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Microsatellites as DNA markers in Sitka spruce

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Abstract Nine microsatellite loci were found by screening a genomic DNA library of Sitka spruce (*Picea sitchensis*) with the four oligonucleotide probes (TG), (CAC), (GATA) and (AT). Pairs of flanking primers were generated for seven microsatellites. Five primer pairs were used to screen up to 58 Sitka spruce clones. The five loci SStg3a, SStg4, SStg4a, SStg4c and SSgata3 were found to have 15, 13, 4, 3 and 6 different length alleles respectively, and in using a combination of them almost all 58 Sitka spruce genotypes could be identified. The five primer pairs were successful in amplifying DNA from two other spruce species (*Picea albutilia* and *Picea smithiana*), while only one primer pair could amplify DNA from the pine species, *Pinus sylvestris* and *Pinus latifolia*. The inheritance of microsatellites in Sitka spruce was co-dominant Mendelian.

Key words Microsatellites · *Picea sitchensis* · Segregation · Sitka spruce

Introduction

DNA molecular markers are used extensively to create genetic and physical genome maps, in taxonomy and population ecology studies, and to identify individual genotypes. Microsatellites or simple sequence repeats are a relatively new type of marker. Microsatellites consist of tandem repeats of 1–6 nucleotide motifs. The repeats are usually in units of ten or more, although repeats as small as six units have been found (Thomas and Scott 1993). The repeats can be perfect tandem repeats, imperfect (interrupted by several non-repeat nucleotides) or compound repeats (Hearne et al. 1992), and are well distributed throughout the genome (Queller et al. 1993). Microsatellites can be amplified by the polymerase chain reaction (PCR) using a pair of primers flanking the repeat sequence. The polymorphism between different individuals is due to the variation in the number of repeat units, and each locus can have many alleles.

One advantage of microsatellites is that they are co-dominant, which make them easily transferable between genetic maps of different crosses, in contrast with RAPDs which are dominant and therefore new maps have to be generated for every cross (Thomas and Scott 1993). Also, several microsatellite-primer pairs can be used simultaneously, thus reducing time and costs (Hughes and Queller 1993). The relatively simple interpretation and genetic analysis of single-locus markers make them superior to multi-locus DNA marker types (i.e., RAPDs), especially for map construction and DNA typing (Thomas and Scott 1993). RAPD primers on the other hand can be used for any species, while microsatellites seem to be conserved within species, with limited possibilities for using heterologous primers between related species (Moore et al. 1991). A disadvantage of microsatellites is that they are difficult to find. Sometimes this can be circumvented by searching databases for microsatellite sequences.

Microsatellites have been widely used for creating high-density maps for human, mouse (Love et al. 1990), and other mammalian genomes (Moore et al. 1991). More recently, they have also been used in plant genetic analyses (Lagercrantz et al. 1993; Morgante and Olivieri 1993; Thomas and Scott 1993).

In the present study, microsatellite loci from Sitka spruce were characterised. It was shown that these loci are well suited for clone identification and paternity analyses in Sitka spruce.

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Materials and methods

Plant materials and DNA isolation

DNA was available of young shoots from 58 Sitka spruce genotypes grown in two seed orchards (numbers 12 and 13), planted in 1981–83 and 1984–85 at Ledmore, Perthshire, Scotland, UK, or in container-pots at the Forestry Authority, Northern Research Station, Roslin, Midlothian, Scotland, UK (Van de Ven and McNicol 1995). DNA was extracted from young needles of *Picea albutilia*, *Picea smithiana*, *Pinus sylvestris* and *Pinus latifolia* obtained from the University Botanical Gardens, Dundee, Scotland. DNA from embryos and megagametophytes from five seeds of a controlled cross, 1101×690 (seed collected in 1989), was also extracted. Seeds were surface-sterilized with 3% hydrogen peroxide, washed five times with sterile distilled water and germinated on wet filter-paper in Petri dishes (Cottrell and White 1995). When seeds had just started to germinate (approximately 7 days) the seed coat was removed and embryo and megagametophyte were separated. DNA was isolated from needles, embryo and megagametophyte according to the method used by Van de Ven and McNicol (1995).

Cloning, selection and sequencing of microsatellite-containing clones

Genomic DNA from Sitka spruce clone 1442 was digested with the restriction enzyme *Mbo*I. Restriction fragments were ligated into plasmid vector pUC19, digested with *Bam*HI, and *E. coli* DH5alpha competent cells (Boehringer Mannheim) were transformed with this vector according to the manufacturer's instructions.

