Maternal inheritance of a chloroplast microsatellite marker in controlled hybrids between *Fraxinus excelsior* and *Fraxinus angustifolia*

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Abstract

Restriction fragment length polymorphism, polymerase chain reaction–restriction fragment length polymorphism and simple sequence repeat (SSR or microsatellites) analyses were performed to detect chloroplast DNA polymorphisms between two ash species, *Fraxinus excelsior* and *F. angustifolia*. Only one SSR locus was found to be polymorphic, confirming the very close relatedness of these species. Inheritance of this marker was studied in hybrids obtained from controlled crosses between the two tree species. Results indicated, for the first time in *Oleaceae*, that chloroplasts are maternally inherited. This chloroplast SSR marker is now used concomitantly with nuclear markers to analyse ash populations in sympatric areas.

Keywords: chloroplast DNA, controlled crosses, *Fraxinus* species, maternal inheritance, microsatellites, RFLP

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Introduction

Since the first report on chloroplast DNA (cpDNA) restriction patterns (Vedel *et al.* 1976) there has been increasing interest in chloroplast genomes for the purposes of population genetics and phylogenetic studies (McCauley 1995). Use of cpDNA restriction fragment length polymorphisms (RFLP) as genetic markers in interspecific hybridization showed that most angiosperm species display maternal inheritance of the chloroplast genome (Reboud & Zeyl 1993). However, cpDNA can also be biparentally inherited, as in *Medicago* and *Pelargonium*, or paternally inherited, as in *Actinidia* (Testolin & Cipriani 1997) and several gymnosperms. Comparison of circular restriction maps and of complete sequences between different plastomes indicates (i) that the majority of angiosperm plastomes share a similar structure and gene order (Suguita 1992); (ii) that there is little intraspecific variation, with an estimated rate of nucleotide substitutions, about one-quarter the rate for plant nuclear DNA; and (iii) that highest frequency of mutations is found in the noncoding regions (Palmer 1992). This has led to the design of pairs of consensus polymerase chain reaction (PCR) primers to amplify such regions which can then be either treated with restriction enzymes in order to detect PCR-RFLP markers (Dumolin *et al.* 1995) or directly sequenced (Hamilton 1999). Noncoding regions often contain simple sequence repeats (cpSSR or microsatellites) that are highly useful markers for size variations that are easy to analyse by using PCR and polyacrylamide gel electrophoresis (Powell *et al.* 1995). Recently, Provan *et al.* (1999a) and Bryan *et al.* (1999) have characterized SSR loci from the chloroplast genome of *Nicotiana tabacum*. These authors found high levels of both intra- and interspecific diversity in solanaceous species and suggested a possible application of tobacco cpSSRs in the study of genetic diversity in other plant species.

In western Europe, the genus *Fraxinus* is mainly represented by the common ash *F. excelsior* (L.) and the narrow-leaved ash *F. angustifolia* (Vahl.). The common ash is used in reforestation programmes because of the rapidity of its...
growth and the toughness and elasticity of its wood, while *F. angustifolia* grows slowly and usually yields wood of inferior quality. In France, *F. excelsior* is widely distributed except in the Mediterranean region where *F. angustifolia* is naturally found. Along the Rhône and Saône valleys the two species coexist and the occurrence of natural hybrids was suggested from observations based on morphological traits (Rameau et al. 1989). However, these traits vary significantly depending on the developmental stage, and cues allowing distinction between these species are not always discriminative. Complementary molecular approaches are required to distinguish unambiguously between *F. excelsior*, *F. angustifolia* and their putative hybrids. Random amplified polymorphic DNA (RAPD) and microsatellite nuclear markers have been developed previously in ash species (Jeandroz et al. 1996; Brachet et al. 1999; Lefort et al. 1999). By an analysis of nuclear microsatellites and RAPD markers, hybrids have been identified recently among the progeny of controlled crosses between *F. excelsior* and *F. angustifolia* (Raquin et al. submitted). Concerning cytoplasmic genome markers, there is only one report describing identical mitochondrial DNA (mtDNA) restriction patterns in *F. excelsior* and *F. angustifolia* (Morand et al. 2001).

In this paper, we tried first, to distinguish between *F. excelsior* and *F. angustifolia* cpDNAs by RFLP, PCR-RFLP and SSR analyses; for SSR studies we used the set of primer pairs deduced previously by Bryan et al. (1999). By an analysis of nuclear microsatellites and RAPD markers, hybrids have been identified recently among the progeny of controlled crosses between *F. excelsior* and *F. angustifolia* (Raquin et al. submitted). Concerning cytoplasmic genome markers, there is only one report describing identical mitochondrial DNA (mtDNA) restriction patterns in *F. excelsior* and *F. angustifolia* (Morand et al. 2001).

