Theor Appl Genet (1996) 92:1003–1008

S. Jeandroz · A. Pugin · A. Bervillé

Cloning and analysis of a 6.8-kb rDNA intergenic spacer region of the European ash (*Fraxinus excelsior* L.)

Received: 6 October 1995 / Accepted: 3 November 1995

Abstract The 6.8-kb rDNA intergenic spacer region of *F. excelsior* was isolated from a CsCl/actinomycin-D gradient and cloned into pUC18 for further characterization. We observed the presence of subrepeats delimited by *Hae*III enzyme sites. These subrepeats were sub-cloned and 11 clones were sequenced. These corresponded to subrepeated elements of either 32 bp or 41 bp that shared a 23-bp common sequence in the 5′ end. Within each family of subrepeats, the percentage of common nucleotides was 84.4% for the 5 32-bp subrepeats and 67.4% for the 6 40-bp subrepeats. Non-repeated *Hae*III fragments of 450 bp and 650 bp were also sub-cloned. To compare homology at the IGS region between the rDNA spacers of *F. excelsior* and the three related species (*F. oxyphylla*, *F. americana*, *F. ornus*), we conducted Southern hybridization analyses using each member of the 32-bp and 40-bp subrepeat families and the unique 450-bp and 650-bp fragments as probes. These analyses indicated that (1) the American ash is more genetically distant from the other three species that the latter are from each other and (2) *F. oxyphylla* and *F. excelsior* are more closely related to each other than to *F. ornus*.

Key words Fraxinus · Evolution · rDNA · IGS structure

Communicated by P. M. A. Tigerstedt

S. Jeandroz[1] · A. Pugin
Université de Franche-Comté, Laboratoire de Biochimie et Biologie Moléculaire, UFR Sciences, 16 route de Gray, 25030 Besançon Cedex, France

A. Bervillé (✉)
I.N.R.A., Station d'Amélioration des Plantes, Laboratoire des Marqueurs Moléculaires, 2 place Viala, 34060 Montpellier Cedex 1, France

Present address:
[1] Centre de Recherche en Biologie Forestiére, Faculté de Foresterie et de Géomatique, Université Laval, Sainte Foy, Québec G1K 7P4, Canada

Introduction

Nuclear genes coding for the 18 S, 5.8 S and 25 S ribosomal RNA (rDNA) of higher eukaryotic ogranisms are organized in tandem repeats. rDNA has been widely studied in animals as well as in higher plants. The tandemly repeated rDNA subunits are composed of transcribed regions (coding regions, internal and external transcribed spacers) which are separated by a non-transcribed intergenic spacer (IGS) that carries the signals for transcription initiation and termination. In plants, the IGS regions are composed of subrepeats interspersed within unique sequences (Appels and Dvorak 1982). The length, copy number, and the number of different subrepeated elements vary considerably from one species to another, as has been shown for *Helianthus* (Choumane and Heizmann 1988), maize (Rocheford et al. 1990), beets (Santoni and Bervillé 1992), and poplar (Faivre-Rampant et al. 1992), or from one individual to another, as has been shown for beets (Santoni and Brevillé 1992). This variability is due to either base changes, as revealed by differences in restriction sites, or to length heterogeneity.

Fraxinus excelsior is preferred over *F. oxyphylla* because of its rapid growth and higher wood quality. However on the basis of morphological characters, it is difficult to distinguish among *F. excelsior*, *F. oxyphylla* and *F. excelsior* × *F. oxyphylla* hybrids. We have previously shown that there is rDNA unit length variation among *Fraxinus* species and that rDNA sequence variation between species is attributable to differences in the IGS (Jeandroz et al. 1995). Ribosomal DNA polymorphism can be used to distinguish between *F. excelsior*, *F. oxyphylla* and their hybrids. In order to develop a more convenient forest research tool for identifying species and hybrids, we have begun looking for species-specific DNA probes. Using an approach similar to that used in radish (Tremousaygue et al. 1988), tomato (Cordesse et al. 1992), and rice (Cordesse et al. 1990), we have further

characterized the IGS region of *F. excelsior* to identify species-specific sequences.

We report here the cloning and analysis of the 6.8-kb IGS region of *F. excelsior* rDNA. We have identified and sequenced two types of subrepeat motifs, thereby demonstrating that these regions are sufficiently variable to be used as probes in Southern hybridization to distinguish both closely and distantly related *Fraxinus* species.

