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Use of Haploid Mixtures and Heteroduplex Analysis Enhance Polymorphisms Revealed by Denaturing Gradient Gel Electrophoresis

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ABSTRACT

PCR-based codominant genetic markers were developed by using primer sequences designed from cDNA clones of loblolly pine (*Pinus taeda* L.). Such markers offer certain advantages relative to simple-sequence repeat (SSR), also known as short-tandem repeat (STR) markers, and include the ability to quantify and map DNA polymorphisms in expressed genes. However, detecting these DNA polymorphisms is more problematic because many DNA polymorphisms in genes involve base substitutions rather than insertions or deletions. Denaturing gradient gel electrophoresis (DGGE) is a sensitive and efficient method for detecting sequence differences among PCR fragments. This paper demonstrates the application of DGGE to genetically map expressed genes in loblolly pine. Also, heteroduplex DNA fragments, formed during the amplification of DNA from heterozygotes and from mixes of haploid DNAs from megagametophytes, enhanced and strengthened genetic interpretations and genotypic classifications.

INTRODUCTION

Genetic markers based on PCR are relatively quick and simple to use. However, to have broad utility, such PCR markers must satisfy two requirements: one, primers must be selected to reliably amplify a specific genomic target and two, polymorphisms must be readily discerned among DNA fragments that represent different haplotypes. Polymorphisms based on simple-sequence repeats (also known as short tandem repeats or microsatellites) are often effective within a species. However, such primers frequently fail to amplify DNA from related species,

presumably because of sequence divergence among primer annealing sites.

We have developed codominant genetic markers by selecting PCR primers that are based on cDNA sequences and then digesting the amplified fragments with restriction endonucleases (4). Because such PCR primers are based on expressed genes, they may allow amplification of orthologous regions in other species (11,13). Although length variants among undigested PCR fragments have been detected in some instances (10,11), results to date suggest that DNA substitutions may be more common (4,13). Restriction endonucleases can detect some of this variation, but screening for polymorphisms is tedious and typically reveals only two alleles for each polymorphic restriction site, which leads us to seek a more efficient strategy for detecting sequence polymorphisms.

This paper describes a technique of using denaturing gradient gel electrophoresis (DGGE) (2,8) to detect polymorphisms in DNA fragments amplified from loblolly pine (*Pinus taeda* L.) using primers developed from cDNA clones. The method provides a rapid and efficient means for detecting multiple alleles and classifying genotypes. In addition, by amplifying mixtures of haploid DNA extracted from megagametophytes and by analyzing properties of the heteroduplexes revealed by DGGE, haplotypes can also be accurately classified. The efficient detection of sequence polymorphisms in amplified DNA will greatly facilitate the mapping of candidate genes or other genes of interest.

MATERIALS AND METHODS

PCR primers were designed from sequences of loblolly pine cDNA clones as previously described (see Table 1 and Reference 4). DNA was isolated from loblolly pine needles and from haploid megagametophytes (a seed tissue), which represented individuals in two three-generation pedigrees used for genetic mapping. Grandparents and parents from the Base Map population (1) are coded B₁–B₆ (Figure 1), and those from the quantitative trait loci (QTL) population (3) are coded Q₁–Q₆. Genomic DNAs (15 ng) were amplified in 25 μ L volumes using a combination of hot-start and touchdown conditions as previously described (4).

DNA polymorphisms were detected either by digesting PCR products with restriction endonucleases (4) or by using DGGE. DGGE gels were made up using either 6% or 8% acrylamide (37.5:1 acrylamide:bis) in 1 \times TAE buffer (7) and contained a linear gradient of denaturant. The gradients were constructed by using a Model 475 Gradient Delivery System, and electrophoresis was done using a D-GENE™ apparatus (both from Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions.

