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Inheritance and molecular variations of PCR-SSCP fragments in pedunculate oak (*Quercus robur* L.)

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Abstract Single-strand conformaiton polymorphism (SSCP) profiles of six PCR-amplified fragments (250–800 bp) were analyzed in three full-sib families of pedunculate oak (*Quercus robur* L.) and their parents. Among the six fragments, four were polymorphic and one exhibited complex patterns that were not changed by varying the SSCP conditions. The number of bands for the analyzed fragments varied between two and four among individuals regardless of fragment size. As shown by segregation data, the variation in the number of bands between trees could only be attributed to the allelic composition (homozygotes vs heterozygotes): a genotype that exhibited two bands was presumptively homozygous, wheras a genotype exhibiting three or four bands was heterozygous. Mendelian proportions were observed in all crosses for each polymorphic fragment. In one cross, we could clearly identify a null allele due to a possible mutation at a primer site. Single-base mutations and short insertion-deletions were shown to be the molecular causes of the SSCP polymorphism observed between different alleles. The use of SSCP as a technique to identify co-dominant markers of PCR fragments (up to 800 bp) is recommended for gene diversity studies or for gene mapping.

Key words *Quercus robur* · PCR · SSCP
Co-dominant marker · Mendelian inheritance

Introduction

The single-stand conformation polymorphism (SSCP) technique is a simple, fast and very sensitive method to identify nucleotide-sequence polymorphisms. Currently used to detect small mutations is human DNA as a disease diagnostic (Dockhorn-Dwornizak et al. 1991; Lenk et al. 1994; Michaud et al. 1992; Orita et al. 1989a; Sarkar et al. 1992), it has been recently applied to the identification of genetic relationships among different species and populations of beetles (Boge et al. 1994), to species identification in bacteria (Widjojoatmodjo et al. 1994) and endomycorrhizal fungi (Simon et al. 1993), and to the detection of mutations in the chloroplast genome (To et al. 1993) or to genetic mapping of the bovine genome (Kirpatrick and Hart 1994) and rice genome (Fukuoka et al. 1994).

SSCP is a technique based on the principle that single-stranded DNA molecules take on a specific sequence-based secondary structure under non-denaturing conditions. Molecules differing by as little as a single base substitution may form different conformations which may result in different mobilities in a non-denaturing polyacrylamide gel. Especially when it is used as a disease diagnostic, SSCP aims at detecting any existing mutations on a given fragment. Consequently, experiments were conducted to optimize protocols to exhaustively identify single mutations (Galvac and Dean 1993), which resulted in the definition of a critical fragment length of 150–200 bp (Orita et al. 1989a; Hayashi et al. 1993; To et al. 1993).

Due to its sensitivity to single mutations, SSCP offers a great potential for searching for polymorphisms within a given fragment in genetic studies. This is the case when informative fragments are transformed into SCARs [sequenced-characterized amplified regions (Paran and Michelmore 1993)] whereas digestion by restriction enzymes does not always reveal polymorphisms. It may also be applied when Expressed sequenced tags (ESTs) are used to design primers for amplifying informative regions.

Our main goal was to test the potential of SSCP as a routine tool to obtain molecular markers in oaks (*Quercus robur*) where large sample sizes are needed for genetic mapping or gene-diversity studies. In this respect,

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the objectives of this contribution were fourfold: (1) to test SSCP for various DNA fragment lengths larger than the critical 200 bp, provided that a search of all of the mutations existing in the fragment is not the goal, (2) to evaluate the reproducibility of the electrophoretic profiles, (3) to check the Mendelian inheritance of the SSCP patterns and (4) to identify the molecular causes of SSCP (i.e. single-base changes, variation in short repeats, microsatellite slippage).

Materials and methods

Plant material

Three intraspecific crosses of *Q. robur* were used for segregation analysis of SSCP markers. Sample sizes of the progeny varied from 26 for the first two crosses, 32P × A6 and 34P × A3, to 48 for the third cross, 33P × A3.

