Microsatellite DNA as shared genetic markers among conifer species

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Abstract: Polymerase chain reaction (PCR) primer pairs for 21 simple sequence repeat (SSR) loci in *Pinus strobus* L. and 6 in *Pinus radiata* D. Don. were evaluated to determine whether SSR marker amplification could be achieved in 10 other conifer species. Eighty percent of SSR primer pairs for $(AC)_n$ loci that were polymorphic in *P. strobus* also amplified SSR loci in two other soft pines of the subgenus *Strobus* but not in seven hard pines of the subgenus *Pinus*, nor in *Picea glauca* (Moench) Voss or *Pseudotsuga menziesii* (Mirb.) Franco. The six *P. strobus* SSR primer pairs that did amplify loci from conifers other than soft pines were those that were specific to loci monomorphic within *P. strobus*. These six loci were also monomorphic within seven other species tested, but four of the loci were polymorphic among species. A comparison of allelic variation among the three soft pine species found only 25 shared alleles among a total of 122 alleles at eight loci. Primer pairs for dinucleotide SSR loci that were polymorphic in *Pinus radiata* also specifically amplified loci from various other hard pines but not from the soft pines or from the other conifers tested.

Résumé : Les auteurs ont évalué le potentiel d'un certain nombre de paires d'amorces de réaction de polymérisation en chaîne de l'ADN permettant d'amplifier 21 loci à motif répété de séquence simple (SSR) chez le *Pinus strobus* L. et 6 loci du même type chez le *Pinus radiata* D. Don., à diriger l'amplification de marqueurs SSR chez 10 autres espèces conifériennes. Quatre-vingt pour cent des paires d'amorces SSR pour les loci de type $(AC)_n$ et qui démontraient du polymorphisme chez le *P. strobus* ont également permis d'amplifier des loci SSR chez deux autres espèces de pin mou du sous-genre *Strobus*. Cependant, aucun résultat positif n'a été obtenu pour sept espèces de pin dur du sous-genre *Pinus*, ni pour le *Picea glauca* (Moench) Voss ou pour le *Pseudotsuga menziesii* (Mirb.) Franco. Les six paires d'amorces SSR dérivées du *P. strobus*, qui permettaient d'amplifier des loci chez les espèces conifériennes autres que les espèces de pin mou, étaient celles responsables de l'amplification de loci monomorphes chez le *P. strobus*. Ces six loci étaient également monomorphes au sein de sept autres espèces testées, mais quatre de ces loci démontraient un polymorphisme interspécifique. En comparant la variabilité allélique parmi les trois espèces de pin mou, les auteurs ont découvert que seulement 25 allèles étaient partagés sur un total de 122 allèles détectés pour huit loci. Les paires d'amorces dirigeant l'amplification de loci SSR dinucléotidiques polymorphes chez le *Pinus radiata* ont permis l'amplification de loci spécifiques chez plusieurs autres espèces de pin dur, mais pas chez les espèces de pin mou ni chez les autres espèces conifériennes étudiées.

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Introduction

For many genetic studies the DNA markers of choice are derived from simple sequence repeat (SSR), or microsatellite, DNA because such markers are highly informative, codominant, unequivocal, and abundant in the genomes of nearly all eukaryotes. SSR markers are assayed using the polymerase chain reaction (PCR), which means that genotype data on numerous loci can be obtained relatively quickly from small quantities of tissue. Consequently, conifer SSR markers are being developed by a number of laboratories and have increasing application in conifer genetics (Smith and Devey 1994; Kostia et al. 1995; Echt et al. 1996; Devey et al. 1996; Karhu et al. 1996; Morgante et al. 1996; van de Ven and McNicol 1996; Echt and Nelson 1997; Fisher et al. 1998; Hicks et al. 1998; Paglia et al. 1998).

