OPTIMIZING EXPRESSED SEQUENCE TAG POLYMORPHISMS BY SINGLE STRAND CONFORMATION POLYMORPHISM IN SPRUCES

D. Fournier [1], D. J. Perry [2,3], Jean Beaulieu [1], J. Bousquet [2] & Nathalie Isabel [1*]

[1)] Ressources naturelles Canada, Service canadien des forêts, 1055 rue du P.E.P.S., C. P. 3800, Sainte-Foy, Québec, Canada, G1V 4C7
* author for correspondence; e-mail: nisabel@cfl.forestry.ca
[2)] Centre de recherche en biologie forestière, Chaire de recherche du Canada en génomique forestière et environnementale, Université Laval, Sainte-Foy, Québec, Canada, G1K 7P4
[3)] present address: Grain Research Laboratory, Plant Molecular Biology, Canadian Grain Commission, 1404-303 Main Street, Winnipeg, Manitoba, Canada, R3C 3G8

Received April 30, 2001; accepted January 31, 2002

ABSTRACT

Over the last decade, individual genetic maps, based largely on RAPD markers, have been constructed for numerous conifer species. The information contained within such maps may be further enhanced if it can be synthesized into a single consensus map at the intra- or interspecific level. However, the development of such consensus maps is hampered by the difficulty in using the same RAPD markers in multiple populations or across closely related species. Thus, the aim of this study was to evaluate the potential of 39 sequence-tagged-site (STS) markers of arbitrary genes (PERRY & BOUSQUET 1998a) for use in genetic mapping at the intra- and interspecific levels. Several white spruce [*Picea glauca* (Moench) Voss] and black spruce [*P. mariana* (Mill.) B.S.P.] trees, belonging to different unrelated pedigrees for which genetic map development is anticipated, were selected. In cases where an STS marker appeared monomorphic on an agarose gel, it was investigated further for single-strand conformation polymorphism (SSCP). By using SSCP in addition to standard agarose gel electrophoresis, the number of informative markers was increased from 10 to 22 in white spruce and from 14 to 19 in black spruce. The number of markers that were informative in both species was increased from 6 to 9. Mendelian segregation was verified for alleles at all loci that were polymorphic in either or both of the species.

Key words: *Picea mariana, Picea glauca,* genetic maps, molecular markers, sequence-tagged-sites, single-strand conformation polymorphism (SSCP)

INTRODUCTION

Several types of PCR-based markers are available for use in genetic mapping. Random-amplified polymorphic DNA (RAPD) markers were first introduced in the early 1990's (WELSH & MCCLELLAND 1990, WILLIAMS *et al.* 1990) and, due to the ease of their implementation, were quickly established as the system of choice to elucidate the genetic architecture of several important traits in conifers (KUANG *et al.* 1998, LEHNER *et al.* 1995, WILCOX *et al.* 1996). However, conifers have very large genomes (DHILLON 1987) and most RAPD markers are amplified in repetitive, primarily non-coding regions (PLOMION *et al.* 1995). Furthermore, RAPD markers have been the target of criticism because reproducibility among different laboratories can be poor (e.g., JONES *et al.* 1997). More recently, the amplified fragment length polymorphism (AFLP) technique (VOS *et al.* 1995) has provided a class of highly polymorphic markers that combine restriction fragment length polymorphism (RFLP) and the polymerase chain reaction (PCR). Like RAPDs, AFLPs are primarily dominant markers; however, they have a significant advantage over RAPDs in their higher reproducibility. Maps can be saturated with the large amounts of data that can be obtained using RAPDs and AFLPs. However, both of these marker types suffer from a difficulty in establishing synteny. It is not clear whether similarly sized fragments in different species, or even different populations of the same species, actually represent the same locus.

The problem of establishing synteny may be largely overcome through the use of specifically targeted markers such as microsatellites (also known as simple-sequence repeats; SSR) (TAUTZ 1989) and other types of sequence-tagged-site (STS) markers (OLSON *et al.* 1989) such as those generated from complementary DNAs (cDNAs). cDNA-based STS markers are particularly interesting since they would reflect the locations of specific genes, and thus may be extremely useful for

