				x										1	24	34	1.7241	0.1892
Mdh-4		x			x									2	58	62	0.1333	0.7150
Me		x	x				x							3	79	87	0.3855	0.5347
Mnr-1	x	x	x		x				x	x		x	x	8	238	234	0.0339	0.8539
Mnr-2							x							1	19	15	0.4706	0.4927
Pgi	x		x	x	x	x			x	x				7	189	211	1.2100	0.2710
Pgm						x						x		2	49	54	0.2427	0.6222
Skdh-1					x									1	24	36	2.4000	0.1213
Skdh-2				x			x	x			x			4	103	114	0.5576	0.4552
Ugpp-1						x				x		x		3	62	96	7.3165	0.0068*
Ugpp-2		x	x			x	x	x	x	x	x	x		9	261	257	0.0309	0.8605
No. of loc	6	7	9	11	8	9	12	8	7	8	10	8	6					

* - significant at alpha = 0.05.

of loci combinations which cosegregated at a LOD score above 1.0 are presented in Table 2, because linkages above a LOD score above 1.0 may be of interest to anyone attempting to study the linkage of isozyme loci in conifers. Three linkage groups were revealed in this study at a linkLOD of 4.0 (Figure 1). Comparing maps constructed at mapLODs of 0.5 and 1.0 revealed no differences in linkage groups or the ordering of markers.

Linkage Group A

The linkage group A includes *Pgi* and *Adh-1* (Figure 1). A linkage between *Pgi* and *Adh* was also reported in ponderosa pine (*Pinus ponderosa*) by O'Malley et al. (1979) and Scots pine (*Pinus sylvestris*) by Szmidt and Muona (1989).

Linkage Group B

This study mapped *Gdh, Idh, Skdh-2, G6pd-2* and *Aco* to linkage group B (Figure 1). All possible two-point analyses between these loci showed strong evidence of linkage (Table 2). Strong linkage between *G6pd-2* and *Aco* was reported in Scots pine by Szmidt and Muona (1989). A linkage group with *Gdh, Idh, G6pd* and *6pgd* was reported by Altukhov et al. (1986) in Norway spruce (*Picea abies* (L.) Karst.). A moderate linkage between *Gdh* and *G6pd* was reported in Polish larch (*Larix decidua* subsp. *polonica* (Racib.) Domin.) by Lewandowski and Mejnartowicz (1991). A strong linkage between *Gdh* and *Idh* loci was reported in black spruce (*Picea mariana*) by Boyle and

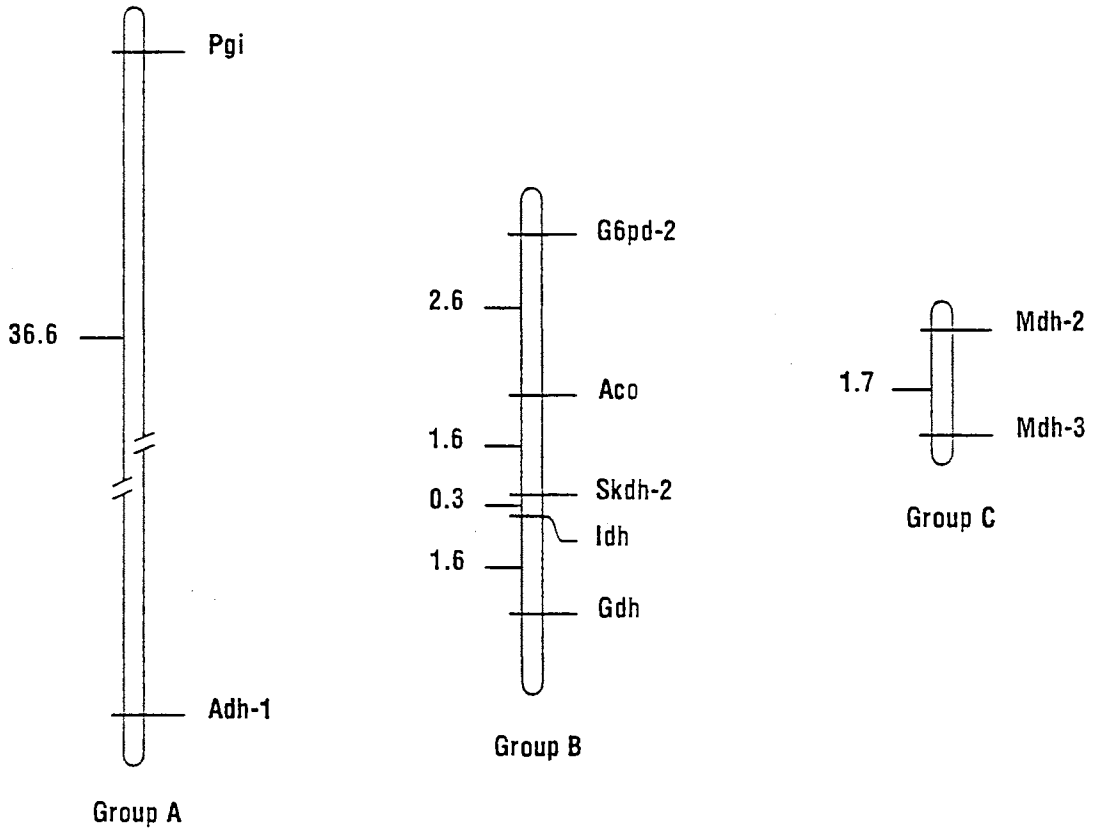

Figure 1. Map of linked loci in shortleaf pine. Kosambi map distances in centiMorgans are shown between loci.

