SHORT COMMUNICATION

COMBINED ANALYSES OF MICROSATELLITE AND RAPD MARKERS DEMONSTRATE POSSIBLE HYBRIDISATION BETWEEN *FRAXINUS EXCELSIOR* L. AND *FRAXINUS ANGUSTIFOLIA* VAHL

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ABSTRACT

Controlled reciprocal crosses were made between the common ash (*Fraxinus excelsior* L.) and the narrow-leaved ash (*Fraxinus angustifolia* Vahl). An appreciable proportion of hybrids were found among the progenies using microsatellites and RAPD markers. This is the first report of controlled hybridisation between these two ash species. Reliable ways of identifying the possible exchanges in *Fraxinus excelsior* stands or seeds are of great importance for the whole forestry community. Furthermore, results of our contribution open the wide question of gene flow in the natural sympatric areas where the two species coexist.

Key words: *Fraxinus excelsior, Fraxinus angustifolia,* controlled crosses, ash, interspecific hybridization, microsatellites, RAPD.

INTRODUCTION

In France, two *Fraxinus* species grow spontaneously, *i.e.* the common ash (*Fraxinus excelsior* L.) and the narrow-leaved ash (*Fraxinus angustifolia* Vahl). The common ash is up to 30 m, has a light-grey bark and large compound leaves, which are divided into four to eight pairs of lanceolate leaflets. In winter, it has clusters of prominent black, velvety buds. The common ash is valued as a timber tree because of its rapid growth and its tough, elastic wood. It is used in reforestation programs and its economic importance is increasing in French forestry. *Fraxinus angustifolia* is up to 20 m, has brown buds and leaves divided into fewer and narrower pairs of leaflets than the common ash. It grows slowly and is usually considered as yielding an inferior wood quality (FUKAREK 1960). However, depending on the developmental stage, variability in morphological characters means that the two species are not always easily distinguished.

The common ash is widely distributed in France, except in the Mediterranean region and in Corsica, where *Fraxinus angustifolia* is naturally found. *Fraxinus angustifolia* is also present along the Rhône and Saône fluvial valleys where it coexists with *Fraxinus excelsior* (PICARD 1983). The occurrence of putative hybrids between the two species was suspected several years ago (WARDLE 1961). In the Rhône and Saône valleys, the occurrence of natural hybrids has been reported based on morphological traits (RAMEAU *et al.* 1989), but their true nature remained to be confirmed. The possible existence of hybrids is of great importance in forestry, where the aim is to guarantee the quality of ash timber, and for *Fraxinus excelsior* seed production, particularly in areas of sympatry, which may suffer from pollen contamination.

Fraxinus angustifolia generally flowers earlier than *Fraxinus excelsior* (PICARD 1983). On our campus near Paris, the common ash is naturally present while the narrow-leaved ash has been introduced. We observed differences between flowering times varying from two weeks (in 1997) to over two months (in 1999 and 2000). Generally the flowering duration of the two species was about two weeks. The common ash of our campus started to flower from mid-March to the beginning of April. The start of the narrow leaved ash flowering period is much more variable depending on the year, from the end of December to the end of February. In 1999, *Fraxinus angustifolia* flowered in mid-January and most flowers were destroyed by frost

but a few flowers appeared at the end of March, during *Fraxinus excelsior* flowering. The pollen of *Fraxinus angustifolia* kept in a meteorological shelter was no more able to germinate *in vitro* five weeks after harvesting. Differences in phenology between the two species show that hybridisation is normally very unlikely but possible some years, depending on meteorological conditions.

Since a reliable way of identifying precisely the two ash species was required, species-specific RAPD markers were developed previously (JEANDROZ *et al.* 1996). In particular, 13 out of 16 putative hybrid trees from the Saône valley sympatric zone showed recombination of these species-specific markers. This suggested that natural hybridisation occurs in this region, in agreement with previous morphological observations. However, these authors recognised that it would be important to study recombination of markers in F_1 hybrids from controlled crosses between the two species.

Many microsatellites markers have been developed in *Fraxinus excelsior* by BRACHET *et al.* (1999) and LEFORT *et al.* (1999). They have shown their potential use in other *Fraxinus* species namely in *Fraxinus angustifolia*.

The aim of the present investigation was to answer to the following question : is hybridisation possible between *Fraxinus excelsior* and *Fraxinus angustifolia* in controlled conditions? Some controlled crosses with either *Fraxinus excelsior* or *Fraxinus angustifolia* as pollen donor were carried out. We found recombination of microsatellite markers in the progenies; this result was confirmed by analysis of RAPD markers which were up to now used to identify these two species (JEANDROZ *et al.* 1996).

