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SHORT NOTE

DETECTION OF RARE POLYMORPHISMS IN MITOCHONDRIAL DNA OF OAKS WITH PCR-RFLP COMBINED TO SSCP ANALYSIS

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ABSTRACT

Five mitochondrial fragments amplified by PCR with consensus primers were cut with restriction enzymes and analysed by SSCP (Single Strand Conformation Polymorphism analysis) in 21 individuals of *Quercus robur* L. The addition of the SSCP step allowed the detection of 12 polymorphisms against 2 with the classical PCR-RFLP technique alone. The SSCP analysis combined to PCR-RFLP appears very useful for the intraspecific studies of mitochondrial DNA in plants where the principal problem is to detect the rare sequence polymorphisms usually present in this genome. The simplicity and rapidity of the PCR-RFLP-SSCP technique will be particularly interesting in population genetic and inheritance studies.

Key words: mitochondrial DNA, PCR, polymorphism, *Quercus* sp., rare mutation, SSCP

Polymorphisms in the plant mitochondrial genome have been mostly studied with the classical RFLP (Restriction Fragments Length Polymorphism) technique. At low taxonomic levels, this has allowed the detection of mutations largely due to rearrangements, a peculiar feature of the evolution of plant mitochondrial DNA (mtDNA). Recently, consensus primers have been proposed which can amplify non-coding DNA fragments of plant mtDNA (intergenic sequences or introns) flanked by conserved coding sequences (DEMESURE *et al.* 1995, DUMOLIN-LAPÈGUE *et al.* 1997). Precisely because these primers have broad taxonomic applications, the relatively frequent rearrangements detected by conventional RFLP are not expected to occur within the amplified sequences. Since plant mtDNA is known to be highly invariant in primary sequence, changing at least three times as slowly as chloroplast DNA (cpDNA) (WOLFE *et al.* 1987), we anticipated that the mtDNA primers would prove less useful than the cpDNA primers for intraspecific studies. Actually, population genetic studies of forest trees realised in our laboratory with the primers described in DEMESURE *et al.* (1995) have confirmed this prediction. After digestion with restriction enzymes, several intraspecific polymorphisms could usually be detected in the cpDNA fragments whereas the mtDNA fragments showed very few polymorphisms: none in *Fagus sylvatica* (Fagaceae; DEMESURE *et al.* 1996), none in *Dicorynia guianensis* (Caesalpiniaceae; unpublished data), none in *Argania spinosa* (Sapotaceae; EL MOUSADIK & PETIT 1996), and two indels in the *Quercus robur* complex (Fagaceae ; DUMOLIN *et al.* 1995 and below). Consequently, it was necessary to use a more resolutive technique to detect additional mtDNA polymorphisms at this level. We show here that the Single-Stranded Conformation Polymorphism (SSCP) technique developed by ORITA *et al.* (1989), which allows the detection of substitutions affecting the conformation of DNA in non-denaturing acrylamide gels, is particularly efficient in these cases, while remaining simple enough, as compared to PCR-RFLP.

Distinct oak chloroplast haplotypes have been identified in a survey of European populations belonging to the *Q. robur* complex (DEMESURE 1996) using four pairs of universal chloroplast primers. The initial sampling of 214 populations, increased to 345, included samples distributed from Sicily to Sweden and from Ireland to Russia. Most of the trees belonged to the closely related species *Q. robur* (L.) and *Q. petraea* (Matt.) Liebl. Total DNA has been extracted following the procedure described in DUMOLIN *et al.* (1995). The 21 chloroplast haplotypes detected were further analysed with five pairs of universal mitochondrial primers. Three pairs, *nad*1 exon2/ *nad*1 exon3, *nad*4 exon1 / *nad*4 exon2, *rps*14/ *cob* were described in DEMESURE *et al.* (1995). Two new pairs, *cox*2 exon1/*cox*2 exon2 and *nad*4 exon2/*nad*4 exon3, are described in DUMOLIN-LAPÈGUE *et al.* (1997). The PCR amplifications

Table 1 List of the mutations detected in the five mitochondrial fragments among 21 oak individuals

Pairs of primers	Fragment	Size (bp)	Mutations detected with PCR-RFLP	Mutations detected with PCR-RFLP-SSCP
*nad*4 exon1 / *nad*4 exon2	*nad*4–1/2	1,700	1 indel	1
*nad*4 exon2 / *nad*4 exon3	*nad*4–2/3	3,500		7
*rps*14 / *cob*	*rps*14/*cob*	1,825	1 indel	1
*nad*1 exon2 / *nad*1 exon3	*nad*1–b/c	1,636		2
*cox*IIa / *cox*IIb	*cox*II	385		1
Total		9,046	2	12