Materials and methods

Plant material

Leaves of *F. excelsior* L., *F. oxyhylla* Bieb., *F. ornus* L., and the American white ash (*F. americana* Marsh.) were collected from selected sampled trees. The leaves were frozen in dry ice and conserved at −80 °C. Specimens from each species were collected from Franche-Comté (east of France), the Besançon Botanical Garden (east) and Saint Gély-du-Fesc (Close to Montpellier, south)

DNA extraction and analysis

Total DNA was extracted from frozen leaves and purified by a CsCl gradient ultracentrifugation as previously reported (Jeandroz et al. 1995). DNA was digested at 37 °C for 5 h with 5 U of restriction enzyme per microgram of DNA according to the manufacturer's conditions. Gel electrophoresis was carried out in 0.8% agarose in 1 × TAE buffer (Sambrook et al. 1989) at 2 V/cm. DNA was transferred to Nylon membranes (Zetaprobe, Biorad) following the manufacturer's protocol.

Probes were labelled by random priming (Boehringer Mannheim) using 20 μCi of [α^{32}P]-dCTP (3000 Ci/mmol). Southern blots were hybridized in 6 × SSC, 5 × Denhardt and 0.5% SDS at 65 °C for 15 h. Membranes were rinsed in 0.1 × SSC at 65 °C and exposed to X-Ray film at −80 °C for 6–18 h.

Ribosomal DNA cloning

The rDNA was separated from the main DNA using a CsCl/actinomycin-D gradient (Gerlach and Bedbrook 1972; Hemleben et al. 1977). An aliquot of each collected fraction was denatured and immobilized onto Nylon filters (Zeta Probe Biorad) according to the manufacturer's protocol. Ribosomal DNA aliquots were identified by hybridization with the entire rDNA unit of flax (pBG35), (Goldsbrough and Cullis 1981). The fractions containing rDNA were pooled, digested by *Eco*RI, then ligated into the *Eco*RI sites of dephosphorylated pUC18 plasmid (Appligene, France). After transformation of *E. coli* DH5 α mcr (Gibco, BRL) host cells, the resulting clones were subjects to colony hybridization (Sambrook et al. 1989) with the rDNA probe.

Isolation and subcloning of the 32-bp and 41-bp subrepeats

The DNA from recombinant plasmids was digested with *Hae*III and electrophoresed on 6% polyacrylamide gel 1 × TAE buffer for 3 h at 12 mA. The 32-bp and 41-bp DNA fragments, visualized under UV after ethidium bromide staining, were eluted from the gel then sub-cloned into the *Sma*I site dephosphorylated pUC18 plasmid. The ligation product was used to transform *E. coli* XL1 Blue (Stratagène, France) competent cells (Sambrook et al. 1989).

Sequencing

Sequencing was performed using a T7 Sanger sequencing kit (Pharmacia, Saint Quentin en Yvelines, France). Computer analysis of the sequence data and sequence comparison were carried out using the 'CLUSTAL' program, PC Gene software (Higgins et al. 1992).

Results

Isolation of a clone containing the intergenic spacer

In order to isolate the IGS region we constructed a library with *Eco*RI fragments that resulted from a complete restriction digestion of a fraction enriched for rDNA. After screening, two clones were selected: pE1G12, which contains a 6.8-kb *Eco*RI fragment corresponding to the region between sites E3 and E1 (Fig. 1A), and pE1C9, which contains the 1.8-kb fragment corresponding to the region between the E1 and E2 sites (Fig. 1A).

Organization of pE1G12: evidence for short repeated sequences

pE1G12 DNA was digested with the *Hae*III restriction enzyme and then electrophoresed on 6% polyacrylamide as described above. While DNA bands ranging in length from 30- to 650-bp were observed, those of 30 bp, 40 bp, 80 bp, and 120 bp fluoresced with a high intensity, indicating the presence of repeated fragments within the IGS region (Fig. 2). Southern blots of pE1G12 DNA partially digested with *Hae*III were hybridized with either the 120-bp *Hae*III fragment or the 40-bp *Hae*III fragment as a probe. The 120-bp *Hae*III fragment (Fig. 3A) hybridized to four fragments of 30 bp, 65 bp, 95 bp and 120 bp, respectively. The 40-bp *Hae*III fragment (Fig. 3B) hybridized strongly to the 40-bp, 65-bp, and 80-bp bands and weakly to the 30-bp, 95-bp, and 120-bp bands. These observations suggested that: (1) the 65-bp, 95-bp and 120-bp fragments were homologous and were a dimer, trimer and tetramer of an approximately 30-bp -long subrepeat element, respectively; (2) the 80-bp fragment was the dimer of a 40-bp subrepeated element; (3) a homology existed between the 30-bp and the 40-bp

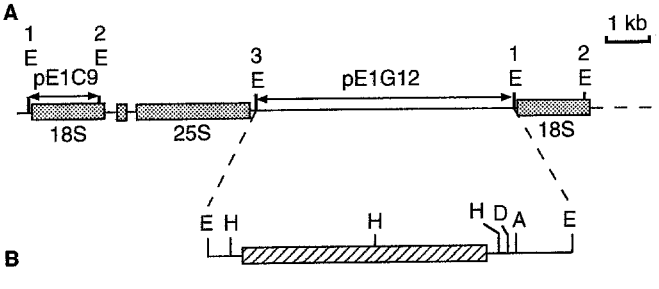

Fig. 1A Assignation of the pE1G12 and pE1C9 isolated fragments and position of the coding sequences. **B** Partial restriction map of the clone pE1G12. *A Acc*I, *D Dra*I, *E Eco*RI, *H Hinf*I. The *box* corresponds to the region containing the *Hae*III subrepeats