The parents of the crosses were adult trees (> 100 years) planted on the campus of the Forestry Research Station at INRA, Pierroton, or in a public park in Arcachon (South West of France). Controlled crosses were made in 1989 and 1992, and acorns were sown in the spring of 1990 and 1993. Buds from adult trees and leaves from 1-year-old seedlings were harvested for DNA extraction. The three crosses were validated by segregation analysis based on isozymes. Segregation ratios obtained in the offspring were consistent with the genotypes of the parents (Zanetto et al. 1996).

DNA extraction, PCR amplification and migration

Genomic DNA was extracted from buds or leaves frozen at −80°C according to Doyle and Doyle (1990) with minor modifications (Moreau et al. 1994).

Oligonucleotide sequences (18–20 bp) were used as primers to amplify fragments of different sizes (Table 1). The sequences of the fragments were obtained by sequencing both extremities of RAPD fragments that showed significant frequency differences between *Q. petraea* and *Q. robur* (Moreau et al. 1994). Fragments additional to those of Moreau et al. (1994) were obtained more recently (unpublished results) and were included in the present study. The six pairs of primers studied amplify fragments varying in size from 250 to 800 bp. PCR fragments were characterized according to the Operon primer used for the random amplification and their length.

Template DNA (5 ng) was amplified by PCR in a total volume of 15 µl containing 0.2 µM of each primer, 67 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 ng of BSA, 0.2% β-mercaptoethanol, 16 mM ammonium acetate, 100 µM of each dNTP, and 0.8 U of *Taq* DNA polymerase (Gibco BRL). The conditions of amplification were programmed on a thermal cycler (Techne PHC3) using 1 cycle at 94°C for 3 min and 30 cycles of 45 s at 94°C, 45 s at the annealing temperature (47.5°C to 57°C) and 90 s at 72°C. To check the amplification, 5 µl of the reaction mixture were resolved electrophoretically in a 1% agarose gel and stained with ethidium bromide.

SSCP conditions

For SSCP analysis, 2 µl of PCR products were added to 5 µl of a solution containing 95% formamide, 10 mM NaOH, 0.05% of xylene cyanol, and 0.05% of bromophenol blue. The samples were heated to 94°C for 4 min then immediately cooled on ice and loaded onto a 0.75 mm × 16 cm × 18 cm non-denaturing acrylamide gel (0.5 × MDE; Bioprobe Systems, 0.6 × TBE). Electrophoresis was run during 14 h at 15 V/cm or 10 V/cm (depending on the size of the fragment) at a constant temperature of 15°C in 0.6 × TBE running buffer. The gel was then silver-stained according to Bassam et al. (1991) as follows: the gels were fixed in 10% acetic acid for 20 min and washed three times with de-ionized water for 2 min. Color impregnation lasted for 30 min with 0.1% silver nitrate and 0.056% formaldehyde. The gels were then rinsed for 10 s with de-ionized water and color development was obtained after a 5–10 min bath in a mixture with 30 g of sodium carbonate per l, 0.056% formaldehyde and 2 mg of sodium thiosulphate per l. The color reaction was stopped with 10% acetic acid. The gels were rinsed for 10 min with de-ionized water and dried between plastic sheets.

Additional conditions were tested for two fragments (I16–500 and B12–750) that showed monomorphic patterns over the five parents (A3, A6, 32P, 33P and 34P): concentration of glycerol (0%, 5% and 10%), concentration of primers (0.2 µM, 0.15 µM and 0.1 µM). Similarly, for I14–250, which exhibited complex patterns, alternative conditions were used by varying the concentration of glycerol (0%, 5% and 10%), the migration temperature (4°C, 15°C and room temperature), and the intensity of running voltage (10–20 V/cm).

Sequencing

Molecular causes of SSCP fragments were investigated for two fragments (P14-450 and I14-800) by sequencing PCR products in two crosses: 33P × A3 for P14-450 and 34P × A3 for I14-800. For each cross, the sequences were obtained for the two parents and the 2–4 different genotypic classes of the offspring. PCR products for cycle sequencing were purified using Promega Wizard™ PCR preps. Then they were cloned in the pGEM-T^R vector System II (Promega) following the manufacturer's instructions. The nucleotide sequences of the cloned PCR products were established using the T7 sequencing™ kit (Pharmacia) and the universal forward and reverse primers. Three clones for homozygous, and eight clones for heterozygous, trees were sequenced.