Colony lifts and hybridisation were performed according to the procedure supplied by Boehringer Mannheim (Anonymous 1993). Four oligonucleotides, $(TG)_{10}$, $(CAC)_5$, $(GATA)_4$ and $(AT)_{10}$, were labelled with Digoxigenin-11-ddUTP using a DIG oligonucleotide 3′-end labelling kit (Boehringer Mannheim) according to the manufacturer's instructions. Hybridisation temperatures for the four oligonucleotides were 58°C, 43°C, 33°C and 25°C respectively. Three consecutive screens were performed for each oligonucleotide, and plasmid DNA was isolated from the positive clones in the final screen using QIAGEN-tip 100 (Qiagen).

DNA sequencing of plasmid clones was performed, from both ends, with a Sequenase Version 2 kit (USB) using M13-universal primers and alpha-^{35}S-dATP (9.25 MBq/mmol). The sequences were run on 5% or 6% polyacrylamide, 7 M urea, 1× Tris-Borate-EDTA gels at 55 watt constant power for 2–6 h.

Primer selection and PCR

Primers flanking the microsatellites, and 100–300 bases apart, were selected using the computer program PRIMER Version 0.5 (Whitehead Institute for Biochemical Research, Cambridge, Massachusetts, 1991) and then synthesised.

PCRs were carried out in a 25 µl volume, each containing 15 ng of genomic DNA, 200 µM of each dNTP, 0.2 µM of each primer, 0.5 units of *Taq*-polymerase, 1 × reaction buffer, 1.5 mM of $MgCl_2$ and 0.05% W-1 (detergent) (GIBCO-BRL). Reactions were overlaid with 25 µl of mineral oil to prevent evaporation. DNA was amplified in a thermal cycler (Omni Gene, Hybaid) using the following program: 1 min at 94°C, 1.5 min at 52°C or 54°C and 2 min at 72°C for 40 cycles followed by an additional 5 min at 72°C. The amplified samples were kept at 4°C before separation on 3–4% NuSieve 3:1 agarose gels. Fragments were visualised under UV-light after staining with ethidium bromide. The PCR products were also separated on sequencing gels containing 6% polyacrylamide, 7 M urea and 1 × TBE at 55 W constant power for 1–3 h. Gels were fixed, dried and stained with silver nitrate using a "DNA Silver Staining System" kit (Promega) according to the manufacturer's instructions.

Results

A total of 19 positive colonies were identified; nine with the TG probe, two with the CAC probe and seven with the GATA probe. No positive colonies were detected with the TA probe after three screenings. Seventeen positive colonies were sequenced and in ten clones no repeat sequences were found, either because the inserts were not completely sequenced as the inserts were too large (eight clones) or because they were false positives (two clones). In total nine microsatellites were found, six clones contained one microsatellite sequence, while one clone contained three separate microsatellites, 93 bases and 96 bases apart. For seven microsatellites a pair of flanking primers could be generated using PRIMER Version 0.5 (Table 1).

All seven primer pairs yielded a single band when used in PCR with the original plasmid DNA. When screening the Sitka spruce clones, one (homozygous) or two (heterozygous) major bands were generated per primer pair. Primer pair SScac4, however, generated from none to more than two major bands. Primer pair SStg3 has not been used in PCR. Table 1 shows the microsatellites and primer pairs which were generated. Two of the microsatellites were perfect repeats [e.g., SStg3a, $(TG)_{27}$], one was a compound repeat with two adjacent repeat runs (SSgata3), and four were imperfect compound repeats, with compound repeats interrupted by two or three non-repeat bases.

A total of 26 or 58 Sitka spruce clones, two spruce and two pine species were analysed using five of the primer pairs. Initially the PCR products were resolved on 3–4% NuSieve gels. Locus SStg3a appeared to have seven alleles by NuSieve gel electrophoresis and 15 alleles by acrylamide electrophoresis. All other loci were analysed by acrylamide electrophoresis. The number of alleles found for the five loci varied from three alleles for SStg4c to 15 alleles for SStg3a (Table 1), with some alleles more common than others. Most of the Sitka spruce clones were homozygous at a locus, with the percentage heterozygosity ranging from 0–47% over five loci.

Figure 1 shows an example of the polymorphism found between Sitka spruce clones, the two other spruce species and the two pine species using primer pair SStg4c. The first lane (m) is a 10-bp ladder and the second lane (4c) the original sequenced allele with a length of 139 bases. The major band was associated with faint bands, possibly resulting from polymerase slippage during PCR (Tautz 1989). Three different alleles (A, B and C) were generated in Sitka spruce with a length of 137, 139 and 141 bases respectively. Two Sitka spruce clones were homozygous BB (320 and 690), one was homozygous AA (1042) and the other clones were heterozygous, either BC (69, 427), AC (1041) or AB (1101, 1615, 1463). *Picea albutilia* was homozygous AA and *Picea smithiana* was homozygous BB. Primer pair SStg4c generated no bands in *Pinus sylvestris* and only a very faint band (141 bases) in *Pinus latifolia*. Each of the other four primer pairs generated one or two alleles for the spruce species (*P. albutilia* and *P. smithiana*), while only SStg4 generated a PCR product with the two pine species.

