Characterization of microsatellite markers in eastern white pine

C.S. Echt, P. May-Marquardt, M. Hseih, and R. Zahorchak

Abstract: An enrichment cloning method was evaluated for the isolation of microsatellite loci from eastern white pine and the resulting markers were examined for polymorphisms. A 200-fold enrichment was achieved for highly abundant $(AC)_n$ repeats, but for much less abundant $(ACAG)_n$ repeats an enrichment of only 20-fold was obtained. Using a single set of PCR conditions, 19 microsatellite loci were identified from 77 primer pairs evaluated. Genotyping of 16 $(AC)_n$ loci in 16 unrelated white pines from the north-central United States revealed an average of 5.4 alleles per locus and an average observed heterozygosity of 0.515. Five loci were scored among megagametophytes from a single pine to obtain a haploid genotype of the segregating female meiotic products. All loci segregated according to Mendelian expectations and linkage was established for two of the loci. It was concluded that $(AC)_n$ loci are highly variable in this species and that SSR (simple sequence repeat) markers can be efficiently developed for genome mapping and population genetics studies.

Key words: *Pinus strobus*, forest genetics, simple sequence repeat, SSR, allelic diversity.

Résumé : Une méthode de clonage par enrichissement a été évaluée en vue de l'isolation de microsatellites chez le pin blanc et les marqueurs obtenus ont été examinés quant à leur polymorphisme. Un facteur d'enrichissement de 200 a été obtenu pour les séquences $(AC)_n$, lesquelles sont très abondantes, tandis que pour les séquences moins abondantes $(ACAG)_n$, un facteur d'enrichissement de 20 seulement a été atteint. En utilisant une seule série de paramètres pour l'amplification PCR, 19 loci de microsatellites ont pu être identifiés avec les 77 paires d'amorces évaluées. L'analyse génotypique de 16 loci $(AC)_n$ chez 16 pins blancs non apparentés provenant de la région centre-nord des États-Unis a indiqué une moyenne de 5,4 allèles par locus et une hétérozygotie moyenne observée se chiffrant à 0,515. La ségrégation de cinq loci a été étudiée parmi des mégagamétophytes provenant d'un seul pin afin d'établir le génotype haploïde des produits de la méiose du côté femelle. Tous les loci ont montré une ségrégation mendélienne et une liaison génétique a été établie pour deux des loci. Il en a été conclu que les loci $(AC)_n$ sont très variables chez cette espèce et que les microsatellites pourraient ainsi être mis au point d'une manière efficiente en vue de travaux de cartographie et d'études de génétique des populations.

Mots clés : *Pinus strobus*, génétique de la forêt, microsatellite, SSR, diversité allélique.
[Traduit par la Rédaction]

Introduction

There are few marker systems currently available or suitable for detailed genetic analyses of eastern white pine (*Pinus strobus*). Eastern white pine is the only native five-needled pine (subgenus *Strobus*) in eastern North America and is an ecologically and potentially economically important forest species. Only a handful of isozymes (Beaulieu and Simon 1994*a*, 1994*b*; Eckert et al. 1981) or restriction fragment length polymorphisms (RFLPs) (Ahuja et al. 1994; Devey et al. 1994) have been identified for use in white pine. Random amplified polymorphic DNA (RAPD) markers are well suited for mapping disease traits (Devey et al. 1995) or for genome mapping in individual pines (Kubisiak et al. 1995; Lu et al. 1995; Plomion et al. 1995; Nelson et al. 1993, 1994), but are of limited use for other types of studies. Our objective was to learn whether simple sequence repeats (SSRs), also known as microsatellites, could provide sufficient variability for genetic studies in eastern white pine and whether SSR marker development was practical. If so, SSR markers could find wide application in genome mapping and linkage analysis, in analyzing genetic structures of natural and breeding populations, and in genetic fingerprinting.

SSRs are abundant sources of genetic variation in many organisms. By designing PCR primers specific to non-repetitive sequences that flank a repeat, small quantities of DNA can be assayed to detect length polymorphisms at an SSR site (Weber and May 1989). A PCR primer pair thus defines an SSR marker locus. The simple repetitive nature of SSRs can lead to strand slippage during DNA replication (Hauge and Litt 1993; Schlötterer and Tautz 1992), thus generating length polymorphisms. Depending on the repeat motif and species involved, mutation rates in mammalian systems can range from 1×10^{-3} to 1.6×10^{-6} per locus per gamete per generation (Ellegren 1995; Weber and Wong 1993; Edwards et al. 1992). The mutation rates of SSRs in plants are not known.