Table 1 Description of the primers (sequence and annealing temperature) used for specific amplification of the different fragments

Primer name	Sequence 5′ 3′	Length of amplified fragment (bp)	Annealing temperature (°C)
I14-250 S	GCG TGG AGG TTG CCA CTG AT	250	57
I14-250 E	CGG CGT CAC TTC AAC TAA TG		
P14-450 S	TGC AAA ACA AAC TAA ACA TT	450	47.5
P14-450 E	AAT CCA GTG GCA AGT TTT AA		
I16-500 S	ATG GAA TAG TGA AAA GAG AC	500	47.5
I16-500 E	TAA AGC ACA TAA GAA TAA GA		
B12-750 S	GTT TAA GCC CAA TTT TTA TT	750	46
B12-750 E	TTT GAA GTT GAT ACA TAT TC		
I14-780 S	GGC GGT ACA ACA AAC TTA TA	780	57
I14-780 E	CAG AGG TCC ATT TGA GAT TA		
U7-790 S	GCC CTA ACA AAT CAT CTC	790	47.5
U7-790 E	TAA TAT AGA AAG GGA AAG		

Results

Reproducibility of SSCP patterns

Six pairs of primers were used to amplify DNA fragments of 250, 450, 500, 750, 780 and 790 bp (Table 1). Four of them (I14-250, P14-450, I14-780 and U7-790) showed polymorphic SSCP fragments whereas the two others (I16-500 and B12-750) did not reveal any polymorphism among the five parents tested. To optimize the detection of mutations on the two monomorphic fragments, or to improve the resolution of SSCP profiles corresponding to I14-250 which gave complex patterns, various concentrations of glycerol and primers, and varying intensities of running voltage and the temperature of migration, were used. As mentioned by several authors (Spinardi et al. 1991; Glavac et Dean 1993; Leren et al. 1993), these factors may influence the separation of the different strands. The modifications of SSCP conditions sometimes changed the electrophoretic profiles but did not improve the detection of mutations or the resolution of the patterns. As a result, the conditions of SSCP were fixed at 15°C without glycerol, and at 15 V/cm except for the I14-250 fragment for which the running voltage was 10 V/cm. For each sample, we obtained two, three or four bands on SSCP gels (Fig. 1) and the number of bands did not vary with the size of the fragment. With these conditions, the segregations observed in the offspring were always consistent with the patterns observed in the parents: the bands present in the parents were also present in the offspring and there was no additional or missing band in the offspring (Fig. 1). The concordance between the parental and offspring profiles is an indirect validation of the reproducibility of the technique.

Mendelian inheritance of SSCP fragments

As an example, we report first the segregation observed in one cross, 32P × A6, for the primer pair U7-790 (Fig. 2B). Four different bands were present in the parents B1, B2, B4 and B5 (B1 and B4 in the female parent 32P, and B1, B2, B4 and B5 in the male parent A6). The offspring also exhibited these four bands but in different combinations (Fig. 2B). We obtained the following results for 26 individuals of the progeny: ten individuals showed B1 and B4 and 16 showed B1, B2, B4 and B5 (Table 2). Hence, B2 and B5 were associated and were segregating in the offspring; B1 and B4 were always associated. The observed proportions of progeny genotypes were not significantly different from those expected under Mendelian inheritance (Table 2).

We concluded that the female parent 32P was homozygous at the allele exhibiting bands B1 and B4 whereas parent A6 was heterozygous, one allele corresponding to bands B1 and B4 and the other corresponding to B2 and B5. As a result, the genotypic types observed in the progeny are: (1,4;1,4) and (1,4;2,5). By convention, we denote the two strands (bands) corresponding to an allele by numbers (2,5 are strands corresponding to bands B2 and B5 for a given allele).