A less favorable aspect is that it is expensive, technically demanding, and time consuming to develop robust and informative SSR markers. SSR marker development is all the more laborious in species, such as conifers, that have large and highly repetitive genomes because of the low proportion of DNA that contains single-locus marker loci. Fortunately, the possibility exists to leverage SSR development efforts by utilizing DNA sequence similarities between related taxa. The evolutionary conservation of DNA sequences that flank SSR sites allows previously developed SSR primers to be used in various other related species. This approach is used in genetic studies of mammals (Moore et al. 1991; Hearne et al. 1992; Roy et al. 1994; Blanquer-Maumont and Crouau-Roy 1995; Forbes et al. 1995; Pepin et al. 1995; Kayser et al. 1996), insects (Harr et al. 1998), and plants (Kijas et al.

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Table 1. Previously unreported SSR primer pair sequences for RPS (*Pinus strobus*) and NZPR (*Pinus radiata*) loci.

Locus	Repeat element[a]	Forward and reverse primer sequences
RPS3	$(AC)_{19}$	F) AATGAAGGACAGTTGGGATGAT
		R) TGCTTCCTTCTCATGTTCTCC
RPS61	$(AC)_{12}$	F) TCCATTTCCATCCTTCTTCG
		R) ACGCAACTACCCAGAAGCAA
RPS105	$(AC)_{13}(AT)_3$	F) TGGACATCCTAGTCGGAACC
		R) AAAATCATTTCTGTATCAGAACAA
RPS152	$(ANAC)_6(N)_{13}(CAGA)_3$	F) AAGGGTTTCATTTTGAGAGG
		R) AAATGGCAATGGGAAATG
NZPR1	$(AG)_{17}$	F) TCTCCATCTATCTCTTACCACTCC
		R) TATTCTAACAAGAGAGGGATGTGG
NZPR4	$(AG)_{20}$	F) CTCCCTCTATGTGTTTCTCC
		R) GAAAATCTTTCTACCCTTCCAG
NZPR5	$(AG)_{20}$	F) CTCCTTTTCTCTCTCAAATCC
		R) GAGATATGGAGTGACATAGTGACTC
NZPR6	$(AG)_{23}$	F) GGAAGAAAAATTGGGCCTTA
		R) CTCTCTATCTCTGCCCCA

[a]Based on sequence of cloned plasmid insert.

1995; Kresovich et al. 1995; Brown et al. 1996; Steinkellner et al. 1997, Westman and Kresovich 1998). For example, two SSR markers developed from *Pinus radiata* D. Don were used to study allele diversity in *Pinus sylvestris* L. (Karhu et al. 1996). In that case, both pine species belong to different taxonomic subsections but are within the same subgenus of *Pinus* (Little and Critchfield 1969). If suitable numbers of informative SSR marker primer pairs developed from one conifer species could be used for genotyping in related species, then overall SSR marker-development costs would be reduced, more SSR markers would be available, and SSR marker analysis could be more widely incorporated in many conifer genetics programs.

Twenty-one primer pairs developed for eastern white pine (*Pinus strobus* L., subgenus *Strobus*) (Echt et al. 1996) were evaluated in two other soft pines of the same subgenus (*Pinus cembra* L., and *Pinus lambertiana* Dougl.), in seven hard pines of the subgenus *Pinus* (*Pinus brutia* Ten., *Pinus halepensis* Mill., *Pinus leucodermis* Antoine, *Pinus pinaster* Ait., *Pinus radiata*, *Pinus resinosa* Ait., and *Pinus taeda* L.), and in two non-pine conifers, *Picea glauca* (Moench) Voss and *Pseudotsuga menziesii* (Mirb.) Franco. Six SSR primer pairs developed for Monterey pine (*P. radiata*) (Smith and Devey 1994; Fisher et al. 1998) were also evaluated in this group of conifers.

