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Inheritance of chloroplast and mitochondrial genomes in pedunculate oak investigated with an efficient PCR method

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Abstract The restriction patterns of two chloroplast fragments and one mitochondrial DNA fragment, amplified by PCR with universal primers, were studied to determine the mode of inheritance of these organelles in 143 progeny of five intraspecific crosses in pedunculate oak (*Quercus robur* L.). The results indicate that both genomes are maternally inherited, an observation which agrees with the commonly observed pattern of inheritance in angiosperms. They confirm that both chloroplast DNA and mitochondrial DNA can be used as a source of seed-specific markers for the study of the geographic structure of oaks. This is the first report of organelle inheritance within the Fagaceae, an important and widespread tree family.

Key words Insertion-deletion · Maternal inheritance · Non coding DNA · *Quercus robur* · Universal primers

Introduction

Oak forests represent a major economical and ecological resource in tropical, mediterranean, and temperate regions of the northern hemisphere. The study of their genetic diversity, and in particular their geographic structure, is a prerequisite for the development of efficient conservation strategies. Interestingly, in this respect, it has been shown theoretically that, in outcrossing plants, maternally transmitted genomes – in contrast to the biparentally inherited nuclear genome – will partition a large amount of their genetic diversity among populations (Petit et al. 1993a).

Most angiosperm species typically display maternal inheritance of the chloroplast genome (cpDNA). However, cpDNA can be biparentally inherited as in *Medicago* (Masoud et al. 1990) and *Pelargonium* (Metzlaff et al. 1981) or even paternally inherited as in *Daucus* (Boblenz et al. 1990) and several gymnosperms such as *Pinus* (Wagner et al. 1987) and *Sequoia* (Neale et al. 1989). Exceptions to the typical pattern of maternal inheritance of mitochondrial DNA (mtDNA) are very rare with only seven reported cases (Faure et al. 1994; Reboud and Zeyl 1994).

To our knowledge, no genetic study of the mode of inheritance of either the chloroplast or the mitochondrial genome has been carried out in the genus *Quercus* or indeed in the family Fagaceae which includes beeches (*Fagus*, *Nothofagus*), chestnuts (*Castanea*) and oaks. The contrast between the high level of differentiation found with cpDNA markers and the low level of differentiation measured with nuclear markers in several oak species (Petit et al. 1993a, b) suggests that cpDNA is maternally inherited. Nevertheless, direct evidence that cpDNA polymorphisms indeed constitute seed-specific markers is desirable. Furthermore, it would be interesting to know if mtDNA could also be used to study acorn transfers. We therefore analysed five intraspecific crosses of *Quercus robur* using a simple and efficient technique: a restriction study of the cpDNA and mtDNA fragments amplified by PCR with pairs of universal chloroplast and mitochondrial primers.

Materials and methods

Plant material

The intraspecific controlled crosses were performed in 1989 and 1992 at the INRA research station of Pierroton/Bordeaux (France). They involved three trees growing in the research station which were used as female parents (32P, 33P and 34P) and three trees originating from Arcachon, 40 km west of the station, used as pollen donors (A3, A5 and A6). The number of offspring studied per cross is given in Table 1. The validity of the crosses was checked by isoenzyme analysis. Segregation and linkage were studied at 12 isozyme loci (Zanetto et al., submitted) and the genotypes of all 143 progeny

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used in this study were consistent with their respective parental genotypes.

DNA extraction

Total genomic DNA was extracted from fresh leaf tissue or buds (about 0.1 g) using a method adapted from Doyle and Doyle (1990). Each sample was ground in an Eppendorf tube with 250 µl of 2 × extraction buffer, as in Doyle and Doyle's technique except that mixed Alkyltrimethylammonium bromide (Sigma M 7635) was used in place of CTAB and that 1% of soluble PVP was added. Then 750 µl of extraction buffer were added and the tubes were incubated at 55°C for 1 h. They were allowed to cool 10 min before adding 400 µl of dichloromethane. After 5 min of centrifugation at 4°C (10000 rpm), the upper phase was transferred to a new tube, mixed with 400 µl of isopropanol, and stored overnight at –20°C. The tubes were centrifuged for 10 min at 4°C (13000 rpm) and the supernatant removed. Then 1 ml of 76% ethanol was added to the pellet and the DNA was precipitated as before. Finally, the pellet was dried for 15 min in a speed-vac at room temperature and combined with 100 µl of pure water. This DNA stock solution was stored at –20°C and diluted 10–100 times before use.