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High information content, definitive allele assignment, and rapid analysis by PCR make SSR markers well suited for both linkage mapping and genetic diversity studies in plants (Akkaya et al. 1995; Plaschke et al. 1995; Bell and Ecker 1994; Saghai-Maroof et al. 1994; Morgante et al. 1994; Wu and Tanksley 1993). SSR markers have been developed in several tree species, such as oak (Dow et al. 1995), *Citrus* (Kijas et al. 1995), and radiata pine (Smith and Devey 1994). Characterization of two SSR markers in radiata pine (*Pinus radiata*), in the subgenus *Pinus*, show that $(AC)_n$ SSR markers can be quite informative in a conifer species. We have found, in loblolly pine (*Pinus taeda*) and eastern white pine, several di-, tri-, and tetranucleotide SSR motifs that are each present at 20 000 – 120 000 copies per haploid genome (C.S. Echt and P. May-Marquardt, submitted for publication).[1] With a 1C (haploid) DNA content of 29 pg (Wakamiya et al. 1993), the most abundant white pine SSRs are expected to be present at an average frequency of one every 220–1280 kilobases (kb), distances that are comparable to those estimated for several angiosperm crop species (Kresovich et al. 1995; Röder et al. 1995; Wu and Tanksley 1993).

Materials and methods

SSR enrichment cloning

Haploid DNA of eastern white pine parent P-312 was digested with a cocktail of *Eco*RV, *Hae*III, *Rsa*I, and *Ssp*I restriction endonucleases. The fragments were ligated to 5′ phosphorylated *Bst*XI linkers (Research Genetics, Inc.) and then size fractionated on agarose gels to 200–700 base pairs (bp). Libraries enriched for SSR-bearing clones were obtained by the methods of Ostrander et al. (1992), with some modifications. In brief, the purified adapted fragments were ligated into the nonpalindromic *Bst*XI site of pJCP1 and recombinant vector was electroporated into JMG-1 (*dut*$^-$ *ung*$^-$ F′) cells to produce a primary uracil-substituted library. Jacqueline Pulido and Geoffrey Duyk of the Harvard Medical School generously provided the vector and host cells. From this library, circular single-stranded DNA was rescued, purified, and used in primer extension reactions containing Taq DNA polymerase, a phosphorylated oligomer of the desired SSR as primer, dNTPs, and no dUTP. The double-stranded products, now enriched for SSR-bearing inserts, were used to transform DH10B (*dut*$^+$ *ung*$^+$) cells. SSR clones were selected for sequencing following two rounds of colony hybridization screening with a ^{32}P end-labeled SSR probe. Either $(AC)_{20}$ or $(ACAG)_{10}$ probes were used and hybridizations were performed at T_d (temperature of dissociation) − 1°C, with stringency washes at about T_d − 45°C.

Sequencing and primer synthesis

Sequencing templates were generated from most clones by PCR amplification of the inserts from primers flanking the cloning site (5′AAAGGGGGATGTGCTGCAAGGCG and 5′GCTTCCGGCTCGTATGTTGTGTGG). Amplified products were then purified by polyethylene glycol precipitation (Rosenthal et al. 1993) and resuspended in water. Alternately, plasmid DNA was purified using Wizard Miniprep columns (Promega Corp.) and recovered in water. The templates were used in forward and reverse dideoxy dye terminator cycle sequencing reactions and analyzed on a 373A automated DNA sequencer according to the manufacturer's instructions (Perkin-Elmer–ABI). Primer pairs for SSR markers were designed with the aid of the PRIMER 0.5 program (Whitehead Institute, Cambridge, Mass.) and synthesized as MapPairs™ by Research Genetics, Inc.