Materials and methods

DNA sources

Terminal buds from vegetative clones of *Pinus strobus* individuals representing 12 eastern U.S. provenances were kindly provided by James K. Bailey of the Pennsylvania Bureau of Forestry, while those representing 12 north-central U.S., and Ontario, Canada, provenances were collected from the Forest Service's Oconto River Seed Orchard in Wisconsin, U.S.A. Buds from individuals representing 24 *Pinus lambertiana* provenances were kindly provided by Dave Johnson of the Institute of Forest Genetics in Placerville, Calif. DNA samples from bud or leaf tissue of *Pinus brutia*, *Pinus cembra*, *Pinus halepensis*, *Pinus lambertiana*, *Pinus leucodermis*, *Pinus strobus*, and *Pinus taeda* were isolated using standard procedures (see Echt and Nelson 1997). Additional DNA for *Pinus lambertiana* was obtained from Dave Neale, Institute of Forest Genetics, Placerville, Calif. *Pinus pinaster* DNA was obtained from Christophe Plomion, Institut national de la recherche agronomique, Gazinet Cédex, France; *Pinus radiata* DNA from Tom Richardson, New Zealand Forest Research, Ltd., Rotorua; *Pinus resinosa* DNA from Linda DeVerno, Canadian Forest Service, Fredericton, New Brunswick, Canada; *Picea glauca* DNA from Heather Cobbin, University of Alberta, Edmonton, Canada; and *Pseudotsuga menziesii* DNA from Sheila Vollmer, Oregon State University, Corvallis, U.S.A. DNA samples from 2–12 individuals representing each species were pooled for use as templates when testing for general success of PCR amplification. When marker polymorphism within species was evaluated, DNA samples from individual trees were used for SSR marker genotyping.

Primer pairs and PCR amplification

SSR marker repeat and primer information for *Pinus strobus* loci RPS1b, RPS2, RPS6, RPS12, RPS18, RPS20, RPS25b, RPS34b, RPS39, RPS50, RPS84, RPS90, RPS118b, RPS124, RPS127, RPS150, and RPS160 are described by Echt et al. (1996). All loci contain $(AC)_n$ repeats. An additional four SSR primer pairs from *Pinus strobus* were also evaluated in the current study (Table 1). Of the six *Pinus radiata* primer pairs evaluated, loci PR4.6 and PR9.3 were developed by Smith and Devey (1994). The other four loci in Table 1, NZPR1, NZPR4, NZPR5, and NZPR6, were developed by Fisher et al. (1998), and their sequences were generously provided by Tom Richardson of New Zealand Forest Research, Ltd. All SSR primer pair sequence information used in this study is also available from the Dendrome Web site at http://dendrome.ucdavis.edu/Data/primer.html. Primer pair oligonucleotides were purchased as MapPairs (Research Genetics, Inc., Huntsville, Alabama, U.S.A.).

The PCR reaction and amplification conditions, agarose and denaturing polyacrylamide gel electrophoresis, and silver staining procedures were as described previously (Echt et al. 1996), except that *Taq* DNA polymerase was used in this study instead of *Tfl* DNA polymerase. Several touchdown PCR (Don et al. 1991; Hecker and Roux 1996) protocols were evaluated to optimize marker amplification for specific combinations of primer pairs and species. The nucleotide lengths of the PCR products were determined

Table 2. PCR cycling programs used to amplify SSR loci in pines and other conifer species.

Locus	Soft pines			Hard pines and other conifers[a]
	Pinus cembra (*N* = 3)	*Pinus lambertiana* (*N* = 3)	*Pinus strobus* (*N* = 3)	
Polymorphic in *Pinus strobus*				
RPS1b	SSRT55	SSRT55	SSRT55	—[b]
RPS2	SSRT50	SSRT50	SSRT50	—
RPS6	SSRT50	SSRT50	SSRT50	—
RPS12	SSRT55	SSRT55	SSRT55	—
RPS18	SSRT50 (w)[c]	SSRT50 (w)	SSRT50	—
RPS20	SSRT50	SSRT55	SSRT55	—
RPS25b	—	SSRT50 (w)	SSRT55	—
RPS34b	SSRT50	SSRT50	SSRT55	—
RPS39	—	SSRT50	SSRT55	—
RPS50	SSRT50	SSRT55	SSRT55	—
RPS84	SSRT50	SSRT50	SSRT50	—
RPS90	SSRT50	SSRT50	SSRT50	—
RPS118b	SSRT50	SSRT50	SSRT50	—
RPS124	—	SSRT50 (w)	SSRT50	—
RPS127	SSRT50	SSRT50	SSRT55	—
Monomorphic in *Pinus strobus*				
RPS3	SSRT55	SSRT50	SSRT50	SSRT50
RPS61	SSRT55	SSRT55	SSRT55	SSRT50
RPS105	SSRT55	SSRT55	SSRT55	SSRT50
RPS150	SSRT55	SSRT55	SSRT55	SSRT55
RPS152	SSRT55	SSRT55	SSRT55	SSRT55
RPS160	SSRT55	SSRT55	SSRT55	SSRT50
Polymorphic in *Pinus radiata*				
NZPR1	—	—	—	SSRT55
NZPR4	—	—	—	RSSRT58
NZPR5	—	—	—	SSRT55
NZPR6	—	—	—	RSSRT58
PR4.6	—	—	—	55(30)
PR9.3	—	—	—	55(30)

