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Organization of nuclear ribosomal DNA and species-specific polymorphism in closely related *Fraxinus excelsior* and *F. oxyphylla*

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Abstract The ribosomal DNA repeat units of two closely related species of the genus *Fraxinus*, *F. excelsior* and *F. oxyphylla*, were characterized. The physical maps were constructed from DNA digested with *Bam*HI, *Eco*RI, *Eco*RV and *Sac*I, and hybridized with three heterologous probes. The presence or the absence of an *Eco*RV restriction site in the 18*s* RNA gene characterizes two ribosomal DNA unit types found in both species and which coexist in all individuals. A third unit type appeared unique to all individuals of *F. oxyphylla*. It carries an *Eco*RI site in the intergenic spacer. Each type of unit displayed length variations. The rDNA unit length of *F. excelsior* and *F. oxyphylla* was determined with *Eco*RV restriction. It varied between 11 kb and 14.5 kb in *F. excelsior* and between 11.8 kb to 13.8 kb in *F. oxyphylla*. Using *Sac*I restriction, at least ten spacer length variants were observed in *F. excelsior*, for which a detailed analysis was conducted. Each individual carries 2–4 length variants which vary by a 0.3-kb step multiple. This length variation was assigned to the intergenic spacer. By using the entire rDNA unit of flax as probe in combination with *Eco*RI restriction, each species can be unambiguously discriminated. The species-specific banding pattern was used to compare trees from a zone of sympatry between the two species. In some cases, a conflicting classification was obtained from morphological analysis and the use of the species-specific rDNA polymorphism. Implications for the genetic management of both species are discussed.

Key words *Fraxinus excelsior* · *Fraxinus oxyphylla* · Nuclear rDNA · Intergenic spacer variation · Interspecific hybridization

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Introduction

In France, the genus *Fraxinus* is represented by two species, *F. excelsior* in the North and *F. oxyphylla* mainly in the South; however, along the Rhône and Saône valleys the two species coexist (Fig. 1). *F. excelsior* is a valuable species used in re-forestation programs, and seed orchards have been established in the sympatric area where putative interspecific hybridization has been frequently reported, based on the analysis of morphological characters (Poinsot 1972). To avoid pollen contamination by *F. oxyphylla*, which is slow growing and usually yields inferior wood quality, *F. oxyphylla* trees must be safely recognized, which is particularly difficult at the young stage. Furthermore, the extent of natural hybridization between the two closely related species needs to be better delineated and validated using complementary molecular approaches.

We chose to study nuclear ribosomal DNA (rDNA) in order to identify species-specific polymorphisms. In forest trees, rDNA has been shown to vary among species within genera in *Alnus* (Bousquet et al. 1989), *Populus* (Smith and Sytsma 1990; Faivre-Rampant et al. 1992 a), *Quercus* (Bellarosa et al. 1990), and *Picea* (Bobola et al. 1992 a, b).

Genes coding for 18*s*, 5.8*s* and 25*s* ribosomal RNA in higher plants are repeated (250–22 000) copies per genome) and generally organized in tandem repeats. Each repeat contains a transcribed region coding for the ribosomal RNA precursor and a non-transcribed region, the intergenic spacer (IGS). The rDNA coding regions tend to be highly conserved between species and genera (Appels and Dvorak 1982; Savard et al. 1993). In

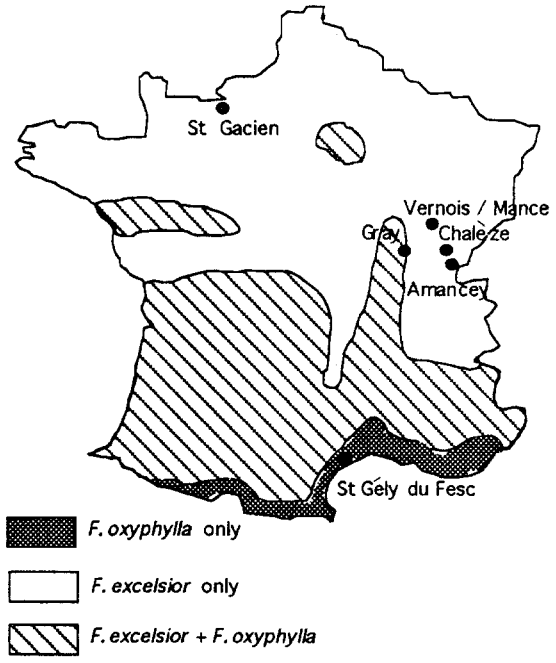

Fig. 1 Distribution of *F. excelsior* and *F. oxyphylla* in France and sampling sites

contrast, several polymorphisms have been reported for the IGS (Barker et al. 1988; Sano and Sano 1989), presumably because of relaxed evolutionary pressures. Variability between rDNA unit types has been found both in sequence and in length (Jorgensen and Cluster 1988). These two types of variation do not appear to show the same evolutionary rate: the change in restriction site caused by a nucleotide substitution usually allows one to differentiate rDNA unit types which could be species-specific, whereas length polymorphism caused by deletion/insertion events can be observed at the intraspecific level (Saghai Maroof et al. 1984; Zhang et al. 1992).