Marker analysis

New primer pairs were tested in PCR by amplifying six *P. strobus* haploid DNA samples. PCR reactions contained 10 ng DNA template in 10 μL of reaction buffer containing 50 mM Tris-Cl (pH 9.0), 20 mM ammonium sulfate, 12% sucrose, 0.2 mM cresol red, 100 μg/mL gelatin, 3.5 mM magnesium chloride, 200 μM each dNTP, 200 nM each primer, and 0.025 U/μL Tfl thermostable DNA polymerase (Epicentre Technologies). A touchdown amplification protocol was run on an MJ Research PTC-100 thermocycler. The first two cycles included a denaturing step at 94°C for 1 min, an annealing step at 60°C for 1 min, 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. Amplification products were examined on horizontal 1.5% TreviGel-500 agarose gels (Trevigen, Inc.) in 1× TAE (Tris-acetate–EDTA) containing 0.2 μg/mL ethidium bromide. Markers that amplified cleanly at or near the expected size were further characterized to detect size variations among 16 different *P. strobus* trees. Positive and negative controls were run for each primer pair. After amplification, one volume of a stop solution (deionized 95% formamide, 10 mM NaOH, 0.05% bromophenol blue, plus 0.05% xylene cyanol) was added to individual PCR reactions. Samples were denatured at 95°C for 2 min before loading 3.0 μL onto pre-electrophoresed vertical denaturing polyacrylamide gels. The composition of the 0.4 mm × 43 cm long gels was 6% acrylamide (acrylamide–bisacrylamide 19:1), 8 M urea, and 1× TBE (Tris-borate–EDTA). For glass plate preparation, Acrylease™ (Stratagene Cloning Systems) was used for a nonstick coating on one plate and γ-methacryloxypropyltrimethoxysilane (Sigma Chemical Co.) for the gel binding treatment on the other. The upper reservoir buffer was 1× TBE and the lower reservoir buffer was 0.66× TBE plus 0.5 M sodium acetate (Sheen and Seed 1988). Gels were electrophoresed at 50°C constant temperature, with 100 W limiting power, for 2–2.5 h. Separated markers were stained with silver following the Promega Silver Sequence™ protocol, with some modifications. All solutions were freshly prepared with 18 MΩ-cm water (d.i. water) and all steps were done at room temperature, but the developer solution was prechilled to 10–12°C to reduce background staining. After electrophoresis, the gel, bound to a glass plate, was fixed for 20 min with 7.5% glacial acetic acid and then given three 2-min rinses with d.i. water. Used fixing solution was stored at 4°C for later use. The gel was stained for 30 min in 6 mM silver nitrate plus 0.056% formaldehyde and then rinsed with d.i. water for 5 s. For development of the stain, 800 mL of chilled developer (0.28 M sodium carbonate, 0.056% formaldehyde, plus 8 μM sodium thiosulfate) was added with gentle agitation and then decanted when the first bands became visible. An additional 1200 mL of developer was added and agitation continued until development was nearly complete. The developing reaction was stopped by adding 1200 mL of chilled used fixing solution and then the gel was rinsed thoroughly with d.i. water and air-dried. Images were recorded either by making contact prints on APC film (Promega Corp.) or by digital capture with a flatbed scanner.

All SSR allele sizes were measured to a 1-bp accuracy using molecular weight standards of either ϕX174 phage DNA

[1] C.S. Echt and P. May-Marquardt. Abundance of microsatellite loci in pine. Submitted for publication.

Table 1. SSR loci, forward and reverse primer sequences, marker sizes expected from cloned sequences[a], and optimum primer annealing temperatures.