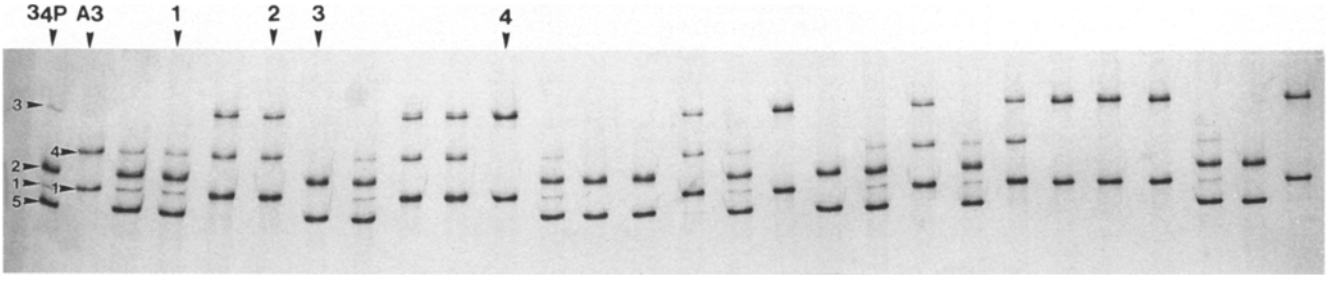
For all other polymorphic fragments (except I14-780, which will be detailed in the next paragraph) allelic compositions could be inferred from comparisons between the segregation of bands in the offspring and the parental profiles (Table 2, Fig. 2). No significant departure from Mendelian proportions was observed (Table 2). The electrophoretic profiles that were identified in the different crosses exhibited some general features that may be specific to SSCP fragments:

The two strands corresponding to a given allele were usually distal on the gels. As suggested by Sheffield et al. (1993), the difference in the migration distance of the strands may be related to the base composition.

There is a definite variation in the mobility for the two strands of a given allele. Two different homozygous parents may share one band (for example 33P and A3 on Fig. 2A). The sensitivity of mutation detection is often much greater for one strand compared to the complementary strand (Michaud et al. 1992; Sheffield et al. 1993). As a result, heterozygous genotypes may exhibit either three or four bands.

The three crosses analyzed for fragment I14-780 revealed unexpected features. The first cross, 32P × A6, combined two heterozygous parents and SSCP markers

Fig. 1 Electrophoretic patterns of SSCP markers for the cross 34P × A3 (primer pair I14-780 was used for PCR amplification). *Lane 1*: female parent 34P, genotype (2,5;1,3); *lane 2*: male parent A3, genotype (1,4;-); other lanes correspond to the offspring. Four types were identified: type 1 in *lane 4* (2,5;1,4), type 2 in *lane 6* (1,3;1,4), type 3 in *lane 7* (2,5;-) and type 4 in *lane 11* (1,3;-)