For each set of primers, an optimum denaturing gradient was determined by running one gel with a 0%–80% denaturing gradient that was oriented perpendicular to the electrical field (8). By convention, the 100% denaturant contains 7 M urea and 40% vol/vol formamide (8). The PCR product (100 μ L)

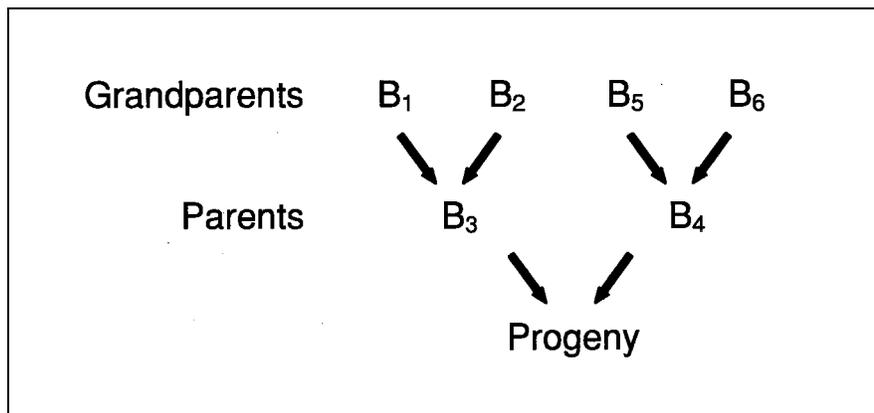


Figure 1. Pedigree structure and identifying codes for individuals in Base Map population of loblolly pine. Codes for individuals in QTL population are similar, except that B₁–B₆ are replaced by Q₁–Q₆.

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was mixed with an equal volume of loading solution (20% Ficoll 400, 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, pH 8.0, 0.1% bromophenol blue, 0.1% xylene cyanol) and then loaded into a single well that spanned the width of the gel. Gels were run at a constant temperature and voltage (58°C, 175 V) for approximately 4 h, until the xylene cyanol dye reached the bottom of the gel; they were then stained with 0.5 µg/mL ethidium bromide. A narrower gradient (e.g., 15%–45% denaturant; Table 1) was visually approximated to maximize separation of the partially denatured DNA fragments (8). DNA polymorphisms were subsequently assessed by loading 4–8 µL of PCR product (mixed 1:1 with loading solution) into each of 16–32 wells in gels, for which the denaturing gradient increased from the top to the bottom, parallel to the electrical field. These gels were run using the same electrophoretic conditions as used for perpendicular gels.

RESULTS AND DISCUSSION

For a given locus, most of the PCR fragments amplified by using primers from cDNA clones were indistinguishable when they were visualized using 2% agarose gels stained with ethidium bromide, as seen in Figure 2A (4). This observation indicates that polymorphisms in fragment length are either relatively rare or they are too small to readily detect through electrophoresis in standard agarose gels. Restriction digests of these PCR fragments reveal some variation in DNA sequence (Figure 2B), but even more polymorphisms are revealed after subjecting undigested fragments to DGGE (Figure 2C). For example, the *Dra*I digestion might suggest that all individuals with only one DNA fragment are identical homozygotes, but DGGE reveals at least three distinct phenotypes (Figure 2B compared with Figure 2C, lanes 4–7, 9 and 11). Furthermore, even among individ-

uals that appear to be heterozygous for a *Dra*I restriction site, DGGE reveals additional phenotypes (Figure 2B compared with Figure 2C, lanes 1–3, 8, 10 and 12).

Among heterozygotes, DGGE usually reveals more than two types of DNA fragments (Figure 2C), which indicates the presence of homoduplex and heteroduplex molecules (9,12). Heteroduplexes are formed by the annealing of complementary DNA strands containing mismatched bases (e.g., different alleles). Heteroduplexes (Figure 2C) have a lower melting point than their homoduplex counterparts, so their mobility is retarded relative to homoduplexes in DGGE gels. For *stsPtIFG-2253*, DGGE reveals at least three alleles among the Base Map (A–C) and QTL (A'–C') parents and grandparents (Figure 2C) known to segregate in a Mendelian fashion (Temesgen et al., manuscript in preparation).

PCR fragments representing homoduplexes amplified from different alleles can appear to be quite similar (Figure 2C, fragments B and C). The fact that these fragments represent distinct alleles is inferred by examining heteroduplexes: homozygous individuals (Figure 2C, lanes 5, 7 and 9) lack heteroduplexes, and for heterozygotes, heteroduplex fragments are readily apparent (e.g., Figure 2C, lanes 4 and 6). This interpretation is confirmed by examining the offspring phenotypes that stem from Base Map parents B₃ and B₄ (Figure 3A). Both parents are heterozygous (B₃ is A/C and B₄ is B/C); four classes of progeny are observed, including C/C homozygotes devoid of heteroduplexes (Figure 3A, lane 3). Here again, heteroduplexes help to discriminate among offspring genotypes: only subtle differences are apparent among homoduplex fragments (Figure 3A, lane 3 compared with lane 6 and lane 4 compared with lane 5), while the differences among heteroduplexes are striking. The usefulness of heteroduplexes in discriminating among homozygotes and heterozygotes has been noted in several other species (6,10,12,14).