establishing anchor positions in genomic maps. A set of 39 cDNA-based STS markers has been developed for black spruce (PERRY & BOUSQUET 1998a). For several markers in this set, polymorphism within three spruce species (*Picea abies*, *P. glauca* and *P. mariana*) has been revealed directly by standard agarose gel electrophoresis, without any further manipulation of the amplified products (PERRY & BOUSQUET 2001, PERRY & BOUSQUET 1998a, b, PERRY *et al.* 1999). Although examining PCR products directly on agarose gels is convenient for a quick survey of large numbers of individuals in population studies (PERRY & BOUSQUET 1998a), this method has low sensitivity when alleles differ only slightly in molecular weight, and it is ineffective in the detection of point mutations. More sensitive but more labour intensive methods for the detection of polymorphism may be warranted to reveal additional polymorphism that would allow the use of a larger number of these STS markers in applications such as genome mapping. In the present study, the aforementioned set of cDNA-based STS markers was examined for the presence of variation that may be observed as single-strand conformation polymorphism (SSCP) in white spruce and black spruce.

MATERIALS AND METHODS

Plant material

Seven white spruces were sampled for the present study. These trees are members of a first-generation breeding population located at the Cap Tourmente National Wildlife Area east of Quebec City. Six of them were involved in a diallel cross designed to examine the genetics underlying the capacity for somatic embryogenesis in this species. The last one is being used in a research program on molecular markers associated with mature wood density. For black spruce, six unrelated trees were sampled in a range-wide provenance trial (BEAULIEU 1998) established at the Valcartier Forest Experiment Station north of Quebec City. Needles and seeds were collected from each tree.

DNA extraction and amplification

For each tree, 100 mg of needles were ground with a mortar and pestle under liquid nitrogen. Needle DNA (diploid) was isolated using a DNEasy Plant Kit (QIAGEN, Mississauga, ON, Canada) according to the manufacturer's instructions. To obtain DNA from megagametophytes (haploid), seeds were first dissected to remove the seedcoat and embryo, DNA was isolated using a CTAB method (ISABEL *et al.* 1993) that was modified to include an extraction with phenol:chloroform: isoamyl alcohol (25:24:1), and ethanol precipitation of the DNA.

Thirty-nine STS markers, originally developed from black spruce cDNAs (PERRY & BOUSQUET 2001), were amplified individually from each of the needle DNA preparations. The primer sequences and PCR conditions were as described previously (PERRY & BOUSQUET 2001).

Agarose gel electrophoresis

PCR products were first examined for polymorphism using agarose gel electrophoresis (2% agarose in TAE, with the exception of *Sb01* for which a 1.2% gel was used). Five to 8 µl of each PCR product were loaded on a gel and electrophoresis was carried out at 200 V for up to 7 h. Following electrophoresis, gels were stained with ethidium bromide and photographed under UV light (254 nm) using an Imager System (Canberra Packard, Mississauga, ON, Canada). The genotype of each putative heterozygote was confirmed by testing allelic segregation among 8 to 46 haploid megagametophytes from the same tree, using a chi-square test.

SSCP analysis

When agarose gel electrophoresis failed to reveal polymorphism, the more sensitive SSCP technique was used. SSCP detection followed methods modified from those described by BODÉNÈS *et al.* (1996). Five µl of a denaturing buffer (95% formamide, 10 mM NaOH, 0.5% xylene cyanol and 0.5% bromphenol blue) were added to 2 µl of each amplified product. Samples were then heat-denatured at 94 °C for 4 min, quenched on ice for 2 to 3 min and then loaded on a non-denaturing acrylamide gel (1.0 mm × 16 cm × 18 cm) composed of 0.5× MDE (FMC BioProducts, Rockland, ME, USA) in 0.6× TBE. Electrophoresis was carried out under constant current (5 mA) at 10 °C for 18 h. Gels were then silver stained (BASSAM *et al.* 1991) using silver stain kit (BioRad, Mississauga, ON, Canada). If no polymorphism was detected under these standard conditions, the temperature of electrophoresis was modified to 4 °C or 15 °C and (or) the length of the run was increased to 24 h. Again, allelic segregation of putative heterozygotes was confirmed by examining amplification products derived from haploid megagametophytes.

RESULTS AND DISCUSSION

Under agarose gel electrophoresis, the relative mobility of double-stranded PCR products is determined primar-

Table 1. Detection of polymorphism with cDNA-based STS primers in *Picea glauca* and *P. mariana*.

