LOBLOLLY PINE SSR MARKERS FOR SHORTLEAF PINE GENETICS

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ABSTRACT.—Simple sequence repeats (SSR) are highly informative DNA-based markers widely used in population genetic and linkage mapping studies. We have been developing PCR primer pairs for amplifying SSR markers for loblolly pine (*Pinus taeda* L.) using loblolly pine DNA and EST sequence data as starting materials. Fifty primer pairs known to reliably amplify polymorphic markers in loblolly pine were screened for their use in shortleaf pine (*P. echinata* Mill.), longleaf pine (*P. palustris* Mill.), and slash pine (*P. elliottii* var. *elliottii* Engelm.). Thirty-four of these generated "high-quality" marker data for a small range-wide sample of shortleaf pines and 32 were polymorphic. Expected heterozygosities for the polymorphic markers averaged 0.71 and ranged from 0.40 to 0.88. A subset of the polymorphic markers should be very useful for determining identity or parentage of unknown trees while the whole set should provide excellent information on genetic diversity, gene flow, and population structure in shortleaf pine.

INTRODUCTION

Microsatellite, or simple sequence repeat (SSR), markers have been used to characterize factors affecting pollen flow in shortleaf pine stands in southern Missouri (Dyer and Sork 2001), and to study local and range-wide population histories in loblolly pine (Al-Rabab'ah and Williams 2002, 2004). Although isozyme markers have proven useful in studying shortleaf pine populations (Edwards and Hamrick 1995, Huneycutt and Askew 1989, Raja and others 1997), it is desirable to develop DNA-based markers for use in detailed studies of local and/or range-wide population dynamics. Most SSR marker development work in pines to date has centered on loblolly pine (*Pinus taeda* L.), a close relative of shortleaf pine (*P. echinata* Mill.). Given these facts, we have begun developing SSR markers for use in various other southern pine species. In the current study we tested a selected set of 50 loblolly pine SSR markers on a sample of 6 shortleaf pine trees. In addition, our test included a loblolly pine control sample and two samples each of longleaf pine (*P. palustris* Mill.) and slash pine (*P. elliottii* var. *elliottii* Engelm.).

MATERIALS AND METHODS

Fifty SSR primer pairs that were developed from loblolly pine DNA and characterized as high-quality genetic markers based on their reliable and repeatable amplification and detection in loblolly pine were tested in this study (Tables 1 and 2). Six shortleaf pine, two longleaf pine, two slash pine and two loblolly pine trees were used in testing the 50 primer pairs. The six shortleaf pine trees were all growing on the Harrison Experimental Forest in southeast Mississippi. Four trees were selected from four different provenances that were growing in a range-wide provenance test (Wells and Wakeley 1970). The sources included Southampton County, VA (source 455), Putnam County, GA (463), St. Helena Parish, LA (473), and Dent County, MO (485). The remaining two shortleaf pine trees were first-generation parent trees growing in a clone bank. Both of these trees originated in the Ozark National Forest in Arkansas.

Genomic DNA was isolated from fresh leaf samples from each tree using a DNeasy^{tm} 96 Plant Kit (Qiagen cat. no. 69181). The 50 primer pairs were screened with genomic DNA using the following PCR protocol for each 12 µl total volume reaction: 20 ng genomic DNA, 200 µM of each primer, 200 µM dNTPs, 1x *Taq* buffer (2.0 mM $MgCl_2$, 10 Mm Tris-HCl, 50 mM KCl), and 0.5 U *Taq* DNA polymerase (Promega). The PCRs were completed using the following touchdown protocol on PTC-200 thermal cyclers (MJ Research): 2 min at 94 °C; followed by 20 cycles of 30 s at 94 °C, 30 s at X, and 30 s at 72 °C, where X = 65 °C in the first cycle, decreasing by 0.5 °C every cycle thereafter; followed by 15 cycles of 30 s at 92 °C, 30 s at 55 °C, and 1 min at 72 °C; followed by a 15-min extension at 72 °C and an indefinite hold at 4 °C. The resulting PCR products were separated on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) as recommended by the manufacturer. ABI PRISM LIZ500 was used as an internal size standard. Allele sizes (in base pairs [bp]) were determined using the local southern algorithm implemented by ABI Prism

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Table 1.—Allelic summary for 34 loblolly pine SSR markers screened for variability in shortleaf pine (n=6 trees), longleaf pine (n=2), and slash pine (n=2). Loci prefixes PtTX (Auckland and others 2002), ript (Echt and Nelson unpublished), and RPtest (Echt and Burns 1999). Min and max are range of allele sizes (base pairs), H_e is Nei's (1978) genetic diversity, and A_n is total number of alleles observed.