Gene families are common in pines and other conifers (5). PCR primers designed from cDNA sequences might amplify DNA from closely related members of some gene families, which

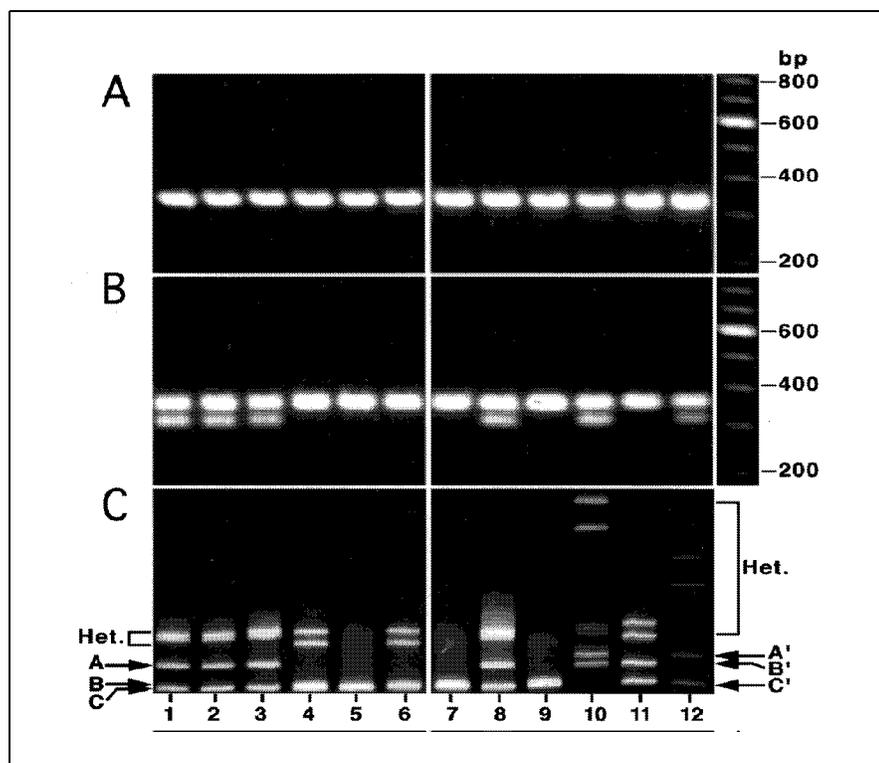


Figure 2. PCR fragments amplified from genomic DNA of loblolly pine using primer set *stsPtIFG2253*. Grandparents and parents (see Figure 1) are shown for each of two mapping populations: individuals B₁–B₆ are in lanes 1–6, and individuals Q₁–Q₆ are in lanes 7–12. (A) Undigested fragments following electrophoresis in 2% agarose and stained with ethidium bromide. (B) Same as (A) except that the PCR fragments were digested with *Dra*I. (C) Undigested PCR fragments separated using DGGE. Different alleles in each population are labeled A–C (Base Map) and A'–C' (QTL). Heteroduplexes (Het.) are indicated by brackets.

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Table 1. Primer Sequences and Sizes of PCR Fragments for Each of Five Loci from Loblolly Pine

Locus	Primer Sequences (For. and Rev., 5'→3')	Size (bp)	Denaturing Gradient	No. Segregating Alleles	
				Base Map Population	QTL Population
stsPtIFG107	GCA GGA CCT TCT GGA CAA TC AGG TGG AGA AAG CCA AGC TC	450	30%–60%	2	3
stsPtIFG500	GGC GAG TTG GCT TTC ATT C CAG CGA GGT ACC AGA TTT GC	500	15%–45%	2	2
stsPtIFG1950	AAA CCA GCA GCC ACA TGA G TAT TAA GAA GGC GGC GGT AC	450	15%–45%	4	3
stsPtIFG2253	CCA ATT TGC ACT TTG CCC CCA AAG CCC AAA TCC ATG	370	20%–45%	3	3
stsPtIFG2274	TGA TCA GAG AGC TGG TGC AG AGA TGA GCA TCA GGT CAG CC	600	15%–45%	3	2

Also shown are the denaturing gradients used for each locus and the number of alleles segregating in each mapping population. All fragments were analyzed in DGGE that consisted of 6% acrylamide gels.

