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Diversity and inheritance of inter-simple sequence repeat polymorphisms in Douglas-fir (*Pseudotsuga menziesii*) and sugi (*Cryptomeria japonica*)

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Abstract We studied inter-simple sequence repeat (ISSR) polymorphism and inheritance in Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] and sugi (*Cryptomeria japonica* D. Don) megagametophytes using primers that anneal to simple repeats of various lengths, sequences, and non-repetitive motifs at the 5′ and 3′ ends. Products were visualized on agarose gels with ethidium bromide staining. More than 60% of the 96 primers tested gave interpretable banding patterns in both Douglas-fir and sugi, and the useful primers were in complete agreement among species. Dinucleotide repeat primers were the majority of those tested, and gave all of the useful banding patterns. The 24 best primers were used for segregation studies, yielding a total of 77 loci distributed among two Douglas-fir families and one sugi family. Approximately 90% of the 24 primers showed polymorphism within at least one of the three families. The average number of variable loci per primer was 1.6. Primers based on $(AG)_n$ repeats gave the largest number of polymorphic loci; 16 primer-family combinations yielded 24 segregating loci. However, primer based on $(GT)_n$ repeats gave the most loci per primer studied (mean of 2.0). All markers displayed apparent dominance (band presence vs absence), and all but three segregation ratios (4%) fit Mendelian expectations. Because they employ longer primers than do RAPDs, have a high degree of polymorphism, conform well to Mendelian expectations, and do not require use of acrylamide gels for analysis, ISSRs may be useful markers for PCR-based genome maps and population studies of conifers.

Key words RAPD · Repetitive DNA · Genome mapping · Microsatellite DNA · Conifers

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Introduction

Simple sequence repeats (SSRs) or microsatellites are abundant in higher eukaryotic genomes. SSRs are tandem repetitive DNA sequences with a repeat length of short motifs (1–5 base pairs) (Litt and Luty 1989; Weber and May 1989; Hamada et al. 1982). SSRs have been proven to be highly informative and to be distributed throughout genomes. Because of their short repeat length and limited iteration at individual loci, they can be readily studied via polymerase chain reaction (PCR)-amplification (Moore et al. 1991; Schlotterer et al. 1991; Lagercrantz et al. 1993; Saghai Maroof et al. 1994). Repeat-anchored primers that amplify regions between SSRs (referred to as inter-simple sequence repeats: ISSRs) are also useful for detecting genetic polymorphism, and have been used to fingerprint closely related individuals (Zietkiewicz et al. 1994). In contrast to methods that amplify SSRs on the basis of primers for flanking singer-copy DNA, ISSR primers anneal directly to SSRs and thus require no prior knowledge of target sequences.

Genomes have been mapped in conifers by means of restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA (RAPD) (reviewed in Neale and Harry 1994; Nelson et al. 1993; Mukai et al. 1995). RFLPs are limited by their slowness, the need for clone banks, and the requirement for large amounts of DNA, the latter precluding megagametophyte-based mapping. In contrast, RAPDs have been widely adopted because of their speed, lack of need for clone banks, and requirement for very little DNA,

enabling megametophyte-based mapping. However, RAPDs also present problems for genome mapping. They often do not show simple Mendelian inheritance (Echt et al. 1992; Halward et al. 1992; Reiter et al. 1992), results can be difficult to replicate, and maps from different families or species are hard to align (Neale and Harry 1994). Because of the length of ISSR primers, they may show greater repeatability and stability of map position in the genome when comparing genotypes within species. In the present paper we show that ISSRs, even when studied in agarose gels without radioactive labeling, have high levels of polymorphism and reliable Mendelian segregation in sugi and Douglas-fir, conifer species important in Japanese and American forestry.

Material and methods

Seed samples and DNA extraction

Open-pollinated seeds from three mother trees, hereafter referred to as families, were analyzed: families include Midori 5 of sugi from Japan, and S31t20 and S45t11 of Douglas-fir. S31t20 is a member of the coastal race of Douglas-fir (variety *menziesii*) from Fish Lake in the western Cascades Mountains of Oregon, USA (Sorensen 1979); S45t11 is a member of the north interior race (variety *glauca*) from Grouse Creek, Idaho, USA.

