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Development of microsatellite markers for white spruce (*Picea glauca*) and related species

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Abstract We report the development of 13 primer pairs that allow the unambiguous amplification of 15 microsatellite (SSR) loci in white spruce (*Picea glauca*). Fourteen of these loci were polymorphic in trees sampled at three geographically separated regions of western Canada. Segregation analysis carried out on these loci confirmed a Mendelian inheritance pattern for all except two, which showed significant segregation distortion. All of these primer pairs amplified SSR loci in at least one of the other *Picea* species tested [black spruce (*P. mariana*), red spruce (*P. rubens*), Norway spruce (*P. abies*), Colorado spruce (*P. pungens*), sitka spruce (*P. sitchensis*) and Engelmann spruce (*P. engelmannii*)]. Given the important commercial and ecological roles of these species, this set of markers will be invaluable for their management, the improvement of commercially important traits, and the study of their ecology and genetics.

Keywords *Picea* · Microsatellites · SSR · PCR

Introduction

The genus *Picea* consists of 40 species, of which seven are native to North America and five to Canada (Farrar 1995): red spruce (*Picea rubens* Sarg.) in the east, sitka spruce [*Picea sitchensis* (Bong.) Carrière] and Engelmann spruce (*Picea engelmannii* Parry ex Engelm.) in the west, and two transcontinental species, white spruce [*Picea glauca* (Moench) Voss] and black spruce [*Picea mariana* (Mill.) BSP.]. All five species are ecologically and economically important in Canada; white and black spruce are key components of the boreal forest, while red spruce is a dominant species of the Acadian forest region in eastern Canada. White and Engelmann spruce, along with their natural hybrids [which are sometimes known collectively as interior spruce (Krajina et al. 1982; Coates et al. 1994)], are economically and ecologically important in Alberta and British Columbia. Sitka spruce is a major commercial species in coastal British Columbia. Species of *Picea* are by far the most-commonly planted trees in Canada (Kuhnke 1989). In 1997, spruce accounted for more than half of the tree-seedlings planted in re-forestation efforts in Canada [3.8×10^8 seedlings (National Forestry Database 2000)]. Introgressive hybridization between white and Engelmann spruce (La Roi and Dugle 1968; Daubenmire 1974; Krajina et al. 1982) and between red and black spruce (Gordon 1976; Bobola et al. 1996; Perron and Bousquet 1997) is of ecological and evolutionary interest and is an important issue in the genetic resource management and improvement of these species (Gordon 1976; Sutton et al. 1991, 1994).

Given the importance of these species, genetic improvement programs have been initiated in Canada for white, black, interior and sitka spruce (Reid and Crown 1995; Morgenstern 1998; Park et al. 1998; Simpson 1998), as well as for the non-native Norway spruce [*Picea abies* (L.) Karst] (Morgenstern 1998; Park et al. 1998; Simpson 1998). However, tree improvement programs can be time-consuming because trait expression is delayed in the phenotype (Zobel and Talbert 1991). For most traits of commercial value, early selection of individual trees only becomes efficient half-way through rotation, so molecular-marker-assisted selection has been proposed as a means of accelerating the rate of genetic improvement (Tuskan 1992). In fact, mapping of the quantitative trait loci responsible for the mature wood density of eastern white spruce (Bousquet et al. 1994) and black spruce (Bousquet, personal communication) is already underway.

Several biochemical and molecular markers have previously been used to examine various aspects of the evo-

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lution and ecology of spruce (as reviewed by Khasa and Dancik 1996). DNA-based marker systems using the polymerase chain reaction [which are now available (Mullis et al. 1994)] offer advantages over previously employed techniques; they have become an attractive option in molecular taxonomy studies and in tree-breeding and improvement programs. The first to be used were the RAPD markers; these have recently been reported to differentiate between black and red spruce (Perron et al. 1995) and between white and Engelmann spruce (Khasa and Dancik 1996). RAPD markers are easy to develop but have limited usefulness for many purposes (Haymer 1994). More recently, expressed-sequence-tag polymorphisms (ESTPs) have been developed for black spruce and related species. However, their relatively low levels of polymorphism [comparable with those of allozymes (Perry and Bousquet 1998a, b, 2001)] restrict their utility in population genetic studies, especially in species with low allozyme variation. Microsatellites (simple sequence repeats, SSRs) are highly polymorphic, codominant markers that have proven useful for a variety of purposes including finger-printing (Smith and Devey 1994), studying population genetics (Haymer 1994; Tsumura et al. 1996; Thomas et al. 1999), assessment of parentage and dispersal (Dow et al. 1995), and examining the effects of domestication (Mörchen et al. 1996).