	Shortleaf				Longleaf			Slash		
Marker	**min**	**max**	**He**	**An**	**min**	**max**	**poly[1]**	**min**	**max**	**poly[1]**
PtTX2123	188	197	0.40	3	197	197	0	194	197	0
PtTX3011	160	212	0.70	5	160	185	1	160	.	0
PtTX3013	121	137	0.63	4	131	140	1	137	141	1
PtTX3029	258	261	0.49	2	267	271	1	262	.	0
PtTX3034	188	208	0.81	7	217	225	1	200	208	1
PtTX3052	239	259	0.70	4	239	259	1	240	259	1
PtTX4058	125	156	0.83	7	146	148	1	146	.	0
PtTX4093	170	323	0.84	8	474	.	0	319	.	0
PtTX4181	368	415	0.83	9	387	411	1	375	.	0
PtTX4205	134	153	0.78	5	134	146	1	.	.	.
PtTX4221	177	193	0.47	2	195	.	0	195	199	1
PtTX4228	141	166	0.76	5	156	160	1	158	162	1
ript0031	243	282	0.82	7	228	228	0	234	.	0
ript0065	130	145	0.51	4	142	142	0	142	144	1
ript0066	117	117	0	1	110	.	0	106	.	0
ript0079	136	171	0.74	4	142	159	1	146	152	1
ript0126	162	214	0.76	6	184	186	1	180	200	1
ript0165	196	209	0.61	3	192	200	1	194	198	1
ript0171	203	213	0.66	4	201	218	1	193	207	1
ript0211	144	160	0.74	5	148	157	1	138	154	1
ript0293	178	189	0.42	2	.	.	.	183	.	0
ript0367	191	213	0.74	4	189	209	1	191	.	0
ript0369	165	178	0.72	4	159	171	1	182	.	0
ript0376	185	200	0.81	7	196	200	1	181	196	1
ript0388	192	212	0.82	7	200	204	1	.	.	.
ript0467	158	185	0.88	9	153	169	1	186	.	0
ript0567	150	180	0.61	5	.	.	.	.	.	.
ript0619	184	206	0.80	6	200	208	1	208	212	1
ript0629	153	167	0.78	6	147	165	1	157	175	1
ript0852	199	205	0.74	5	191	.	0	203	209	1
ript0968	177	222	0.78	6	206	210	1	198	214	1
ript0984	212	237	0.75	6	217	.	0	217	.	0
RPtest9	273	295	0.79	5	278	.	0	257	.	0
RPtest11	218	218	0	1	218	.	0	218	.	0
Mean			0.67	4.9						
Total[2]							22			16

Note: SSR markers tested and not recommended for use in shortleaf pine: PtTX3047, PtTX3063, PtTX3087, PtTX3110, ript0032, ript0106, ript0123, ript0158, ript0255, ript0263, ript0287, ript0647, ript0649, ript0814, ript0990, and ript1077.

[1]poly = polymorphic score: 1 = more than 1 allele observed, 0 = 1 allele, . = 0 alleles.

[2]32 of these 34 markers are polymorphic in shortleaf pine.

Table 2.—GenBank accession and dbSTS numbers for markers not previously described.

Marker	Acc#	dbSTS#
ript0031	BV683043	814084
ript0065	BV683047	814088
ript0066	BV683048	814089
ript0079	BV683053	814094
ript0126	BV683062	814103
ript0165	BV683070	814111
ript0171	BV683072	814113
ript0211	BV683076	814117
ript0293	BV683078	814119
ript0367	BV683081	814122
ript0369	BV683082	814123
ript0376	BV683083	814124
ript0388	BV683084	814125
ript0467	BV683150	814191
ript0567	BV683089	814130
ript0619	BV683091	814132
ript0629	BV683094	814135
ript0852	BV683115	814156
ript0968	BV683124	814165
ript0984	BV683125	814166

GeneMapper® software version 3.7. Due to mobility shift problems associated with the 250 bp and 340 bp bands, these sizes were excluded from all sizing calls. Allelic data were analyzed by standard genetic methods using SAS (SAS Institute).