Seeds of sugi were soaked overnight in a 3% H_2O_2 solution, placed on wet filter paper in sterile dishes, and incubated for 3 weeks at 25 °C. Megagametophytes were extracted from each seed when germination began and the seed coat had broken. DNA was extracted with a modified SDS method: megagametophytes were placed in 200 µl of extraction buffer (0.1 M Tris-HCl pH 8.0, 10 mM EDTA, 0.5% SDS, 0.1 mg proteinase K), homogenized with a Minitor homogenizer, incubated at 37 °C for 2 h, then extracted with TE-saturated phenol. RNase was added to a final concentration of 50 µg/ml, incubated at 37 °C for 1 h, then extracted twice again with TE-saturated phenol. DNA was precipitated with a one-tenth vol of 3 M NaOAc and 2.5 vol of cold ethanol, washed twice with 70% ethanol, then dissolved in 200 µl of TE. Seeds of Douglas-fir were soaked in distilled water overnight before megagametophytes were removed from the seeds. Megagametophyte DNA was then extracted with a modification of the CTAB protocol of Wagner et al. (1987) in which the DNA was further purified by four phenol : chloroform : isoamyl alochol (25 : 24 : 1) extractions and a final ethanol precipitation.

Oligonucleotide primers

We examined 96 SSR primers from the University of British Columbia Biotechnology Laboratory primer set 9 during our initial screen (Fig. 1). Primers were mostly 16- to 17-mers. Twenty-four of these primers that gave clear banding patterns were used to study segregation.

PCR amplification and electrophoresis

PCR amplification was performed in 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 1.3% BSA, 0.2 mM dNTPs, 2% formamide, 1 µM of primer, 1 unit of *Taq* polymerase (Promega), and 10 ng of genomic DNA per 20-µl reaction. Initial denaturation was for 7 min at 94 °C, followed by 45 cycles of 30 s at 94 °C, 45 s at 52 °C, 2 min at 72 °C, and a final 7-min extension at 72 °C. PCR was performed in a PCT-100 thermocycler (MJ Research). PCR products were analyzed on 2% agarose gels in 1 × TBE buffer (Maniatis et al. 1982), then stained with ethidium bromide, and scored for band presence or absence.

Dinucleotide motifs

801 ATA TAT ATA TAT ATA TT
802 ATA TAT ATA TAT ATA TG
803 ATA TAT ATA TAT ATA TC
804 TAT ATA TAT ATA TAT AA
805 TAT ATA TAT ATA TAT AC
806 TAT ATA TAT ATA TAT AG
807 AGA GAG AGA GAG AGA GT
808 AGA GAG AGA GAG AGA GC
809 AGA GAG AGA GAG AGA GG
810 GAG AGA GAG AGA GAG AT
811 GAG AGA GAG AGA GAG AC
812 GAG AGA GAG AGA GAG AA
813 CTC TCT CTC TCT CTC TT
814 CTC TCT CTC TCT CTC TA
815 CTC TCT CTC TCT CTC TG
816 CAC ACA CAC ACA CAC AT
817 CAC ACA CAC ACA CAC AA
818 CAC ACA CAC ACA CAC AG
819 GTG TGT GTG TGT GTG TA
820 GTG TGT GTG TGT GTG TC
821 GTG TGT GTG TGT GTG TT
822 TCT CTC TCT CTC TCT CA
823 TCT CTC TCT CTC TCT CC
824 TCT CTC TCT CTC TCT CG
825 ACA CAC ACA CAC ACA CT
826 ACA CAC ACA CAC ACA CC
827 ACA CAC ACA CAC ACA CG
828 TGT GTG TGT GTG TGT GA
829 TGT GTG TGT GTG TGT GC
830 TGT GTG TGT GTG TGT GG
831 ATA TAT ATA TAT ATA TYA
832 ATA TAT ATA TAT ATA TYC
833 ATA TAT ATA TAT ATA TYG
834 AGA GAG AGA GAG AGA GYT
835 AGA GAG AGA GAG AGA GYC
836 AGA GAG AGA GAG AGA GYA
837 TAT ATA TAT ATA TAT ART
838 TAT ATA TAT ATA TAT ARC
839 TAT ATA TAT ATA TAT ARG
840 GAG AGA GAG AGA GAG AYT
841 GAG AGA GAG AGA GAG AYC
842 GAG AGA GAG AGA GAG AYG
843 CTC TCT CTC TCT CTC TRA
844 CTC TCT CTC TCT CTC TRC
845 CTC TCT CTC TCT CTC TRG
846 CAC ACA CAC ACA CAC ART
847 CAC ACA CAC ACA CAC ARC
848 CAC ACA CAC ACA CAC ARG
849 GTG TGT GTG TGT GTG TYA
850 GTG TGT GTG TGT GTG TYC
851 GTG TGT GTG TGT GTG TYG
852 TCT CTC TCT CTC TCT CRA
853 TCT CTC TCT CTC TCT CRT
854 TCT CTC TCT CTC TCT CRG
855 ACA CAC ACA CAC ACA CYT
856 ACA CAC ACA CAC ACA CYA
857 ACA CAC ACA CAC ACA CYG
858 TGT GTG TGT GTG TGT GRT
859 TGT GTG TGT GTG TGT GRC
860 TGT GTG TGT GTG TGT GRA