In this paper, we report the development of 15 microsatellite markers in spruce. The loci are polymorphic and reliably amplified in up to seven species. Further, the codominant alleles at the 13 loci that we tested segregated from one another in maternally derived haploid megagametophytic tissue. These markers are, therefore, well suited for use in population genetic studies, forensics, evolutionary and ecological research, and genetic improvement programs.

Materials and methods

Tissue collection

Needle tissue was collected from 15 white spruce trees in three geographically separated locations within the provinces of Alberta and Saskatchewan, Canada. The tissue was stored at −70°C over the course of the project. Cones were collected from the same trees; seeds from each cone were separately extracted, then stored at 4°C until used as a source of the megagametophytic tissue needed to test for Mendelian inheritance of the SSR loci. The eight samples of Engelmann spruce were collected at one location in Idaho, the six samples of sitka spruce at locations in coastal British Columbia, the eight samples of Colarado blue spruce from planted specimens on the campus of the University of Alberta, the eight samples of black spruce from three locations in Ontario, the eight samples of Norway spruce from a provenance trial established at Hudson's Place in Ontario, and the eight samples of red spruce from a provenance trial in Valcartier, Quebec.

Isolation of genomic DNA from needle tissue

Method 1. To prepare DNA for amplification, approximately 1 g of needle tissue was ground to a fine powder in liquid nitrogen. This powder was dissolved in 6 ml of lysis buffer [1.4 M NaCl, 1 M LiCl, 100 mM Tris–HCl (pH 8.0), 20 mM EDTA, 2% CTAB and 1% PEG]. The tube was gently inverted ten times, then incubated at 65°C for 45 min, with occasional gentle agitation. After incubation, insoluble debris was removed by centrifugation at 1500 g for 15 min; the supernatant was extracted twice with 1 vol of cold chloroform. Following the addition of 6 ml of iso-propanol, the DNA was precipitated overnight at −20°C, then recovered by centrifugation, as described above. The pellet was washed with 6 ml of 70% ethanol, allowed to air dry and re-suspended in 150 l of sterile distilled water. DNA obtained by this method from any of the spruce species was readily amplified.

Method 2. To constrct the genomic library (see below), DNA was extracted from a 10-g sample of needle tissue according to Murray and Thompson (1980) with modifications. A liquid nitrogen powder of the tissue was re-suspended in 200 ml of 50 mM Tris-HCl (pH 8.0), 5 mM of EDTA, 0.35 M sorbitol, 0.1 mg/ml of BSA and homogenized in a polytron. The mixture was filtered through two layers of cheese-cloth and the organelles were collected by centrifugation at 6500 g for 20 min. The pellet was re-suspended in 5 ml of 50 mM Tris-HCl (pH 8.0), 5 mM of EDTA, and 0.35 M sorbitol to which 1 ml of 5% N-lauryl sarcosine was added. Following incubation at room temperature for 60 min, 1 ml of 5 M NaCl and 0.8 ml of 10% CTAB in 0.7 M NaCl were added and the extract was incubated at 60°C for 10 min. An organic extraction was carried out with chloroform-octanol (24:1); the aqueous phase was then subjected to CsCl equilibrium gradient centrifugation at 53000 rpm in a Beckman VTi65 rotor for 16 h. The ethidium bromide was removed by successive butanol extractions (Sambrook et al. 1989) and the sample dialyzed into 10 mM of Tris-HCl (pH 8.0) and 1 mM of EDTA.