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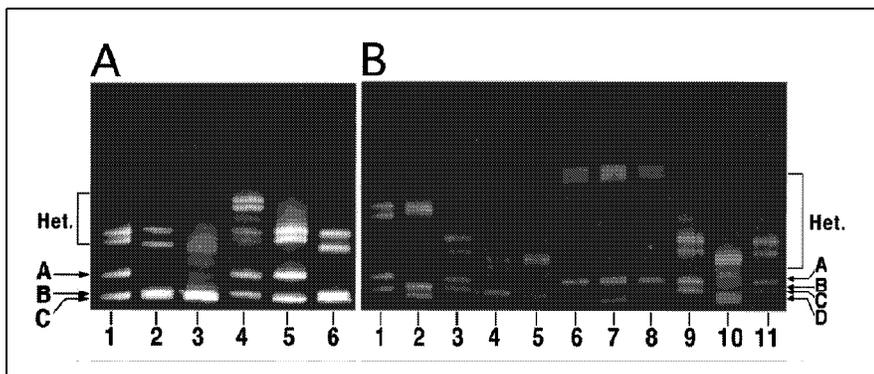


Figure 3. DGGE reveals the segregation of multiple alleles among parents and their progeny. (A) PCR fragments from Base Map parents B₃ (lane 1) and B₄ (lane 2) and four of their progeny (lanes 3–6) amplified using primer set, stsPtIFG2253. Progeny were selected to represent all four phenotypic classes. (B) PCR fragments from QTL parents Q₃ (lane 1) and Q₄ (lane 2), and nine of their progeny (lanes 3–11) amplified using primer set, stsPtIFG1950.