Trinucleotide motifs

861 ACC ACC ACC ACC ACC ACC
862 AGC AGC AGC AGC AGC AGC
863 AGT AGT AGT AGT AGT AGT
864 ATG ATG ATG ATG ATG ATG
865 CCG CCG CCG CCG CCG CCG
866 CTC CTC CTC CTC CTC CTC
867 GGC GGC GGC GGC GGC GGC
868 GAA GAA GAA GAA GAA GAA
869 GTT GTT GTT GTT GTT GTT
870 TGC TGC TGC TGC TGC TGC
871 TAT TAT TAT TAT TAT TAT

Tetra- and pentanucleotide motifs

872 GAT AGA TAG ATA GAT A
873 GAC AGA CAG ACA GAC A
874 CCC TCC CTC CCT CCC T
875 CTA GCT ACG TAG CTA G
876 GAT AGA TAG ACA GAC A
877 TGC ATG CAT GCA TGC A
878 GGA TGG ATG GAT GGA T
879 CTT CAC TTC ACT TCA
880 GGA GAG GAG AGG AGA
881 GGGG TGG GGT GGG GTG

5'degenerate motifs

882 VBV ATA TAT ATA TAT AT
883 BVB TAT ATA TAT ATA TA
884 HBH AGA GAG AGA GAG AG
885 BHB GAG AGA GAG AGA GA
886 VDV CTC TCT CTC TCT CT
887 DVD TCT CTC TCT CTC TC
888 BDB CAC ACA CAC ACA CA
889 DBD ACA CAC ACA CAC AC
890 VHV GTG TGT GTG TGT GT
891 HVH TGT GTG TGT GTG TG

Other motifs

892 TAG ATC TGA TAT CTG AAT TCC C
895 AGA GTT GGT ACG TCT TGA TC
898 GAT CAA GCT TNN NNN NAT GTG G
899 CAT GGT GTT GGT CAT TGT TCC A
900 ACT TCC CCA CAG GTT AAC ACA

Fig. 1 Primers used in the present study (derived from University of British Columbia set 9). *R* purine; *Y* pyrimidine; *N* any nucleotide; *B* C, G, or T; *D* A, G, or T; *H* A, C, or T; *V* A, C, or G

Genetic segregation analysis

The megagametophyte tissue of conifers is haploid; therefore DNA fragments from a heterozygous mother tree are expected to show 1 : 1 segregation among seeds. We used eight DNA samples from each family in a preliminary segregation analysis, providing a 99.2% probability of detecting heterozygosity in the absence of segregation distortion [$1-(1/2)^{n-1}$, where n indicates number of megagametophytes sampled]. After detecting heterozygosity, up to 40 additional samples per tree were analyzed for segregation.