MATERIAL AND METHODS

Controlled crosses

Five *Fraxinus excelsior* (three hermaphrodite trees and two males) and two hermaphrodite *Fraxinus angustifolia*, located at the Orsay University campus were used as parents in the controlled crosses. Unfortunately, no pure female trees were present on the campus, so we chose three *Fraxinus excelsior* hermaphrodites as mother trees and two males as pollen donor trees. Before using hermaphrodite trees we verified that no samaras were produced after self pollination. Only two *Fraxinus angustifolia* hermaphrodite trees planted on the campus could be used. The one which naturally bore samaras was used as mother tree, and the other one which bore no or very few samaras was used as pollen donnor.

Branches of donor trees were cut in order to collect pollen. These branches were isolated, put into water and stored at room temperature until flowering. Pollen was then harvested and frozen at -70°C before use. No decrease in the *in vitro* pollen germination rate of the two species was observed whatever the length of the freezing storage (at least until 11 month of storage). Frozen pollen was used in a previous study on interspecific hybridisation with poplar species (RAQUIN *et al.*,1993).

Branches of pollen receptor trees were bagged before bud opening and dusted with pollen as soon as stigmas were clearly visible over the anthers. Because of the large number of small flowers per inflorescence, it was impossible to emasculate hermaphrodite flowers. Two pollinations were performed at seven days interval *i.e.* one pollination before anther opening and the other one during or after anther opening. Three weeks later, bags were removed when the ovaries began to enlarge. At this period, we thought that the receptive stage of the stigmas was over. Fruits were collected in mid-August. Seedlings for molecular analysis were obtained as described by RAQUIN *et al.* (2002).

Molecular analysis

DNA extractions from leaves of parent trees and young seedlings were performed using the Dneasy Plant Mini Kit (Qiagen).

Microsatellite analysis

Among the microsatellites markers developed for *Fraxinus excelsior* by BRACHET *et al.* (1999) and LEFORT *et al.* (1999), seven markers which also amplified in *Fraxinus angustifolia* were used: Femsatl 1, 4, 8, 11, 12, 19, and M230. PCR conditions were as in BRACHET *et al.* (1999); LEFORT *et al.* (1999). The genotype of the seven parents trees and of the 87 seedlings was determined using the seven microsatellite markers.

RAPDs analysis

Six RAPD primers producing seven species-specific markers (JEANDROZ *et al.* 1996) were used to amplify the DNA of the F_1 hybrids previously detected by the microsatellites markers: three for *Fraxinus excelsior* (OpL03 600 bp, OpN04 800 bp, OpO07 1400 bp) and four for *Fraxinus angustifolia* (OpH04 1600 bp, OpL03 750 bp, OpL14 800 bp, OpO08 1000 bp). DNA amplifications were conducted following previously published protocols (JEANDROZ *et al.* 1996) and duplicate

amplifications were conducted for trees with ambiguous RAPD fingerprints to ensure reproducible results.

RESULTS

We realized five different controlled crosses. Three crosses used hermaphrodite *Fraxinus excelsior* as mother trees pollinated by the single *Fraxinus angustifolia* pollen donor and two crosses used the other *Fraxinus angustifolia* as mother tree pollinated by the two different *Fraxinus excelsior* males.

Microsatellite analysis of seedlings

The segregation of three to four microsatellite loci has been examined on seedlings obtained from controlled crosses between the two *Fraxinus* species (Fig. 1). The segregation of all the variable markers in the F_1 progenies was consistent with a biparental diploid mode of inheritance.

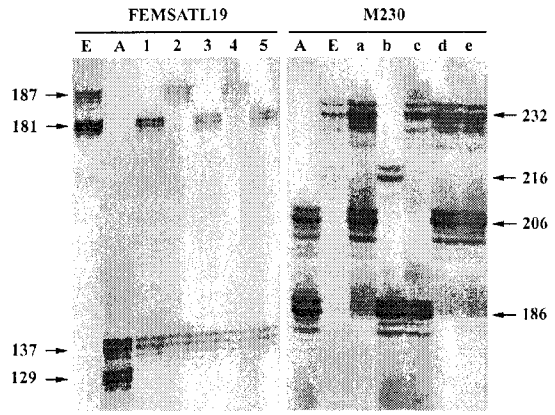

Figure 1. Analysis of two crosses between *Fraxinus excelsior* and *Fraxinus angustifolia*. **I**: Cross 3E5 × 3A1, E: 3E5, A: 3A1, nr. 1 to 5: seedlings (microsatellite marker Femsatl 19); **II**: Cross 3A2 × 1E2, E: 1E2, A: 3A2, letters **a** to **e**: seedlings (microsatellite marker M230) (sizes of alleles are given in bp).

For the four controlled crosses involving *Fraxinus angustifolia* as a pollen donor, among the 42 analysed seedlings, 36 were identified as hybrids whatever the microsatellite marker. For the two controlled crosses involving *Fraxinus excelsior* as a pollen donor, among the 45 analysed seedlings, 16 were identified as hybrids.