Loci	Detection of polymorphism							
	Picea glauca				*Picea mariana*			
	Agarose	No. of het.[a] (# ind./7)	SSCP	No. of add. het.[b] (# ind./7)	Agarose	No. of het.[a] (# ind./12)	SSCP	No. of add. het.[b] (# ind./12)
Sb01	Yes	3	–	–	Yes	4	–	–
Sb06	No	–	No	–	Yes	1	Yes	4
Sb07	No	–	No	–	Yes	1	–	–
Sb08	No	–	Yes	4	No	–	No	–
Sb11	No	–	Yes	1	Yes	2	–	–
Sb12	Yes	0[c]	–	–	No	–	No	–
Sb14	No	–	Yes	3	No	–	Yes	3
Sb16	Yes	7	–	–	Yes	1	–	–
Sb17	Yes	3	–	–	Yes	1	–	–
Sb18	Yes	0[c]	–	–	No	–	No	–
Sb21	No	–	Yes	2	No	–	Yes	2
Sb24	Yes	7	–	–	Yes	5	–	–
Sb29	Yes	5	–	–	Yes	3	–	–
Sb31	No	–	Yes	3	Yes	2	Yes	2
Sb32	Yes	4	Yes	1	No	–	No	–
Sb35	Yes	4	–	–	Yes	3	–	–
Sb36	No	–	Yes	1	Yes	1	Yes	1
Sb41	Yes	0[c]	–	–	No	–	No	–
Sb42	No	–	Yes	3	Yes	3	Yes	4
Sb49	No	–	Yes	5	No	–	Yes	3
Sb51	No	–	Yes	1	No	–	No	–
Sb52	Yes	1	Yes	0	No	–	Yes	4
Sb58	No	–	No	–	No	–	Yes	4
Sb60	Yes	2	Yes	1	No	–	No	–
Sb62	No	–	Yes	1	No	–	No	–
Sb64	No	–	Yes	1	No	–	No	–
Sb66	No	–	No	–	Yes	1	–	–
Sb70	No	–	Yes	1	No	–	No	–
Sb71	Yes	4	–	–	No	–	No	–
Sb72	No	–	No	–	Yes	1	–	–

[a] number of heterozygous parents
[b] number of additional heterozygous parents
[c] only homozygous parents of different allelic classes were observed

ily by the length, with differences observed among alleles corresponding to deletions of portions of the DNA sequence and (or) insertions of DNA within this sequence. Alleles of several of these STS markers in black spruce have been shown to differ by the presence or absence of direct repeats (PERRY & BOUSQUET 2001). Alleles that differ by size are generally codominant and, following agarose gel electrophoresis, homozygotes are identified by the occurrence of a single PCR product while heterozygotes usually generate two or three bands, with the third displaying lower mobility and resulting from the formation of heteroduplex DNA (one strand of the double-stranded DNA contributed by each of the two alleles).

Polymorphism on agarose gels was noted for 13 loci in white spruce and for 14 loci in black spruce (Table 1). Most of the alleles revealed by agarose gel were codominant with previously noted exceptions (PERRY & BOUSQUET 2001, PERRY & BOUSQUET 1998a, b) of dominant length polymorphism at *Sb42* in black spruce, *Sb24* and *Sb71* in white spruce, and *Sb35* in both species and a null amplification allele at *Sb51* in black spruce. Two previously unreported alleles (*Sb32*, ~750 bp; *Sb71*, ~2400 bp) were observed in white spruce. In all cases, allelic segregation among megagametophytes of heterozygous trees was in accordance with Mendelian expectations ($p > 0.05$; data not shown).

