MOLECULAR RECOGNITION OF THE CLOSELY RELATED *FRAXINUS EXCELSIOR* AND *F. OXYPHYLLA* (OLEACEAE) BY RAPD MARKERS

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ABSTRACT

RAPD markers were designed to recognize two closely related European ash species, *Fraxinus excelsior* and *F. oxyphylla* and their putative hybrids. From the use of 210 primers, nine species-specific fragments were identified. These markers were found to be monomorphic or nearly monomorphic in either *F. excelsior* or *F. oxyphylla* or were present in frequencies which differed greatly between the two species. Southern blot analyses were conducted to verify the homology of the diagnostic fragments. Fingerprints obtained for 16 putative hybrids from the zone of sympatry showed recombination of the species-specific markers, indicating potentially high level of natural hybridization.

Keywords: RAPD, *Fraxinus excelsior*, *Fraxinus oxyphylla*, natural hybridization

INTRODUCTION

Two *Fraxinus* species are found in France: *F. excelsior* which is widely distributed throughout the country, except in the South along the Mediterranean coast, and where *F. oxyphylla* is naturally found. *F. oxyphylla* is also present along the Saône and Rhône fluvial valleys where the two species are sympatric. In this region, the two species have been reported to hybridize naturally (RAMEAU *et al.* 1989) and adequate recognition of the two species and their hybrids with morphological characters is difficult. *F. excelsior* seed orchards located in the zone of sympatry might suffer more or less extensively of pollen contamination from *F. oxyphylla* naturally surrounding trees, which are slow growing and usually yield inferior wood quality. Hence, reliable ways of identifying potential sources of contaminants as well as measuring the effective levels of pollen contamination in *F. excelsior* seed orchards must be developed.

Previous studies with ribosomal DNA have shown that *F. oxyphylla* may be distinguished from *F. excelsior* by the presence of a species-specific polymorphism located in the intergenic spacer (IGS) (JEANDROZ *et al.* 1995). However, because this polymorphism in the IGS is a dominant marker for *F. oxyphylla*, hybrid/introgressant trees might not be distinguished from *F. oxyphylla* with this marker alone, and fixed species-specific dominant or co-dominant markers for *F. excelsior* would be necessary to recognize reliably hybrids or introgressants from pure parental types, involving a more intensive sampling of the genome.

Random amplified polymorphic DNA markers are appropriate tools to address such a problem because of the many loci that can be surveyed. This approach has been used successfully to identify genetic markers useful for the study of hybridization between closely related plant and tree taxa (ARNOLD *et al.* 1991, PERRON *et al.* 1995). Numerous primers may be screened and the RAPD markers showing fixed differences between parental species may help identify hybrid or introgressant forms. However, because of the shortcomings of RAPD markers related to their most frequent dominant nature (WILLIAMS *et al.* 1990), several markers have to be developed for each species in order to confidently identify the recombination of all or some of the species-specific markers in hybrid or introgressant individuals (PERRON *et al.* 1995). The homology of such RAPD diagnostic fragments within and between species within genera should also be verified by using Southern blot analysis, particularly when banding patterns vary in intensity or when species relative divergence is unknown or important (PILLAY & KENNY 1995; RIESEBERG 1996).

In this work, monomorphic or near monomorphic RAPD markers that are specific to either *F. excelsior* and *F. oxyphylla* were developed. As such, these markers are not much useful for population genetics

studies, but we show that they may be used with confidence to identify putative hybrid/introgressant trees.

MATERIAL AND METHODS

Plant material

Trees used for the construction of bulk samples and for the identification of species-specific molecular markers were selected in regions from outside the zone of sympatry to avoid as much as possible bias induced by possible natural hybridization in the sampling. The sample for *F. excelsior* was composed of 20 trees from three provenances, St Gacien, in the northwest part of France, and Amancey and Chalèze in the east of France. For *F. oxyphylla,* 19 trees were obtained from Montpellier and the surrounding region in the southern part of France. Sixteen trees of ambiguous morphological classification (JEANDROZ *et al.* 1995) were also sampled from the site of Sennecey le Grand (Bourgogne), which is located in the east of France, along the Saône river, in the sympatric zone where natural hybridization between the two species is presumed to take place (RAMEAU *et al.* 1989).

Construction of the bulk samples and screening of RAPD primers

A preliminary screening for RAPD markers of potential diagnostic value for distinguishing between the two parental species was conducted with three bulk samples for each species, in each of which DNA samples from six to seven individuals were combined. These six bulk samples were screened with 210 primers. Only major and reproducible fragments were scored and retained for further analysis. A total of 42 primers which showed presumed species-specific DNA fragments between the two species were further screened on individual tree DNAs that composed the bulk samples for final validation.