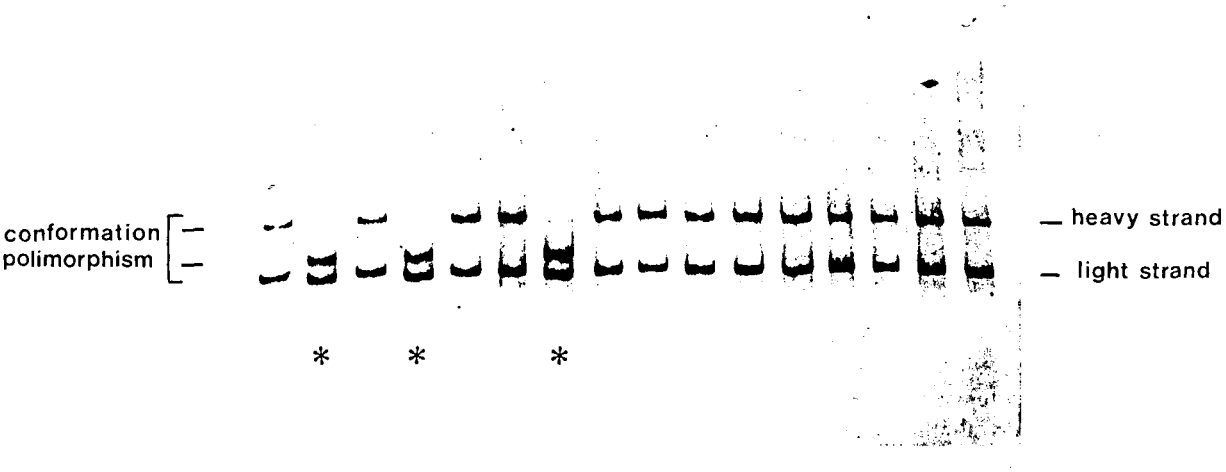

Figure 1 Example of a conformation polymorphism. The mtDNA fragment comprised between the genes *rps*14 and *cob* (1,825 bp) was amplified and digested with *Hinf*1. The SSCP technique was then applied on the resulting fragments. On this picture of the silver stained SSCP gel, the two strands of the heaviest restriction fragment (500 bp) can be seen. Three individuals (*) differ from the others by a conformation polymoprhism affecting strongly the heaviest strand, and only slightly the lightest one.

were performed as detailed in DEMESURE *et al.* (1995). The observed lengths of the fragments are given in Table 1. Note that, if we compare the size of the amplification product for *cox*II with those obtained in a survey of about 40 plant species (RABBI & WILSON 1993), it appears that *Quercus* lacks the intron.

Except in the case of this smaller *cox*II fragment, 5 µl of the PCR products were digested with 5 units of a single 4-bases recognition restriction endonuclease, *Hin*fI. The DNA restriction fragments were then separated by electrophoresis on 8% polyacrylamide gels and stained with ethidium bromide as described in DUMOLIN *et al.* (1995). Whereas silver staining is necessary for single strand analysis, ethidium bromide staining is sufficient for PCR-RFLP analysis. This technique allowed the detection of only two length mutations: a relatively large deletion (400 bp) in the *rps*14/*cob* fragment of one individual and a 6 bp indel in the *nad*4-1/2 fragment that was used in a previous study of mtDNA inheritance in *Quercus robur* (DUMOLIN *et al.* 1995).

For the SSCP analysis, the digested PCR products (2 µl) were added to a denaturing solution (5 µl) containing 95% formamide, 10mM NaOH, 0.05% of xylene cyanol and 0.05% of bromophenol blue. After 4 minutes at 94°C, to separate the two strands, the solution was immediately cooled on ice. The single-stranded DNA fragments were separated in a 0.75 mm × 16 cm × 18 cm, non-denaturing Mutation Detection Enhancement (MDE) gel (0.5 × MDE, Bioprobe Systems; 0.6 × TBE). Electrophoresis lasted 16 to 18 h at 7.5 to 10 $V \cdot cm^{-1}$ at a constant temperature of 15 °C in 0.6× TBE buffer. The gels were then silver-stained with the method of BASSAM *et al.* (1991). After fixation with acetic acid, the gels were impregnated with silver nitrate and formaldehyde. Colour development was then obtained with sodium carbonate, formaldehyde and sodium thiosulfate and the reaction was stopped with acetic acid. The gels were finally rinsed with deionized water and dried between plastic sheets (see

details in BODÉNÈS *et al.* 1996).

Patterns having twice as many DNA fragments are expected with this technique as compared to standard PCR-RFLP. The polymorphisms may be principally caused by substitutions or by very small insertion /deletion events that could not be detected by PCR-RFLP alone. Even if the mutations should always involve both DNA strands, the conformation polymorphisms can affect differentially the two strands (BODÉNÈS *et al.* 1996). This is shown in Figure 1 where the conformation polymorphism affected strongly the heaviest strand and only slightly the lightest one. To avoid scoring twice the same mutation, a single mutation was inferred when there were two polymorphisms present in the same individual(s) and affecting fragments of similar mobility in the gel. With this conservative procedure, a minimum of 10 polymorphisms could be identified with the SSCP analysis (Table1). Adding this step to the PCR-RFLP analysis allowed to distinguish 11 mitochondrial haplotypes among the 21 indi-viduals representing the chloroplast haplotypes. The two polymorphisms detected with PCR-RFLP were also observed by SSCP analysis.