Table 2. The recombination fraction (R), its standard error (S_R) and LOD scores for combinations of isoenzyme loci that exhibit a significant cosegregation at a LOD score > 1.0, in 13 shortleaf pine trees studied.

Combination	Recombination		LOD	Combination	Recombination		LOD
Locus 1 - Locus 2	R	S_R	Value	Locus 1 - Locus 2	R	S_R	Value
Mnr-1 - Ald-2	0.381	0.046	1.3472	*G2d - Skdh-2*	0.379	0.052	1.1117
Mnr-1 - Ugpp-2	0.434	0.028	1.1078	*G2d - Aco*	0.351	0.050	1.7660
Mnr-1 - Dia-1	0.266	0.057	2.9506	*G2d - Mnr-2*	0.312	0.081	1.0015
Got-2 - Pgi	0.435	0.024	1.4721	*Mdh-4 - Me*	0.300	0.059	2.1441
Got-2 - Ugpp-2	0.429	0.024	1.7167	*Acp-2 - Pgm*	0.279	0.068	1.8875
Got-2 - 6pgd-2	0.395	0.029	2.6907	*Gdh - Idh*	0.024	0.012	40.9117*
Got-2 - Acp-1	0.400	0.037	1.4866	*Gdh - Skdh-2*	0.018	0.010	41.9860*
Pgi - Adh-1	0.312	0.037	4.9348*	*Gdh - G6pd-2*	0.053	0.019	27.2953*
Pgi - Mdh-2	0.333	0.062	1.4019	*Gdh - Aco*	0.029	0.016	24.5391*
Pgi - Mdh-3	0.350	0.063	1.1179	*Mdh-2 - Mdh-3*	0.017	0.017	15.2658*
Ald-2 - Mdh-4	0.350	0.061	1.1908	*Idh - Skdh-2*	0.004	0.004	61.9557*
Adh-1 - Acp-1	0.370	0.048	1.4849	*Idh - G6pd-2*	0.046	0.018	28.0138*
Adh-1 - Acp-2	0.345	0.064	1.1599	*Idh - Aco*	0.025	0.012	40.0413*
G2d - Ugpp-2	0.405	0.029	2.0966	*Skdh-2 - G6pd-2*	0.038	0.016	29.9299*
G2d - 6pgd-2	0.436	0.029	1.0315	*Skdh-2 - Aco*	0.019	0.010	40.8150*
G2d - Acp-1	0.394	0.046	1.0618	*G6pd-2 - Aco*	0.042	0.024	15.6936*
G2d - Idh	0.372	0.049	1.3455	*Mnr-2 - Dia-2*	0.176	0.065	3.3541

* - Pairs of loci with significant cosegregation at a LOD score of 4.0

Morgenstern (1985) and in Chinese fir (*Cunninghamia lanceolata* Hook.) by Geburek and Wang (1990). A moderate linkage between *G6pd* and *Idh* was reported in pitch pine (*Pinus rigida* Mill.) by O'Malley et al. (1986).

Linkage Group C

The third linkage group (C) included 2 loci: *Mdh-2* and *Mdh-3* (Figure 1). To the best of our knowledge this linkage has been previously reported only once, in Norway spruce (Poulsen et al. 1983). As evident from Table 1, only individual 4 provided the information for testing linkage between these two loci. The analysis suggested a tight linkage between *Mdh-2* and *Mdh-3*.

Other linkages

A moderate linkage was detected between *Mnr-2* and *Dia-2* (R=0.176, LOD=3.35) and a weak linkage between *Mnr-1* and *Dia-1* (R=0.266, LOD=2.95) and *Got-2* and *6pgd-2* (R=0.395, LOD=2.69). A linkage between *Got-2* and *6pgd* was reported in balsam fir (*Abies balsamea*) by Neale and Adams (1981)) and in Chinese fir by Geburek and Wang (1990).

DISCUSSION

From Table 1 it is evident that there is a strong relationship between the thirteen trees studied, allowing reliable estimates of linkage between loci that were found to be heterozygous in different individuals. All loci showed 1:1 Mendelian segregation with the exception of *Ugpp-1*. Segregation distortion of alleles in *Ugpp-1* may be due to several reasons, as discussed by previous linkage studies. Differential viability of gametes carrying different isozymes (Adams and Joly 1980; Rudin and Ekberg 1978), linkage to lethals (Sorensen 1967) or simply sampling or scoring errors could result in non-random segregation of alleles.

We found three different linkage groups among the 28 loci studied. Maps constructed at mapLODs of 0.5 and 1.0 agreed completely with each other regarding linked markers and their ordering, which suggested internal consistency in the data. In general, our results agree with studies reported in other conifers like ponderosa pine (O'Malley et al. 1979), balsam fir (Neale and Adams 1981), Polish larch (Lewandowski and Mejnartowicz 1991), black spruce (Boyle and Morgenstern 1985), Norway spruce (Poulsen et al. 1983; Altukhov et al. 1986), pitch pine (O'Malley et al. 1986) and Scots pine (Szmidt and Muona 1989). Linkage between *Pgi* and *Adh* loci was reported in ponderosa pine (O'Malley et al. 1979) and Scots pine (Szmidt and Muona 1989) and we confirm it as a strong linkage in shortleaf pine.