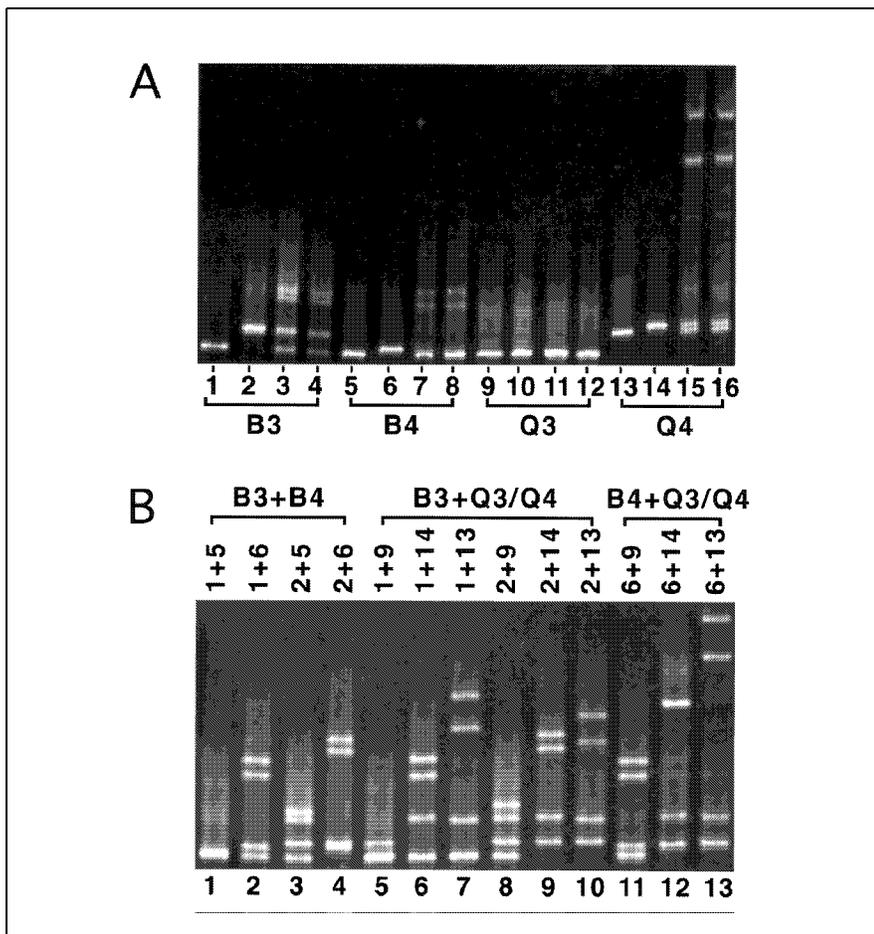


Figure 4. PCR-amplified mixes of haploid DNAs followed by DGGE enhance allelic classifications. Amplifications were done using primer set, stsPtIFG2253. (A) Reconstructing parental phenotypes using DNA from megagametophytes: B₃ (lanes 1–4), B₄ (lanes 5–8), Q₃ (lanes 9–12) and Q₄ (lanes 13–16). Each set of four lanes is organized as follows: the first and second lanes are haploid DNAs from individual megagametophytes; the third lane is a mixture of haploid DNAs and the fourth lane is diploid DNA from the parent. (B) Paired mixtures of haploid DNAs. Numbers at the top of each lane describe specific mixtures of haploids referenced by lane number in Panel A. For example, lane 1 (Panel B) contains a mixture of haploids shown in Panel A, lanes 1 and 5. Lanes 1–4 are mixtures of B₃ with B₄, while lanes 5–10 are mixtures of B₃ with either Q₃ or Q₄; and lanes 11–13 are B₄ with either Q₃ or Q₄.

could complicate genetic analyses of these fragments (4). Consequently, we expect that the use of DGGE to analyze genes will be limited to some degree. Nevertheless, amplified fragments that represent gene families can be genetically tractable when using DGGE. On the basis of restriction fragment length polymorphisms from Southern blots, the cDNA clone pPtIFG1950 belongs to a modestly complex gene family (4). Even though individual DNA fragments are not as crisply defined and the background smearing is increased (perhaps in part because of multiple heteroduplexes), phenotypic classifications based on the most abundant fragments are relatively clear (Figure 3B). QTL parents Q₃ and Q₄ are both heterozygous for stsPtIFG1950 (Figure 3B, lanes 1 and 2), and four phenotypic classes are apparent among their progeny (Figure 3B: lanes 3, 9 and 11 compared with lanes 5 and 10 compared with lanes 6–8 compared with lane 4).

DNA polymorphisms were surveyed for several additional loci using DGGE (Table 1). Amplified fragments ranged in size from 370–600 bp, with loci segregating two to four alleles among progeny within the Base Map and QTL populations. The upper limit of fragment size for using DGGE to detect polymorphisms is reported to be approximately 600 bp (2). Our experience suggests polymorphisms among 500–600 bp fragments are routinely resolved using DGGE, and fragments as large as 950 bp can be analyzed (Temesgen, et al., manuscript in preparation).

Haploid tissues from conifer megagametophytes provide unique advantages for understanding marker inheritance (4). To better characterize heteroduplex PCR fragments, we mixed haploid DNA isolated from megagametophytes that were dissected from the seeds of various individuals. As long as PCR fragments represent a single gene, then amplification of haploid DNA should preclude the formation of heteroduplexes. Similarly, the DGGE phenotypes of diploid heterozygotes can be selectively reconstructed by mixing haploid DNAs before amplification (Figure 4 and Reference 10). Using primers for stsPtIFG2253, homoduplex molecules are the predominant product among the PCR fragments amplified

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from megagametophyte DNAs from the four Base Map and QTL parents (Figure 4A, lanes 1–2, 5–6, 9–10 and 13–14). In addition to homoduplexes, mixtures of DNAs from two types of megagametophytes also produce prominent heteroduplexes with overall phenotypes that are indistinguishable from those of the corresponding diploid parents (Figure 4A, lanes 3 compared to 4, 7 compared to 8 and 15 compared to 16). For parent Q₃, which is known to be homozygous for stsPtIFG2253 based on other tests, the DGGE banding patterns from megagametophytes, DNA mixtures and diploid tissues are indistinguishable (Figure 4A, lanes 9–12).

In addition to reconstructing parental phenotypes, DNA mixtures from different haploids can be used to reconstruct progeny phenotypes. For example, paired combinations of DNA from B₃ and B₄ megagametophytes (Figure 4B, lanes 1–4) can be used to reconstruct the four phenotypic classes seen among their diploid offspring (Figure 3A). In this case, B₃ and B₄ share a common allele, as evidenced by their homozygous progeny (Figure 3A, lane 3 and Figure 4B, lane 1).