[a]The included species were *Pinus brutia* (*N* = 3), *Pinus halepensis* (*N* = 3), *Pinus leucodermis* (*N* = 3), *Pinus pinaster* (*N* = 4), *Pinus radiata* (*N* = 4), *Pinus resinosa* (*N* = 3), *Pinus taeda* (*N* = 3), *Picea glauca* (*N* = 12), and *Pseudotsuga menziesii* (*N* = 2).
[b]No amplification was observed.
[c]w, weak amplification was observed.

by ABI Prism GeneScan analysis on an ABI 373A automated DNA sequencer using fluorescently labeled markers according to manufacturer's instructions (PE Applied Biosystems, Foster City, California, U.S.A.).

The standard touchdown PCR thermal cycling protocol used for *P. strobus* primers was SSRT50. The first two thermal cycles included a denaturing step at 94°C for 60 s, an annealing step at 60°C for 60 s, and an extension step at 70°C for 35 s. The next 18 cycles consisted of a denaturing step at 93°C for 45 s, an annealing step at 59°C for 45 s (which subsequently was decreased by 0.5°C every cycle until a final temperature of 50.5°C was reached), and an extension step at 70°C for 45 s. Conditions for the last 20 cycles were 92°C for 30 s, 50°C for 30 s, and 70°C for 60 s, followed by a final extension at 70°C for 5 min. A modification of the SSRT50 protocol, SSRT55, was used to increase primer specificity and decrease background amplification in certain cases. The first two cycles of SSRT55 used a 65°C (rather than 60°C) annealing temperature. In the next 18 cycles the annealing temperature started at 64°C, and decreased to 55.5°C by 0.5°C increments (rather than from 59 to 50.5°C), with the final 20 cycles having an annealing temperature of 55°C.

Three thermal cycling protocols were used with *Pinus radiata* primers, SSRT55, RSSRT58, and 55(30). The latter two were derived from protocols used by the laboratories that developed the *Pinus radiata* SSR markers (P.J. Fisher and T.E. Richardson, personal communication; Smith and Devey 1994). For RSSRT58, after an initial denaturation of 94°C for 2 min, the first five cycles had of temperature steps of 93, 62, and 72°C for 30 s each. The next five cycles had steps of 93, 60, and 72°C for 30 s each, while the last 30 cycles had steps of 92, 58, and 70°C for 30 s each, followed by a final extension step at 70°C for 5 min. In the 55(30) protocol, after an initial denaturation step of 95°C for 5 min, the next 30 cycles had steps of 94, 55, and 72°C, each step lasting 60 s, ending with a final extension step at 72°C for 8 min.

Hybridization analysis

To confirm the presence of a microsatellite repeat in the *Pinus strobus* $(AC)_n$ markers that were monomorphic within a species, DNA probe hybridization assays were done on the amplified fragments. Following PCR amplification with selected primer pairs, 1 µL of PCR product was dotted to nylon membranes (Immobilon-S, Millipore) and air-dried. Denaturation, neutralization, and cross-linking of the DNA on the nylon membranes was done according to New England Biolabs Phototope protocols. Hybridization of an alkaline phosphatase conjugated oligonucleotide $(GT)_{13}$ probe and chemiluminescent signal detection were done as previously described (Echt and May-Marquardt 1997).