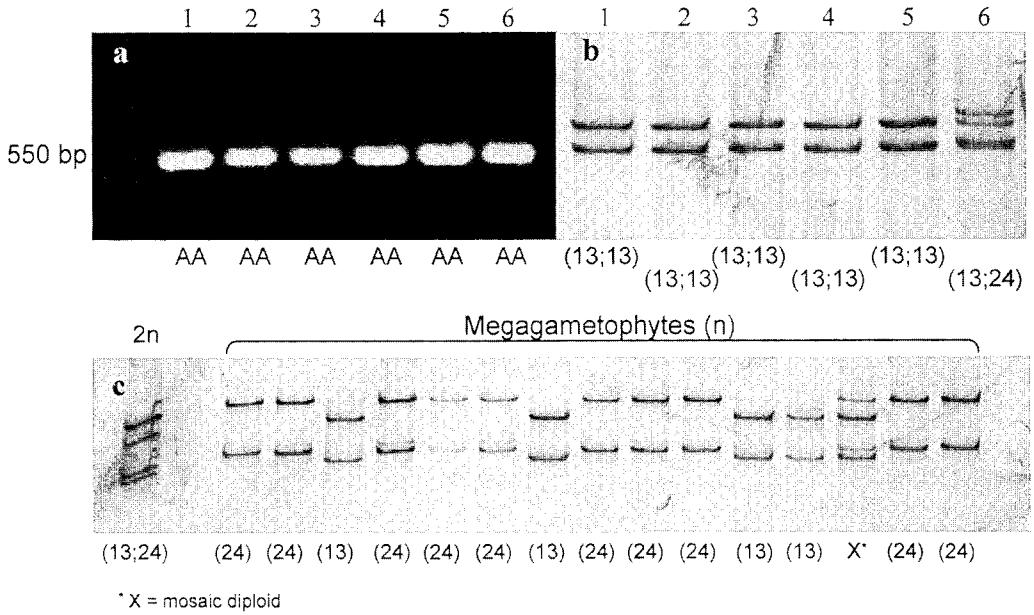
On agarose gels, loci *Sb01*, *Sb16*, *Sb17*, *Sb24*, *Sb29*, and *Sb35* were variable in both the white spruce and the black spruce examined here. Consequently, these

markers should be useful for comparing maps developed for both species. On the other hand, *Sb12*, *Sb18*, *Sb32*, *Sb41*, *Sb60* and *Sb71* showed polymorphism only within white spruce while *Sb06*, *Sb07*, *Sb11*, *Sb31*, *Sb36*, *Sb42*, *Sb52*, *Sb66* and *Sb72* were variable only within black spruce. While these markers may still be useful for mapping, they will be less informative.

When agarose gels failed to reveal polymorphism, we resorted to the more sensitive SSCP technique in an attempt to detect more subtle polymorphisms such as point mutations or small insertions and (or) deletions. While agarose gel electrophoresis is only capable of separating PCR products on the basis of size, the SSCP technique can detect differences in the secondary structure of single-stranded DNAs, and often allows discrimination between alleles that differ by as little as a single base substitution (HAYASHI & YANDELL 1993, SHEFFIELD *et al.* 1993).

SSCP analysis revealed additional polymorphism at 15 loci in white spruce and at 9 loci in black spruce (Table 1). Again, allelic segregation among megagametophytes of heterozygous trees was in accordance with Mendelian expectations ($p > 0.05$; data not shown). Following non-denaturing acrylamide gel electrophoresis, an individual SSCP allele generally displayed two distinct bands corresponding to the two different single-stranded DNA molecules produced by denaturing the double-stranded PCR product. Therefore, when diploid DNA was used as the PCR template, a homozygote was expected to produce a double-banded pattern while trees producing three- or four-banded patterns were inferred to be heterozygotes. PCR amplification of haploid megagametophyte DNA derived from putative heterozygotes generally produced double-banded patterns as expected of a single allele, and the segregation of allelic bands among different megagametophytes of the same parent was evident (e.g., Figs. 1 and 2). In rare instances, all of the three or four bands that were present in the diploid products of a heterozygote were also present in products derived from a single haploid megagametophyte from that same parent (e.g., Fig 1c, megagametophyte 13). This phenomenon may be attributed to the occasional occurrence of mosaic diploid megagametophytes in conifers (O'MALLEY *et al.*, 1988) or by contamination of megagametophyte DNA by diploid embryonic one in rare cases where diploid zygotic embryos could have not been entirely excised from megagametophytes.

The electrophoretic conditions that were found to give good resolution in each case are listed in Table 2. A migration time of 18 h was usually sufficient, but in some instances migration for 24 h gave better results. Temperature during electrophoresis has been shown to influence the patterns obtained (GLAVAC & DEAN 1993); our results are in agreement with this observation. We began by using a temperature of 10 °C, but in most cases we obtained better resolution at 4 °C or 15 °C. We found that the optimal conditions for each locus must be determined empirically and that no relationship appears to exist between product size and optimum temperature. For some loci, the optimum temperature seemed to be species-specific, as the best



* X = mosaic diploid

Figure 1. Detection of polymorphism in *Picea glauca* using marker *Sb21*. **a.** All parents (1–6) appeared homozygous on agarose gel with a fragment at 550 bp. **b.** Parent 6 seemed to be heterozygous on acrylamide gel (4 °C, 18 h). **c.** Example of segregation among megagametophytes of parent 6 (4 °C, 18 h).