Extending this idea, we used haploid mixtures to examine allelic identities among samples or populations that have not been intermated. Homoduplex fragments from B₄ (Figure 4A, lane 5) closely resemble those from Q₃ (Figure 4A, lanes 9–10), but heteroduplex fragments are apparent from mixes of these haploid samples (Figure 4B, lanes 5 and 11). Heteroduplexes are apparent among all of the nine paired combinations (Figure 4B, lanes 5–13), which indicates that the two mapping populations share no common alleles. This difference is significant because it reveals the sensitivity of DGGE to detect polymorphisms: a total of six distinct haplotypes are represented within only four loblolly pine parents.

DGGE has proven to be an efficient method for detecting polymorphisms that encompass multiple alleles among PCR fragments amplified from expressed genes. While requiring only a few analyses, DGGE appears to reveal more variation than do restriction digests (4). Heteroduplex molecules formed during the amplification of heterogeneous DNA samples can be de-

tected by using non-denaturing polyacrylamide gels (9,14), by using DGGE (6,12) and even by using agarose gels in some cases (10,11). However they are detected, heteroduplexes enhance the ability to discriminate among phenotypes, thereby enhancing genotypic classifications and reducing errors (6,12). Given the importance of heteroduplexes in classifying genotypes, being able to reconstruct diploid phenotypes from haploids is particularly useful, besides being a powerful tool for classifying alleles. Because PCR primers from expressed genes are often useful across a variety of related species (11,13, Harry, et al., manuscript in preparation, Brown, et al., manuscript in preparation), we expect these markers to be useful for a variety of applications, including the mapping of genes, consensus mapping and in comparing genetic maps across species.

REFERENCES

1. Devey, M.E., T.A. Fiddler, B.-H. Liu, S.J. Knapp and D.B. Neale. 1994. An RFLP linkage map for loblolly pine based on a three-generation outbred pedigree. *Theor. Appl. Genet.* 88:273-278.
2. Grompe, M. 1993. The rapid detection of unknown mutations in nucleic acids. *Nat. Genet.* 5:111-117.
3. Groover, A., M.E. Devey, T. Fiddler, J. Lee, R. Megraw, T. Mitchell-Olds, B. Sherman, S. Vujcic, C. Williams and D.B. Neale. 1994. Identification of quantitative trait loci influencing wood specific gravity in outbred pedigree of loblolly pine. *Genetics* 138:1293-1300.
4. Harry, D.E., B. Temesgen and D.B. Neale. 1998. Codominant PCR-based markers for *Pinus taeda* developed from mapped cDNA clones. *Theor. Appl. Genet.* 97:327-336.
5. Kinlaw, C.S. and D.B. Neale. 1997. Complex gene families in pine genomes. *Trends Plant Sci.* 2:356-359.
6. Lanneluc, I., P. Mulsant, B. Harrison and R.C. Drinkwater. 1996. Denaturing gradient gel electrophoresis detection of polymorphism in a PCR fragment of sheep epidermal growth factor gene. *Anim. Genet.* 27:99-101.
7. Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual*. CSH Laboratory Press, Cold Spring Harbor, NY.
8. Myers, R.M., T. Maniatis and L.S. Lerman. 1987. Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Methods Enzymol.* 155:501-527.
9. Nagamine, C.M., K. Chan and Y.C. Lau. 1989. A PCR artifact: generation of heteroduplexes. *Am. J. Hum. Genet.* 45:337-339.
10. Perry, D.J. and J. Bousquet. 1998. Sequence-tagged-site (STS) markers of arbitrary genes: Development, characterization and analysis of linkage in black spruce. *Genetics* 149:1089-1098.
11. Perry, D.J. and J. Bousquet. 1998. 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.
12. Sheffield, V.C., J.S. Beck, B. Nichols, A. Cousineau, A.C. Lidral and E.M. Stone. 1992. Detection of multiallele polymorphisms within gene sequences by GC-clamped denaturing gradient gel electrophoresis. *Am. J. Hum. Genet.* 50:567-575.
13. Tsumura, Y., Y. Suyama, K. Yoshimura, N. Shirato and Y. Mukai. 1997. Sequence-tagged-sites (STSs) of cDNA clones in *Cryptomeria japonica* and their evolution as molecular markers in conifers. *Theor. Appl. Genet.* 94:764-772.
14. White, M.B., M. Carvalho, D. Derse, S.J. O'Brien and M. Dean. 1992. Detecting single base substitutions as heteroduplex polymorphisms. *Genomics* 12:301-306.

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