### Materials and methods

The samples included in this study are described in Table 1. Two *Fraxinus excelsior* trees (1E2 and 3E2) and two *Fraxinus angustifolia* trees (3A1 and 3A2) located at the Orsay University campus were chosen as parents in the controlled crosses 3E1 × 3A1 and 3A2 × 1E2 (Table 1). Since the flowering time is different between the two species, pollen was harvested from 3A1 and 1E2 trees and frozen at −70 °C. Branches from mother trees 3E2 and 3A2 were bagged before the stigmas emerged and dusted with pollen as soon as stigmas were visible on the anthers. Bags were removed about 3 weeks later and fruits collected in mid-August. *In vitro* culture from seeds were performed as described in Raquin et al. (2002). Chloroplast DNA used in RFLP analyses was prepared from chloroplasts isolated from young leaves in a high ionic strength medium (Bookjans et al. 1984). Lysis of chloroplast particles, RNase and proteinase K treatments, purification of cpDNA on genomic tip 20/G disposable column (Qiagen) and cpDNA restriction analysis were performed as described previously for ash mtDNA (Morand et al. 2001).

Total DNA was extracted with a DNeasy Plant Mini Kit (Qiagen) from leaf tissues harvested from plantlets cultured in *in vitro* and from adult parent trees. PCR-RFLP analyses were performed on total DNA as described by Dumolin et al. (1995) with the four primer pairs developed by Hamilton (1999). Chloroplast microsatellites were amplified with 36 primer pairs. First, chloroplast microsatellite patterns were obtained with a pair of primers that were derived from the *Nicotiana tabacum* chloroplast sequence: forward primer GTG CGT GTG AGT AAA TAG GAG, reverse primer GAA CGT GTC ACA AGC TTA ATT CTG.

Table 1. Origin of the plant materials

<table>
<thead>
<tr>
<th>Species</th>
<th>Population</th>
<th>Location</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fraxinus excelsior</em></td>
<td>Athies</td>
<td>4°07’44&quot;/49°03’10&quot;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>La Romagne</td>
<td>4°18’56’/49°40’47&quot;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Marly</td>
<td>2°05’41’/48°52’03’</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>St Gobain</td>
<td>3°22’33’/49°35’48’</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>St Martin d’Ablons</td>
<td>3°52’07’/49°00’47’</td>
<td>3</td>
</tr>
<tr>
<td><em>Fraxinus angustifolia</em></td>
<td>Cogolin</td>
<td>6°31’50’/43°15’12’</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>La Môle</td>
<td>6°27’58’/43°12’33’</td>
<td>18</td>
</tr>
<tr>
<td>Individuals from Orsay campus</td>
<td>5</td>
<td>2°11’18’/48°41’56’</td>
<td>5</td>
</tr>
<tr>
<td><em>F. excelsior</em></td>
<td>1E1; 1E2; 3E1; 3E2; 3E5</td>
<td>2°11’18’/48°41’56’</td>
<td>2</td>
</tr>
<tr>
<td><em>F. angustifolia</em></td>
<td>3A1; 3A2</td>
<td>2°11’18’/48°41’56’</td>
<td>2</td>
</tr>
</tbody>
</table>

Controlled hybrids (Orsay) *F. excelsior × F. angustifolia* (3E2 × 3A1) 2°11’18’/48°41’56’ 8
*F. angustifolia × F. excelsior* (3A2 × 1E2) 2°11’18’/48°41’56’ 9
Putative natural hybrids Tavaux 5°24’46’/47°02’39’ 20

Locations are indicated by co-ordinates of longitude/latitude; n is the number of individuals analysed.

Results and Discussion

The different *Fraxinus excelsior* and *Fraxinus angustifolia* genotypes cannot be distinguished by either the cpDNA restriction patterns obtained by digestion with *Hpa*II (Fig. 1) and several other enzymes or the cpDNA PCR-RFLP patterns, with 10 different restriction enzymes (not shown). This result agrees with previously published mtDNA restriction patterns which also failed to distinguish between the two ash species (Morand et al. 2001).

Among the 36 pairs of primers defining 36 microsatellite loci (with a repeat unit of at least 10 A/T mononucleotide repeats) in *Nicotiana tabacum* (Bryan et al. 1999), 11 pairs (NTCP4, -5, -11, -15, -16, -21, -22, -25, -32, -33 and -38) did not produce amplification products for either species of ash. Seven primer pairs (NTCP7, -14, -18, -19, -27, -28 and -37) produced amplification products for both species only after lowering (5–10°C) the annealing temperature. Several bands were amplified with three primer pairs (NTCP2, -8 and -18). Finally, among 15 primer pairs (NTCP1, -3, -6, -9, -10, -12, -13, -20, -23, -24, -26, -29, -30, -34 and -39) that produced a fragment at the same annealing temperature as in *N. tabacum*, 14 pairs amplified a fragment of the approximate expected size, while one (NTCP6) detected a fragment twice as large as expected. Size variations between *F. excelsior* and *F. angustifolia* for the 25 loci for which PCR products could be generated were found with only two primer pairs, NTCP1 and NTCP40, each encompassing an (A14) SSR. All *F. excelsior* genotypes, like *N. tabacum*, were characterized by an NTCP1 fragment 89 bp in size, while *F. angustifolia* genotypes showed an NTCP1 fragment of 88 bp (Fig. 1). The NTCP40 fragment allowed us to distinguish between the Cogolin and La Môle populations of *F. angustifolia* with sizes of 163 and 162 bp, respectively (18 individuals of each population were analysed) (not shown). However, the NTCP40 fragment obtained with several individuals of different *F. excelsior* populations was the same size as in Cogolin individuals.