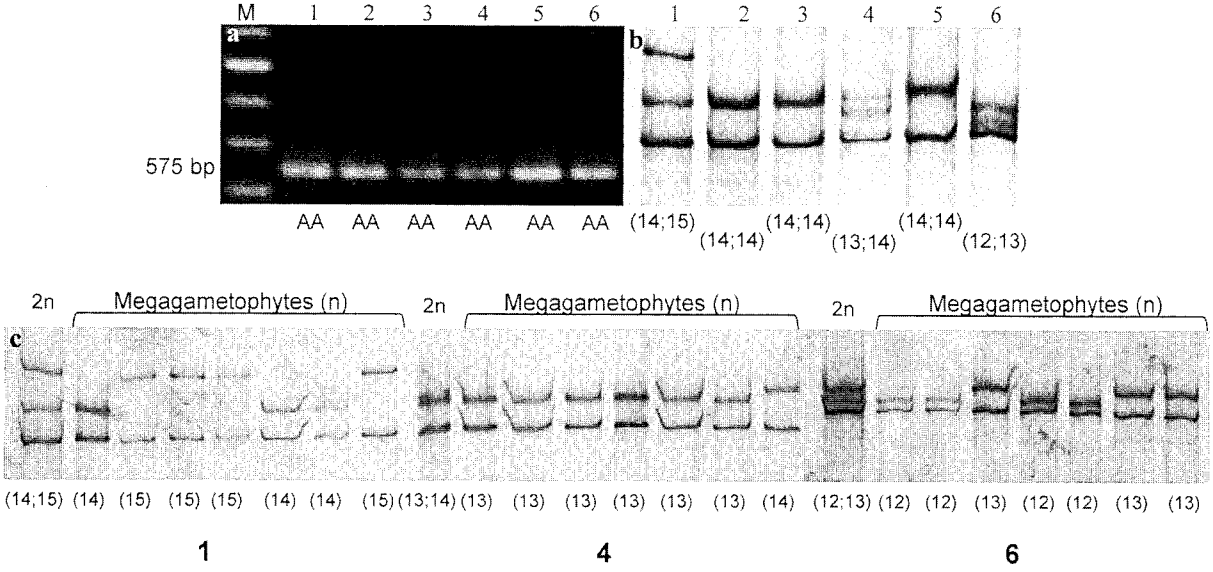


Figure 2. Detection of polymorphism in *Picea mariana* using marker *Sb06*. **a.** All parents (1–6) appeared homozygous on agarose gel with a fragment at 575 bp. **b.** Parents 1, 4 and 6 seemed to be heterozygous on acrylamide gel (15°C, 18 h). **c.** Examples of segregation among megagametophytes of parents 1, 4 and 6 (15°C, 18 h).

Table 2. Comparison of polyacrylamide gel electrophoretic conditions with respect to their power to reveal polymorphism.

Marker	Size (bp)	*Picea glauca*			*Picea mariana*		
		Migration temperature[1]			Migration temperature[1]		
		4°C	10°C	15°C	4°C	10°C	15°C
Sb06	550	/	/	/	G 18 h	P 18 h	P 18 h
Sb08	600	P 18 h	P 18 h	G 18 h	/	/	/
Sb11	695	ND	G 18 h	ND	/	/	/
Sb14	450	G 24 h	P 18 h	P 18 h	G 18 h	P 18 h	P 18 h
Sb21	575	P 18 h	P 18 h	G 24 h	P 18 h	P 18 h	G 18 h
Sb31	450	G 18 h	P 18 h	P 18 h	P 18 h	P 18 h	G 18 h
Sb32	750	ND	G 18 h	P 18 h	/	/	/
Sb36	425	P 18 h	P 18 h	G 18 h	P 18 h	P 18 h	P 18 h
Sb42	590	G 18 h	P 18 h	P 18 h	G 18 h	P 18 h	P 18 h
Sb49	350	G 18 h	P 18 h	P 18 h	G 18 h	P 18 h	P 18 h
Sb51	375	G 18 h	P 18 h	P 18 h	/	/	/
Sb52	925	G 18 h	P 18 h	P 18 h	G 18 h	P 18 h	P 18 h
Sb58	425	/	/	/	G 18 h	P 18 h	P 18 h
Sb60	375	P 18 h	P 18 h	G 18 h	/	/	/
Sb62	675	G 18 h	P 18 h	P 18 h	/	/	/
Sb64	520	G 18 h	P 18 h	P 18 h	/	/	/
Sb70	425	P 18 h	P 18 h	G 18 h	/	/	/
Polymorphic markers		8	2	5	6	0	2

/, monomorphic marker; G, good resolution; P, poor resolution; ND, not determined
[1] Migration was run under constant current (5 mA)

temperature for resolving polymorphism in white spruce was not necessarily the best temperature in black spruce (see *Sb31* in Table 2).