Locus	Repeat	PCR primers	Size (bp)	Annealing temperature (°C)
RPS1b	$(AC)_{10}$	GCCCACTATTCAAGATGTCA GATGTTAGCAGAAACATGAGG	207	55
RPS2	$(AC)_{15}$	CATGGTGTTGGTCATTGTTCCA TGGAGGCTATCACGTATGCACC	163	55
RPS6	$(AC)_{14}$	TTTTCTAATCAGTGTGCGCTACA CACCGCTGCCCTATTTTACA	162	55
RPS12	$(AC)_{17}$	TCAATGTGGAGATGGTGATT ACTTCTGACCTAACCAGAAACC	185	57
RPS18	$(AC)_{14}$	TTTCTAATCAGTGTGCGCTACAT CACCGCTGCCCTATTTTACA	160	54
RPS20	$(AC)_{16}(AT)_6$	ACTTCCCCACAGGTTAACACA AACAAGATAGGCGGGATTCA	138	54
RPS25b	$(AC)_{17}AG(AT)_9$	CACATATGGCAGAACACACA GATCGTCGCACTATCGAAC	107	55
RPS34b	$(AC)_{14}$	CAGTGTTCTCTTATCACAGCG GCACTATAATGAAATAGCGCA	145	55
RPS39	$(AC)_{17}$	GCCAGCTCCAACCAGAATC GGCTCGCTGACCCAATAA	172	57
RPS50	$(AC)_{17}$	CCCAGAAATCTGTTTTAGAGC ACACATGAAATGTCAGAATGC	170	50
RPS60	$(AC)_{19}(AT)_7$	ACGATAATGGCGGTGAGAACAA CCACCTGTCCTTCGTACATCCA	269	57
RPS84	$(CT)_{10}(AC)_{11}$	CCTTTGGTCATTGTATTTTTGGAC CTTCCTTTTCCTTCTTGCTCCAC	147	52
RPS90	$(AC)_{23}$	ACCCATTGTGGTGTGTTTGTG CCTCCGACCATAAACCTTAATG	164	55
RPS118b	$(AC)_{23}$	CATTGTGGTGTGTTTGTGAA CCACCTCCGACCATAAAC	160	52
RPS119	$(AC)_{10}(AT)_5$	TTGTGAGAAGATACTTCCTCCA CCTTGTCTTCTAAAAAACACTTTT	205	55
RPS124	$(AC)_{12}$	AGAGTTCTCACTTCACAATAGGTG ATTTCACACAATTTTGAGTGTTT	149	56
RPS127	$(AC)_{10}(AT)_5$	ACTTCCTCCAAGTTACTATTGTCA CCTTGTCTTCTAAAAAACACTTTT	193	55
RPS150	$(GAG)_4$	TCCATCAGTGAGCAGTGG CACTTGGGCTTCCTCTTC	248	52
RPS160	$(ACAG)_3$ $AGGC(AGAC)_3$	ACTAAGAACTCTCCCTCTCACC TCATTGTTCCCCAAATCAT	246	55

[a]GenBank accession Nos. U60239–U60258.

digested with *Hae*III or pGEM DNA markers (Promega Corp.) mixed with poly(dA) (Pharmacia Biotech Inc.) that had been sonicated to produce a 1-bp ladder. Haploid linkage analysis was performed using MAPMAKER (Lander et al. 1987), generously provided by S. Tingey, Du Pont Inc., on a Macintosh computer. An upper recombination fraction of 0.30 and a minimum LOD limit of 4.0 were used for detecting linkages.

Results

Enrichment cloning and sequencing

Initial attempts by our laboratory to develop $(AC)_n$ markers involved cloning size-selected *Sau*3AI fragments into M13mp18 (C.S. Echt, unpublished data). Only 5 $(AC)_n$ clones, with an average insert size of 400 bp, were obtained out of 60 000 plaques screened. This frequency of recovery was much lower than expected and led us to use the SSR enrichment cloning method described in Materials and methods. The enriched library had an average insert size of 303 bp and an $(AC)_n$ clone frequency of 30%. Using an estimate for white pine of one $(AC)_n$ site every 221 kilobase pairs (kbp), for $n > 11$ (C.S. Echt and P. May-Marquardt, submitted for publication, see footnote 1), an $(AC)_n$ clone frequency of 0.14% would be expected from a representative nonenriched library. Enrichment thus provided over a 200-fold increase in the frequency of $(AC)_n$ clones.

One hundred and forty clones were selected by colony hybridization, 99 were sequenced, and 84 unique sequences were obtained. Seventy-eight of these contained $(AC)_n$

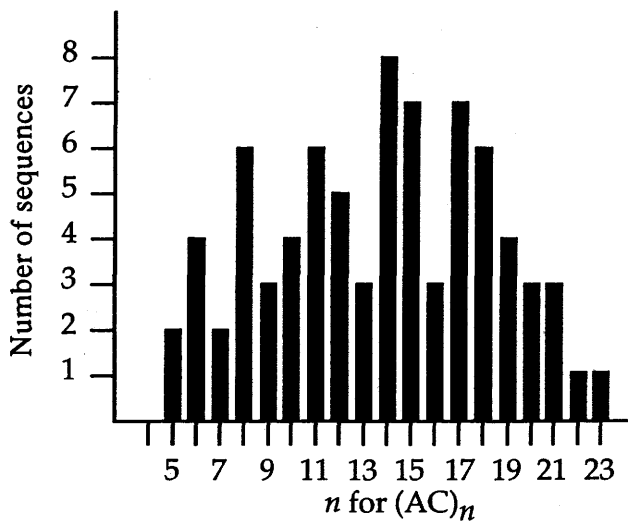
Table 2. SSR allele number, sizes, and observed heterozygosity among clones of 16 *P. strobus* individuals from the north-central United States Great Lakes region.