Results

Pinus strobus SSR primer pairs

Pinus strobus SSR loci were previously classified as either polymorphic or monomorphic based on their genotypes in 16 unrelated *P. strobus* individuals (Echt et al. 1996). Primer pairs for 15 polymorphic $(AC)_n$ loci were tested in two other soft pines, in seven hard pines, and in two other conifers (Table 2). Of these primer pairs, 12 amplified specific products in the expected size ranges from *Pinus cembra* DNA, and all did so from *Pinus lambertiana* DNA (Table 2). When amplification occurred, PCR yields were generally high, but lower yields were observed for three loci in *Pinus cembra* and *Pinus lambertiana* (Table 2). With few exceptions, only single bands, some possibly containing several allelic fragments within a narrow size range, were observed on agarose gels for each species. This is consistent with amplification of single loci by most of the primer pairs.

Allelic variation at eight loci was compared among 24 individuals from each of the three soft pine species (Table 3). Evidence for amplification of two loci occurred with only two primer pairs: RPS6 in *Pinus lambertiana*, and RPS50 in both *Pinus cembra* and *Pinus lambertiana*. For the eight loci surveyed, a total of 122 alleles were observed among the three species, but only 25 alleles were shared between any two, or among all three, species. The primer pair for RPS39 did not amplify a product in *Pinus cembra*, and RPS127 was not tested in this species. The genotyping survey revealed that the primer pair for RPS119 described by Echt et al. (1996) is actually specific for locus RPS127. In the prior study a duplicate clone for this locus was sequenced, and different primer pairs were designed from each sequence, resulting in amplification of differently sized markers for the locus.

Table 3. SSR allele size range, in bp, and number of alleles (in parentheses) in three soft pine species, using primer pairs derived from *Pinus strobus*.

RPS locus	Species: *Pinus cembra* (N = 24)	*Pinus lambertiana* (N = 24)	*Pinus strobus* (N = 24)	No. of shared alleles[a]
2	149–157 (4)*	143–183 (12)	151–171 (6)	4
6	134–169 (8)*	155–185 (10)[b]	160–187 (6)	2
12	144–158 (5)	154–164 (6)	150–189 (13)	6
34b	120–148 (11)*	134–168 (11)	143–146 (4)	3
39	None	157–171 (5)*	169–179 (3)	2
50	169–173 (3)[c]	157–177 (9)[d]	159–185 (12)	7
84	132–170 (7)*	131–142 (6)*	147–162 (5)	0
127	Not tested	187–201 (7)	191–193 (2)	1

*PCR amplification did not occur in several samples, suggesting that some trees were homozygous for a null allele. Null alleles were not counted as alleles.

[a]The number of alleles common among any two or all three species, as determined by allele lengths.

[b]Does not include alleles at a second locus that amplified in the 138- to 142-bp range in several samples.

[c]Does not include alleles at a second locus that amplified in the 139- to 141-bp range in half of the samples.

[d]Does not include a 140-bp fragment from a second locus that amplified in all samples.

The 15 primer pairs for polymorphic soft pine $(AC)_n$ loci did not amplify marker fragments in the seven hard pines tested, nor in the other two conifers (Table 2). Weak and inconsistent amplification was occasionally observed for some of the primer pairs, but PCR conditions could not be optimized to reliably amplify single bands in the expected size ranges for any of them.

Primer pairs for the six *Pinus strobus* loci classified as monomorphic (Table 2) all strongly amplified single specific DNA fragments from various hard pines and other conifers, as well as from *Pinus lambertiana* and *Pinus cembra* (Table 4). Only the primer pair for RPS150 strongly amplified fragments from every species tested. However, in *Pseudotsuga menziesii* it amplified six fragments, while in all other species it amplified only a single fragment. The primer pair for RPS152 generated two fragments of equal intensity in *Pinus pinaster*, suggesting the presence of a single polymorphic locus in that species. Amplification of two loci, each themselves monomorphic, could not be ruled out, however, as segregation data from individual *Pinus pinaster* progeny were not obtained.