Even though the linkage group involving *Gdh, Idh, G6pd* and *Aco* loci is well known in pines and has been reported in various combinations and with varying degrees of linkage in Scots pine (Szmidt and Muona 1989), Norway spruce (Altukhov et al. 1986), Polish larch (Lewandowski and Mejnartowicz 1991), black spruce (Boyle and Morgenstern 1985), Chinese fir (Geburek and Wang 1990) and pitch pine (O'Malley et al. 1986), we believe that the strong linkage we detected between the *Gdh, Idh, Skdh-2, G6pd-2* and *Aco* loci in shortleaf pine is of special significance in studying the mechanism of hybridization and introgression between shortleaf pine and loblolly pine. Huneycutt and Askew (1989) reported that the *Idh* locus was monomorphic for shortleaf pine (average mobility 17mm from origin) and loblolly pine (average mobility 22mm from origin), and segregates in a 1:1 ratio (polymorphic) for their F1 hybrids. They concluded that the electrophoretic separation of *Idh* isoenzymes is a highly accurate technique for identifying F1 hybrids. However, they also recognized the fact that identification of hybrid generations beyond F1 using *Idh* may not be reliable, because Mendelian segregation would allow only one half of the progeny to be heterozygous at the *Idh* locus when a natural backcross or a cross between two F1's takes place. Also, a recent study of genetic variation in shortleaf pine (Raja et al. 1997) showed a very high percentage (16.7%) of trees sampled across the United States were polymorphic at the *Idh* locus, leading us to rethink the adequacy of using the *Idh* locus alone, in identifying hybrids. The tight linkage of the *Idh* locus with *Gdh, Skdh-2, G6pd-2* and *Aco* loci in shortleaf pine that we identified through this study would

enable the potential use of these loci as additional markers in identifying hybrids more reliably, as well as possibly allowing us to trace them beyond the F1 generation. The presence of *Skdh-2* in linkage group B has not been previously reported in any other conifer.

Our study suggests a tight linkage between *Mdh-2* and *Mdh-3*. However, since the linkage has been reported only once before (Poulsen et al. 1983) and our result is based on the data from one shortleaf pine individual, we advise caution. Additional results to support our findings may be helpful in confirming this linkage in shortleaf pine. The moderate linkage detected between *Mnr-2* and *Dia-2* and the weak linkage between *Mnr-1* and *Dia-1* and between *Got-2* and *6pgd-2* are positive indications but also require additional investigation and confirmation. Since *Got-2* and *6pgd* linkage was previously reported in other conifers (Neale and Adams 1981; Geburek and Wang 1990), it may be a credible linkage.

Our study finds linkages between isoenzyme loci, many of which were previously reported in other conifers even though their combinations and degrees of linkage varied. This may be an indication that not only are important genes with vital functions conserved across species, but that their linkages are conserved as well. Exploring the significance of chromosomal conservatism in conifers, i.e. the significance of why several "house-keeping" genes are on one chromosome, may provide valuable insights into the mechanisms of natural selection, evolution and speciation.

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CHAPTER III

REGENERATION METHODS AFFECT GENETIC VARIATION AND STRUCTURE IN SHORTLEAF PINE (*Pinus echinata* Mill.)

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ABSTRACT

The effects of regeneration methods on genetic diversity and structure in shortleaf pine (*Pinus echinata* Mill.) were examined by quantifying the changes in genetic composition of shortleaf pine stands following harvest by monitoring changes in allele number and frequency at heterozygous loci over time. The results were also compared to the genetic composition of seed used for artificial regeneration following clear-cutting. Both natural regeneration treatments resulted in higher genetic variation in post-treatment seed, indicating a richer pollen cloud after management. Artificial regeneration showed fewer alleles per locus and fewer polymorphic loci compared to both natural regeneration treatments. Frequency of alternate alleles increased at 13 loci in the seed-tree stand after treatment, which is an indication of less inbreeding or consanguineous mating. Single tree selection resulted in an increase in alternate allele frequencies at 10 loci and at 4 loci alternate allele frequencies decreased, indicating that the treatment may result in more inbreeding than seed tree. Artificial regeneration showed a considerable increase in alternate allele frequencies at 17 loci and hence can be considered outbred. The above mentioned observations were confirmed by comparing H_o, H_e and F values for the two stands before and after treatment. The seed tree method resulted in a decrease in inbreeding, whereas the first selection cut for single tree selection did not alter it. Artificial regeneration showed a negative F value indicative of high levels of heterozygosity and outbreeding. The natural regeneration

treatments did not result in genetic drift whereas the artificial regeneration showed considerable change in the genetic composition of the potential regeneration.