DNA amplification

DNA was extracted from leaves following published methods (BOUSQUET *et al.* 1990). The DNA concentration of each sample was adjusted by dilution as to be similar among samples. Reaction mixtures (12.5 µl) were essentially as described by ISABEL *et al.* (1995) and contained 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5mM $MgCl_2$, 0.1 mM of each dNTP (Pharmacia), 50 pmol of primer (Operon), 5 ng of total DNA and 0.5 U of *Taq* polymerase (Boehringer Mannheim). Amplifications were conducted in a Perkin Elmer Gene Amp PCR System 9600 under conditions described by PERRON *et al.* (1995). Amplification products were analyzed by gel electrophoresis (1% agarose, 0.5% Synergel, Mandel) in TPE buffer (SAMBROOK *et al.* 1989).

Southern blot and hybridization conditions

Following electrophoresis, PCR amplification products from single trees were transferred onto Nylon membranes under alkaline conditions (SAMBROOK *et al.* 1989). RAPD fragments used as probes were from single tree amplifications and were purified using a QUIA gel extraction kit (Quiagen). Probe labeling and detection were performed with the DIG DNA labeling and detection kit following manufacturer's recommendations (Boehringer Mannheim).

RESULTS

Of the 210 primers screened with the bulk samples, 42 primers that showed presumed species-specific DNA fragments were retained for individual tree analysis. These 42 primers resulted in the scoring of 177 fragments, for an average of four markers per primer. A total of 36 (20%) fragments were monomorphic and shared by the two species, 88 (50%) were polymorphic in both species, 18 (10%) were monomorphic in one species and polymorphic in the other species, and 26 (15%) were absent in one species and polymorphic in the other species. Only nine fragments (5%) derived from eight primers were found to be monomorphic to either *F. excelsior* or *F. oxyphylla* or showed frequen-

Table 1 Frequencies (± s.e.) of the nine most discriminatory RAPD fragments between *F. excelsior* and *F. oxyphylla*. Differences between species were all significant at P<0.0001 (frequency differences between spercies were tested using the Fisher exact test).

Diagnostic RAPD fragments	Marker frequency	
	Fraxinus excelsior (n = 20)	*Fraxinus oxyphylla* (n = 19)
OpL03 600 bp	1.0	0.05 (0.05)
OpN04 800 bp	1.0	0.05 (0.05)
OpO07 1400 bp	0.90 (0.07)	0
OpA07 1100 bp	0	0.85 (0.08)
OpH04 1600 bp	0	1.0
OpL03 750 bp	0	1.0
OpL14 800 bp	0.05 (0.05)	0.95 (0.05)
OpO08 1000 bp	0	0.95 (0.05)
OpO18 1600 bp	0	1.0

Table 2 Fingerprints obtained for 16 putative hybrids from a population (Sennecey le Grand) of the zone of sympatry

Trees	*F. excelsior* markers			*F. oxyphylla* markers					
	OpL03 600 bp	OpN04 800 bp	OpO07 1400 bp	OpA07 1100 bp	OpH04 1600 bp	OpL03 750 bp	OpL14 800 bp	OpO08 1000 bp	OpO18 1600 bp
F. excelsior [1]	1	1	1	0	0	0	0	0	0
F. oxyphylla [1]	0	0	0	1	1	1	1	1	1
Se01*	1	1	0	0	1	0	0	1	0
Se02*	1	1	0	0	1	0	1	1	0
Se03*	1	1	0	0	1	0	1	1	0
Se05*	0	1	0	0	1	1	0	1	0
Se06	0	0	0	0	1	1	0	1	0
Se07*	1	1	1	0	1	0	0	1	0
Se08*	1	1	1	0	1	1	0	1	0
Se09	0	0	0	0	1	1	0	1	0
Se11*	0	1	0	0	1	1	1	1	0
Se14*	0	1	0	0	1	0	1	1	1
Se15*	1	1	1	0	1	0	0	1	0
Se16*	1	0	1	0	1	1	1	1	0
Se17*	0	1	0	0	1	0	1	1	0
Se19*	1	0	1	0	1	0	0	1	0
Se20*	1	0	0	0	0	1	1	0	0
Se21	0	0	0	1	1	1	0	1	0

[1] Theoretical fingerprints for pure *F. excelsior* and *F. oxyphylla*

* Recombination of markers from both species observed, indicating putative hybrid/introgressant trees

cies which differed greatly between the two species (Table 1 and Fig. 1). The homology of each of these fragments was confirmed by Southern blot hybridizations of the original RAPD fingerprints (see also below) (Fig. 1). Of the nine fragments identified, three fragments (OpH04-1600, OpL03-750 and OpO18-1600) were found to be monomorphic in *F. oxyphylla* and absent in *F. excelsior* (Table 1). Three other fragments were nearly monomorphic in one species and absent from the other: OpO07-1400 present in *F. excelsior*, and OpA07-1100 and OpO08-1000 present in *F. oxyphylla*. The last three fragments had frequencies that differed substantially between the two species: OpL03-600 and OpN04-800 were monomorphic in *F. excelsior* and at low frequency (≤ 0.05) in *F. oxyphylla*, and OpL14-800 was nearly monomorphic in *F. oxyphylla* and at low frequency in *F. excelsior* (Table 1).