Marker polymorphism was surveyed in *Pinus brutia* (N = 24), *Pinus cembra* (N = 24), *Pinus halepensis* (N = 24), *Pinus lambertiana* (N = 24), *Pinus leucodermis* (N = 24), *Pinus resinosa* (N = 48), and *Pinus taeda* (N = 7) for each of the primer pairs that amplified a monomorphic locus from *Pinus strobus*. Despite that only monomorphic fragments were observed within each species in which amplification occurred, four of the markers (RPS61, RPS105, RPS152, and RPS160) were variable in length between species (Table 4). Hybridization of a $(GT)_{15}$ probe to the PCR products confirmed the presence of an $(AC)_n$ repeat at loci RPS3, RPS61, and RPS105. In all but two cases the repeat was present in the amplified fragments. No $(AC)_n$ repeat could be detected in the RPS3 fragment from *Pinus taeda* nor in the RPS61 fragment from *Pinus pinaster*. The size of the fragment in the latter case was 170 base pairs (bp), shortest among the seven species from which amplification occurred for the RPS61 primer pair (Table 4), suggesting that the repeat may have been too short to successfully hybridize with the probe. The RPS3 fragment from *Pinus taeda*, however, was the same size as it was in *Pinus strobus* and *Pinus resinosa* (Table 4), indicating that a substitution of all or part of the repeat sequence may have occurred. The repeats in loci RPS150, RPS152, and RPS160 were not of the $(AC)_n$ class, and their presence in the amplified fragments was not confirmed by hybridization.

Pinus radiata SSR primer pairs

Primer pairs for six dinucleotide SSR loci that are polymorphic in *Pinus radiata* were evaluated in 11 other conifer species, with the results summarized in Tables 2 and 5. The *Pinus radiata* primers did not amplify loci from the three soft pines tested, nor from the two non-pine species (Table 5). They did amplify loci from various other hard pine species, but no primer pair amplified a product from all of the hard pine species tested. As determined by agarose gel analysis, all of the fragments that were amplified from other species were near the expected size of the *Pinus radiata* marker. For PR4.6, PR9.3, NZPR5, and NZPR6, variation in fragment sizes on agarose gels was observed among species, but the presence of the SSR in the amplified fragments was not confirmed by hybridization. Primer pairs were tested on pools of DNA from individuals within each species, and fragment sizes were not determined.

Discussion

The identification of informative PCR-based markers is difficult in species with large, complex genomes because of the relative scarcity of unique, nonrepeated, DNA sequences. Consequently, only a small fraction of SSR clones selected from genomic libraries can be converted to informative SSR markers. For example, suitable markers were obtained from only 20–24% of the primer pairs designed for *Pinus strobus* (Echt et al. 1996), *Picea abies* (L.) Karst. (Pfeiffer et al. 1997), and *Triticum aestivum* L. (Ma et al. 1996). It is expected that, if some SSR motifs were associated with highly conserved regions of the genome, primer pairs for such loci would work in a broader range of species or genera. This certainly is the case with conifer chloroplast microsatellite markers. The high degree of sequence conservation among conifer chloroplast genomes allows PCR primer pairs designed from the *Pinus thunbergii* chloroplast DNA sequence to amplify homologous sites in distantly related species (Cato and Richardson 1996; Powell et al. 1995; Vendramin et al. 1996; Echt et al. 1998; Vendramin and Ziegenhagen 1998).

When *Pinus strobus* SSR primer pairs were used in closely related species, 86% of them amplified loci from *Pinus cembra*, and all amplified loci from *Pinus lambertiana* (Table 2). When only polymorphic *Pinus strobus* SSR loci are considered, 80% of the primer pairs amplified loci from *Pinus cembra* (Table 2). *Pinus lambertiana* and *Pinus strobus* are taxonomically classified in the *Strobi* subsection.

Table 4. Sizes, in base pairs, of PCR products from various conifer species amplified using primer pairs specific for monomorphic *Pinus strobus* SSR loci.