INTRODUCTION

Increased public desire to maintain genetic diversity in forests has resulted in a growing concern over the influence of forest management practices on genetic variation in forests. Continuing demand for forest products, the increasing demand for use of forested areas for non-traditional purposes, and the general public desire to maintain landscape diversity, biodiversity, conserve wildlife, protect old growth forests, control ecodegradation and global climate change has put a complex array of often conflicting demands, priorities and conditions on forest managers. The choice of suitable management strategies applicable to the climatic, political and public situation of the forests under their care has become exceedingly difficult. Therefore, an evaluation of within-species genetic diversity of existing stands compared to that of stands regenerated by various management schemes would help in understanding man's effect on these stands, and may suggest suitable management strategies.

The trend on federal lands has been to move from artificial regeneration methods like clear-cutting to natural regeneration systems such as seed-tree and single tree selection methods. The genetic consequences of various natural and artificial regeneration methods have been hypothesized (Daniel *et al.* 1979) but there have been very few efforts to quantify changes in genetic variation at a molecular level.

Neale and Adams (1985) studied the mating system of an uncut and a shelterwood stand of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) in

Oregon. Their results suggested related matings other than selfs probably are occurring in uncut stands but not in shelterwoods. However, since they were not able to detect significant differences, they proposed an expanded study of this nature with lower residual stand densities.

Two studies in Europe evaluating genetic changes during different life stages in Scots pine (*Pinus sylvestris* L.) found that inbreeding was reduced from 12% in embryos to less than 1% in 3-year old natural regeneration established from those seeds (Muona *et al.* 1987) and that excess homozygosity found in embryos disappeared from the surviving regeneration by age 10-20 (Yazdani *et al.* 1985), both indicating that elimination of inbred individuals occurs during stand establishment and early competition.

A study of change in population structure in a parent and adjacent progeny stand of loblolly pine (*Pinus taeda* L.) (Roberds and Conkle 1984) found that although allele frequencies did not differ between the parent and progeny stands, genetic population structure was not the same, demonstrating that local genetic structure can differ between successive generations in a stand. Such a change may occur due to various regeneration methods and is the kind of knowledge required to address the question of management effects on genetic diversity.

A study comparing genetic variation and heterozygosity in seed orchards and natural stands of Norway spruce (*Picea abies* (L.) Karst.) reported no change in gene frequency distributions, percent polymorphic loci (p) and mean number of alleles per locus (A), but found higher levels of heterozygosity in

seed orchard trees compared to natural stands (Bergmann and Ruetz 1991). However, a similar study examining the progenies from seed orchard and natural stands in Sitka spruce (*Picea sitchensis* (Bong.) Carr.) reported significantly higher values for *p* and *A* for seeds from seed orchards, but the mean heterozygosities were not significantly different (Chaisurisri and El-Kassaby 1994).

These studies suggest certain trends regarding the genetic consequences of management but do not give a clear picture since methods and results were variable. In this study, we report the changes in genetic variation and structure at 31 isoenzyme loci in two shortleaf pine stands managed by the seed-tree and single tree selection systems. The results are also compared to the potential genetic variation that would be found in these stands had they been artificially regenerated using bulked seed from two different seed orchards. We chose the seed-tree and single tree selection systems for our study since they are in use and represent the two extremes of selection pressure, seed-tree being most intense with residual pine basal area of 3.7m^2 per hectare, and single tree selection being the least with residual pine basal area of 14.2m^2 per hectare (Wittwer *et al.* 1997).

MATERIALS AND METHODS

Plant material, electrophoresis and enzyme detection procedures

Seeds from 48 trees in each of two 15-hectare shortleaf pine stands in the Ouachita Mountains of Montgomery County, Arkansas were collected in the fall of 1993. Approximately 70% of the basal area in these stands was shortleaf pine and 30% deciduous species, predominantly *Quercus* and *Carya* species (Wittwer *et al.* 1997). The average age of dominant shortleaf pine trees in both stands was 64 years. Each stand was subdivided into quarters of approximately equal area arranged perpendicular to the elevation gradient and each quarter further subdivided into thirds along the elevation gradient, as part of a large ecosystem management research study on the Ouachita and Ozark National Forests in west-central Arkansas and eastern Oklahoma (Guldin *et al.* 1993). A plot center was marked in each of the 12 subdivisions and 4 healthy trees of at least 20 cm diameter at breast height with abundant cones were selected in each subdivision for this study. Seed-tree and single tree selection harvest / regeneration systems were applied to the two stands, respectively, about three months before the first seed collection. Two years later the seed crop representing genetic variation after management was imposed, was collected.

The seed samples were assayed to detect changes in genetic variation due to management. Twenty-five seeds from each of the 48 trees from each stand for both pre- and post-treatment were assayed for 34 isoenzyme loci that were found polymorphic through an earlier study (Raja *et al.* 1997). Fifty seeds each