Results

From the primers that gave amplification products in our initial screen, we selected the 24 that gave the clearest bands; all of these annealed to dinucleotide repeats (Tables 1 and 2). Although most primers that gave amplification products appeared to include polymorphic bands, there were often too many bands for interpretation on a 2% agarose gel. We studied 24 primers for Midori 5, 22 for S31t20, and 21 for S45t11.

Table 1 Segregation of ISSR fragments in families S31t20 and S45t11 of Douglas-fir

Family	Primer	Fragment size (bp)	Segregation $a:b$[a]	χ^2	Signif.[b]
S31t20	$(AC)_8$ YG	680	16:7	3.52	NS
		500	16:7	3.52	NS
		480	11:12	0.04	NS
		440	9:14	1.09	NS
	$(AC)_8$ YT	650	9:17	2.46	NS
		290	14:12	0.15	NS
	$(AG)_8$ C	600	10:12	0.04	NS
	$(AG)_8$ G	580	10:12	0.04	NS
		480	13:9	0.73	NS
	$(AG)_8$ T	1050	11:11	0.00	NS
		650	10:12	0.04	NS
	$(AG)_8$ YA	400	13:12	0.04	NS
	$(AG)_8$ YT	260	13:12	0.04	NS
	$(CA)_8$ A	850	7:10	0.53	NS
		650	10:7	0.53	NS
	$(CA)_8$ RT	600	10:15	1.00	NS
	$(CT)_8$ RC	850	9:14	1.09	NS
		480	16:7	3.52	NS
	$(CT)_8$ RG	500	12:10	0.18	NS
	$(GA)_8$ A	520	8:9	0.06	NS
	$(GA)_8$ C	280	8:12	0.80	NS
	$(GA)_8$ T	850	11:9	0.20	NS
		520	11:9	0.20	NS
	$(GA)_8$ YC	520	10:14	0.67	NS
	$(GA)_8$ YG	700	15:9	1.50	NS
		600	14:10	0.67	NS
		400	16:8	2.67	NS
	$(GA)_8$ YT	650	14:11	0.36	NS
		440	15:10	1.00	NS
	$(GT)_8$ YC	520	11:15	0.62	NS
		440	13:13	0.00	NS
		270	14:12	0.15	NS
	$(GT)_8$ YG	290	12:13	0.04	NS
		260	9:16	1.96	NS
		130	12:13	0.04	NS
S45t11	$(AC)_8$ YA	700	12:19	1.58	NS
		340	15:16	0.03	NS
	$(AC)_8$ YG	870	16:15	0.03	NS
	$(AC)_8$ YT	880	13:18	0.81	NS
	$(AG)_8$ C	1000	14:17	0.29	NS
	$(AG)_8$ T	460	17:14	0.29	NS
	$(AG)_8$ YC	230	15:16	0.03	NS
	$(AG)_8$ YT	560	14:17	0.29	NS
	$(CA)_8$ A	450	16:15	0.03	NS
	$(CA)_8$ RT	340	13:18	0.81	NS
	$(CT)_8$ RC	850	16:16	0.00	NS
		480	18:14	0.50	NS
	$(GA)_8$ C	680	18:13	0.81	NS
	$(GA)_8$ YG	220	17:15	0.13	NS
	$(GA)_8$ YT	800	10:21	3.90	<0.05
		440	20:11	2.61	NS
	$(GT)_8$ YA	800	19:13	1.13	NS
	$(GT)_8$ YC	310	11:20	2.61	NS
	$(GT)_8$ YG	260	18:13	0.81	NS

[a] "*a*" indicates band presence, "*b*" indicates band absence
[b] Tested at the 5% level of statistical confidence; NS, not significant