Isolation of megagametophyte DNA

Megagametophyte tissue was dissected from seeds soaked in distilled water overnight and homogenized in 100 µl of 50 M Tris-Cl (pH 8.0), 25 mM of EDTA, 0.35% sorbitol, 0.1% β-mercaptoethanol, 1% sarcosyl, then incubated at room temperature for 10 min. Forty microliters of 5 M NaCl and 30 µl of 8.6% CTAB in 0.7 M NaCl were added and the mixture was incubated for 30 min at 65°C. Two phenol/chloroform (1:1) and one chloroform extraction were performed and the DNA was precipitated in 95% ethanol, washed with 70% ethanol, dried and finally re-suspended in 30 µl of distilled H_2O.

SSR libraries and primer design

Two-hundred clones, from either an *Hae*III or *Alu*I white spruce genomic library enriched for AG and AC motifs, were obtained from Dr. Craig Newton (BC Research, Inc.). The DNA used for the construction of the library was isolated by Method 2 (above) and the inserts were ligated into the pGEM3Z+ vector (Promega). Sixty independent clones were sequenced to confirm the existence of microsatellite arrays; 56 of them contained the expected di-nucleotide SSR. In 16 clones, there was insufficient flanking sequence to permit the design of primers; of the remaining 40 clones, primers for 34 were designed using the software package DNAMAN 4.11 (Lynnon BioSoft, Inc). Optimization of these primer pairs was carried out on DNA extracted from needle tissue by Method 2 above.

Amplification and detection of microsatellite loci

Both radioactive and non-radioactive methods for detecting the amplification products of the SSR loci were employed. In the former, forward primers were 5′ end-labeled using T4 polynucleotide kinase (Gibco BRL) and 5 µl of ^{32}P dATP (Amersham, 3000 Ci/mmol). Amplifications were carried out in a volume of 10 µl that included 1 U of Taq polymerase, 0.4 µM of forward

(labeled) and reverse primers, 2 mM of $MgCl_2$ and 1 µl of 10× buffer [500 mM KCl, 100 mM Tris-HCl (pH 8.3), 2 mg/ml BSA] and 2 mM of each dNTP. The PCR conditions were as follows: a pre-incubation at 94°C for 5 min was followed by 25 cycles consisting of 30 s at 94°C (denaturation), 30 s at the appropriate annealing temperatures (see Table 1) and 30 s at 72°C (extension). Reactions were stopped using 10 µl of loading buffer [0.3% bromophenol blue and xylene cyanol FF, 10 mM EDTA (pH 7.5), 97.5% de-ionized formamide] and stored at –20°C. The products were fractionated on 6% denaturing polyacrylamide gels containing 7 M urea; these gels were then dried and exposed to X-ray film (Kodak). Products were sized using a molecular-weight ladder generated from a sequencing reaction of a known DNA sequence.

Amplified SSR products that were produced using non-radioactive primers were fractionated on denaturing acrylamide gels as described above and visualized on fixed gels by staining with silver nitrate [Promega: Silver Sequence Staining protocol as modified by Echt et al. (1996)].

Results and discussion

In a previous study of lodgepole pine (Hicks et al. 1998), we found that only 25% of the clones that were identified as positives in a hybridization screen of a non-enriched library actually contained a SSR. In contrast, 96% of the random set of clones that we sequenced from the enriched library used in this study contained a SSR, which indicates the utility of an enrichment process. These results corroborate those reported earlier for pine (Echt et al. 1996) and larch (Khasa et al. 2000). Table 1 shows the sequences and optimal annealing temperatures of the 11 primer pairs that were derived from the sequencing of 34 clones from the library. In addition to a di-nucleotide locus (UAPgTG64), one of these clones contained a tri-nucleotide repeat (UAPgGAT64). Another locus (UAPgAC/AT6) contained a complex SSR with a mixed repeat motif, a phenomenon encountered rather commonly in conifers (Echt et al. 1996; Pfeiffer et al. 1997; Hicks et al. 1998). One additional locus, UAPgCT3, was isolated from a non-enriched white spruce library (Cobban 1997); the final locus, UAPsTG25, was isolated from sitka spruce (Van de Ven and McNicol 1996). All 13 primer pairs allowed reliable amplification in white spruce of the SSR motifs shown