from the bulked seeds of the Ouachita and Ozark seed orchards were also analyzed to represent artificial regeneration for these stands, had they been clear-cut and planted with seedlings from those seed orchard seeds. Seed extraction and storage procedures, sample preparation, starch gel electrophoresis, enzymes staining and isoenzyme detection procedures followed protocols described by Raja *et al.* (1997). Thirty-four loci belonging to 20 enzyme systems were assayed, from which 31 loci were resolved and consistently scorable in this study. The enzyme systems were aconitase (*Aco*, EC 4.2.1.3, 1 locus), acid phosphatase (*Acp*, EC 3.1.3.2, 2 loci), adenylate kinase (*Adk*, EC 2.7.4.3, 2 loci), alcohol dehydrogenase (*Adh*, EC 1.1.1.1, 1 locus), aldolase (*Ald*, EC 4.1.2.13, 2 loci), diaphorase (*Dia*, EC 1.6.4.3, 1 locus), glutamic dehydrogenase (*Gdh*, EC 1.4.1.3, 1 locus), glutamate-oxaloacetate transaminase (*Got*, EC 2.6.1.1, 2 loci), glucose-6-phosphate dehydrogenase (*G6pd*, EC 1.1.1.49, 2 loci), glycerate-2-dehydrogenase (*G2d*, EC 1.1.1.29, 1 locus), isocitric dehydrogenase (*Idh*, EC 1.1.1.42, 1 locus), malic dehydrogenase (*Mdh*, EC 1.1.1.37, 4 loci), malic enzyme (*Me*, EC 1.1.1.40, 1 locus), menadione reductase (*Mnr*, EC 1.6.99.2, 2 loci), phosphoglucose isomerase (*Pgi*, EC 5.3.1.9, 1 locus), phosphoglucomutase (*Pgm*, EC 2.7.5.1, 1 locus), 6-phosphogluconate dehydrogenase (*6Pgd*, EC 1.1.1.44, 2 loci), sorbitol dehydrogenase (*Sdh*, EC 1.1.1.14, 1 locus), shikimate dehydrogenase (*Skdh*, EC 1.1.1.25, 2 loci) and uridine diphosphoglucose pyrophosphorylase (*Ugpp*, EC 2.7.7.9, 1 locus).

Data Analysis

Megagametophytes and embryos from each seed were scored for each locus. Identification of pollen genotype was accomplished by comparing megagametophyte and embryo data. Haploid pollen allele frequencies and diploid embryo genotypic frequencies were then calculated. Pollen allele frequencies for pre-treatment and post-treatment, and pre-treatment and artificial regeneration were compared with χ^2 tests (Snedecor and Cochran 1967, p. 250). When expected values were too small for χ^2 tests, Fisher's exact test was used (Sokal and Rohlf 1981, p. 740). Genetic diversity was estimated by percent polymorphic loci '*p*', mean number of alleles per locus '*A*' and mean number of alleles per polymorphic locus 'A_p'. Diploid embryo data from each stand were pooled for pre-treatment, post-treatment, and artificial regeneration to calculate the observed (H_o) and expected (H_e) heterozygosities, and the fixation index using the formula :

[1] $$F = 1 - H_o / H_e$$

Levels of genetic differentiation between stands were estimated using Wright's F statistics, F_{ST} (Wright 1965; 1969; 1978; Nei 1977). Haploid pollen allele frequencies, diploid embryo genotypic frequencies, *p*, *A* and A_p were calculated using the FREQ procedure of SAS computer program, and H_o, H_e and

F_{ST} were calculated using the BIOSYS-1 computer program (Swofford and Selander 1981).

RESULTS AND DISCUSSION

Genetic Diversity

The 20 enzyme systems assayed identified 75 electrophoretic variants at 31 loci. Twenty nine of the 31 loci assayed exhibited polymorphism in at least one stand. Seven of the 75 electrophoretic variants found were seen only in one stand, 4 in the seed-tree stand (at loci *Adh*, *Adk-2*, *Ald-2* and *G6pd-1*) and 3 in the single tree selection stand (at loci *Acp-1*, *Mdh-3* and *Sdh*).

Table 1 presents the haploid pollen allele frequencies by locus with χ^2 and probability values for testing allele frequency differences between pre- and post-treatment for both the seed-tree and single tree selection method, as well as for the two pre-treatment stands with artificial regeneration. The seed-tree method resulted in a gain of 8 alleles at 7 loci (*Adh*, *Adk-2*, *G6pd-1*, *G6pd-2*, *Got-1*, *6Pgd-2* and *Pgi*) and a loss at one (*Mdh-4*) following treatment. The single-tree selection method resulted in a gain of 9 alleles at 7 loci (*Acp-1*, *Adh*, *Got-1*, *Got-2, Mdh-1, Mdh-4* and *Me-1*) and a loss at one (*Sdh*). Artificial regeneration resulted in a gain of 6 alleles at 6 loci (*Acp-1, Adh, Got-1, Got-2, Mdh-1* and *Me-1*) and a loss of 12 alleles at 11 loci (*Acp-2, Ald-2, G6pd-1, G6pd-2, Mdh-1, Mdh-3, Mdh-4, Me-1, Pgd-2, Pgi* and *Sdh*). It is important to note that all results reported here are based on the seed available for regeneration of these stands, and may not necessarily represent the genetics of advanced regeneration.

The seed-tree and the single tree selection system resulted a similar increase in *P* (about 8%), *A* (about 12%) and A_p (about 8%) following treatment

Table 1. Pollen allele frequencies (F) by locus for seed-tree, single tree selection, and artificial regeneration. Chi-square (Chi^2) values and significance levels (P) for testing Pre- (PR), post-treatment (PS) and artificial regeneration allele frequency differences are also presented.