Locus	Repeat sequence	No. of alleles	Allele size range (bp)	Repeat unit no. (*n*)	H_o
RPS1b	$(CA)_n(GACACA)_3$	2	193–207	3, 10	0.125
RPS2	$(AC)_n$	4	149–171	8–19	0.312
RPS6	$(T)_6...(AC)_n...(T)_6$	4	159–164	13–15	0.500
RPS12	$(AC)_n$	11	163–209	5–29	0.812
RPS18	$(AC)_n...(A)_6$	4	162–166	12–16	0.500
RPS20	$(AC)_n(AT)_6$	8	138–174	16–34	0.625
RPS25b	$(AC)_nAG(AT)_9$	9	97–115	12–21	0.812
RPS34b	$(AC)_n$	3	145–149	14–16	0.500
RPS39	$(AC)_n$	2	172–174	17, 18	0.562
RPS50	$(AC)_n$	8	160–188	8–22	0.687
RPS60	$(AC)_n(AT)_7$	8	261–279	15–24	0.375
RPS84	$(CT)_{10}(AC)_n$	5	145–163	10–19	0.375
RPS90	$(AC)_n$	5	138–164, null	10–23	0.500
RPS118b	$(AC)_n$	7	148–164	15–23	0.687
RPS124	$(AC)_n$	4	147–153	10–13	0.500
RPS127	$(AC)_n(AT)_5$	2	194–196	10, 11	0.375
RPS150	$(GAG)_n$	1	248	4	0.0
RPS160	$(ACAG)_nAGGC$ $(AGAC)_n$	1	246	3	0.0

sequences ranging from 5 to 23 repeat units (Fig. 1), with an average repeat length of 13.6 units. Fifty-seven (73%) of the $(AC)_n$ repeats had $n > 10$. By standard definitions (Weber 1990), 11 (14%) of the repeat sequences were imperfect and 19 (24%) were compound. Sixteen of the compound repeats involved the sequence $(AT)_n$ and one each involved $(AG)_{10}$, $(CG)_6$, or $(ACCC)_3$.

Selection for $(ACAG)_n$ sequences was much less successful, with an enriched library frequency of 0.3% giving an enrichment factor of 22, based on the genome frequency estimate of one $(ACAG)_n$ every 1930 kbp for $n > 5$ (C.S. Echt and P. May-Marquardt, submitted for publication, see footnote 1). Only 30 clones were selected and sequenced and 13 fell into two repetitive sequence classes. For all $(ACAG)_n$ sequences, the longest uninterrupted repeat length was $n = 3$ and all repeats were either compound or imperfect.

Fig. 1. Length distributions of $(AC)_n$ in sequences obtained from enrichment cloning. Values for *n* correspond to the longest perfect repeat run.