Polymorphism at the NTCP1 locus was used to analyse chloroplast inheritance in hybrids obtained from controlled crosses by Raquin et al. (submitted). As shown in Fig. 1, the hybrids possessed the same chloroplast marker as the female parent irrespective of the sense of crossing. This result is the first evidence for maternal inheritance of chloroplasts in *Fraxinus* species as well as in the *Oleaceae* family. In other previously analysed forest trees, the chloroplast genome was shown to be either maternally inherited, as in oaks (Dumolin et al. 1995), according to the commonly observed pattern in angiosperms, or paternally inherited, as in the majority of gymnosperms (Vendramin et al. 1999).

The NTCP1 and NTCP40 loci are located in the rps19/rpl2 and the rpl2/trnH intergenic regions, respectively, of the angiosperm chloroplast genome. It is noteworthy that these regions are found close to (NTCP1) or at (NTCP40)
the junction between inverted repeat A and the large single copy region and between internal repeat B and the large single copy region (Sugiura 1992). Recently, sequence comparisons of these junctions among 26 Eucalyptus DNA samples revealed extensive intraspecific variation (Vaillancourt & Jackson 2000). These data suggest that the junction regions will provide very useful cpDNA polymorphisms for future studies in Fraxinus species. Indeed, sequence comparisons between NTCP1 fragments from F. excelsior, F. angustifolia and N. tabacum show (i) seven substitutions between both species of ash and tobacco; and (ii) a difference of one adenine between the two ashes, which is located in the microsatellite and explains the size difference between the corresponding fragments (Fig. 2). The very low level of variation between the two ash species suggests that they are closely related. A high level of cpDNA variation was reported in oaks (Dumolin et al. 1995) and Albus (Vendramin et al. 1999) and the absence of cpSSR variation was found in only one case, that of Pinus torreyana, confirming that this species descended from an original highly monomorphic population following the last glaciation bottleneck (Provan et al. 1999b).

It is noticeable that the very low variation of cpSSR either within or between F. excelsior and F. angustifolia contrasts with the high variation of nuclear microsatellites in both species (Brachet et al. 1999; Lefort et al. 1999). However, linking of this asymmetry between the two genomes to a much higher level of pollen gene flow as compared to seed gene flow requires further analyses and differences in patterns of variation. Preliminary analysis of NTCP1 polymorphism in 20 individuals belonging to the Tavaux population located in the Saône valley sympatric area indicates that 19 individuals are characterized by an F. angustifolia pattern and only one by an F. excelsior pattern. Recently, by using nuclear (RAPD and microsatellites) markers, we have demonstrated the possibility of interspecific hybridization between the two species of ash located at Orsay from direct and reciprocal controlled crosses (Raquin et al. submitted). Analyses of the Tavaux population with these nuclear markers will be performed (i) to distinguish between parents and putative natural hybrids; and (ii) to know if the ratio 19 : 1 in favour of F. angustifolia cpDNA indeed represents a preferential gene flow under natural conditions of F. excelsior pollen onto stigmas of F. angustifolia rather than the reciprocal cross. Chloroplast DNA polymorphism in the two ash species requires further assessment, for example, by analysis of microsatellites smaller than 10 nucleotides which are located at or close to the junctions between internal repeats and large single copy regions, and by sequencing of these junctions. Polymorphism at the NTCP1 locus (and possibly at additional loci) can now be used with RAPD and nuclear microsatellite markers to analyse ash populations in sympatric areas, characterize natural hybrids, and evaluate the genetic purity of F. excelsior seed samples.

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References


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Fig. 2 Alignment of cpDNA sequences at the NTCP1 locus for (T) Nicotiana tabacum, (E) Fraxinus excelsior and (A) F. angustifolia. Accession numbers are (T) EMBL CHNTXX, positions 86 760–86 758; (E) GenBank AF3394599; (A) GenBank AF3394600. Bold face type indicates base substitution relative to N. tabacum, the dot in (A) indicates the only base deletion. NTCP1 primer sequences are underlined.


This work is part of a broader project on the evolution of genetic diversity of trees by N. Frascaria-Lacoste’s team. One part of the project aims at quantifying levels of hybridization and introgression between Fraxinus excelsior and F. angustifolia. M. E. Morand-Prieur is a PhD student with an ENGREF graduate, C. Raquin and F. Vedel are CNRS researchers, S. Brachet is an ENGREF doctor-engineer, D. Shachak is a lecturer at Paris Sud University and N. Frascaria-Lacoste is a lecturer in forest genetics at the ENGREF institution.

ash Fraxinus excelsior using 5’ anchored PCR. Molecular Ecology, 8, 160–163.


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