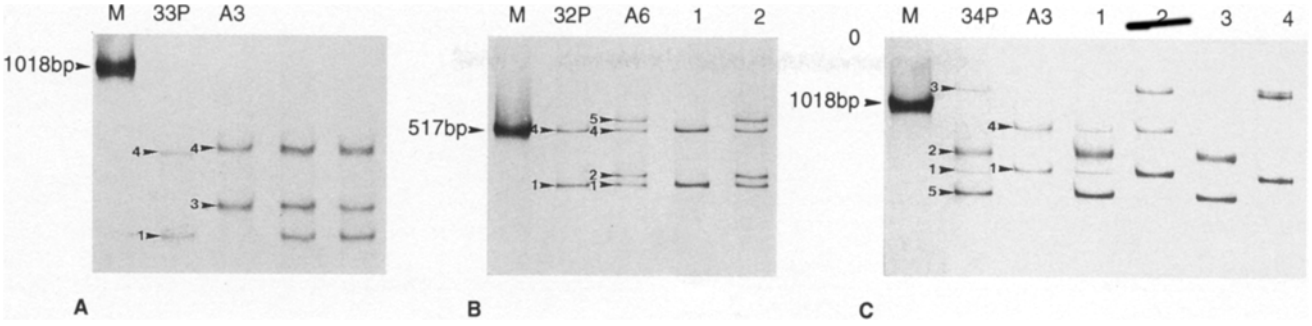


Fig. 2A–C Electrophoretic SSCP patterns of parental trees and their different types of offspring for three analyzed fragments. **A** Fragment P14-450. *Lane 1*: molecular-weight ladder; *lane 2*: female parent 33P, genotype (1,4; 1,4); *lane 3*: male parent A3, genotype (3,4; 3,4); *lanes 4 and 5* correspond to the one segregation type observed in the offspring (1,4; 3,4). **B** Fragment U7-790. *Lane 1*: molecular-weight marker; *lane 2*: female parent 32P, genotype (1,4; 1,4); *lane 3*: male parent A6, genotype (1,4; 2,5); *lanes 4 and 5* correspond to the two segregation types observed in the offspring 1 (1,4; 1,4) and 2 (1,4; 2,5). **C** Fragment I14-780. *Lane 1*: molecular-weight ladder; *lane 2*: female parent 34P, genotype (1,3; 2,5); *lane 3*: male parent A3, genotype (1,4; -); *lanes 4 to 7* correspond to the four segregation types observed in the offspring 1 (1,4; 2,5), 2 (1,4; 1,3), 3 (2,5; -) and 4 (1,3; -)

show Mendelian segregation (Table 2). The two other crosses presented unexpected ratios if co-dominance is assumed. For example, for cross 34P × A3 (Fig. 1) the allele of male parent A3 (presumably homozygous) is only present in half of the offspring. The patterns obtained clearly indicated that one allele was not amplified due to a mutation on the primer site. We had therefore to assume the existence of a null allele in A3.

Molecular bases of SSCP

The sequences of the two PCR fragments are unknown in the Genebank/EMBL data bases. For fragment P14-450, in cross 33P × A3, two alleles were sequenced (Fig. 2 A), and three for the fragment I14-780 in the cross 34P × A3 (Fig. 2 C). For the two fragments, numerous substitutions were identified (six for P14-450 and 30 for I14-780) and one insertion-deletion of 8 bp for I14-780 (Figs. 3 and 4).

For the fragment I14-780, the substitutions are distributed all over the sequence of 783 bp and the insertion-deletion is close to the upstream primer. Thirty mutations and one insertion-deletion of 8 bp distinguish the three alleles. However, alleles 2 and 3 differ only for the insertion-deletion and two mutations whereas allele 1 differs for 30 mutations from allele 2 and for 28 mutations and the insertion-deletion from allele 3 (Fig. 3).

For P14-450, the six substitutions that distinguish the two alleles are located close to the extremities of the fragment (Fig. 4).

Discussion

SSCP: a reproducible method to detect polymorphism in PCR fragments

The main objective of this study was to test SSCP as a technique to detect polymorphisms within PCR fragments of different size. From six analyzed anonymous fragments of 250–800 bp, four showed polymorphic patterns. Under the same SSCP conditions, these patterns were reproducible with usually two, three or four bands. The two other fragments of 500 and 750 bp did not reveal SSCPs even with different conditions of migration. The I14-250 fragment gave complex SSCP patterns. It has been shown that environmental conditions can play a role in the sensitivity of SSCP by changing the degree of mobility shift. Effects of temperature, ionic strength, DNA concentration, loading vol-

Table 2 Allelic interpretation of SSCP patterns for the three crosses and their offspring

Fragment	Crossing F × M	Parents genotypes F/M	Segregation classes[a]	Frequency	Test χ^{2b}	*df*
P14-450	32P × A6	2,5; 3,6/1,4; 1,4	2,5; 1,4/3,6; 1,4	17/9	2.45	1
	34P × A3	3,4; 3,4/3,4; 3,4	3,4; 3,4	26	–	–
	33P × A3	1,4; 1,4/3,4; 3,4	1,4; 3,4	46	–	–
I14-780	32P × A6	2,4; 2,5/2,5; 1,3	2,4; 2,5/2,4; 1,3/2,5; 2,5/2,5; 1,3	4/7/6/9	1.98	3
	34P × A3	2,5; 1,3/1,4; –	2,5; 1,4/2,5; –/1,3; 1,4/1,3; –	7/6/8/5	0.75	3
	33P × A3	2,5; 2,5/2,4; –	2,5; 2,4/2,5; –	23/24	0.02	1
U7-790	32P × A6	1,4; 1,4/1,4; 2,5	1,4; 1,4/1,4; 2,5	10/16	1.37	1
	34P × A3	2,5; 3,5/1,4; 1,4	2,5; 1,4/3,5; 1,4	12/12	0	3
	33P × A3	1,4; 1,4/1,4; 1,4	1,4; 1,4	26	–	–