The IGS is organized into sub-repeats that vary in number and length, resulting in various IGS length units referred to as spacer length variants (Rogers and Bendich 1987; Baldridge et al. 1992). Such polymorphism has been observed between poplar, oak, and spruce species as well as among individuals of the same species (Bellarosa et al. 1990; Smith and Sytsma 1990; Bobola et al. 1992a, b; Faivre-Rampant et al. 1992a), while there was no detectable heterogeneity in rDNA among individuals of the same species in flax (Goldsborough and Cullis 1981), radish (Tremousaygue et al. 1988), and alder (Bousquet et al. 1989).

In the present paper, the organization of nuclear DNA in *Fraxinus* was investigated and a species-specific nuclear rDNA unit type was identified using heterologous probes from flax (Goldsborough and Cullis 1981) and poplar. The distribution of this polymorphism was also investigated in a limited sample of trees and putative hybrids from a stand where the two species coexist.

Materials and methods

Plant material

Trees' acronyms and their origins are given in Table 1. To characterize the nuclear rDNA of each species, trees and stands were sampled so as to be from outside the sympatric zone where hybridization between species is less likely to occur. Leaves of *F. excelsior* were collected from trees located in six natural populations, five in Franche Comté, eastern France, and one in Normandie, western France. Leaves were immediately frozen in dry ice and stored at −80 °C. For *F. oxyphylla*: (1) leaves were collected from seven trees located in Montpellier in southern France and (2) seeds were collected from at least four trees at Saint Gély du Fesc (near Montpellier) and were grown in greenhouse. Leaves were subsequently collected from seedlings and frozen at −80 °C.

Trees were also sampled in a single population (Gray, Franche-Comté) where the two species coexist. Because of putative hybridization at this site, these trees were classified *a priori* as *F. excelsior*, *F. oxyphylla*, or hybrids, using three morphological characters (shape of leave, bud color and seed size). Leaves were immediately frozen in dry ice and stored at −80 °C.

DNA preparation

Leaves (20–40 g) were ground at 4 °C in a Waring Blender (high speed for 1 min) in 150 ml of extraction buffer (3 M sorbitol, 0.1 M Tris-HCl pH 7.5, 5 mM EDTA and 20 mM sodium bisulphite). The homogenate was filtered and centrifuged at 1000 g for 20 min. The pellet

Table 1 List and origin of the plant material used

Species	Locality	Trees sampled
F. excelsior	Amancey (Franche-Comté)	A1, A2, A9, A5, A7, A10, A22
	Chaleze (Franche-Comté)	C5, C9, C10, C11, C12, C13, C14, C21
	Vernois/Mance (Franche-Comté)	V12, V15, V19, V20, V21, V22, V23, V24
	Gray (Franche-Comté)	EG1, EG2
	St Gacien (Normandie)	S9, S20, S21, S22, S24, S25
	Besancon (Franche-Comté)	Fac
	Salins les Bains (Franche-Comté)	Sal1
F. oxyphylla	St Gely du Fesc (Herault)	O1, O3, O10, O12, O16, O18, O31, O45, SGF3
	Montpellier(Herault)	Cros, Cros1, Cros2, Cros3, Eco2, Hort, Pisc
	Gray (Franche-Comté)	OG3, OG4
Putative hybrids	Gray (Franche-Comté)	HG1, HG2

was resuspended in 5 ml of extraction buffer. Five microliters of lysis buffer (0.2 M Tris-HCl pH 7.5, 5 mM EDTA, 2 M NaCl, 2% CTAB) and 0.5 ml of 20% (w/v) sarkosyl were added. The homogenate was incubated at 65 °C for 15 min and then extracted with chloroform:isoamyl alcohol (24:1, v/v). DNA was recovered from the aqueous phase by cold ethanol precipitation and then solubilized in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). CsCl was added to a refractive index of 1.398 at 20 °C. The final concentration of ethidium bromide was adjusted to 5 µg/ml. After centrifugation at 45 000 rpm for 15 h (VTi50), the DNA band was withdrawn and diluted in TE buffer. Ethidium bromide was extracted by isobutanol. DNA was precipitated with cold ethanol and then resus-pended in TE buffer.

Restriction, gel electrophoresis and Southern blotting

Total DNA (5 µg) was digested at 37 °C for 5 h with 5 U of enzyme per µg of DNA according to the manufacturer's conditions. For mapping, total DNA was restricted with *Bam*HI, *Eco*RI, *Eco*RV, and *Sac*I (Boehringer), either separately or in pairwise combination. Agarose-gel electrophoresis was carried out in TAE buffer (Sambrook et al. 1989) at 2 V/cm. DNA was transferred to nylon membranes using the manufacturer's recommendations (Zetaprobe, Biorad).