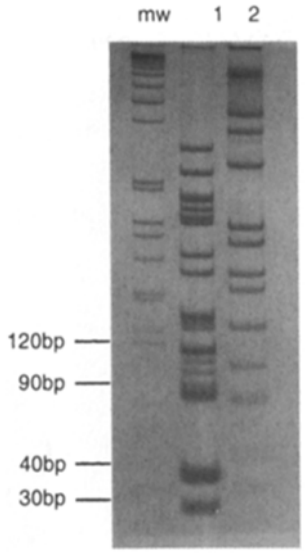

Fig. 2 Evidence for subrepeats elements in the pE1G12 clone (6.8-kb *Eco*RI insert + vector pUC18). *Lane 1 Hae*III restriction pattern, *lane 2 Sau*3AI restriction pattern. The 6% polyacrylamide gel was visualized under UV after staining by ethidium bromide

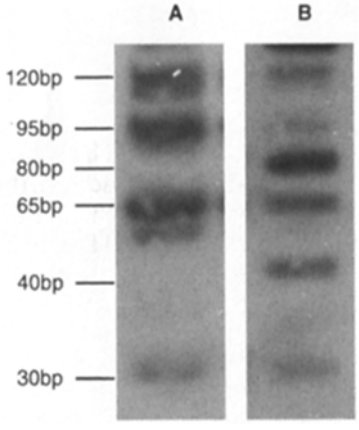

Fig. 3A, B Autoradiograms of pE1G12 DNA after partial digestion with*Hae*III, polyacrylamide gel electrophoresis, and Southern blotting. **A** Hybridized with the pE1G12/*Hae*III 120-bp fragment as a probe, **B** hybridized with the pE1G12/*Hae*III 41-bp fragments as a probe

elements; (4) the intergenic spacer of *F. excelsior* contained two related families of subrepeats of different sizes: one of approximately 30 bp and another of approximately 40 bp.

To confirm our interpretation, we eluted the 30-bp and the 40-bp fragments from an acrylamide gel and then subcloned. The 11 subclones which were sequenced showed a high degree of homology between the two families of subrepeats (Table 1). All of the clones shared a 23-bp common sequence at the 5′ end. All of the subrepeats were heterogeneous both in size and in sequence. Their size varied from 31 bp to 32 bp in one class and form 41 bp to 43 bp in the other; sequence comparisons indicated an 84% and 67% sequence similarity, respectively (Table 1).

The pE1G12 insert was isolated from vector and digested with different endonucleases to construct a restriction map. The enzyme *Hinf*I yielded two fragments of 2.7 kb and 2.3 kb and numerous other frag-

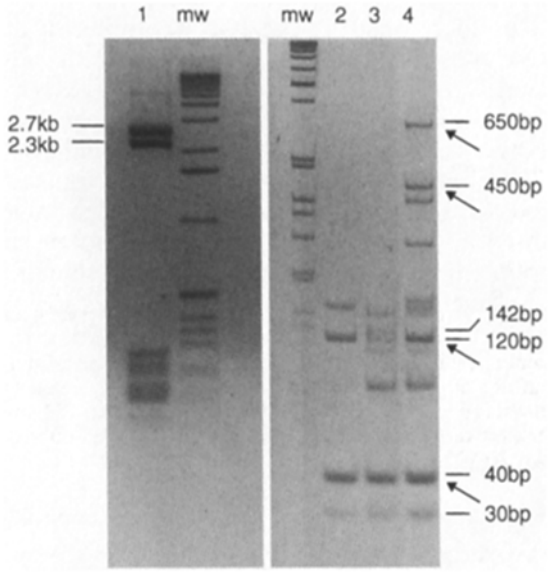

Fig. 4 Restriction profiles of the 6.8-kb *Eco*RI fragment and the 2.3-and 2.7-kb *Hinf*I subfragment. (*Lane1*) the 6.8-kb *Eco*RI fragment was isolated from pE1G12 and restricted with *Hinf*I. *2* The *Hinf*I 2.3-kb DNA fragment from (*lane 1*) was isolated and digested with *Hae*III. *3* The *Hinf*I 2.7-kb DNA fragment from (*lane 1*) was isolated and digested with *Hae*III. (*4*) The 6.8-kb *Eco*RI DNA fragment was isolated and digested with *Hae*III. DNA fragments were separated on 1.5% agarose gel (*1*) or 6% polyacrylamide (*2–4*) and visualized under UV illumination after ethidium bromide staining. The *arrows* indicate the *Hae*III 41-bp, 120-bp, 450-bp and 650-bp fragments used as probes in Fig. 5