Table 1 Properties of the white spruce microsatellite loci: primer sequences, optimum annealing temperatures, size and number of alleles, and observed heterozygosity

Locus name (motif)	Primer sequences (5′-3′) (F-forward; R-reverse)	Annealing temp. (°C)	Allele range (bp)	# of alleles (# trees scored)	H_O[a]
UAPgCT3 (CT_{15})	F-TTGAAAAAGAGGTTAGGAAGGGA R-TTCTTAAAGAAGCAGGGCATTG	60	238–304	11 (11)	0.73
UAPgAC/AT6 (AC_{10}/AT_7)	F-GTTTGGAGAGATAGAGATTGTAC R-TTTTTGACGGCTGGAAACTTC	60	114–128	6 (17))	0.94
UAPgGT8 (GT_{22})	F-AATGCTTGGTGCATAAGG R-AACACTGTGGTTCTTCCG	58	204–230	8 (8)	0.50
UAPgCA24 (AC_{23})	F-ATGCTCTTCTTAACCACCTG R-GACAATTCCTACCTCCACAC	55	201–285	11 (11)	0.64
UAPsTG25 (TG_{27})	F-TCAAGCTCTCCAACCCAGAT R-TGTCGAGTTTGACTTGTACCAA	62	94–100	32 (11)	0.45
UAPgTG64 (TG_{16})	F-AATTTCCTTCCTCTATGTCGAC R-CAATATGATGTGTAATTCCTTCC	56	104–134	11 (17)	0.35
UAPgGAT64 (GAT_8)	F-TGTTAAATAAGGAAGGAATTACAC R-CACTTACCCTCTTCAGGTCC	57	86–101	3 (18)	0.33
UAPgTG87 (TG_{30})	F-GCACCAATAATCAAATCATGCC R-TTTGGAACACTACACATCAACC	60	110–200	18 (14)	0.79
UAPgCA91 (CA_{20})	F-TCTGTTCTTCATACGTCTCAC R-GGAAATTGGCACTCTGTATTC	60	118–158	8 (10)	0.40
UAPgAG105 (AG_{11})	F-CAACTACCTTGAGCCAATCA R-GTCCGGCATTATTGATCATT	56	167–175	7 (15)	0.73
UAPgCT144 (CT_{18})	F-CACTCGATCACTTTCTCATC R-CAAGATAGTAATGGTGAGGC	58	134–180	10 (16)	0.44
UAPgAG150 A (AG_{19})	F-ACCAATGCTTTTACCAAACG R-TTGATTGCAAGTGATGGTTG	54	150–164	8 (17)	0.41
UAPgAG150B	As above	54	124–132	5 (18)	0.39
UAPgCT189 A (CT_{23})	F-TGCACTCCTTGCGGAAATTCTTC R-GTTGTTGACTAAGGTTGAAGGGG	65	138–208	14 (13)	0.54
UAPgCT189B	As above	65	114	1 (15)	0.00

[a] H_o (observed heterozygosity) is the fraction of heterozygotes in the sample

Table 2 Segregation analysis of megagametophytic tissue for 13 microsatellite loci in *P. glauca* heterozygotes

Locus	Maternal parent genotype (A/B)	Progeny genotypes		Chi-square (χ^2)
		No. A alleles	No. B alleles	
UAPgAG3	304/250	16	13	0.31
UAPgAC/AT6	127/125	13	17	0.53
UAPgGT8	218/212	10	9	0.05
UAPgAC24	247/227	23	24	0.02
UAPgTG25	100/96	13	19	1.13
UAPgTG64	124/108	15	13	0.14
UAPgGAT64	89/86	13	16	0.31
UAPgTG87	157/141	12	12	0
UAPgCA91	128/118	14	20	1.06
UAPgAG105	175/163	18	33	4.41[a]
UAPgCT144	180/160	8	9	0.06
UAPgAG150 A	156/150	7	20	6.26[a]
UAPgAG150B	130/126	9	9	0
UAPgCT189 A	178/156	13	12	0.04