Amplification and electrophoresis

The parents and progenies were characterised by restriction studies of two cpDNA fragments and one mtDNA fragment amplified by PCR with pairs of universal chloroplast and mitochondrial primers (see Demesure et al. 1995 for the details of the amplification technique). A description of the primers is given in Table 2. Each of the three amplified fragments was digested by a single restriction enzyme: TF with *Alu*I, DT with *Taq*I, and *nad*4-1/2 with *Hinf*I. These particular fragments were chosen because they had been shown to detect polymorphisms in western France in a survey of the cytoplasmic diversity of several oaks species (B. Demesure, unpublished results). We separated the DNA fragments by electrophoresis on 8% polyacrylamide gels. The 1-kb ladder of GibcoBRL (Life Technologies) was used as molecular-weight marker. Gels were photographed on a UV box with Polaroid 665 films after staining with ethidium bromide. The negatives of the picture were scanned by a camera and analysed with the Whole Band Analyzer Software (version 3.2) of the Bio Image system in order to estimate precisely the size of the fragments.

Table 1 Number of offspring studied per cross

Female parent	Male parent		
	A3	A5	A6
32P	22	16	26
33P	49	–	–
34P	30	–	–

Results

Chloroplast inheritance

For each fragment, only a subset of the crosses were informative. Polymorphic patterns were revealed between the parents of four out of five crosses for the TF-*Alu*I combination and two out of five for the DT-*Taq*I combination. These two polymorphisms are each due to an insertion/deletion of a DNA sequence (respectively about 50 bp and 7 bp). The sizes of the polymorphic fragments are given in Table 3. All the progenies had the same DNA fragments than the female parent (Figs. 1 and 2).

Mitochondrial inheritance

Polymorphic patterns were revealed between the parents of four out of five crosses for the *nad*4-1/2–*Hinf*I combination. This polymorphism is also due to a small insertion/deletion which was estimated to be of six bases (Table 3). All the progenies of the four informative crosses had the same maternal fragment of 499 bp (Fig. 3).

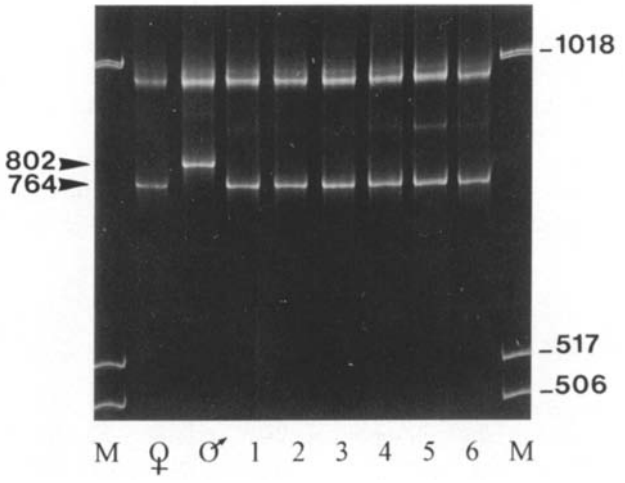

Fig. 1 Maternal inheritance of chloroplast DNA in a progeny from the cross 33P × A3. A 1730-bp cpDNA fragment (*trn*T to *trn*F) was amplified by PCR and digested by the endonuclease *Alu*I. The male parent A3 differs from the female parent 33P and the progeny (*lanes 1–6*) by an insertion of 50 bp in the second restriction fragment. *M* molecular-weight marker

Table 2 Description of the three pairs of cpDNA and plant mtDNA universal primers used in this study