Hybridization experiments allowed to determine whether the equivalently sized fragments were homologous. Among the 16 candidate fragments that were used as probes, including the nine fragments found to be specific or nearly specific to one species (see above), only two fragment probes detected instances of non homology among equivalently sized fragments, and homology among individuals within species was the rule for each of the species-specific fragments. The 10 nonspecific co-migrating fragments tested were also found homologous between species. In contrast, a few individual fragments that were not visible with ethidium bromide staining cross-hybridized for three fragment probes (data not shown). Southern blot analysis also allowed to clarify the question of fragment homology when banding patterns were of varying intensity. For instance, variation could be noted in the intensity of the fragment OpO18-1600, specific to *F. oxyphylla* (Fig. 1B); the autoradiogram showed similar saturated signals, suggesting complete homology between the fragments. Southern blot analyses did not reveal any co-dominant markers.

The eight primers leading to the nine species-specific markers were used to amplify the DNA of 16 trees from a sympatric stand (Sennecey le Grand). For two primers (OpO07 and OpN04), the presence of the species-specific fragments and their homology were further confirmed by Southern blot analysis. The fingerprints obtained are reported on Table 2. These fingerprints showed recombination of the species-specific markers in 13 out of the 16 trees, suggesting quite extensive levels of natural hybridization in this population.