[a] Indicates null alleles
[b] Critical value of χ^2 at $P = 0.05$ (1 *df*: 3.84; 3 *df*: 7.81)

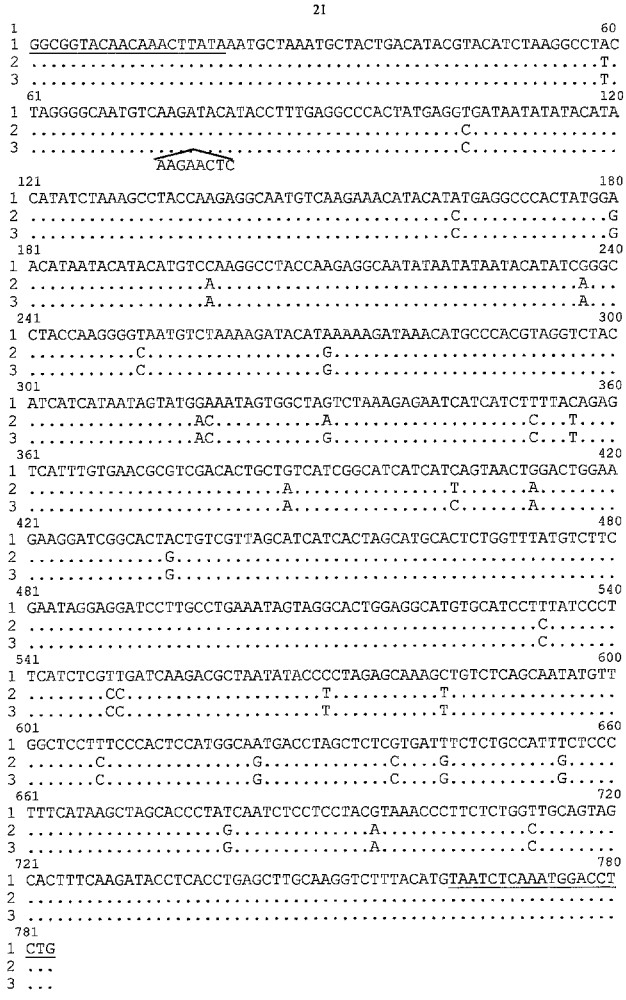


Fig. 3 Sequences of the three alleles of fragment I14-780. For alleles 2 and 3 only base substitutions to allele 1 are indicated. Allele 3 presents an insertion of eight bases at position 78. Sequences of the pair of primers are *underlined*

Fig. 4 Sequences of the two alleles of fragment P14-450. For allele 2, only base substitutions to allele 1 are indicated. Sequences of the pair of primers are *underlined*

ume, denaturant agents (Maekawa et al. 1993), neutral compounds, gel matrix (ratio of acrylamide/bis acrylamide, concentration of acrylamide) (Sarkar et al. 1992), concentration of primers in the PCR (Cai and Touitou 1993), and running voltage have all been shown to induce different conformations (Hayashi et al. 1993; Hongyo et al. 1993). These factors should be modified if the objective is to identify all mutations existing within a fragment (Orita et al. 1989a; Michaud et al. 1992; Leren et al. 1993). For the two monomorphic fragments, we modified the conditions according to these references but obtained the same monomorphic results. An alternative, that was not tested in the present study, would have been to design primers amplifying parts of these fragments, e.g. segments of 200–300 bp. The fact that we did not observe any conformation polymorphism does not mean that no polymorphism existed in these fragments. It may just indicate that the primers that we used were not adequately placed in comparison to polymorphic sites.