RESULTS AND DISCUSSION

Of the 50 SSR primer pairs tested, 34 were characterized as "high quality" for use in population genetic analyses of shortleaf pine (Table 1; GenBank and dbSTS accession numbers for previously unreported loci are given in Table 2). These markers met criteria based on clean PCR amplification with low failure rates. Of the 32 markers that were polymorphic in shortleaf pine (Table 1), 30 and 29 cleanly amplified longleaf pine and slash pine DNA, respectively. Within this select group, 22 markers for longleaf pine and 16 markers for slash pine appeared to be polymorphic within these two species (Table 1). Although our diversity measure (i.e., expected heterozygosity, He) is based on only six shortleaf pine trees, it does suggest that these markers are at least as heterozygous as they are for loblolly pine. In fact, the average diversity of the 32 polymorphic markers is 0.71 for this sample of shortleaf pine compared to 0.68 for a similar range-wide sample of loblolly pine (Nelson unpublished). The correlation between shortleaf pine and loblolly pine diversity values is 0.42, suggesting that one cannot predict with any certainty diversity rates for individual SSR markers between these two species. Genetic drift since divergence from a common ancestor is a likely explanation for these results.

The transferability of loblolly pine SSR markers to shortleaf pine, longleaf pine, and slash pine was evaluated by comparing amplification and polymorphism results for two samples from each species. Four shortleaf pine trees were evaluated—two from Arkansas and one each from Louisiana and Georgia. As described in the Materials and Methods, two each of longleaf pine and slash pine trees of Mississippi origin were evaluated. Results of this comparison are provided in Table 3. Transfer rate of polymorphic markers was higher for shortleaf pine (70 percent and 72 percent) than for longleaf pine (54 percent) or slash pine (44 percent). The rate for longleaf pine is comparable to that reported by Shepherd and others (2002), who found 53 percent (19 of 36) of loblolly pine SSR markers polymorphic in slash pine and Caribbean pine (*P. caribea* Morelet). In contrast, Liewlaksaneeyanawin and others (2004) reported only 22 percemt (23 of 107) to be polymorphic in lodgepole pine (*P. contorta* Dougl. ex Loud.). The close phylogenetic relationship of loblolly pine to the other southern U.S. pines, particularly shortleaf pine, and the more distant relationship of loblolly pine to lodgepole pine (Little and Critchfield 1969) is a likely explanation for these results.

Polymorphism (i.e., two or more alleles detected) rates based on pairs of trees from the same area are also higher for shortleaf pine from the two sample areas (78 and 81 percent, calculated from Table 3) than for longleaf pine and slash pine (68 and 54 percemt, respectively). These rates are consistent with previous isozyme data that show longleaf pine to be less genetically diverse than shortleaf pine (Schmidtling and Hipkins 1998). The degree to which polymorphism can be predicted based on only two samples (four chromosomes in a diploid species) was evaluated by

Table 3.—Transfer of 50 loblolly pine SSR markers to shortleaf pine, longleaf pine, and slash pine.

Species	Origin of Sample Pair	Number (%) Amplified	Number (%) Polymorphic
Shortleaf	Arkansas	46 (92)	36 (72)
Shortleaf	Louisiana and Georgia	43 (86)	35 (70)
Longleaf	Mississippi	40 (80)	27 (54)
Slash	Mississippi	41 (82)	22 (44)

comparing the two-sample estimates from shortleaf pine with the six-sample (12 chromosomes) estimate. Based on six samples, 46 of the 50 markers were cleanly amplified and 41 were polymorphic (data not shown), indicating that the two-sample data underestimated the polymorphism rate by about 10 percent. Thus we might expect the actual polymorphism rates for these markers in longleaf pine and slash pine to be higher.

CONCLUSION

Loblolly pine SSR primer pairs proved to be a very good source of genetic markers for shortleaf pine, as well as for longleaf pine and slash pine. Thirty-two out of 50 PCR primer pairs successfully amplified high quality, polymorphic SSR markers in shortleaf pine. We are currently evaluating additional loblolly pine SSR markers. Based on the results of this study, we expect that a relatively high proportion of these markers will be transferable to shortleaf pine. With an average expected heterozygosity of about 0.70, these markers should provide a valuable tool in species restoration and conservation programs that require population genetic data and analysis.

ACKNOWLEDGMENTS

We thank Larry Lott and Dennis Deemer (Southern Institute of Forest Genetics), respectively, for making sample collections and assisting in scoring with GeneMapper, and Tom Kubisiak for helpful comments in review.

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