[a] Significant deviations between observed and expected values occur when $\chi^2 \geq \chi^2_{0.05[1]}=3.84$

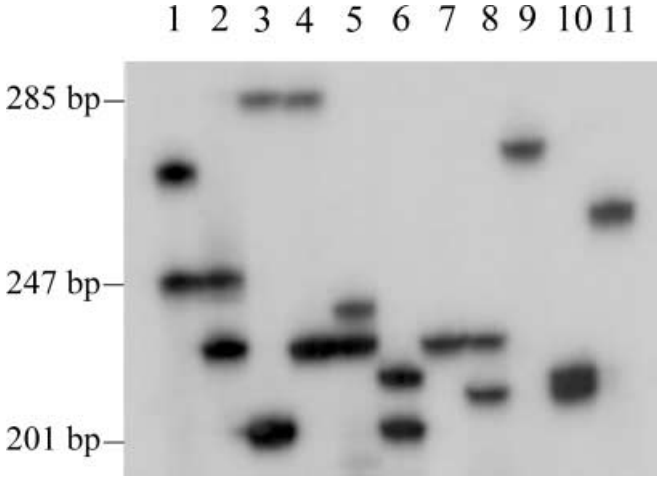

Fig. 1 The genotypes of 11 trees from the test sample shown for the SSR locus UAPgCA24. *Lanes 1–6* comprised DNA from needle tissue collected from individuals in one geographic location, while the DNA in *lanes 7–11* was obtained from trees in a second location, which was genetically isolated from the first. The alleles were visualized using radioactive primers as described in the Materials and methods

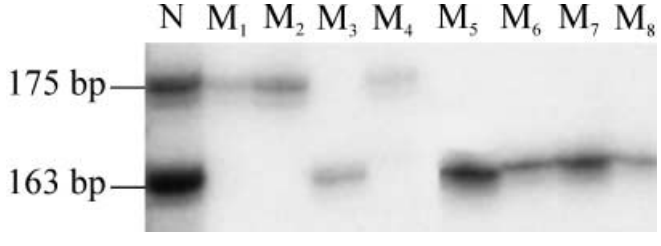

Fig. 2 Segregation analysis at the SSR locus AUPgAG105. DNA was obtained from needle tissue (*N*) and haploid gametophytic tissue (M_1–M_8) of a tree heterozygous for alleles of 175 bp and 163 bp

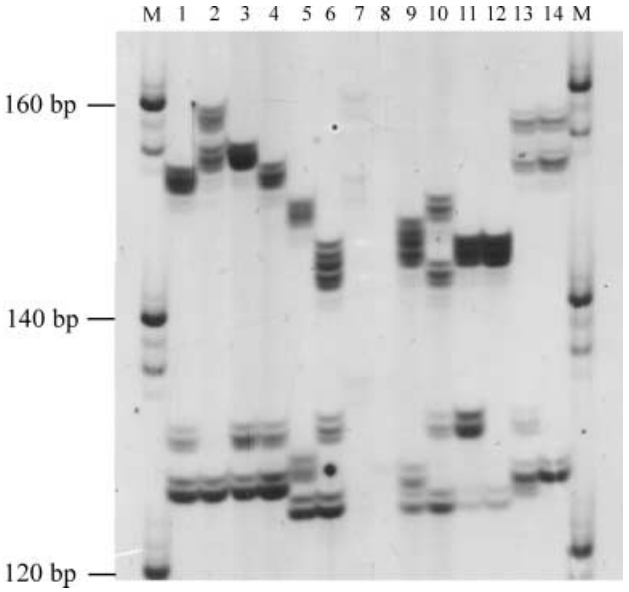

Fig. 3 Cross-species amplification of the UAPgAG150A,B loci. DNA samples were amplified from two trees of white (*lanes 1*, *2*), Engelmann (*3*, *4*), black (*5*, *6*), Colorado (*7*, *8*), Norway (*9*, *10*), red (*11*, *12*), and sitka (*13*, *14*) spruce. M is a DNA sizing marker

in column 1 of Table 1. In several instances, spurious amplification products were produced, but they fell well-outside the range of alleles encountered in our sample populations of *P. glauca*. With the exception of UAPgTG87, UAPgCT144 and UAPgCT189 A, the loci exhibited few of the stutter bands usually ascribed to polymerase slippage (Tautz 1989) and commonly associated with the amplification of SSR loci.