To improve the resolution of the complex pattern of fragment I14-250 we tested different conditions, in particular the concentrations of primers in the PCR reaction. Complex patterns may result from the interaction of PCR primers, or partial PCR reaction products, with the single-stranded DNA (Cai and Touitou 1993) which can reduce the efficiency of SSCP detection. Performing either unbalanced PCR or regular PCR followed by product purification removed this artifact. Kasuga et al. (1995) observed that PCR primers may also influence SSCP profiles. They assumed that even for conventional SSCP analysis, removing residual primers would help to eliminate the uncertainty in band mobility and band identity associated with primer-single-strand DNA interactions. We did not observe any modification of the profiles when the concentration of primers was changed.

Mendelian co-dominant inheritance of SSCPs

For each fragment, different single-strand bands gave Mendelian segregation in the crosses. Observed ratios of progeny genotypes were not significantly different from the expected Mendelian segregations. Mendelian segregation of SSCP markers was verified animal genetic studies (Kirkpatrick et al. 1994), in genetic mapping on rice (Fukuoka et al. 1994), and also in human genetic studies (Orita et al. 1989b). For each genotype, an allelic interpretation was proposed corresponding of the observed patterns. It is worthwhile noting than an allele may exhibit different sequences, since not all mutations are detected within a fragment by SSCP. A genotype with two bands was homozygous and a genotype with three or four bands was heterozygous. A heterozygote with three bands resulted from the cross of two gametes having one single-strand DNA in common (no mobility shift), the second single-strand DNA migrating at a different level (mobility shift). In different human genetic studies, homozygous individuals usually exhibit two bands and heterozygous individuals present more than

two bands (Dockhorn-Dworniczak et al. 1991; Condie et al. 1993; Leren et al. 1993; Tasi et al. 1993; Lenk et al. 1994). These results indicate that one SSCP profile does not necessarily correspond to a given genotype class, e.g. two bands to a homozygote and four bands to a heterozygote. We therefore recommend the testing of segregation in a cross to validate a genetic hypothesis. Our results show that, even for large fragments, SSCP can be used as a technique to identify co-dominant fragments.

Molecular causes of SSCPs

In our study, the number of bands ranged from two to four regardless of fragment size. The variation from two to four bands among trees was interpreted as a result of allelic composition (homozygote vs heterozygote) as inferred from segregation data. To identify the nature of the polymorphism (i.e. single base changes, sequence rearrangements) between different alleles, two PCR fragments were sequenced, P14-450 (which contains composite repeat sequences) and I14-780, each one for one cross. Several substitutions were detected, distributed all over the PCR fragment for I14-780 or especially located close to the extremities for P14-450. One insertion-deletion together with two base substitutions distinguished one allele from the others for I14-780.

Although it is not possible to predict the mobility shift induced by single-base changes, it has been suggested (Glavac and Dean 1993; Sheffield et al. 1993) that: (1) the type of mutation (transition vs transversion) did not seem to play a major role (single strands with different mutations at the same position usually gave identical shifts, Sheffield et al. 1993), (2) the neighboring bases round a point mutation are more important than the type of base change, (3) the mutations not detected by SSCP analysis are likely to be interspersed within the sequence (there were no long stretches of sequence where numerous mutations were not detected, Sheffield et al. 1993).

For the two PCR fragments that were sequenced, it was not possible to assign the origin of mobility shift to a precise substitution. Among the 30 base substitutions between two alleles for I14-780, some may be silent and would not modify the conformation. However, it is likely that the insertion-deletion of 8 bp may be responsible for a change in mobility.

The efficiency of SSCP is very dependent on the size and sequence of the PCR fragment and may therefore vary significantly from one fragment to another. Even if there is a striking relationship between SSCP sensitivity and fragment size (lower and upper limit, Sheffield et al. 1993), larger fragments can be analyzed. For example, the sequenced fragment I14 of 780 bp presents several substitutions and one insertion-deletion and only a few of the substitutions may be detected by SSCP.

Despite the sensitivity of SSCP to the sequence composition and the sequence itself, we did not observe an increase in the complexity of the electrophoretic patterns with the size of the PCR fragment and the number of substitutions. Among the potential conformations that exist for a given fragment, one preferentially occurs for a given primary sequence.

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