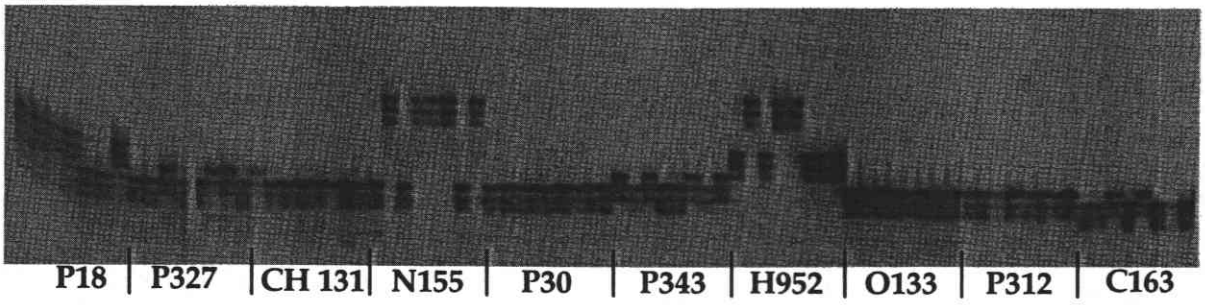


SSR marker diversity

Primer pairs were made from 70 of the $(AC)_n$ sequences and 7 of the $(ACAG)_n$ sequences. Under a single set of PCR conditions, 19 suitable markers were obtained (Table 1). The locus designation RPS is an abbreviation for "Rhinelander, *Pinus strobus*." A marker was deemed suitable when a single locus amplified efficiently at the expected size and with a minimum of stutter bands when assayed on silver-stained denaturing polyacrylamide gels. All markers reported in Table 1 contained the target SSR sequence, as determined by Southern hybridizations of the PCR products with oligonucleotide probes. The marker rps150, selected from the $(ACAG)_n$ library, also contained a $(GAG)_4$ repeat, which was listed as the main repeat sequence in Table 1 because of its longer repeat length.

Allelic diversity at 16 $(AC)_n$ loci was measured in 16 unrelated trees growing in the states of Wisconsin and Minnesota (Table 2). Heterozygosity was detected by determining the haploid genotype of eight megagametophytes from each tree. Examples of segregating alleles for a locus are shown in Fig. 2. All loci were polymorphic within the group of 16 trees examined. There was an average of 5.4 alleles per locus (86 alleles total), and observed heterozygosity values (H_o) ranged from 0.125 to 0.812, with an average value of 0.515. The two non-$(AC)_n$ loci, RPS150 and RPS160, were not polymorphic. There were five $(AC)_n$ loci, not among those listed in Table 1, whose PCR products differed in size by 10–34 bp from what was

Fig. 2. Segregation of alleles of locus RPS84 among megagametophytes of 10 eastern white pine trees. The smallest allele observed for this locus was 145 bp and contained 10 (AC) repeats (tree C163), while the longest allele was 163 bp and contained 19 repeats (trees N155 and H952). Spacing between the stutter bands was 2 nucleotides and the top-most fragment is the correct allele size based on the cloned insert sequence (obtained from tree P-312).



expected and all were monomorphic among the 16 trees examined.

Five loci were genotyped in the eastern white pine parent P-18 and all loci met Mendelian expectations for 1:1 segregation among the 72 megagametophytes scored. For loci RPS12, RPS50, RPS84, RPS90, and RPS118b, the respective χ^2 values were 0.00, 0.68, 0.00, 0.25, and 0.01. Linkage was found between RPS90 and RPS118b, giving a recombination fraction of 0.23 with a LOD of 4.32.

Discussion

The enrichment method described by Ostrander et al. (1993) worked well in white pine for $(AC)_n$ loci but not for $(ACAG)_n$ loci. $(ACAG)_n$ sequences were initially chosen for selection because they had previously been enriched with success from several mammalian genomes. Poor recovery of $(ACAG)_n$ from the white pine genome was probably due to the comparatively low genomic density of these sites and association of the sequence with repetitive DNA. This motif appears to be rare in plants in general, as it was not found in a DNA database survey (Wang et al. 1994). Given the success of the $(AC)_n$ enrichment, and the conversion of about 20% of $(AC)_n$ clone sequences to SSR markers, further development of selected classes of SSR markers is warranted in eastern white pine. Through application of screening methods to identify noninformative clones before sequencing, the sequence-to-marker success rate could be improved. For example, hybridization with labeled total genomic DNA could identify clones containing repetitive DNA (Smith and Devey 1994), while PCR screening of the inserts using various combinations of universal and SSR-specific primers could identify clones having the SSR site too close to an end of the cloned fragment. Approximately 12% of the clones we sequenced had the SSR run too close to an insert end to allow for suitable primer-pair selection.

Our initial nonenriched M13 library had a very low frequency (8×10^{-5}) of $(AC)_n$-bearing clones, apparently because most *Sau*3AI fragments carrying $(AC)_n$ sites exist in the 1–6 kbp range (Smith and Devey 1994; C.S. Echt, unpublished data) and were thus excluded from the 200–600 bp size selected fragments used for cloning. This points to the importance of characterizing restriction enzyme and SSR combinations by Southern blot analysis before SSR library construction. The combination of blunt end generating restriction enzymes we used for the enriched library did work for generating small $(AC)_n$-bearing fragments, but may need to be optimized for different SSR motifs.