Probe labelling and hybridization conditions

The blots were sequentially hybridized with three heterologous probes(Fig. 2): (1) the entire rDNA unit of flax (pBG35): (2) a subclone of the unit, a 800 bp *Bam*HI fragment corresponding to the 25*s* rRNA gene (pRH83) (these two clones were kindly supplied by N. Ellis, see Goldsborough and Cullis 1981); (3) a PCR-amplified DNA fragment (Amp 18*s*) of poplar corresponding to the entire 18*s* rRNA gene using primers described by White et al. (1990). Probes were labelled using a random-primed DNA labelling kit (Boehringer Mannhein) with 20 µCi and α^{32} P-dCTP (3000 Ci/mmol). Nylon membranes were pre-hybridized in 6 × SSC, 5 × Denhardt and 0.5% at 65 °C for 15 h. Membranes were rinsed in 0.1 × SSC and exposed to X-Ray films for 6–18 h.

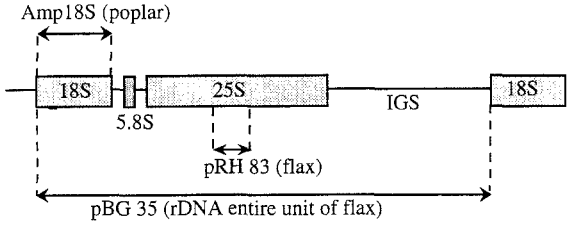

Fig. 2 Location, name and size of heterologous probes used

Results

Mapping rDNA in *F. excelsior*

Total DNA from the individual S21 was restricted with *Eco*RV, *Bam*HI, *Sac*I, and *Eco*RI, alone or in pairwise combinations (Fig. 3). The *Eco*RV lanes showed two major bands at 13.5 kb and 12.3 kb, and two minor bands at 10 kb and 2.3 kb. The 2.3-kb fragment did not appear when hybridized with the 25*s* probe (Fig. 3 B), but was revealed when probed with the 18*s* probe or the entire rDNA unit (Fig. 3A, C). This indicates that the 2.3-kb fragment carried part of the 18*s* rRNA gene and the 10-kb fragment carried the 25*s* rRNA gene and the IGS. The sum of the two fragments of 10 kb and 2.3 kb corresponds to the length of one of the major bands. The 13.5-kb and 12.3-kb fragments most likely correspond to two length variants of the same rDNA restriction-type unit which is characterized by only one *Eco*RV site (V1) per unit. This unit type will be referred to as unit I. The fragment of 10 kb and 2.3 kb correspond to a second rDNA restriction-type unit which carries an additional *Eco*RV site (V2). This type will be referred to as unit II. Units I and II were found to coexist in the same individual, S21. However, from the intensity of the hybridization signal (Fig. 3 C), unit I appears as a major type while unit II is a minor type, in terms of copy number.

The *Bam*HI digests of individual S21 showed two major bands at 4 kb and 1.1 kb when probed with the entire flax rDNA unit (Fig. 3 C). The 4-kb fragment includes part of the 25*s* rRNA gene (Fig. 3 B, lane B) whereas the 1.1-kb fragment carries only part of the 18*s* rRNA gene (Fig. 3 A, lane B), indicating that a *Bam*HI

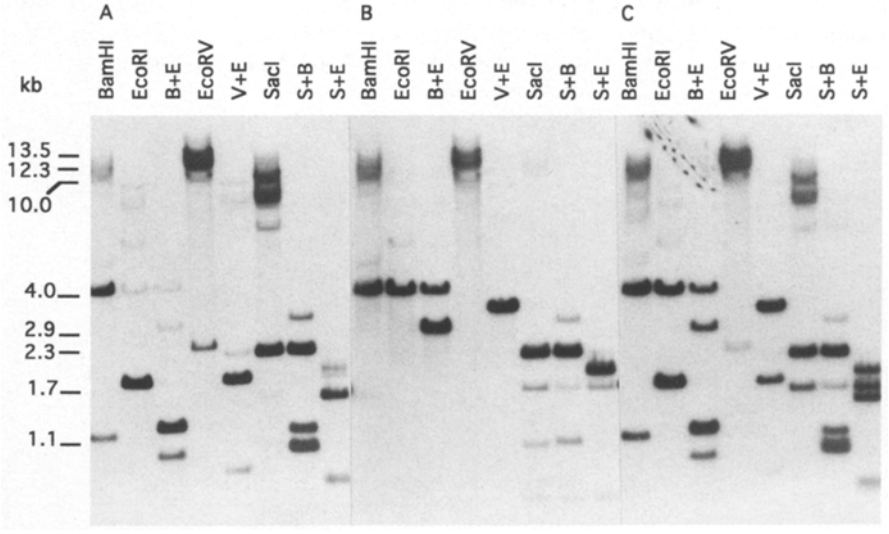

Fig. 3A–C Hybridization results for *F. excelsior* invidual S21 with single and double digests. *E*: *Eco*RI; *B*: *Bam*HI; *S*: *Sac*I and *V*: *Eco*RV. **PanelA**: probed with the 18*s* from poplar. **Panel B**: probed with the 25*s* from flax. **Panel C**: probed with the entire rDNA unit from flax

restriction site is present in the 18 *s* gene. The *Bam*HI digest also yielded larger fragments (5.1 kb, 7.2 kb, 8.4 kb and 12.3 kb), which most likely resulted from partial digestion due to possible DNA methylation (Fig. 3 C); *Bam*HI has been reported to be sensitive to DNA methylation (Gerlach and Bedbrook 1979). These additional fragments corresponded to 5.1 kb (1.1 + 4.0), 12.3 kb (5.1 + 7.2) and 13.5 kb (5.1 + 8.4), as expected for partial digestion. The fragments of 7.2 kb and 8.4 kb confirmed the two length variants (12.3 and 13.5 kb) detected with *Eco*RV digests. Also, the weak hybridization signals for the 7.2 and 8.4-kb fragments, which were only observed using the entire rDNA probe (Fig. 3 C), indicate that the length polymorphism in unit I is located within the IGS, and that the IGS of *Fraxinus* has diverged considerably from that of flax.