Species	Locus RPS3 (AC)	RPS61 (AC)	RPS105 (AC)	RPS150 (GAG)	RPS152 (AGAC)	RPS160 (ACAG)
Soft pines[a]	268	179	149	243	149	245
Hard pines						
Pinus brutia	—[b]	176	141	243	—	241
Pinus halepensis	—	176	141	243	—	245
Pinus leucodermis	—	176	141	243	183	249
Pinus pinaster	—	170	141	243	182, 212	249
Pinus radiata	—	—	141	243	—	—
Pinus resinosa	268	173	141	243	149	245
Pinus taeda	268	172	141	243	—	—
Other conifers						
Picea glauca	—	—	—	243	—	—
Pseudotsuga menziesii	—	—	—	Multiple products	—	—

Note: Repeat motifs are given in parentheses. The numbers of individuals used in the pooled DNA samples for each species were as indicated in Table 2.
[a]The fragment sizes amplified from each primer pair were the same in *Pinus cembra*, *Pinus lambertiana*, and *Pinus strobus*.
[b]Amplification did not occur.

Table 5. Ability of SSR primer pairs from polymorphic *Pinus radiata* $(AG)_n$ loci to amplify single DNA fragments from various conifer species.

Species	Locus PR4.6 (210)	PR9.3 (100)	NZPR1 (140)	NZPR4 (145)	NZPR5 (115)	NZPR6 (200)
Soft pines						
Pinus cembra	—[a]	—	—	—	—	—
Pinus lambertiana	—	—	—	—	—	—
Pinus strobus	—	—	—	—	—	—
Hard pines						
Pinus brutia	—	+[b]	—	—	—	+
Pinus halepensis	—	+	—	+	—	—
Pinus leucodermis	+	+	—	—	+	+
Pinus pinaster	—	+	—	—	—	+
Pinus resinosa	+	—	—	—	+	—
Pinus taeda	+	+	+	—	+	+
Other conifers						
Picea glauca	—	—	—	—	—	—
Pseudotsuga menziesii	—	—	—	—	—	—

Note: The approximate size of the fragment (bp) in *Pinus radiata* is given in parentheses.
[a]Amplification did not occur.
[b]A fragment in the expected size range was amplified.

while *Pinus cembra* is in the *Cembrae* subsection, of the section *Strobus* (Little and Critchfield 1969). The greater taxonomic distance of *Pinus cembra* from *Pinus strobus* was evident in fewer successful PCR amplifications in *Pinus cembra* than in *Pinus lambertiana* when using *Pinus strobus* SSR primer pairs. Greater DNA sequence divergence at primer target sites is expected between members of different subsections than among members of the same subsection.

In contrast, only 29% of all *Pinus strobus* primer pairs amplified fragments from hard pine species (section *Pinus*), and none of the polymorphic *Pinus strobus* primer pairs did so (Table 2). The *Pinus strobus* primer pairs were not tested with other soft pine species in the *Parrya* section of the subgenus *Strobus*, and the *Pinus radiata* primer pairs were not tested with other hard pines in the section *Pinea* of the subgenus *Pinus*, so no conclusion can be made about how well the SSR markers are shared among members of a subgenus. But the results show that many are shared at least among members of the same taxonomic section.

Our results differ somewhat from those reported by Fisher et al. (1998) in their characterization of some of the same *Pinus radiata* primer pairs. In contrast to the single-locus PCR products we found, they reported that the primer pairs for NZPR1, NZPR4, and NZPR5 amplified three to five

fragments of widely differing sizes from *Pinus radiata*. They also reported that NZPR6 amplified a fragment from *Pinus strobus*, while we obtained no amplification from any soft pine DNA using the *Pinus radiata* primer pairs. All of these discrepancies may be accounted for by the more stringent primer annealing temperatures we employed during PCR cycling that would have resulted in higher sequence discrimination between the primers and the target sequences. The lower annealing temperatures employed by Fisher et al. (1998) may have allowed amplification of additional loci with minor nucleotide differences in their primer target sites.