Table 1 Sitka spruce microsatellites

Locus	Microsatellite sequence[a]	Primers (5′-3′)	Length of PCR product[b]	Number of alleles	Hetero-zygosity[c]
SSgata3	$(TA)_9(RA)_{16}(GATA)_{14}$	CTGTGTACTTTTTCATGGCC CTTTGTATCAAACTCCCCCT	346 bp	6	23%
SStg3a	$(TG)_{27}$	TCAAGCTCTCCAACCCAGAT TGTCGAGTTTGACTTGTACCAA	136 bp	15	47%
SStg4	$(TA)_4(TG)_{11}(TA(TG)_3$	CTCACCTCCGGTTTCCATTA CATTGTCCCCCACCATTTAC	207 bp	13	24%
SStg4a	$(TA)_6TA(TG)_2TC(TG)_2$	ACAATGTCAGGCATCGCTTA GTCCCTTCCCCTTTACAATG	122 bp	4	0%
SStg4c	$(TG)_8$	TAACCCCGAGGTACTCAACC ATTCGGTTAACTTGTTCGGC	139 bp	3	41%
SScac4	$(CAC)_2CAA(CAC)_3CCAA(C)_4(A)_4$	TTGGGGAGTAGTTAAAGTAACGAA AATGCGAAACCAGTTCAGG	119 bp	nd	nd
SStg3	$(TA)_6TC(TG)_{22}$ $(TATG)_5TATAAATA(TG)_8TT(TG)_2$ $(TATG)_5TATAAATA(TG)_8$	TTCACATGCACCCCTTTTTA TCGACTTACAATACACACAACATTC	225 bp	nd	nd
SSgata39	$(TA)_8$	nd			
SStg4b	$(TA)_n$	nd			

[a] R: G or T
[b] Length of the sequenced allele from Sitka spruce clone 1442
[c] Heterozygosity calculated over 57 Sitka spruce clones for loci SStg3a, SStg4, SStg4a and SStg4c, and over 26 clones for the SSgata3 locus; nd: not determined

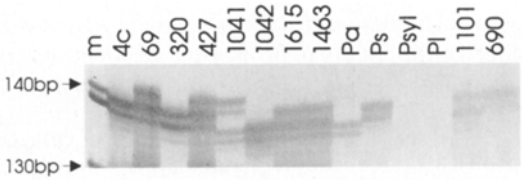

Fig. 1 Silver-stained polyacrylamide gel of microsatellite SStg4c. Numbers at the top: Sitka spruce clones; *m* 10 bp marker; *4c* PCR using the plasmid DNA containing SStg4c as a template; *Pa P. albutilia*; *Ps P. smithiana; Psyl P. sylvestris*; *Pl Pinus latifolia*

All alleles found in these spruce and pine species were also present in Sitka spruce.

All 58 clones were scored for the presence or absence of an allele at a certain locus. Data were obtained only for 26 Sitka spruce clones, employing primer pair SSgata3. Using a combination of all data most Sitka spruce clones could be identified. Five of the clones, which were only screened with four out of the five primer pairs, could not be distinguished (clones 492, 1427 and 1609; 321 and 727).

Segregation of microsatellites in Sitka spruce

Five primer pairs were used to amplify sequences from five embryos and their megagametophytes from a cross between clones 1101 690. According to Tulsieram et al. (1992) five seeds would be sufficient for identifying segregating loci in the maternal parent tree. Table 2 gives the segregation of microsatellite loci detected with the five primer pairs (SStg3a, SStg4, SStg4a, SStg4c and SSgata3). For SStg4a both parents have the same alleles (BB) and thus all embryos and megasporophytes also have allele B. For SStg4 (Fig. 2) the female parent, 1101, has alleles EE (approximately 227 bases) while the male parent, 690, has alleles II (207 bases). The five megagametophytes have the same allele as the female parent (E) and the five embryos have one allele from the female and one allele from the male parent (EI). SStg4c generated two different alleles in

Table 2 Segregation of microsatellite loci in a controlled cross between clones 1101 and 690