The *Sac*I restriction patterns, obtained with the entire rDNA unit (Fig. 3 C), showed two fragments of 2.2 kb and 1.6 kb corresponding to coding regions (Fig. 3A and B) and two larger fragments identified as part of the IGS overlapping the 18 *s* rRNA gene (Fig. 3 A). These two fragments correspond to the two spacer length variants of unit I identified with *Eco*RV digests. These results also suggest that this length polymorphism is located within the IGS. A total of three *Sac*I restriction sites were inferred to explain the presence of the 2.2 and 1.6 kb fragments.

The *Eco*RI lanes showed two fragments of 4 kb and 1.7 kb when probed with the entire rDNA unit (Fig. 3 C). The 4-kb fragment carried the 25 *s* rRNA gene (Fig. 3B) while the 1.7-kb fragment carried part of the 18 srRNA gene (Fig. 3 A).

From the results obtained with single and double digests, physical maps of the two rDNA restriction type units found in individual S21 were constructed (Fig. 4). Physical maps were also constructed with three other *F. excelsior* individuals (Fac, C5 and Sal1) from three different stands (see Table 1). The restriction patterns of the transcribed regions were identical and the two restriction-type units were found in each of the individuals (data not shown). Unit I always appeared as the major one while unit II was always the minor one. Therefore, except for the polymorphism corresponding to spacer length variation, the restriction maps for these four individuals could be considered to be representative of the species.

Total rDNA unit length and spacer length variation was investigated with 13 additional individuals of *F. excelsior* from four different stands (Amancey, Chalèze, St Gacien and Vernois/Mance). To do so, we monitored the size of restriction-type unit I, which is found in all four individuals previously analysed and which is characterized by a single *Eco*RV site. Total DNA was restricted with *Eco*RV and probed with the entire flax rDNA unit. A variable unit length ranging from 11 kb to 14.5 kb was observed (data not shown). Because the lengths of the coding regions were constant among the four individuals used to obtain restriction maps, it is likely that the observed length variations were mainly due to differences in the IGS length. Spacer length variations were further characterized by examining *Sac*I fragment patterns, because rDNA units contained three conserved *Sac*I sites in all individuals checked (see above), which delimit two smaller fragments of conserved length (the 2.2 and 1.6 kb), and larger fragments (6.6 to 9.5 kb) containing the intergenic region and the 5′ end of the 18 *s* gene (Fig. 4). These larger fragments are easier to distinguish that the large *Eco*RV fragments because they are 3.8 kb smaller. *Sac*I digests indicated ten size classes for the rDNA spacer with a step of approximately 0.3 kb (Fig. 5), which correspond to the unit length variants determined by the *Eco*RV digests. Up to four spacer length variants were observed in a single individual and the average was 3.7. Four classes of spacer length were more often represented, corresponding to 6.6-, 7.5-, 8.0-, and 9.0-kb fragments and to unit lengths of 10.5, 11.4, 11.9, and 12.3 kb, respectively.

Mapping rDNA in *F. oxyphylla*

Total DNA from individual O1 (St Gély du Fesc) was restricted with the four previously used restriction enzymes. The fragments patterns for the coding regions were similar to those obtained with *F. excelsior*, although some differences were observed (Fig. 6).

The *Eco*RV digests (Fig. 6 C) yielded one major fragment of 12.3 kb corresponding to the entire rDNA

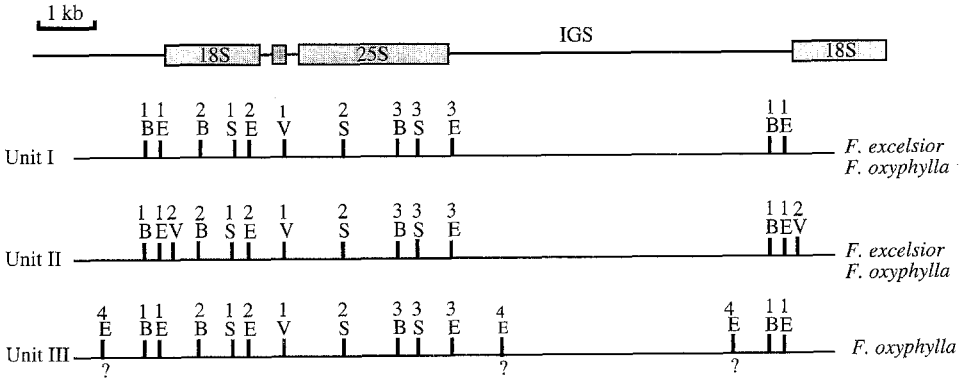

Fig. 4 Physical maps of the three rDNA unit restriction types encountered in *F. excelsior* and *F. oxyphylla*. *E*: *Eco*RI; *B*: *Bam*HI; *S*: *Sac*I and *V*: *Eco*RV. Unit III appeared unique to *F. oxyphylla*. The maps exclude the length variation observed in the IGS region. *Question marks* indicate the uncertainty of the position of the additional *Eco*RI restriction site for *F. oxyphylla*