Homozygosity for null alleles may have resulted in the failure of five *Pinus strobus* SSR primers to amplify a marker from polymorphic SSR loci in certain *Pinus cembra* and *Pinus lambertiana* individuals (Table 3). Null alleles in this context are allelic sequence variants at the primer target sites that prevent primer annealing during PCR, resulting in no marker amplification. Support for this interpretation was provided by the higher proportion of loci exhibiting failed amplification in *Pinus cembra*, which is more distantly related to *Pinus strobus* than is *Pinus lambertiana*. Loci may also be heterozygous for null alleles, which may explain the significantly higher inbreeding coefficient, F_{IT}, observed for *Pinus lambertiana* SSR loci ($F_{IT} = 0.40$) than for the same *Pinus strobus* loci ($F_{IT} = 0.14$) (Echt 1999). The higher F_{IT} value for *Pinus lambertiana* results from a higher than expected proportion of homozygous genotypes in general, yet exists despite a greater number of SSR alleles in *Pinus lambertiana* than in *Pinus strobus* (Table 3). Isozyme data and seed viability data from controlled crosses indicate that *Pinus lambertiana* does not tolerate the high levels of inbreeding that would be associated with an inbreeding coefficient of 0.40 (T. Conkle, personal communication), so the unexpected frequencies of homozygous SSR genotypes most likely result from appreciable numbers of null-allele heterozygotes. A null-allele heterozygote would be phenotypically scored in diploid tissue as a homozygote for any particular amplified SSR allele in the population with which it was paired, thus giving a false measure of the frequency of homozygous genotypes. Segregation tests on progeny from putative null allele heterozygote parents have not yet been done to confirm this hypothesis.

Several *Pinus strobus* SSR primer pairs amplified fragments that were monomorphic within species but polymorphic between species (Table 4). For RPS3 in *Pinus taeda* and RPS61 in *Pinus pinaster* the SSR could not be detected by probe hybridization, although the SSR was detected in the other species from which a locus-specific fragment was amplified. It is possible that the SSRs in these loci in *Pinus taeda* and *Pinus pinaster* were replaced by different sequences, as the amplified fragment sizes were not short enough for there to have been an outright deletion of the native repeats (Tables 1 and 4). For example, the RPS3 fragment from *Pinus taeda* was the same length as that from *Pinus strobus*, yet the $(AC)_n$ repeat was not detectable in *Pinus taeda*. Similarly, the RPS61 fragment from *Pinus pinaster* was only 2–7 bp shorter than the orthologous fragments in other species and did not contain a detectable $(AC)_n$ repeat, although the SSR was detectable in the other species. Substitution of SSR elements, and nonunit repeat mutations, among pine species has been reported by Karhu et al. (1997). These results indicate that caution must be taken when comparing allele sizes between distantly related species generated by primer pairs for SSR loci, as allele length difference may not be confined to simple contraction or expansion of the repeat sequence. Likewise, SSR marker alleles that share identical sizes among species (Table 3) may in fact not be identical in their DNA sequences.

The absence of length polymorphisms within species is unexpected, as probe hybridizations indicated that some loci in species from which amplification occurred clearly still retained a SSR element (e.g., RPS61 and RPS105). One possible reason why monomorphic SSR loci are more highly conserved across greater taxonomic distances than are polymorphic loci is because of natural selection. SSR repeat length at a locus may be constrained by the same selective forces that constrain variation in the adjacent PCR primer target sequences. However, with no apparent function for these loci and without comparative DNA sequence data, the selection hypothesis must remain merely speculative. Whatever the mechanism, primer-pair sequences that amplify SSR loci from species of the two pine subgenera have been conserved since the time that the hard and soft pines diverged over 130 million years ago (Millar 1993).

While monomorphic loci are not useful for linkage or population genetic analyses, those that are polymorphic among species could be used as species-specific markers. Although additional testing on a broader sampling of individuals in each species is needed, the putative species-specific markers we identified may be of use in phylogenetic studies or to genotype interspecies hybrids.

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