The number of loci that appeared monomorphic under standard agarose gel electrophoresis but polymorphic following SSCP analysis totaled twelve in white spruce and five in black spruce (Table 1). Thus, in the case of white spruce, SSCP generates more than twice

the number of informative markers obtained by agarose gel electrophoresis. In black spruce, the increase in informative markers was over one third. Three of these markers (*Sb14*, *Sb21* and *Sb49*) revealed SSCP polymorphism in both species. These represent additional markers that may be useful for comparing maps developed for both species. Only nine of the 39 markers tested (*Sb19*, *Sb28*, *Sb34*, *Sb46*, *Sb50*, *Sb53*, *Sb56*, *Sb67* and *Sb68*) were classified as monomorphic following both agarose gel electrophoresis and SSCP analysis.

Clearly, the application of the SSCP technique is useful for uncovering significant amounts of additional polymorphism at cDNA-based STS marker loci in white spruce and black spruce. Similar observations have also been reported for SSCP markers in the genus *Pinus* (PLOMION *et al.* 1999). Whereas the use of agarose gels may, in some instances, be a simple and convenient approach to detect polymorphic STS markers, the value of STS markers as anchor positions for comparative mapping purposes should justify the additional time and expense of using the more sensitive silver-stained acrylamide gels for SSCP detection. Given the high rate of polymorphism detection observed here (30 of the 39 markers tested were polymorphic in one or both species), it should not be difficult to locate large numbers of cDNA-based markers on saturated RAPD or AFLP marker-based maps. cDNA-based markers such as these have the added benefit of representing expressed genes. If large enough numbers of cDNA-based markers – perhaps derived from specific tissues or ontogenetic stages – are incorporated into genomic maps, the information gained from QTL analyses may lead directly to a more detailed understanding of the genetic basis of phenotypic features in conifers.

ACKNOWLEDGEMENTS

The authors thank Peter Copis, from the Petawawa Research Forest, as well as René Pâquet and Isabelle Gosselin, from the Canadian Forest Service, Laurentian Forestry Centre, for providing the samples. They are also indebted to Julie Dubé and Manuel Lamothe for technical assistance, to Pamela Cheers for her editing work, and to Michel Cusson for critically reading the manuscript and providing useful suggestions. This research was supported by a grant to N. Isabel and J. Beaulieu from the Canadian Biotechnology Strategy and a NSERC strategic grant to J. Bousquet, J. Beaulieu and N. Isabel.

REFERENCES

BASSAM, B. J., CAETENO-ANOLLES, G. & GRESSHOFF, P. M. 1991: Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal. Biochem.* **196**:80–83.

BEAULIEU, J., CORRIVEAU, A. & DAOUST, G. 1989: Phenotypic stability and delineation of black spruce breeding zones in Quebec. Forestry Canada, Quebec Region, Inf. Rep. LAU-X-85E.

BODÉNÈS, C., LAIGRET, F. & KREMER, A. 1996: Inheritance and molecular variations of PCR-SSCP fragments in pedunculate oak (*Quercus robur* L.). *Theor. Appl. Genet.* **93**:348–354.

DHILLON, S. S. 1987: DNA in tree species. *In*: Bonga JM, Durzan DJ (eds) Cell and tissue culture in forestry. Vol. 1 General Principles and Biotechnology, pp. 298–313. Martinus Nijhoff Publishers, Dordrecht.

GLAVAC, D. & DEAN, M. 1993: Optimization of the single-strand conformation polymorphism (SSCP) technique for detection of point mutations. *Hum. Mutat.* **2**: 404–414.

HAYASHI, K. & YANDELL, D. W. 1993: How sensitive is PCR-SSCP? *Hum. Mutat.* **2**:338–346.

ISABEL, N., TREMBLAY, L., MICHAUD, M., TREMBLAY, F. M. & BOUSQUET, J. 1993: RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis-derived populations of *Picea mariana* (Mill.) B.S.P. *Theor. Appl. Genet.* **86**:81–87.