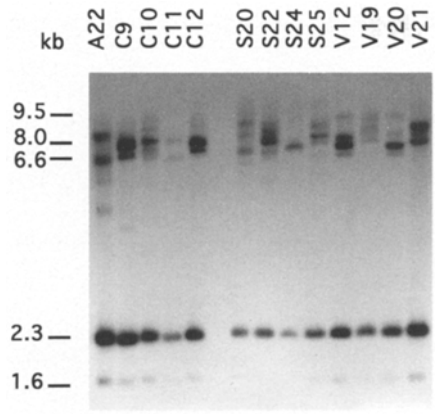

Fig. 5 Hybridization results for 13 individuals of *F. excelsior* restricted with *Sac*I and hybridized with the entire rDNA unit from flax

unit and two minor fragments, a 2.3-kb fragment carrying a part of the 18*s* rRNA gene and a 10-kb fragment carrying the 25*s* rRNA region and the IGS (Fig. 6). This indicated that the two rDNA restriction-type units observed in *F. excelsior* were also present in *F. oxyphylla* (types I and II). Again, from the relative intensity of the hybridization signals, unit I appeared as a major type and unit II as a minor type. The length of the *F. oxyphylla* rDNA unit was also characterized with *Eco*RV digests from eight additional individuals from St Gély du Fesc(O1, 3, 10, 12, 16, 18, 31, 45). It varied from 11.8 kb to 13.8 kb and showed heterogeneity both within and between individuals (data not shown). The small number of individuals analysed did not allow us to determine the step of the spacer length variation.

The *Eco*RI restriction digests of individuals O1 showed three fragments (4 kb, 1.7 kb and 1.0 kb) (Fig. 6 C) instead of two in *F. excelsior* (4 kb and 1.7 kb). The additional 1-kb fragment hybridized only with the entire rDNA unit and more weakly, which indicates an additional *Eco*RI site (E4) located in the IGS, either near the 18*s* or the 25*s* gene (Fig. 4). Therefore, a third rDNA restriction1type unit (unit III) which carried the E4 site could be defined (Fig. 4). Restriction analysis was also conducted with two additional individuals, SGF3 (from St Gély du Fesc) and Cros (from Montpellier). Restriction patterns of the transcribed regions were similar but a 0.7-kb *Eco*RI fragment was observed instead of the 1.0-kb fragment, when using the entire rDNA unit as probe. It is unlikely that this new fragment indicates a change of restriction site. Rather, the position of the third *Eco*RI site is most likely conserved among individuals of *F. oxyphylla*, if we accept that this fragment length polymorphism (0.7–1.0 kb) is likely to be caused by spacer length variation, as observed in *F. excelsior* (step of 0.3 kb). In all three *F. oxyphylla* individuals here analysed, restriction types I and II were always present in addition to the third restriction-type unit III. Therefore, *F. oxyphylla* could be distinguished from *F. excelsior* by the presence of unit III.

Validation of the species-specific DNA polymorphism

The results obtained with a small number of well characterized *F. excelsior* (4) and *F. oxyphylla* (3) individuals from outside the sympatric zone suggested that these two species may be distinguished by the presence or absence of rDNA restriction-type unit III. To confirm this difference between species, restriction with *Eco*RI followed by hybridization with the entire rDNA unit of flax was extended to numerous trees of each species from outside the sympatric zone: 26 *F. excelsior* individuals from Amancey (A1, A2, A9, A10, A22), Chalèze (C5, C9, C10, C11, C12, C13, C14, C21), St Gacien (S9, S20, S22, S24, S25), and Vernois (V12, V15, V19, V20, V21, V22,