Table 2 Segregation of ISSR fragments in family Midori 5 of sugi

Family	Primer	Fragment size (bp)	Segregation $a:b$[a]	χ^2	Signif.[b]
Midori 5	$(AC)_8$ YA	550	25:23	0.08	NS
	$(AC)_8$ YG	1090	21:27	0.75	NS
		290	23:25	0.08	NS
		950	22:26	0.33	NS
	$(AC)_8$ YT	580	29:19	2.08	NS
	$(AG)_8$ C	320	28:20	1.33	NS
	$(AG)_8$ T	1200	17:31	4.08	<0.05
		720	22:26	0.33	NS
		460	17:31	4.08	<0.05
		340	27:21	0.75	NS
	$(AG)_8$ RA	960	18:14	0.50	NS
		520	14:18	0.50	NS
	$(AG)_8$ YA	1050	23:25	0.08	NS
	$(AG)_8$ YT	870	19:29	2.08	NS
	$(CA)_8$ RT	650	26:22	0.33	NS
	$(GA)_8$ T	650	16:15	0.03	NS
		480	13:18	0.81	NS
	$(GA)_8$ YG	680	23:25	0.08	NS
	$(GT)_8$ YC	400	19:29	2.08	NS
	$(GT)_8$ YG	880	21:27	0.75	NS
		820	24:24	0.00	NS
		650	30:18	3.00	NS
		580	26:22	0.33	NS

[a] "*a*" indicates band presence, "*b*" indicates band absence
[b] Tested at the 5% level of statistical confidence; NS, not significant

Unstable or weak bands were not scored for segregation analysis, although there appeared to be many such variable bands in both species (data not shown).

Amplified DNA fragments varied in size from approximately 200 bp to 1000 bp (Fig. 2). Segregation in Douglas-fir was easier to interpret than in sugi. In S31t20, none of the 35 segregating bands that were generated by the 19 primers departed from a 1:1 segregation ratio (Table 1). In S45t11, of 19 segregating bands detected with 16 primers, all but one fit a 1:1 ratio (Table 1). In Midori 5, of 23 segregating bands amplified with 13 primers, two departed from Mendelian expectations (Table 2). In all, 3 of the 77 bands studied in the three families showed distortion, which is very close to expectation due to chance alone at the 5% probability level (3.8). When data were pooled over loci, there was also no evidence of distortion or of heterogeneity among loci (Table 3). Null and banded phenotypes were equally represented within the three families; band : null ratios of 412:395, 292:301, and 503:535 were observed in S31t20, S45t11, and Midori 5, respectively. The ratio was 1207:1231 when pooled over all loci and families.

Of the 24 primers studied in the three families, the $(AG)_n$ primers were most abundant (16) and thus gave rise to the largest number of polymorphic loci (24) (Table 4). However, the $(GT)_n$ primers showed the highest ratio of polymorphic loci per primer (2.0; 14/7), followed by $(AC)_n$ at 1.9 and $(CT)_n$ at 1.7. The mean number of polymorphic loci per primer was 1.6. There were no consistent differences between the species in diversity; the average number of loci per primer were 1.8, 1.2, and 1.8 in S31t20, S45t11, and Midori 5, and the proportion of polymorphic primers of the 24 tested were 92%, 87%, and 100%, respectively. As expected from previous studies (cf. Li and Adams 1989), the Douglas-fir tree of the coastal variety was more heterozygous than was the one from the interior variety, having 35 hetero-