The primers in Table 1 were retained because all amplified polymorphic loci in the set of 15 trees that comprised our three sample populations. For every locus, unambiguous genotypes could be assigned to trees in the test set. Figure 1 shows an example in which the DNA from 11 trees was amplified to display the alleles at UAPgAC24. Three homozygotes (lanes 7, 9 and 11) and eight heterozygotes were identified in this sample. The size range and number of alleles, found at all 15 loci in the sample of trees studied are shown in Table 1. The primer pairs that amplified the loci UAPgAG150A and

Table 3 Cross-species amplification in spruce using 13 SSR primer sets. Note**:** n, sample size; AP, amplification pattern: ++, strong amplification; +, weak amplification; 0, no amplification; mb, multiple bands. mNa, minimum number of alleles detected; SR, size range detected (base pairs)

Locus		Engelmann spruce (*n*=8)	Red spruce (*n*=8)	Black spruce (*n*=8)	Sitka spruce (*n*=6)	Norway spruce (*n*=8)	Colorado blue spruce (*n*=8)
UAPgCT3	AP	++	+	++	+	++	+
	mNa	7	5	6	2	6	3
	SR	248–296	206–214	206–228	220–232	204–227	204–222
UAPgAC/AT6	AP	++	++	++	++	++	+
	mNa	4	1	3	1	2	3
	SR	114–122	120	118–122	114	118–120	114–124
UAPgGT8	AP	++	0	0	0	++	+
	mNa	7				7	2
	SR	194–224				198–236	188–218
UAPgCA24	AP	+	++	+	0	++	+
	mNa	3	6	1		7	1
	SR	198–224	184–212	246		180–310	212
UAPsTG25	AP	++	++	++	++	++	++
	mNa	6	3	5	2	5	3
	SR	94–118	94–106	94–118	96–104	94–102	96–102
UAPgTG64	AP	++	++	++	+	++	0
	mNa	7	2	4	2	3	
	SR	86–138	114–144	158–168	104–106	128–144	
UAPgGAT64	AP	++	++	++	++	++	+
	mNa	8	2	2	1	2	2
	SR	85–108	102–112	102–117	102	105–108	99–102
UAPgTG87	AP	mb	mb	mb	mb	mb	Mb
	mNa						
	SR						
UAPgCA91	AP	++	++	++	++	++	+
	mNa	7	7	2	5	3	1
	SR	132–160	114–132	112–114	108–126	108–122	114
UAPgAG105	AP	++	++	++	++	++	+
	mNa	3	2	6	2	2	2
	SR	157–167	155–157	157–167	159–161	155–159	159–160
UAPgCT144	AP	++	++	++	++	++	++
	mNa	6	4	3	5	4	3
	SR	132–183	148–154	168–178	132–164	130–166	136–156
UAPgAG150 A	AP	++	++	++	++	++	+
	mNa	2	1	4	2	5	5
	SR	151–153	145	143–149	153–157	142–149	143–162
UAPgAG150B	AP	++	++	++	++	++	+
	mNa	4	2	4	2	4	4
	SR	116–130	124–130	124–130	126–130	124–130	124–132
UAPgCT189 A	AP	++	0	+	++	0	0
	mNa	7		1	3		
	SR	138–186		136	134–148		
UAPgCT189B	AP	++	++	++	++	++	+
	mNa	1	2	1	2	2	1
	SR	114	114–116	118	114–116	114–116	114

UAPgCT189A each amplified a second locus, for which the repeat motifs were not ascertained. One of these loci, UAPgAG150B, was polymorphic whereas the other, UAPgCT189B, was monomorphic. The number of alleles/locus in our small sample of trees ranged from 1 to 18, with those loci whose amplification was associated with stutter bands (see above) tending to exhibit the largest number of alleles. The levels of observed heterozygosity (H_O) fell between 0.33 and 0.94 ($\bar{x}$=0.52). High levels of heterozygosity appear to characterize the SSR loci of conifers in general (Echt et al. 1996; Thomas et al. 1999).