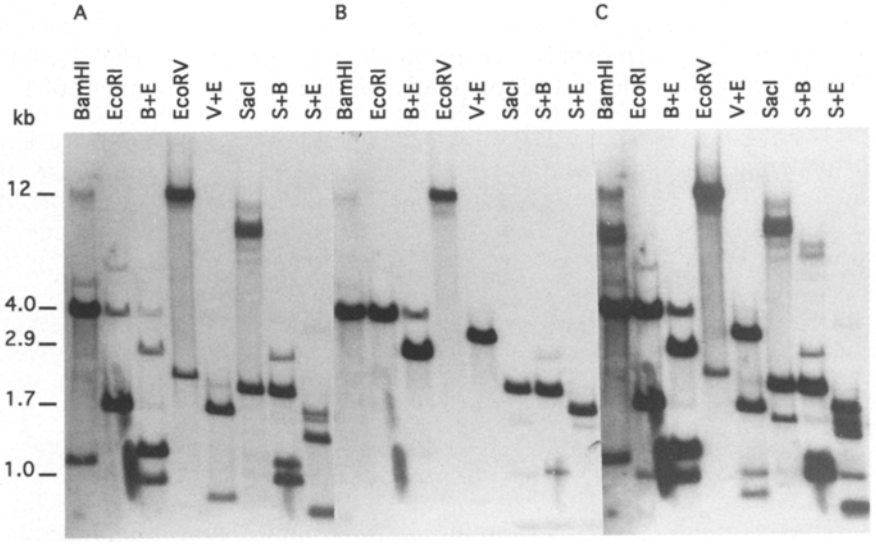

Fig. 6A–C Hybridization results for *F. oxyphylla* individual O1 with single and double digests. *E*: *Eco*RI; *B*: *Bam*HI; *S*: *Sac*I and *V*: *Eco*RV. **Panel A**: probed with the 18*s* from poplar. **Panel B**: probed with the25*s* from flax. **Panel C**: probed with the entire rDNA unit of flax

Table 2 Morphological and molecular characterization of individuals sampled from the Gray (Franche-Comté) zone of sympatry

Trees	Morphological characters						Molecular marker
	Leaves		Buds		Fruits (samara)		0.7–1.0-kb *Eco*RI fragment
	With tight and numerous teeth	With spaced and sharp teeth	Black	Brown	Seed shorter than half	Seed longer than half fruit	
F. excelsior theoretical value	+	−	+	−	+	−	−
F. oxyphylla theoretical value	−	+	−	+	−	+	+
F. oxyphylla OG3	−	+	−	+	−	+	+
F. oxyphylla OG4	−	+	−	+	−	+	+
F. excelsior EG1	+	−	+	−	+	−	−
F. excelsior EG2	+	−	+	−	+	−	+
Hybrid HG1	I.F.[a]	I.F.	I.F.	I.F.	I.F.	I.F.	−
Hybrid HG2	I.F.	I.F.	I.F.	I.F.	I.F.	I.F.	+

[a] I.F.: intermediate form

V23, V24); and 14 *F. oxyphylla* individuals from St Gély du Fesc (O1, O3, O10, O12, O18, O31, O45) and Montpellier(Cros, Cros1, Cros2, Cros3, Eco2, Hort, Pisc).

All *F. excelsior* individuals assayed displayed the 4.0-kb and 1.7-kb *Eco*RI fragment but shorter fragments were never recovered. All *F. oxyphylla* individuals analysed showed the 4.0-kb and 1.7-kb *Eco*RI fragments with an additional 1.0-kb or 0.7-kb *Eco*RI fragment. In addition, all individuals from St Gély du Fesc, except SGF3 (see above), showed the 1.0-kb fragment, while all individuals from Montpellier showed the 0.7-kb fragment. Therefore, with respect to the number of individuals analysed, our results suggest that the presence or absence of the rDNA restriction-type unit III might be used to distinguish the two species.

The presence or absence of restriction-type unit III was monitored in a limited sample of individuals from a stand where the two species coexist. Based on morphology, six individuals were chosen *a priori* so to be typical of one or the other species, or else intermediate, as would be expected from putative hybrids (Table 2). As expected, 0.7-kb *Eco*RI fragment which characterized *F. oxyphylla* was detected in *F. oxyphylla* individuals OG3 and OG4. This 0.7-kb fragment was also detected in HG2 but not in HG1, both characterized by intermediate morphology and classified as putative hybrids. More interesting was the unexpected presence of this fragment in one individual (EG2) which, based on morphology, was identified *a priori* as *F. excelsior*.

Discussion

The general organization of nuclear rDNA in *Fraxinus* agrees with that of many other plant species. The restriction sites E1, B2, E2, V1 and B3, located within the transcribed regions of *Fraxinus* rDNA, are highly conserved in higher plants, including the Graminaceae (Gerlach and Bedbrook 1979; Appels and Dvorak 1982) and the Crucifereae (Delseny et al. 1990). The total unit length, which varied from 11.5 to 14.5 kb for *F. excelsior*, and from 11.8 to 13.8 kb for *F. oxyphylla*, is also in the range of that found in higher plants, particularly in woody angiosperms such as poplar (Faivre-Rampant et al. 1992 a, b) or oak (Bellarosa et al. 1990).

IGS length polymorphism

*Sac*I restriction patterns revealed that spacer length variability is the rule in all the individuals of *F. excelsior* and *F. oxyphylla* assayed. In *F. excelsior*, where a detailed analysis of spacer length variants was conducted, at least ten types of length variants were observed and up to four spacer length variants were encountered in one individual. This high level of polymorphism has also been reported for barley (Saghai Maroof et al. 1984; Zhang et al. 1992): 20 spacer length variants allowed for the identification of 17 rDNA phenotypes. In other taxa, such as *Musa* (banana) (Lanaud et al. 1992), *Aegilops* (Kim et al. 1992) and *Pisum sativum* (pea) (Polans et al. 1986), similar results have been obtained. The number of spacer length variants per individual seems species-dependent; for instance, one for *Aegilops* (Kim et al. 1992), up to ten for *P. sativum* (Polans et al. 1986), and up to four for *Fraxinus* (this study). Therefore, in *F. excelsior* (and in *F. oxyphylla*) length variation in the IGS is frequent and not fixed within the species, so that it could be used as a marker of diversity at the intraspecific level, as recently reported in *Hevea* (Besse et al. 1993). In contrast, in *Picea* the length of the IGS shows significant variation that could be used to discriminate closely related species such as black spruce and red spruce, while no restriction-site polymorphism was found between these two species (Bobola et al. 1992b).