Locus	Clone			Microsatellite loci				
	1101	690		1	2	3	4	5
SStg3a	AA		Megasporophyte	A	A	A	A	A
		BB	Embryo	AB	AB	AB	AB	AB
SStg4	EE		Megasporophyte	E	E	E	E	E
		II	Embryo	EI	EI	EI	EI	EI
SStg4a	BB		Megasporophyte	B	B	B	B	B
		BB	Embryo	BB	BB	BB	BB	BB
SStg4c	AB		Megasporophyte	A	A	A	B	B
		BB	Embryo	AB	AB	AB	BB	BB
SSgata3	FF		Megasporophyte	F	F	F	F	F
		FF	Embryo	FF	FF	FF	FF	FF

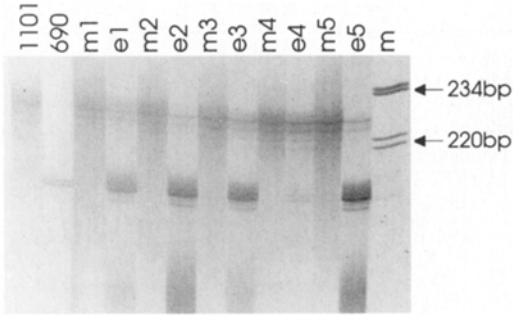

Fig. 2 Segregation of microsatellite locus SStg4. *Clone 1101* female parent; *clone 690* male parent; *m1-5* megagametophyte; *e1-5* embryo; *m* molecular-weight marker VI (Boehringer Mannheim)

1101 (AB) and alleles BB in 690. Three of the megasporophytes had allele A and their embryos had alleles AB. The other two megasporophytes had allele B and their embryos had alleles BB.

Discussion

Microsatellites were found in Sitka spruce using the probes TG, CAC and GATA, but not with the TA probe. Lavi et al. (1994) could not detect any repeat sequences in avocado using an AT probe, although AT/TA repeats are among the more abundant repeats in plants (Lagercrantz et al. 1993; Morgante and Olivieri 1993; Wang et al. 1994). The $(AT)_{10}$ probe may easily bind to itself, which would reduce the amount of probe available for detecting repeat sequences in the genomic library. As the Tm value of this probe is relatively low, it is difficult to obtain the right hybridisation conditions.

Nine repeats were found in seven plasmid clones, of which most were imperfect compound repeats. In one of the plasmid clones three separate repeats were detected, which was also found in other species (Lavi et al. 1994). Microsatellite analyses by acrylamide gels gives better resolution of the individual alleles than does 4% NuSieve gel-electrophoresis.

The highest number of alleles (15) and the highest number of different genotypes observed (27) between the 58 Sitka spruce clones was found with the longest dinucleotide locus SStg3a ($(TG)_{27}$). The lowest number of alleles (three) and the lowest number of observed genotypes (six) was found with the shortest dinucleotide locus SStg4c ($(TG)_8$). This corresponds with results from Weber (1990) and Thomas and Scott (1993) who found that longer dinucleotide loci are genetically more variable and detect more alleles than those with a short repeat length. Others have concentrated on loci containing ten or more repeats to increase the probability of finding more variable loci (Lagercrantz et al. 1993). Six alleles were generated in 27 of the Sitka spruce genotypes analysed with SSgata3. The number of alleles would probably increase if all 58 genotypes had been analysed.

Polymorphic microsatellite loci can be detected across species boundaries, but seem to be limited to the conservation of the primer sequences flanking the variable region (Tautz and Schlötterer 1994). Cross species amplification has been found in mammals (Hearne et al. 1992) and plants (Lagercrantz et al. 1993; Thomas and Scott 1993). Lagercrantz et al. (1993) used five primer pairs developed for *Brassica napus* on other *Brassica* species. They found that all primer pairs could amplify DNA from related species, but that only one of the primer pairs was able to amplify DNA from the more distant species *B. nigra*. This result is similar to ours, as DNA from two other spruce species could be amplified with the four primer pairs used. But only one of the primer pairs could successfully amplify DNA from two pine species belonging to the same family as spruce (*Pinaceae*).

Not all 58 Sitka spruce clones analysed could be identified using a combination of four primer pairs. Possibly, if primer pair SSgata3 had been analysed fully all clones could have been distinguished. In an earlier study, using six RAPD primers, all these 58 genotypes could be identified (Van de Ven and McNicol 1995).

As in many other species, microsatellites in Sitka spruce were co-dominant and inherited in a Mendelian fashion.

We conclude from this study that microsatellite loci are useful markers in detecting variation in Sitka spruce and have the potential to provide a unique DNA identification for individual genotypes. The microsatellites developed for Sitka spruce are informative when applied to other spruce species but less so for the more distant pine species. Microsatellites are likely to be useful tools in paternity and gene-flow studies of Sitka spruce.

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