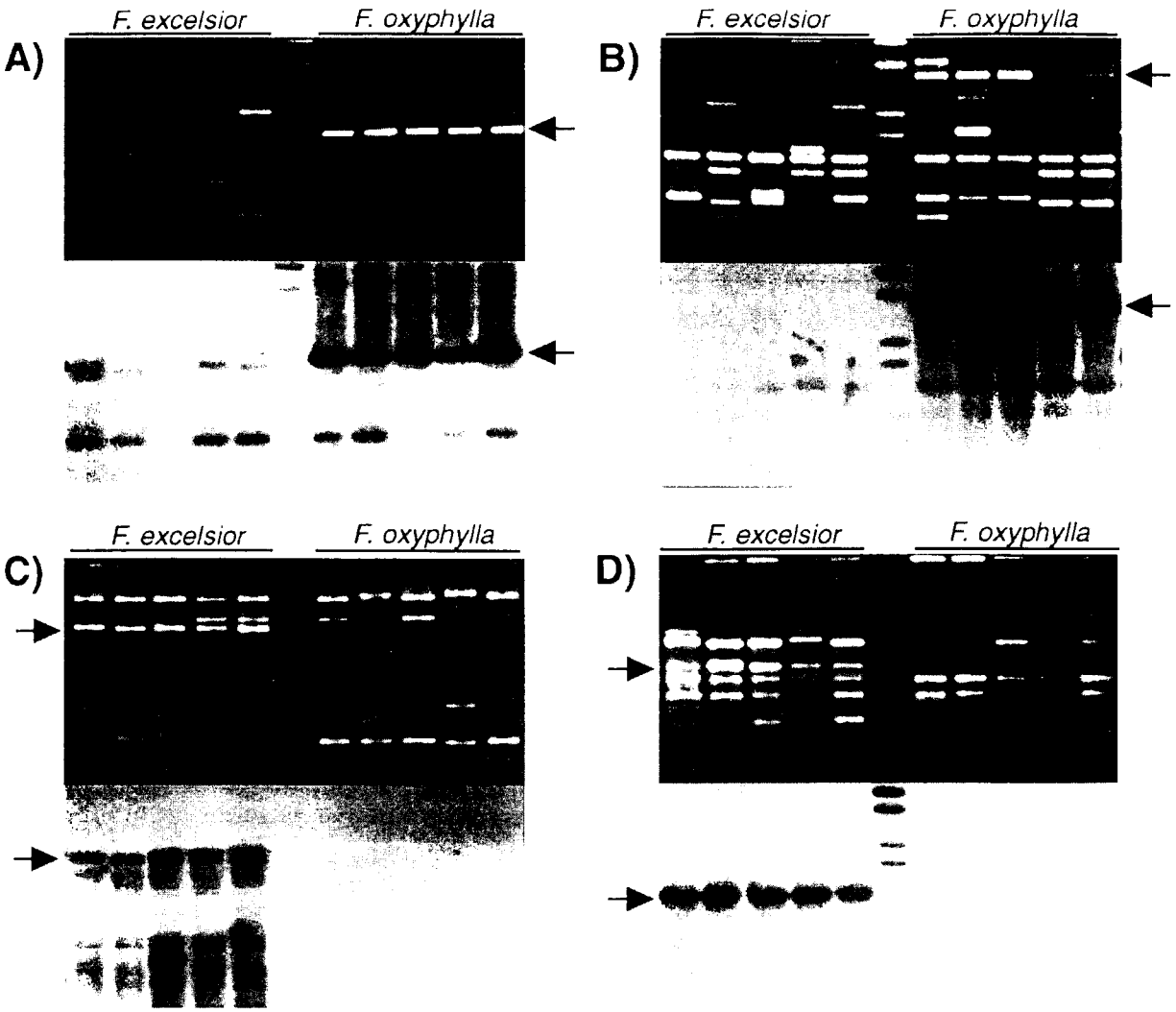

Figure 1 Gel electrophoresis (above) and corresponding autoradiogram of Southern blot (below) of RAPD fragments obtained: (A) primer OpO08, (B) primer OpO18, (C) primer OpO07, and (D) primer OpN04. These primers produced fragments (indicated by arrows) nearly monomorphic and specific to either *F. oxyphylla* or *F. excelsior*. These species-specific fragments were used as probes on Southern blots to determine whether the equivalently sized fragments were homologous.

DISCUSSION

Only nine out of 177 RAPD fragments scored with the 42 selected primers showed fixed or almost fixed polymorphism between the closely related *F. excelsior* and *F. oxyphylla*, which indicates a high level of genetic similarity between the two species. Likewise, the phylogenetic reconstruction of the genus *Fraxinus* from ITS sequences also supported a high affinity between *F. excelsior* and *F. oxyphylla* (JEANDROZ *et al.* 1997). Similar results with RAPD markers have been reported between closely related *Picea* species (PERRON *et al.* 1995; KHASA & DANCIK 1996). However, the success of finding such species-specific markers is largely dependent upon the degree of differentiation between the species surveyed, as more divergent species lead to easier identification of species-specific markers.

In most genetic studies using RAPD markers, it is assumed that co-migrating fragments in different individuals or populations of the same species are homologous. This assumption might become false as matters related to interspecific differentiation are addressed and as species divergence increases. RIESEBERG (1996) showed that 9.1% of the co-migrating fragments tested did not cross-hybridize in interspecific comparisons. Similarly, THORMAN *et al.* (1994) reported that three out of the 15 fragments used as probes did not hybridize with all fragments of the same mobility among the species tested. In the *F. excelsior* – *F. oxyphylla*

species complex, most of the fragments of same size cross-hybridized and appeared to be homologous between individuals within and between species, indicating again minimal interspecific divergence. In our study, it might be concluded that DNA fragment size similarity was a good predictor of fragment homology.

Southern blots were also a reliable tool to assist scoring the presence/absence of RAPD fragments, particularly when fragments of similar size showed differences in staining intensity. The Southern blot analyses showed that most co-migrating fragments with varying staining intensity that were tested had high DNA homology. Moreover, the apparent absence of a RAPD fragment after ethidium bromide staining did not always correspond to a total absence because, in some cases, fragments could be revealed by autoradiography at the expected locations. In such cases, presence/absence could be interpreted as differences in the amount of product amplified. This inconsistency of fragment amplification may be due to competition effects or mismatch between the primer and the DNA template (WAGNER *et al.* 1994). Furthermore, in the absence of pedigree material derived from interspecific controlled crosses, Southern blot analysis is a valuable way to check for the dominance (presence/absence) or the co-dominance (homologous fragments of different size in each of the two species) of the diagnostic fragments. In our study, no co-dominant fragments were found, which appears to be the rule rather than the exception with RAPD markers (WILLIAMS *et al.* 1990).

A lack of controlled crosses between *F. excelsior* and *F. oxyhylla* prevented us from studying the recombination of species-specific markers in true F_1 hybrids. It is likely that some of the species-specific markers identified in this study are not homozygous for all parental type individuals and therefore, hybrid/introgressant trees are likely to show some fragment absences when derived from mating events involving trees heterozygous for the markers (see PERRON *et al.* 1995). This could explain why markers OpA07-1100 and OpO18-1600 were absent from a great proportion of the putative hybrid/introgressant trees sampled from the sympatric population analyzed. This is one severe limitation of dominant RAPD markers, which render mandatory the use of several markers to detect hybrid/introgressant individuals, and makes difficult the precise estimation of introgression levels.

However, the observed recombination of the species-specific markers in a large proportion of the individuals sampled from a sympatric population lends support to the hypothesis of extensive natural hybridization between the two species. Therefore, DNA markers developed in this study are likely to be useful in delineating the extent of the hybrid zone in this species complex, to estimate levels of interspecific pollen contamination in seed orchards and to identify with confidence sources of pollen contamination.

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