Primer 1	Primer 2	Abbreviation	References
Chloroplast primers			
*trn*T [tRNA-Thr(UGU)]	*trn*F [tRNA-Phe(GAA)]	TF	Taberlet et al. 1991
*trn*D [tRNA-Asp(GUC)]	*trn*T [tRNA-Thr(GGU)]	DT	Demesure et al. 1995
Mitochondrial primer			
*nad*4 exon1	*nad*4 exon2	*nad*4-1/2	Demesure et al. 1995

Table 3 Size of the chloroplast and mitochondrial polymorphic fragments (in bp) present in the parents of the five crosses. The informative crosses are represented with bold-faced characters. Note the presence of a reciprocal situation with the DT-*Taq*I combination (crosses 32P × A6 and 33P × A3)

Fragment	Restriction enzyme	Crosses (♀ × ♂)				
		32P × A3	32P × A5	32P × A6	33P × A3	34P × A3
TF	*Alu*I	**764/802**	**764/802**	764/764	**764/802**	**764/802**
DT	*Taq*I	323/323	323/323	**323/316**	**316/323**	323/323
*nad*4-1/2	*Hinf*I	**499/493**	**499/493**	499/499	**499/493**	**499/493**

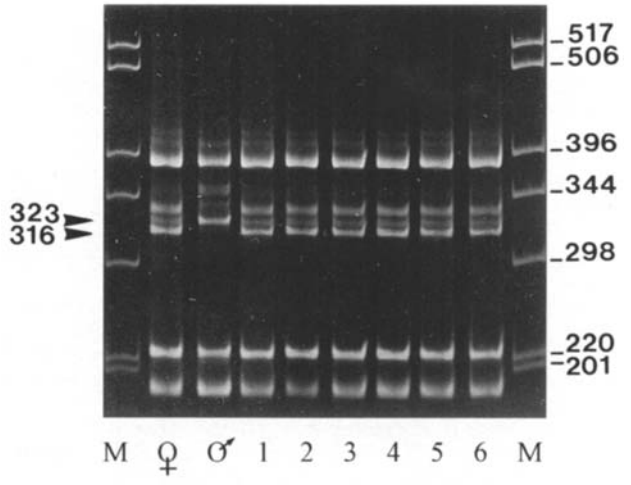

Fig. 2 Maternal inheritance of chloroplast DNA in a progeny from the cross 33P × A3. A 1800-bp cpDNA fragment (*trn*D to *trn*T) was amplified by PCR and digested by the endonuclease *Taq*I. The male parent A3 differs from the female parent 33P and the progeny (*lanes 1–6*) by an insertion of 7 bp in the second restriction fragment. *M* molecular-weight marker

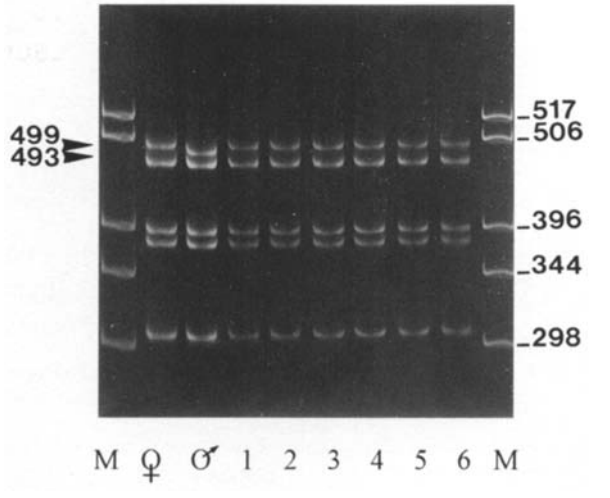

Fig. 3 Maternal inheritance of mitochondrial DNA in a progeny from the cross 33P × A3. A 1700-bp cpDNA fragment (the first intron of the *nad*4 gene) was amplified by PCR and digested by the endonuclease *Hinf*I. The male parent A3 differs from the female parent 33P and the progeny (*lanes 1–6*) by a deletion of 6 bp in the first restriction fragment. *M* molecular-weight marker