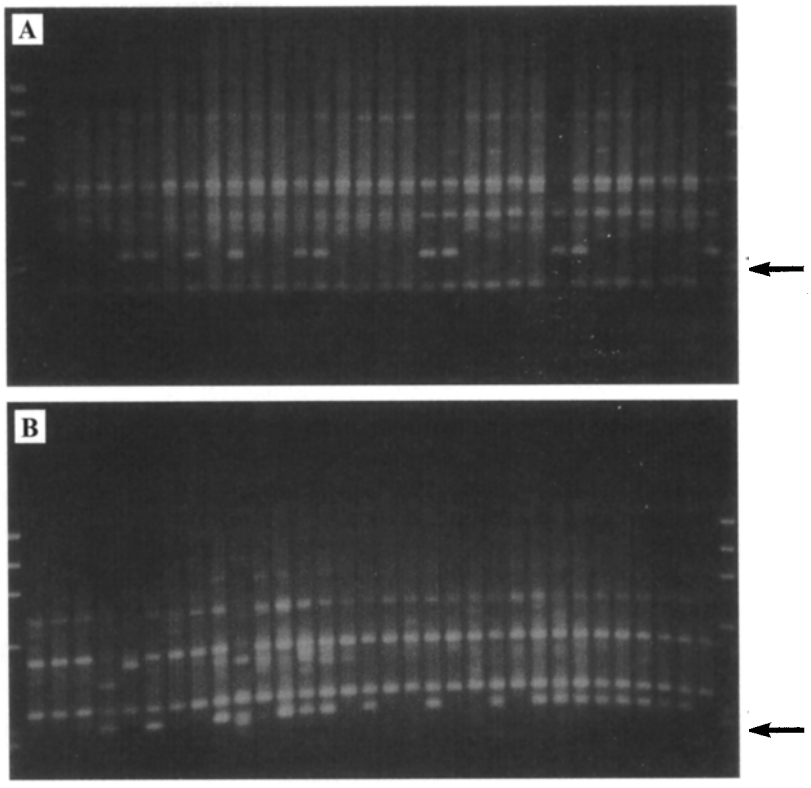

Fig. 2 A,B PCR-amplified inter-simple sequence repeat patterns on 2% agarose gels. Size markers at the ends of the gel and ϕX174/*Hae*III (1353, 1078, 872, 603, 310, 281, 234, and 194 bp). **A** Douglas-fir S45t20 family with the $(GT)_8$ YC primer. **B** Sugi Midori 5 family with the $(AG)_8$ C primer

Table 3 Pooled and heterogeneity tests of segregation[a]

Family	Pooled χ^2	Ratio (a:b)	Heterogeneity χ^2	df
Douglas-fir S31t20	0.358 (NS)	412:395	29.57 (NS)	34
S45t11	0.137 (NS)	292:301	16.55 (NS)	18
Sugi Midori 5	0.987 (NS)	503:535	23.54 (NS)	22

[a] NS, not significant at the 5% level; *df*, degrees of freedom

zygous loci from 19 primers as compared with 19 heterozygous loci from 16 primers.

Discussion

PCR conditions for ISSR amplification

We informally studied several factors to enhance the ISSR-PCR reaction. We found that formamide was critical to reducing background and smear in the PCR products, thus enabling interpretation of bands. Formamide influences primer-template annealing and melting temperature, and concentrations above 10% formamide are known to inhibit *Taq* polymerase (Gelfand 1989). In the present study, formamide concentrations of 1–2% aided amplification (unpublished data), and 2% dramatically enhanced the clarity of banding patterns for most primers. Formamide concentrations of 3% or above completely inhibited amplification.

The number of PCR amplification cycles affected banding patterns: 27, 30, 35, 45 and 50 cycles gave useful amplification; however, 45 and 50 cycles yielded bands of higher intensity and stability than did the lower number of cycles. We examined six template DNA concentrations: 2.5, 5, 10, 15, 20, and 25 ng. Concentrations of 10 ng per reaction yielded the same amplification products as did 25 or 50 ng. Even 2.5 and 5 ng of template DNA yielded good PCR products, although amplification was inconsistent compared to the higher concentrations. In related studies, higher template amounts (e.g. 25–50 ng) have often been used (Moore et al. 1991; Saghai Maroof et al. 1994; Zietkiewicz et al.

Table 4 Number of primers and loci (in parentheses) yielding polymorphisms in two Douglas-fir families and one sugi family

Family	$(AG)_n$	$(GA)_n$	$(CA)_n$	$(AC)_n$	$(CT)_n$	$(GT)_n$	Total
Douglas-fir S31t20	5 (7)	6 (10)	2 (3)	2 (6)	2 (3)	2 (6)	19 (35)
Douglas-fir S45t11	4 (4)	3 (4)	2 (2)	3 (4)	1 (2)	3 (3)	16 (19)
Sugi Midori 5	5 (9)	2 (3)	2 (3)	3 (5)	0 (0)	2 (5)	13 (23)
Total	14 (20)	11 (17)	5 (6)	8 (15)	3 (15)	7 (14)	48 (77)
Ratio[a]	1.4	1.5	1.2	1.9	1.7	2.0	1.6

[a] Loci per primer

1994). Because megagametophyte DNA is limited in amount (often 1 μg per seed), DNA concentration per reaction may need to be optimized for 1–5 ng levels in order to complete saturated genome maps.