Locus	Allele	Treatment												
		Seed-tree				Single Tree Selection				Artificial Regeneration				
		F(PR)	F(PS)	Chi^2	P	F(PR)	F(PS)	Chi^2	P	F	Chi^2	$P(1)^a$	Chi^2	$P(2)^b$
Aco	A	0.073	0.110	8.29	<0.01	0.099	0.093	0.21	0.65	0.130	4.15	0.04	0.92	0.34
	B	0.927	0.890			0.901	0.907			0.870				
Acp-1	A	0.005	0.016			-	0.006			0.060				
	B	0.995	0.984	6.59	<0.01	1.000	0.980	16.08	<0.01	0.940	28.9	<0.01	50.0	<0.01
	C	-	-			-	0.014			-				
Acp-2	A	0.003	0.013			0.024	0.048			-				
	B	0.994	0.964	21.27	<0.01	0.948	0.944	16.07	<0.01	0.810	159.9	<0.01	56.12	<0.01
	C	0.003	0.023			0.028	0.008			0.190				
Adh	A	-	0.012			-	0.001			0.010				
	B	1.000	0.987	13.92	<0.01	1.000	0.999	0.98	0.32	0.990	10.36	<0.01	8.62	<0.01
	C	-	0.001			-	-			-				
Adk-1	B	1.000	1.000	-	-	1.000	1.000	-	-	1.000	-	-	-	-
Adk-2	A	-	0.003	2.19	0.14	-	-	-	-	-	-	-	-	-
	B	1.000	0.997			1.000	1.000			1.000				
Ald-1	B	1.000	1.000	-	-	1.000	1.000	-	-	1.000	-	-	-	-
Ald-2	A	0.001	0.002	0.33	0.57	-	-	-	-	-	0.10	0.76	-	-
	B	0.999	0.998			1.000	1.000			1.000				
Dia	A	0.044	0.138	50.30	<0.01	0.021	0.107	49.43	<0.01	0.200	39.97	<0.01	72.37	<0.01
	B	0.956	0.862			0.979	0.893			0.800				
G2d	A	0.489	0.488	0.002	0.96	0.440	0.440	0.00	1.00	0.510	0.16	0.69	1.77	0.18
	B	0.511	0.512			0.560	0.560			0.490				
G6pd-1	A	-	0.007			0.004	0.015			-				
	B	0.993	0.986	7.01	0.03	0.996	0.985	5.95	0.02	1.000	0.70	0.40	0.36	0.54
	C	0.007	0.007			-	-			-				
G6pd-2	A	-	0.006	6.15	0.01	0.006	0.002	1.41	0.24	-	-	-	0.61	0.44
	B	1.000	0.994			0.994	0.998			1.000				
Gdh	A	0.072	0.071	0.02	0.90	0.086	0.082	0.11	0.74	0.080	0.09	0.77	0.04	0.84
	B	0.928	0.929			0.914	0.918			0.920				
Got-1	A	-	0.019			-	0.008			0.060				
	B	0.998	0.871	133.1	<0.01	0.986	0.910	51.71	<0.01	0.840	143.8	<0.01	82.62	<0.01
	C	0.002	0.110			0.014	0.082			0.100				
Got-2	A	0.002	0.043			-	0.042			0.220				
	B	0.794	0.665	62.35	<0.01	0.775	0.627	60.76	<0.01	0.540	196.3	<0.01	177.8	<0.01
	C	0.204	0.292			0.225	0.331			0.240				