Discussion

We studied the inheritance of chloroplast and mitochondrial genomes in pedunculate oaks using the restriction patterns of PCR amplified cpDNA and mtDNA fragments. The results clearly indicate that both genomes are at least predominantly maternally inherited. Note that it is important to distinguish between strict maternal inheritance and occasional biparental inheritance because even a low level of paternal leakage (for instance 1%) may have a strong impact on genetic subdivision in populations of hermaphrodite plants (cf. Fig. 3 in Petit et al. 1993a). This is a consequence of the largely asymmetric pollen and seed flow typical of many plant species, pollen grains being generally dispersed over longer distances than seeds. It seems useful, therefore, to quantify the higher limit that the paternal contribution could take given the number of progenies analysed. For this purpose, we used a binomial model of organelle inheritance (Milligan 1992). According to this model, our data allow us to conclude that, at the 95% confidence limit, the chloroplast and mitochondrial genomes are predominantly maternally inherited with respectively less than 2.0 and 2.5% of a paternal contribution in the studied crosses.

These results agree with the commonly observed maternal inheritance of the chloroplast and mitochondrial genomes in angiosperms. However, very few angiosperm trees have been studied to-date, notably *Populus* (mtDNA: Radetzky 1990; cpDNA: Mejnartowiez 1991; Rajora and Dancik 1992), *Eucalyptus* (cpDNA: Byrne et al. 1993), *Citrus* (mtDNA: Luro 1993). In all these examples, maternal inheritance was demonstrated.

Note that we used intraspecific crosses to infer the mode of inheritance of cpDNA and mtDNA in oaks. Interspecific crosses may be misleading if fertilization problems affect the transmission of organelles. However, given the importance of hybridisation in the genus *Quercus* (Rushton 1993), it would be interesting to investigate the inheritance of organelles in crosses between different oak species.

The study of organelle transmission is often limited by the lack of intraspecific polymorphisms. The universal chloroplast primers that were recently described have allowed the detection of numerous intraspecific polymorphisms in all species investigated so far (Demesure et al. 1995). However, the four mitochondrial primers which

were described in the same paper did not reveal a single intraspecific polymorphism in oaks, a probable consequence of the slower substitution (and probably insertion/deletion) rate of mtDNA as compared to cpDNA (Palmer 1992). In the present study, we were able for the first time to find a polymorphism in *Q. robur* within the first intron of the mitochondrial gene *nad*4. This confirms the interest of these mitochondrial universal primers for studies at a low taxonomic level. PCR-derived methods are simple, rapid and economical compared to those which use labelled probes to detect specific DNA sequences transferred to membranes. A large number of samples can be amplified simultaneously (96 samples per thermocycler), so that the large data set required for population or inheritance studies can be quickly gathered (Erlich and Arnheim 1992). Note in addition that small insertions/deletions, one of the most frequent class of mutations in non-coding sequences, are relatively easy to detect if sufficiently resolutive methods of electrophoresis are used. Indeed, insertions/deletions are readily detected both directly or after digestion by one or a few restriction enzymes which cut DNA frequently, contrary to substitution events which can be detected only if they modify a restriction site. Finally, biparental inheritance is unambiguously detected if the parents differ by an insertion/deletion, contrary to point mutations where heteroplasmic individuals can be confused with incomplete digestion of the polymorphic sites. In inheritance studies, PCR-derived markers have already been used successfully to detect a low paternal contribution of mtDNA in mice (Gyllensten et al. 1991). More recently, they were used to study the mode of inheritance of cpDNA in *Iris* (Cruzan et al. 1993) and in *Turnera* (Shore et al. 1994). The availability of a large set of universal primers for both organelle genomes (Demesure et al. 1995) should now facilitate the use of PCR-derived methods in chloroplast and mitochondrial inheritance studies in plants.

In conclusion, intraspecific polymorphisms of cpDNA and mtDNA in pedunculate oak allowed the demonstration of the maternal inheritance of both organelles in this species using an efficient PCR method. The genomes of these two organelles can be used as a source of seed-specific markers for studies of the geographic structure in oaks. Moreover, it will now become possible to compare two different maternal phylogenies within a single species, a situation unique to plants.

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