The lower limit of the number of $(AC)_n$ repeat units was usually found at $n = 8$, although RPS12 had one allele with $n = 5$, assuming that the reduction in length of this allele was due entirely to loss of simple sequence repeat units. As seen with locus RPS39, the longer repeat lengths of $n = 18$ or $n = 17$ are not always highly variable, as is generally the rule with human SSRs (Weber 1990). In Table 2 the number of repeat units is given as a function of the $(AC)_n$ repeat, but in some compound repeats, other repeat motifs could have been involved, such as the $(AG)_{10}$ element of RPS84 or the $(AT)_9$ element of RPS25b. A high incidence of associated $(AT)_n$ sequences was also observed among $(AC)_n$ and $(AG)_n$ loci examined in radiata pine (Smith and Devey 1994), and the $(AT)_n$ motif may be the most common among plants (Wang et al. 1994). Loci RPS6 and RPS18 had alleles that differed by 1 nucleotide, a size variation not consistent with a step-wise model for dinucleotide repeat mutations (Valdes et al. 1993). These loci also contained $(A)_6$ repeats, however, so it is assumed that a mutation in the mononucleotide repeat was responsible for the unique allele sizes.

Smith and Devey (1994) examined allelic diversity at two $(AC)_n$ loci in 40 radiata pines (*P. radiata*) distributed among four populations. They found H_o values ranging from 0 to 0.85, with a mean of 0.625, and an average of 6 alleles per locus. These values are comparable to those we determined for *P. strobus*, where H_o ranged from 0.125 to 0.812, with a mean of 0.515, and an average of 5.4 alleles per locus. In contrast, isozyme diversity in eastern white pine is notably lower. Among 300 individuals distributed in 10 populations in Quebec, Canada, 18 isozyme loci had a mean H_o of 0.176 with an average of 1.96 alleles per locus (Beaulieu and Simon 1994*b*).

Segregation data was obtained for only five white pine SSR loci. All segregated normally and linkage was established between two markers (RPS90 and RPS118b). Preliminary genome mapping has placed all five SSR loci

in linkage groups in a RAPD marker map of eastern white pine (C.S. Echt, unpublished data). The abundance, ease of genotyping, and high allelic diversity found for $(AC)_n$ loci in pines should make them useful markers for genome mapping and population genetics studies.

The SSR marker detection system we describe, using silver staining of denaturing polyacrylamide gels, is quick and convenient. We calculate that the cost of reagents used for a single silver staining reaction, plus one half sheet of APC film for gel documentation, amounts to $US 11. If two gels are processed in a single staining reaction and digital imaging is used for documentation, then the cost is reduced to $US 4.50. The use of sharktooth combs having a 4×-offset microtitre format allows 133 sample lanes (not including standards) to be loaded on a 33 cm wide gel, and by loading two or more markers of different size ranges in each lane, over 500 genotypes could be generated per day. This system could thus generate data economically and at sufficient rates to support most genetic studies.

Although we were not successful in obtaining markers based on $(ACAG)_n$ repeats, it is possible that other tri- and tetra-nucleotide repeats will yield usable markers. The next step is to perform enrichment cloning for abundant tri- and tetra-nucleotide SSR motif clones and to learn whether these repeats are as informative as those of $(AC)_n$. The advantage of the longer repeat SSRs is that they are more amenable to both automated and manual analysis than are the dinucleotide repeats, in large part owing to reduction or elimination of stuttering artifacts (Hauge and Litt 1993; Edwards et al. 1991).

Conservation of SSR loci has been observed among mammalian genomes (Blanquer-Maumont and Crouau-Roy 1995; Moore et al. 1991) and among *Citrus* species (Kijas et al. 1995). This raises the possibility that some white pine SSR markers could be used in other conifers, thus benefiting conifer SSR marker development efforts and extending the potential uses of the markers to comparative genome mapping. Preliminary data from our laboratory, however, indicate that most SSR primers can only be expected to amplify markers among members of the same pine subgenus, and will have low success rates when used in more distantly related species. It is our opinion that the best strategy for pine SSR marker development is to develop markers for soft pines (subgenus *Strobus*) and hard pines (subgenus *Pinus*) separately. The use of white pine SSR markers for comparative genome mapping across the genus is thus probably quite limited, but should prove useful among closely related species. No single marker system is optimally suited for all studies, however, and the use of SSR markers would be expected to be augmented by other types of markers as the study dictates.

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