Idh	A	0.056	0.058	0.064	0.80	0.041	0.058	2.92	0.09	0.040	0.44	0.51	0.001	0.98
	B	0.944	0.942			0.959	0.942			0.960				
	A	0.994	0.995			1.000	0.997			0.960				
Mdh-1	B	0.001	0.003	2.28	0.32	-	0.002	2.85	0.24	0.040	32.92	<0.01	34.58	<0.01
	C	0.005	0.002			-	0.001			-				
	A	0.009	0.027			0.022	0.012			0.010				
Mdh-2	B	0.987	0.956	16.32	<0.01	0.974	0.974	7.67	0.02	0.960	9.22	0.01	10.00	<0.01
	C	0.004	0.017			0.004	0.014			0.030				
	A	0.995	0.971			0.963	0.983			0.980				
Mdh-3	B	0.003	0.024	16.38	<0.01	0.034	0.012	9.54	<0.01	0.020	5.54	0.06	0.83	0.66
	C	0.002	0.005			0.003	0.005			-				
	A	-	-			0.004	0.001			-				
Mdh-4	B	0.999	1.000	0.99	0.32	0.996	0.998	2.06	0.36	1.000	0.10	0.76	0.35	0.55
	C	0.001	-			-	0.001			-				
	A	0.002	0.006			0.042	0.036			-				
Me-1	B	0.996	0.986	5.92	0.05	0.958	0.945	15.61	<0.01	0.960	25.31	<0.01	36.63	<0.01
	C	0.002	0.008			-	0.019			0.040				
	A	0.030	0.048			0.032	0.059			0.173				
Mnr-1	B	0.952	0.856	65.84	<0.01	0.930	0.853	25.58	<0.01	0.707	70.29	<0.01	45.05	<0.01
	C	0.018	0.096			0.038	0.088			0.120				
Mnr-2	A	0.009	0.024	8.17	<0.01	0.004	0.040	26.95	<0.01	0.107	45.51	<0.01	63.24	<0.01
	B	0.991	0.976			0.996	0.960			0.893				
	A	0.125	0.177			0.101	0.128			0.150				
6pgd-1	B	0.857	0.740	62.48	<0.01	0.867	0.792	23.28	<0.01	0.780	12.17	<0.01	6.33	0.04
	C	0.018	0.083			0.032	0.080			0.070				
	A	-	0.001			0.049	0.044			-				
6pgd-2	B	0.926	0.875	15.52	<0.01	0.883	0.840	11.74	<0.01	0.810	15.74	<0.01	21.73	<0.01
	C	0.074	0.124			0.068	0.116			0.190				
	A	-	0.005			0.011	0.018			-				
Pgi	B	0.909	0.869	11.72	<0.01	0.908	0.894	1.93	0.38	0.790	14.04	<0.01	18.12	<0.01
	C	0.091	0.126			0.081	0.088			0.210				
Pgm	A	0.989	0.985	0.57	0.45	0.945	0.929	1.69	0.19	0.950	9.80	<0.01	0.05	0.82
	B	0.011	0.015			0.055	0.071			0.050				
Sdh	A	1.000	1.000	-	-	0.997	1.000	2.18	0.14	1.000	-	-	0.25	0.62
	B	-	-			0.003	-			-				
Skdh-1	A	0.059	0.065	0.30	0.58	0.095	0.094	0.002	0.96	0.110	3.96	0.05	0.22	0.64
	B	0.941	0.935			0.905	0.906			0.890				
Skdh-2	A	0.003	0.021	14.07	<0.01	0.026	0.003	15.72	<0.01	0.010	1.27	0.26	0.93	0.34
	B	0.997	0.979			0.974	0.997			0.990				
Ugpp-2	A	0.893	0.797	35.40	<0.01	0.854	0.739	32.14	<0.01	0.530	99.55	<0.01	62.21	<0.01
	B	0.107	0.203			0.146	0.261			0.470				

a : Chi^2 value and P for testing differences between seed-tree (PR) and artificial regeneration.
b : Chi^2 value and P for testing differences between single tree selection (PR) and artificial regeneration.

(Table 2). However, the absolute values of *P* and *A* were consistently lower for the single-tree selection system compared with the seed-tree system. It is to be noted that the lowest values for *P, A* and A_p were observed for the seeds representing artificial regeneration (Table 2).

By comparing the change in number of alleles, and the genetic diversity estimates *P, A* and A_p, it can be inferred that seed from the seed-tree and single tree selection systems resulted in a richer pollen cloud after treatment whereas seed orchard seed for artificial regeneration had a less diverse pollen cloud.

Genetic Structure

A significant increase in the frequency of alternate alleles following treatment was observed at 13 loci in the seed-tree stand, at 10 loci in the single tree selection stand, and at 17 loci in the seed representing artificial regeneration (Table 1). It is interesting to note that while the frequency of alternate alleles was not reduced at any loci in the seed-tree stand and the artificial regeneration, a significant reduction was observed at 4 loci in the single tree selection stand.

Observed (H_o) and expected (H_e) heterozygosities calculated from pooled diploid genotypic frequency data and the fixation index F for each stand are presented in Table 3. Observed heterozygosities were lower than the expected for seed-tree and single tree selection stands prior to treatment. Following treatment the seed-tree method resulted in a shift in the observed heterozygosity

Table 2: Percentage of polymorphic loci (P), Mean number of alleles per locus (A) and mean number of alleles per polymorphic locus (Ap) for all treatments

	Treatment				
	Seed-tree		Single tree selection		Artificial Regeneration
	Pre	Post	Pre	Post	
P	80.6	87.1	77.4	83.9	74.2
A	2.06	2.29	2.00	2.26	1.90
Ap	2.32	2.48	2.29	2.50	2.22

Table 3: Observed (H_o) and expected (H_e) heterozygosities, and inbreeding values (F) for each treatment.

	Treatment				
	Seed-tree		Single tree selection		Artificial Regeneration
	Pre	Post	Pre	Post	
H_o	0.124	0.101	0.099	0.117	0.197
H_e	0.136	0.104	0.110	0.130	0.177
F	0.088	0.029	0.100	0.100	-0.133

closer to the expected, whereas the single tree selection method did not result in a change. Consequently, a 3-fold reduction in F value was seen due to seed-tree method, while no change was detected in the single tree selection method. Artificial regeneration had considerably higher observed heterozygosity than expected, and consequently a negative F value.

A diagrammatic representation of genetic differentiation (F_{ST}) between stands is shown in Figure 1. The two stands prior to treatment had the same amount genetic differentiation as the two stands after treatment (F_{ST} = 0.007). When each stand was analyzed across time, the seed-tree stand had an F_{ST} of 0.005 and the single tree selection stand, 0.004, again no change or difference. Compared to the two pre-treatment stands, artificial regeneration had 4 to 7 fold higher F_{ST} values (0.025 with seed-tree and 0.036 with single tree selection). Artificial regeneration of these stands with seed orchard seed would result in a significant change in the genetic structure of the stands.