Restriction-site polymorphism

The restriction-site polymorphism observed allowed us to define three distinct rDNA units. Two of these restriction-type units, units I and II, differ by the gain or loss of the *Eco*RV site V2, which is located in the transcribed region. These two rDNA unit types coexist in all individuals of the two *Fraxinus* species. The intensity of the hybridization signals indicated that their relative proportion differs: unit I appears as a major locus whereas unit II is a minor locus. This organization suggests indeed that these two restriction-type units correspond to two different loci, as observed in pea (Polans et al. 1986). The presence of several rDNA-type units in the same genus or species has often been described (Delseny et al. 1990; Kim et al. 1992; Santoni and Bervillé 1992). Unit III differs from the other two by the presence of an additional *Eco*RI site (E4) located in the IGS, which was only observed in *F. oxyphylla* individuals. In individual O1 (*F. oxyphylla*), used to obtain the restriction map, and in the other trees of St Gély du Fesc, the *Eco*RI site E4 is usually located at about 1 kb away from the *Eco*RI site E3, but we found that equivalent sites (i.e. at 0.7 kb from site E3) are present in other individuals from another provenance (Montpellier). At present, we have no ex- planation for this almost fixed difference between the individuals of these two populations, and sampling of additional populations would be required to monitor the distribution of these two variants.

The results obtained in this study are in agreement with previous studies on other plant species concerning the rate of evolutionary change in rDNA regions (Jorgensen and Cluster 1988; Schaal and Learn 1988), where both restriction-site and length polymorphism are more frequent in the IGS region than in the coding regions. The *Eco*RV restriction-site polymorphism located in the 18 *s* gene and found in both *Fraxinus* species is most likely homologous to both species, deriving from an ancient event preceeding the divergence of the two. It is unlikely that this polymorphism resulted from parallel evolution in each species. In contrast, the *Eco*RI restriction-site polymorphism found in the IGS is specific to *F. oxyphylla* and is most likely more recent, following the divergence of these two closely related species. In the Crucifereae, unique regions located downstream from repeated IGS elements (Tremoussaygue et al. 1988), as well as repeated IGS subunits in tomato (Levesque et al. 1990) have shown to be species-specific, as they did not hybridize across related species.

Implications for genetic management

The genetic purity of progenitors chosen to establish breeding populations and seed production areas, such as seed orchards, is essential. It is also important that undesirable pollen contamination in the seed production areas be limited, particularly when hybridization with an undesirable closely-related species is likely. In our case, *F. excelsior* progenitor trees and seed orchards must be certified free from any undesirable contributions from *F. oxyphylla*, in terms of misidentified trees or in terms of pollen contamination. Although no polymorphism was found specific to *F. excelsior*, rDNA restriction-type unit III has been shown to be specific to *F. oxyphylla*. This marker could be used in a negative selection strategy scheme where, for instance, *F. oxyphylla* trees or *F. oxyphylla* gametic contributions to potential hybrids could be identified, so that undesirable trees and hybrids could be removed from breeding populations, seed orchards, and around seed orchards.

Indeed, we have shown that one tree identified as *F. excelsior* based on morphology was most likely misclassified because of the presence of the *F. oxyphylla* species-specific rDNA polymorphism in this tree. Additionally, one tree identified as a putative hybrid from morphological character was probably misclassified because of the absence of the *F. oxyphylla* species-specific rDNA polymorphism in this putative hybrid. However, because rDNA restriction-type unit III is a dominant marker for *F. oxyphylla*, hybrid trees cannot be distinguished from *F. oxyphylla*, and a fixed species-specific dominant marker (or co-dominant) for *F. excelsior* would be necessary to safely identify hybrids from parental types. This could be pursued by screening for species-specific RAPDs (Perron et al. 1995), SCARs (sequence characterized amplified regions) (Paran and Michelmore 1993), SWAPPs (sequencing with arbitrary primer pairs) (Burt et al. 1994), or DNA microsatellites (Morgante and Olivieri 1993; Smith and Devey 1994).

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References

Appels R, Dvorak J (1982) The wheat ribosomal DNA spacer region: its structure and variation in populations and among species. Theor Appl Genet 63:337–348

Baldridge GD, Dalton MW, Fallon AM (1992) Is higher-order structure conserved in eukaryotic ribosomal DNA intergenic spacers? J Mol Evol 35:514–523

Barker RF, Harberd NP, Jarvis MG, Flavell RB (1988) Structure and evolution of the intergenic region in a ribosomal DNA unit of wheat. Plant Mol Biol 201:1–17

Bellarosa R, Delre V, Schirone B, Maggini F (1990) Ribosomal RNA genes in *Quercus* spp. (Fagaceae). Plant Syst Evol 172:127–139

Besse P, Seguin M, Lebrun P, Lanaud C (1993) Ribosomal DNA variations in wild and cultivated rubber tree (*Hevea brasiliensis*). Genome 36:1049–1057

