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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 ten 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 2 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.

Key words Isoenzyme · Segregation and linkage · Mapping · JOINMAP · Shortleaf pine, *Pinus echinata*

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. 1993, 1994, 1995b; 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 2 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

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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% 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 the segregation and linkage of 28 isoenzyme loci in ten 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 15 populations were collected in the Fall of 1993 by Dr. R. Schmidtling of the USDA Forest Service Southern Research Station, Gulfport, Mississippi 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 6 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, both of which are 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 ten 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 12 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 60 megagametophytes analyzed, at each heterozygous locus for each parent, was entered into the computer as backcross data ($H \times A$). 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 1 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 1 parent; *Pgm* and *Mdh-4* segregated in 2 parents and the remaining 18 in 3 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 9 loci and 18 unlinked loci at

Table 1 Distribution of heterozygous isozyme loci (x) in the 13 shortleaf pine trees studied with segregation χ^2 test for a 1:1 ratio

Isozyme locus	Parent trees													Number of trees segregating	Allele A	Allele B	Segregation χ^2 for 1:1 ratio	*P*
	1	2	3	4	5	6	7	8	9	10	11	12	13					
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
Number of Segregating loci	6	7	9	11	8	9	12	8	7	8	10	8	6					

*Significant at $\alpha = 0.05$

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 the 13 shortleaf pine trees studied

Combination Locus 1–Locus 2	Recombination		LOD value	Combination Locus 1–Locus 2	Recombination		LOD value
	R	S_R			*R*	S_R	
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

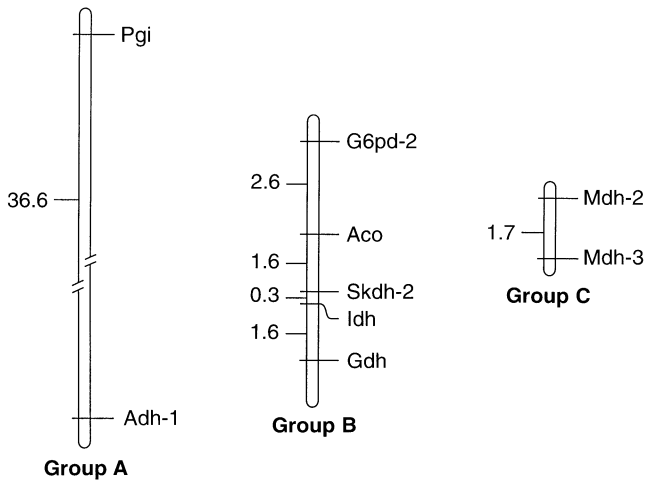

Fig. 1 Map of linked loci in shortleaf pine. Kosambi map distances in centiMorgans are shown between loci

a linkLOD of 4.0. *Ugpp-1* was excluded from analysis since it failed the χ^2 test for 1:1 segregation. The 18 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 ÷ 100), the associated standard errors (S_R) and actual LOD scores 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 (Fig. 1). A comparison of 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* (Fig. 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 (Fig. 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 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* (Fig. 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 2 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 13 trees studied, allowing reliable estimates of linkage between loci that were found to be heterozygous in different individuals. All loci showed a 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. The 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 with respect to linked markers and their ordering, which suggested internal consistency in the data. In general, our results agree with those of 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 the *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 the *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 17 mm from origin) and loblolly pine (average mobility 22 mm from origin), and segregated in a 1:1 ratio (polymorphic) for their F_1 hybrids. They concluded that the electrophoretic separation of *Idh* isoenzymes is a highly accurate technique for identifying F_1 hybrids. However, they also recognized the fact that identification of hybrid generations beyond F_1 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 F_1s takes place. Also, a recent study of genetic variation in shortleaf pine (Raja et al. 1997) showed that 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 F_1 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 1 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 found 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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