Of the polymorphic loci, the tri-nucleotide locus UAPgGAT64 exhibited the fewest alleles. This locus is very tightly linked with UAPgTG64; the two comprise

what has been termed a juxtaposed microsatellite system (JMS). Several systems of this kind have been reported in humans (Hanis and Bertin 1992; Chakraborty et al. 1994; Pena et al. 1994). In the dystrophin gene, significant linkage dis-equilibrium has been observed; this dis-equilibrium could make the JMS useful in prenatal diagnosis and carrier detection (Chakraborty et al. 1994). Estoup et al. (1999) have commented on the use of JMS polymorphisms in estimating genetic admixture and have reported an empirical assessment of their utility in brown trout (Estoup et al. 2000). In the present context, the JMS locus that we have detected could be useful in forensic applications.

Segregation analyses of the SSR loci were carried out on the haploid megagametophytic tissue obtained from the seeds of heterozygous trees. Figure 2 shows a typical result in that each megagametophyte exhibits just one of the two alleles present in the heterozygous parent. Locus UAPgCT189B could not be tested because it was monomorphic in our sample of trees (Table 1). We used the chi-square test with 1 *df* (Sokal and Rohlf 1981) to detect deviation from the 1:1 ratio expected for a Mendelian trait. The data for all the loci tested generally confirm a 1:1 segregation of the two alleles (Table 2). Locus UAPgAG105 (with the 175/163 allelic combination) and locus UAPgAG150A (with the 156/150 allelic combination) showed significant segregation distortion. Such deviations can be caused by a number of factors, including meiotic drive, selection between meioses, linkage to a deleterious allele at another locus or experimental error.

All of the 13 SSR primers listed in Table 1 amplified white spruce DNA; most successfully amplified SSR loci in several of six other related species – Engelmann spruce, Colorado spruce (*Picea pungens* Engelm.), sitka spruce, black spruce, red spruce and Norway spruce. Figure 3 shows the products of the two loci amplified by the primer pair UAPgAG150. For all seven species, alleles of the A locus fell between 140 and 160 bp, and those of the B locus between 120 and 140 bp. Both loci were readily detected in every species except Colorado blue spruce (lanes 7, 8), for which a longer exposure of the gel was required before unambiguous genotypes could be assigned. Further optimization of this primer pair for use in this species was not attempted.

The complete data set of the amplification results using the 13 SSR primer pairs on the six additional species is included in Table 3. With the exception of locus UAPgTG87, whose amplification in all six species produced a number of bands that could not be interpreted, every locus was amplified well in at least two species. The primers that amplified UAPsTG25 and UAPgCT144 amplified these loci unambiguously in all the species tested. The cross-taxa amplification of SSR loci at the sub-genus level, which we have observed, appears to be widespread in plants (Thomas and Scott 1993; Dayanandan et al. 1997; Gullberg et al. 1997; Khasa et al. 2000). We expect that additional cross-taxa amplification of at least some of the SSRs that we have described will occur within spruce. The most-likely candidates are the two highly conserved loci, UAPsTG25 and UAPgCT144. In fact, we attempted to amplify these loci in two other genera of the conifera. DNA from at least five trees of lodgepole pine (*Pinus contorta* var. *latifolia*), western larch (*Larix occidentalis*), and alpine larch (*Larix lyallii*) were subjected to amplification using these primers. Lodgepole pine DNA failed to amplify; about one-half of the alpine larch samples produced a weak amplification product but most of the western larch samples yielded an unambiguous SSR banding pattern. These results suggest that the utility of the primers we describe here is probably restricted within the conifers but that their reliability, confirmed Mendelian inheritance and their high degree of polymorphism will make them very useful for applied and theoretical studies within the related North American, European and Asian spruce species.

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