We calculated that the melting temperature (DNASIS, Hitachi Software, Japan) of most of the ISSR primers studied was between 55° and 60 °C. Some $(AT)_n$ primers, however, had much lower melting temperatures (20° to 33 °C), and primers with high GC-content had melting temperatures above 60 °C. Three annealing temperatures, 45 °C, 52 °C and 54 °C, were examined for all primers, and 30 °C was examined for the A- and T-rich primers. An annealing temperature of 52 °C gave superior banding patterns with most primers; 45 °C tended to yield high background and broad bands. Annealing at 54 °C worked poorly compared to annealing at 52 °C. Annealing as low as 30 °C for the AT-rich primers gave little discernible product. Gupta et al. (1994) also found that an $(AT)_n$ primer annealed at 35 °C gave no discernible product in loblolly pine (*Pinus taeda* L.) or in several other plant and animal species. Because AT-repeats are abundant in plant genomes (Wang et al. 1994), it would be worthwhile to carefully explore PCR conditions to enable their amplification.

ISSRs in conifers

Approximately 90% of ISSR primers out of the 24 selected showed polymorphism within the three families. Dinucleotide repeat primers were far superior to the others. However, trinuculeotide repeat primers could also be useful if amplification conditions were optimized to reduce background. Tetra- and penta-nucleotide repeat primers do not appear to be suitable with the methods employed.

According to Wang et al. (1994), in plant nuclear DNA the dinucleotide sequence $(AT)_n$ is most abundant, followed by $(A)_n/(T)_n$ and $(AG)_n/(CT)_n$. Common tri- and tetra-nucleotide repeat motifs include $(AAT)_n/(ATT)_n$, $(AAC)_n/(GTT)_n$, $(AGC)_n/(GTC)_n$, $(AAG)_n/(CTT)_n$, $(AATT)_n/(TTAA)_n$, $(AAAT)_n/(ATTT)_n$, and $(AC)_n/(GT)_n$ sequences. In the present study, the abundant $(AG)_n/(CT)_n$ sequences were most useful; they are also known to be abundant in pine genomes (approximately 50 000 repeats per genome; C. Echt, personal communication).

The ISSR loci we studied showed virtually complete agreement with the expectations of Mendelian markers, which will facilitate their use in genome mapping and population studies. Their simple inheritance contrasts with the variable and sometimes large amounts of segregation distortion observed with RAPD markers. For example, only 33% of RAPD markers showed Mendelian inheritance in Norway spruce (*Picea abies* Karst.) (Bucci and Menozzi 1990), approximately 69% in *Picea glauca* Moench (Tulsieram et al. 1992), and 86% in *Populus* (Bradshaw et al. 1994).

The primers employed appear to be of broad utility for studies of conifers. Sugi and Douglas-fir are members of two distinct conifer families, the Cuppressaceae Bartling (including the Taxodiaceae) and the Pinaceae Lindley, respectively. Their ancient phylogenetic differentiation has been supported by recent molecular studies of their chloroplast genomes (Strauss et al. 1988; Tsumura et al. 1993; Brunsfeld et al. 1994). Nonetheless, the 24 selected primers were in complete agreement in the two species.

Our study indicates that analysis of ISSRs can be accomplished with the ease of analysis of RAPDs, and may thus present a readily accessible means for studying SSR-associated polymorphism. Their advantages over primers to sequences that flank SSRs are that they require no prior knowledge of genomes or the use of polyacrylamide gels and radioactivity for resolution. Their main disadvantage is lack of co-dominance and the consequent resolution of effectively biallelic loci (band presence vs absence). Once optimized, the higher annealing temperature and primer length of ISSR, compared to RAPD, primers should result in more reliable amplification and greater stability of map position among genotypes within species. However, these contentions require explicit testing. Nonetheless, ISSRs can be conventient supplements to RAPD markers, and their Mendelian inheritance will facilitate the saturation of genomic maps and their use in population studies.

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