JONES, C. J., EDWARDS, K.J., CASTAGLIONE, S., WINFIELD, M.O., SALA, F., VAN DE WIEL, C., BREDEMEIJER, G., VOSMAN, B., MATTHES, M., DALY, A., BRETTSCHNEIDER, R., BETTINI, P., BUIATTI, M., MAESTRI, E., MALCEVSCHI, A., MARMIROLI, N., AERT, R., VOLCKAERT, G., RUEDA, J., LINACERO, R., VAZQUEZ, A., & KARP, A. 1997: Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Mol. Breed.* **3**:381–390.

KUANG, H., RICHARDSON, T. E., CARSON, S. D. & BONGARTEN, B. C. 1998: An allele responsible for seedling death in *Pinus radiata* D. Don. *Theor. Appl. Genet.* **96**:640–644.

LEHNER, A., CAMPBELL, M. A., WHEELER, N. C., PÖYKKÖ, T., GLÖSSL, J., KREIKE, J. & NEALE, D. B. 1995: Identification of a RAPD marker linked to the pendula gene in Norway spruce (*Picea abies* (L.) Karst. f. *pendula*). *Theor. Appl. Genet.* **91**: 1092–1094.

OLSON, M., HOOD, L., CANTOR, C. & BOTSTEIN, D. 1989: A common language for physical mapping of the human genome. *Science* **245**:1434–1435.

O'MALLEY, D. M., GURIES, R. P. & WHEELER, N. C. 1988: Electrophoretic evidence for mosaic 'diploids' in megagametophytes of knobcone pine (*Pinus attenuata* Lemm.). *Silvae Genet.* **37**:85–88.

PERRY, D. J. & BOUSQUET, J. 1998a: Sequence-tagged-site (STS) markers of arbitrary genes: development, characterization and analysis of linkage in black spruce. *Genetics* **149**:1089–1098.

PERRY, D. J. & BOUSQUET, J. 1998b: Sequence-tagged-site (STS) markers of arbitrary genes: the utility of black spruce-derived STS primers in other conifers. *Theor. Appl. Genet.* **97**:735–743.

PERRY, D. J. & BOUSQUET, J. 2001: Genetic diversity and mating system of post-fire and post-harvest black spruce: an investigation using codominant sequence-tagged-site (STS) markers. *Can. J. For. Res.* **31**:32–40.

PERRY, D. J., ISABEL, N. & BOUSQUET, J. 1999: Sequence-tagged-site (STS) markers of arbitrary genes: the amount and nature of variation revealed in Norway spruce. *Heredity* **83**:239–248.

PLOMION, C., BAHRMAN, N., DUREL, C.-E. & O'MALLEY, D.M. 1995: Genomic mapping in *Pinus pinaster* (maritime pine) using RAPD and protein markers. *Heredity* **74**:661–668.

PLOMION, C., HURME, P., FRIGERIO, J.-M., RIDOLFI, M., POT, D., PIONNEAU, C., AVILA, C., GALLARDO, F., DAVID, H., NEUTELINGS, G., CAMPBELL, M., CANOVAS, F.M., SAVOLAINEN, O., BODÉNÈS, C. & KREMER, A. 1999: Developing SSCP markers in two *Pinus* species. *Mol. Breed.* **5**:21–31.

SHEFFIELD, V. C., BECK, J. S., KWITEK, A. E., SANDSTROM, D. W. & STONE, E. M. 1993: The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. *Genomics* **16**:325– 332.

TAUTZ, D. 1989: Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Res.* **17**:6463–6471.

VOS, P., HOGERS, R., BLEEKER, M., REIJANS, M., VAN DE LEE, T., HORNES, M., FRIJTERS, A., POT, J., PELEMAN, J., KUIPER, M. & ZABEAU, M. 1995: AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **23**: 4407– 4414.

WELSH, J. & MCCLELLAND, M. 1990: Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18**:7213–7218.

WILCOX, P. L., AMERSON, H. V., KUHLMAN, E. G., LIU, B.-H., O'MALLEY, D. M. & SEDEROFF, R. R. 1996: Detection of a major gene for resistance to fusiform rust disease in loblolly pine by genomic mapping. *Proc. Natl. Acad. Sci. USA* **93**:3859–3864.

WILLIAMS, J. G. K., KUBELIK, A. R., LIVAK, K. J., RAFALSKI, J. A. & TINGEY, S. V. 1990: DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**:6531–6535.