When comparing the change in frequency of alternate alleles, it is seen that the increase in frequency of alternate alleles was greatest for artificial regeneration and least for the single-tree selection. H_o, H_e and F values together with allele frequency comparisons confirm that artificial regeneration results in highly heterozygous, outbred regeneration. Regeneration from the seed-tree method showed a reduction in F value, indicative of less inbreeding or consanguineous mating. While the seed-tree method seems to reduce inbreeding, the single tree selection method seems to maintain the existing level

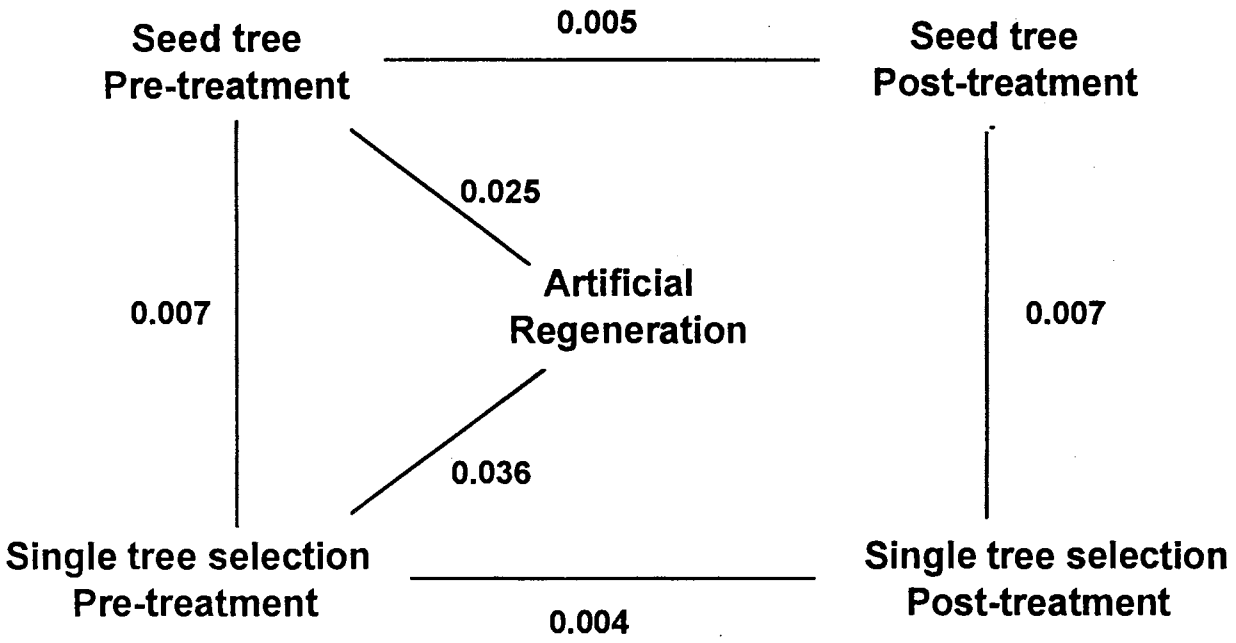

Figure 1. Diagrammatic representation of genetic differentiation (F_{ST}) between stands of shortleaf pine.

of inbreeding. This, of course, may change following additional selection cuts in the single tree selection stand. When the genetic difference between the two stands prior to treatment is considered as base line genetic difference (0.7%), it can be inferred that the two treatments do not introduce any genetic drift between stands or within stands across time. However, artificial regeneration introduces 2.5 to 3.6% genetic differentiation compared to the previously existing stand. This is a significant change when considering the fact that the total genetic differentiation in shortleaf pine across its natural range is only 9% or lower (Raja *at al.* 1997; Edwards and Hamrick, 1995).

CONCLUSION

Isoenzyme markers were powerful enough to detect changes in genetic diversity and structure in shortleaf pine due to management. The pollen cloud was enriched when the seed-tree and single tree selection regeneration methods were applied to the stands. The pollen diversity in the seed orchard was less than that achieved by the two natural regeneration treatments and less than that of the stands prior to treatment. The seed-tree method increased the frequency of heterozygotes, thereby reducing inbreeding, while the single tree method did not alter the level of heterozygotes or inbreeding, and artificial regeneration would result in a highly heterozygous, outbred population. The two natural regeneration systems do not introduce genetic drift, but artificial regeneration seems to introduce a high genetic change compared to the previously existing stands. These results confirm the trend noted by Neale and Adams (1985).

However, we advise some caution in interpreting these results. While the seed-tree stand and artificial regeneration seed sampled reflects genetic changes after the final cut had been applied to the stands, the single tree selection stand sampled the genetic changes after just the first cutting had been applied. In the single tree selection system, the regeneration that restocks the stand comes from periodic harvest cuts and hence the genetic composition of the stand's seed after several harvests could possibly be somewhat different from that after the first harvest cut. The results are also limited by the fact that

we sampled seed and not the actual regeneration. We recommend an extended study evaluating established regeneration at later stages of all three regeneration systems. The genetic differences we detected were in the seeds, many of which may disappear during seedling establishment owing to natural selection as suggested by Muona *et al.* (1987) and Yazdani *et al.* (1985).

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