It should be noted that the technique is not likely to be exhaustive. First, in most cases, the digestion of the PCR fragments generates too many restriction fragments to be observed in a 16 cm high polyacrylamide gel. The heaviest restriction fragments were preferentially studied here. Second, the size of the restriction fragments (300 to 650 bp) analysed by SSCP is higher than the limit up to which SSCP is considered to be exhaustive (about 150–200 bp, ORITA *et al.* 1989, TO *et al.* 1993). However, the goal here was simply to identify polymorphisms routinely by PCR in particularly slowly evolving sequences such as the plant mtDNA. The conventional SSCP technique (*i.e.*, not combined with RFLP) has been previously used to study cytoplasmic genomes. Point mutations were detected in the chloroplast genome of tobacco (TO *et al.* 1993) or pine (WATANO *et al.* 1995) and in the mitochondrial genomes of *Gliricidia sepium*, a leguminous tree (DAWSON *et al.* 1996). Clearly, when dealing with large PCR fragments (1600 to 3500 bp in our case), cutting them with restriction enzymes increases the chance to detect polymorphisms as was demonstrated for the human genome (IWAHANA *et al.* 1992). The interest of the technique described here resides in its relative simplicity since only small gels are used which are then silver-stained, without the need to use radioactive elements or autoradiography. The method can also be combined with sequencing, for example when haplotypes have been identified in a population survey. With the availability of pairs of conserved plant mtDNA primers and only one more step as compared to the PCR-RFLP method, the SSCP technique should now facilitate mtDNA population or inheritance studies in plants.

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REFERENCES

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

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

DAWSON, I. K., SIMONS, A. J., WAUGH, R. & POWELL, W. 1996: Detection and pattern of interspecific hibridization between *Gliricidia sepium* and *G. maculata* in Meso-America revealed by PCR-based assays. *Molecular Ecology* **5**:89–98.

DEMESURE, B., SODZI, N. & PETIT, R. J. 1995: A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Molecular Ecology* **4**:129–131.

DEMESURE, B. 1996: *Analyse de la diversité chloroplastique en utilisant des fragments PCR chez des Fagacées:* Fagus sylvatica *L. et* Quercus *ssp.* Doctorate thesis, University of Bordeaux I.

DEMESURE, B., COMPS, B. & PETIT, R. J. 1996: Chloroplast DNA phylogeography of the common beech (*Fagus sylvatica* L.) in Europe. *Evolution* **50**(6):2515–2520.

DUMOLIN, S., DEMESURE, B. & PETIT R. J. 1995: Inheritance of chloroplast and mitochondrial genomes in pedunculate oak investigated with an efficient PCR method. *Theoretical and Applied Genetics* **91**:1253–1256.

DUMOLIN-LAPEGUE, S., PEMONGE, M. H. & PETIT R. J. 1997: An enlarged set of consensus primers for the study of organelle DNA in plants. *Molecular Ecology* **6**:in press.

EL MOUSADIK, A. & PETIT, R. J. 1996: Chloroplast DNA phylogeography of the argan tree of Morocco. *Molecular Ecology* **5**:547–555.

IWAHANA, H., YOSHIMOTO, K. & ITAKURA, M. 1992: Detection of point mutations by SSCP of PCR-amplified DNA after endonuclease digestion. *Biotechniques* **12** (1): 64–66.

ORITA, M., IWAHANA, H., KANAZAWA, H., HAYASHI, K. & SEKIYA, T. 1989: Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proceedings of the National Academy of Sciences USA* **86**:2766–2770.

RABBI, M. F. & WILSON, K. G. 1993: The mitochondrial *cox*II intron has been lost in two different lineages of dicots and altered in others. *American Journal of Botany* **80**:1216–1223.

TO, K.-Y., LIU, C.-I., LIU, S.-T. & CHANG, Y.-S. 1993:

Detection of point mutations in the chloroplast genome by single-strand conformation polymorphism analysis. *The Plant Journal* **3**:183–186.

WATANO, Y., IMAZU, M. & SHIMIZU T. 1995: Chloroplast DNA typing by PCR-SSCP in the *Pinus pumila – P. parviflora* var. *pentaphylla* complex (Pinaceae). *Journal of Plant Research* **108**:493–499.

WOLFE, K. H., LI, W.-H. & SHARP, P. M. 1987: Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proceedings of the National Academy of Sciences USA* **84**:9054 –99058.