Bobola MS, Smith DE, Klein AS (1992a) Five major nuclear ribosomal repeats represent a large and variable fraction of the genomic DNA of *Picea rubens* and *P. mariana*. Mol Biol Evol 91:125–137

Bobola MS, Eckert RT, Klein AS (1992b) Restriction-fragment variation in the nuclear ribosomal DNA repeat unit within and between *Picea rubens* and *Picea mariana*. Can J For Res 22:255–263

Bousquet J, Girouard É, Strobeck C, Dancik BP, Lalonde M (1989) Restriction-fragment length polymorphisms in the rDNA region

among seven species of *Alnus* and *Betula papyrifera*. Plant Soil 118:231–240

Burt A, Carter DA, White TJ, Taylor JW (1994) DNA sequencing with arbitrary primers. Mol Ecol 3:523–525

Delseny M, McGrath JM, This P, Chevre AM, Quiros CF (1990) Ribosomal RNA genes in diploid and amphidiploid *Brassica* and related species: organization, polymorphism and evolution. Genome 33:733–744

Faivre-Rampant P, Jeandroz S, Lefèvre F, Lemoine M, Villar M, Bervillé A (1992a) Ribosomal DNA studies in poplars. Genome 35:733–740

Faivre-Rampant P, Bodergat R, Bervillé A (1992b) Une méthode de classemment des clones de peuplier (*Populus*) dans les sections Tacamahaca, Aigeiros, Leuce et Leucoides par des fragments de restriction des unités ribosomiques. CR Acad Sci Paris 35:133–138

Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res 7:1869–1885

Goldsborough PB, Cullis CA (1981) Characterization of the genes for ribosomal RNA in flax. Nucleic Acids Res 9:1301–1309

Jorgensen RA, Cluster PD (1988) Modes and tempos in the evolution of nuclear ribosomal DNA: new characters for evolutionary studies and new markers for genetic and populations studies. Ann Missouri Bot Gard 75:1238–1247

Kim WK, Innes RL, Kerber ER (1992) Ribosomal DNA repeat unit polymorphism in six *Aegilops* species. Genome 35:510–515

Lanaud C, Tezenas du Montcel H, Jolivot MP, Glaszman JC, Gonzales de Leon D (1992) Variation of ribosomal gene spacer length among wild and cultivated banana. Heredity 68: 147–156

Levesque H, Vedel F, Mathieu C, De Courcel AGL (1990) Identification of a short rDNA spacer sequence highly specific of a tomato line containing the *Tm*-1 gene introgressed from *Lycopersicon hirsutum*. Theor Appl Genet 80:602–608

Morgante M, Olivieri AM (1993) PCR-amplified microsatellites as markers in plant genetics. Plant J 3:175–182

Paran I, Michelmore RW (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor Appl Genet 85:985–993

Perron M, Gordon AG, Bousquet J (1995) Development of species-specific RAPD fingerprints for the black spruce-red spruce complex. Theor Appl Genet (in press)

Poinsot H (1972) Flore de Bourgogne. Librairie de l'Université, Dijon, France

Polans NO, Weeden NF, Thomson WF (1986) Distribution, inheritance and linkage relationships of ribosomal DNA spacer length variants in pea. Theor Appl Genet 72:289–295

Rogers SO, Bendich AJ (1987) Ribosomal DNA genes in plants: variability in copy number and in the intergenic spacer. Plant Mol Biol 9:509–520

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer length polymorphisms in barley. Mendelian inheritance, chromosomal location and population dynamics. Proc Natl Acad Sci USA 8:8014–8018

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Sano Y, Sano R (1989) Variation of the intergenic spacer region of ribosomal DNA in cultivated and wild rice species. Genome 33:209–218

Santoni S, Bervillé A (1992) Characterization of the nuclear ribosomal DNA units and phylogeny of *Beta* L. wild forms and cultivated beets. Theor Appl Genet 83:533–542

Savard L, Michaud M, Bousquet J (1993) Genetic diversity and phylogenetic relationships between birches and alders using ITS, 18 *s*, and *rbc*L gene sequences. Mol Phyl Evol 2:112–118

Schaal BA, Learn GH (1988) Ribosomal DNA variation within and among plant populations. Ann Missouri Bot Gard 75:1207–1216

Smith DN, Devey ME (1994) Occurrence and inheritance of microsatellites in *Pinus radiata*. Genome 37:977–983

Smith RL, Sytsma RJ (1990) Evolution of *Populus nigra* (sect. Aigeiros): introgressive hybridization and the cloroplast contribution of *Populus alba* (sect. Populus). Am J Bot 77:1176–1187

Tremousaygue D, Grellet F, Delseny M, Delourme R, Renard M (1988) The large spacer of a nuclear ribosomal RNA gene from radish: organization and use as a probe in rapeseed breeding. Theor Appl Genet 75:298–304

White TJ, Bruns T, Lee S, Taylor S (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols. A guide to methods and applications. Academic Press, San Diego, pp 482

Zhang Q, Saghai-Maroof MA, Yang PG (1992) Ribosomal DNA polymorphisms and the Oriental-Occidental genetic differentiation in cultivated barley. Theor Appl Genet 84:682–687