RAPD analysis

For the six RAPD primers, the presence of the species-specific fragments was tested on the 52 hybrids. Two of them (OpL03 600 bp, OpH04 1600 bp) confirmed the microsatellite analysis. RAPDs obtained with these primers for ten hybrids are shown in Fig. 2.

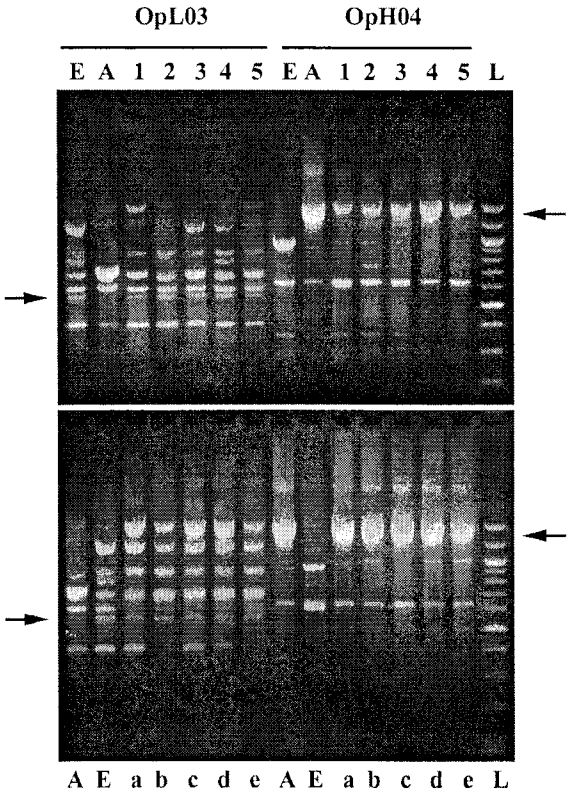

Figure 2. Confirmation of the microsatellite analysis by the RAPD analysis. Amplification of ten seedlings of crosses between *Fraxinus excelsior* and *Fraxinus angustifolia* by RAPD primers *i.e.* OpL03 and OpH04. **A**: *F. angustifolia*, **E**: *F. excelsior*, **L**: 100 bp DNA Ladder (Biolabs), nr. 1 to 5: seedlings from the cross 3E2 × 3A1, letters **a** to **e**: seedlings from the cross 3A2 × 1E2. Specific parental DNA fragments are located by arrows.

DISCUSSION

Analyses of microsatellites and RAPD markers clearly indicate that hybridisation is possible between *Fraxinus excelsior* and *Fraxinus angustifolia*. These results confirm the JEANDROZ *et al.* (1996) supposition about the occurrence of hybrid trees in the Saône valley sympatric area.

Hybrids exhibited mostly intermediate morphological characteristics of the two species. For example, hybrids from crosses involving *Fraxinus excelsior* as a mother tree expressed branching on the shoots of the year. This was never seen in pure *Fraxinus excelsior*. Reciprocally, buds of hybrids from crosses involving *Fraxinus angustifolia* as a mother tree were much

darker and more rounded than those of *Fraxinus angustifolia*. It would be interesting to follow the development of these hybrids in terms of growth and fertility.

These analyses have also led to the detection of pollen pollution in the different crosses. This was likely due to the bad quality of the bags that led to pollen contamination from surrounding trees. Indeed, in recent experiments we have eliminated this pollen pollution by using high quality bags. On the other hand we never observed up to now, seedlings which can be ascertained as selfings.

The scarcity of narrow-leaved ashes (three individuals on the campus) and the winter weather conditions strongly reduced the possibilities of crosses with *Fraxinus angustifolia* in Orsay. Experiments have now to be carried out in the sympatric area (for example the Saône fluvial valley) in order to compare the success rate of the reciprocal crosses between the two species. Nevertheless our study has shown that these interspecific crosses are possible either with *Fraxinus angustifolia* or with *Fraxinus excelsior* as the pollen donor. In sympatric zones, because of phenology differences between the two species, natural hybrids are more likely issued from crosses involving *Fraxinus angustifolia* as pollen donor. However, under very unusual weather conditions, *Fraxinus excelsior* could pollinate *Fraxinus angustifolia* as we have observed in our campus. Further studies in sympatric areas are thus needed to better understand patterns of gene exchange between these two species as well as the structure of local hybridisation areas.

In the context of progeny analysis, this paper shows that microsatellites are clearly sufficient to answer at the question of hybridisation. Anyway, in the study of natural hybridisation, the two specific RAPD markers could help to recognize the potential hybrids. Nevertheless, the development of other reliable markers is, of course, clearly needed. For example, the use of maternally inherited DNA markers would be very interesting for this prospect.

The potential ease with which *Fraxinus excelsior* and *Fraxinus angustifolia* crossed in this case as well as previous studies on phylogenetic reconstruction of the genus *Fraxinus* from rDNA ITS sequences (JEANDROZ *et al.* 1997) support the idea of a close relationship between the two species. Our findings here suggest that reproductive barriers do not separate these taxa.

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