Table 1 Nucleotide sequence of the short sub-repeats. Gaps were introduced to optimize alignments between repeats

Codes	Sequences	Sizes (bp)
EXC40_2	CCAAGACACGGTGCCTGGAAATCCCTGTGATGCCTGAACATGG	43
EXC40_3	CCAAGACACGGTGCCTGGAAATCCCTGTGATGCCTGA-CATGG	42
EXC40_7	CCAAGACACGGTGCCTG-AAATCCCTGTGATGCCTGA-CATGG	41
EXC40_5	CCAAGACGCGGT-CTTGAAAATCCAAGTGATGGCTGA-CACGG	41
EXC40_8	CCTAGACACGATGCCTGAAAATCAAGGTGATGGCT--ACACGG	41
EXC33_1	CCA-GACACGGTGCCTGAAAATCCAGG----------CACGG	31
EXC33_2	CCAAGACACGGTGCCTGAAAATCCAGG----------CACGG	32
EXC33_4	CCAAGACACGGTGCCTGAAAATCCAGG----------CACGG	32
EXC33_6	CCTAGACACGATGCCTGAAAATCCAGG----------CACGG	32
EXC33_7	CCATGACACGGTGTCTGAAAATCCAGG----------CACGG	32
EXC33_8	CCAAGACACGGTGCCT-AAAATCCAGG----------CACGG	31

ments of under 300 bp in size (Fig. 4, lane 1). The 2.7-kb and 2.3-kb fragments were isolated and digested separately with *Hae*III, the enzyme which delimited the subrepeats. The results showed that 2.3-kb fragment had four fragments (Fig. 4, lane 2), with each fragment corresponding to a band observed after the *Hae*III digestion of pE1G12 (Fig. 4, lane 4). The 2.3-kb fragment was determined to consist entirely of 32-bp and 41-bp subrepeats. The 2.7-kb fragment had seven bands after digestion with *Hae*III (Fig. 4, lane 3), with six of these bands corresponding to pE1G12/*Hae*III fragments. The 142-bp fragment, which was not present after pE1G12/*Hae*III digestion, corresponded to a *Hinf*I-*Hae*III, *Hinf*I-*Hinf*I, or *Hinf*I-*Eco*RI fragment. The remaining six bands were considered to be multimers of the 32-bp and 41-bp subrepeat classes. These results and results from *Dra*I and *Acc*I digestions (results not shown) enabled us to reconstruct the IGS organization of the 6.8-kb cloned fragment (Fig. 1B).

Total DNA of *F. oxyphylla*, *F. ornus*, *F. americana* was also digested with *Hae*III and electrophoresed on 6% polyacrylamide gels. After ethidium bromide staining, DNA bands of comparable sizes (approximately 30 bp and 40 bp) again fluoresced with a high intensity (results not shown). These bands have not been sequenced, but they indicate the presence of similar subrepeat elements in these species.

Genome specificity of the 30-bp and 40-bp subrepeat elements and of the 450 and 650-bp *Hae*III fragments

In order to investigate the species specificity of different regions of the intergenic spacer, total DNA prepared from *F. excelsior* and from three related species (*F. oxyphylla*, *F. ornus* and *F. americana*) were separately restricted with *Eco*RI. Hybridizations were performed with the 32-bp and 41-bp *Hae*III subrepeats or with the 450-bp and 650-bp *Hae*III fragments (Fig. 4, lane 4) as a probe, respectively. The 450-bp *Hae*III fragment hybridized strongly to the DNA of *F. oxyphylla* and *F. ornus*, but less strongly to the DNA of the *F. americana* (Fig. 5A). The 650-bp *Hae*III fragment hybridized with *F. oxyphylla* and *F. ornus*, but not to the *F. americana* (Fig. 5B). When the 32-bp and 41-bp subrepeats were used as probes (Fig. 5C and D) we observed homologies between *F. excelsior* and *F. oxyphylla*, although the hybridization was weaker with *F. oxyphylla*. No significant homology was found between the DNA of *F. ornus* or of *F. americana* and both subrepeats as a probe. Hybridization signals were not observed even when the stringency of the rinsing conditions was reduced to 2 × SSC. When a clone containing the 18 S rDNA gene of *F. excelsior* was used as a probe, equally intense signals of equal size were observed for all the species (Fig. 5E).

Discussion

Characterization of two IGS subrepeats types

To our knowledge, this is the first report of the cloning and characterization of a rDNA spacer from a woody plant. The main structural feature of this region is the presence of subrepeats. These repeated elements are characteristic of the largest DNA spacer in plants or animals and have been described and sequenced in

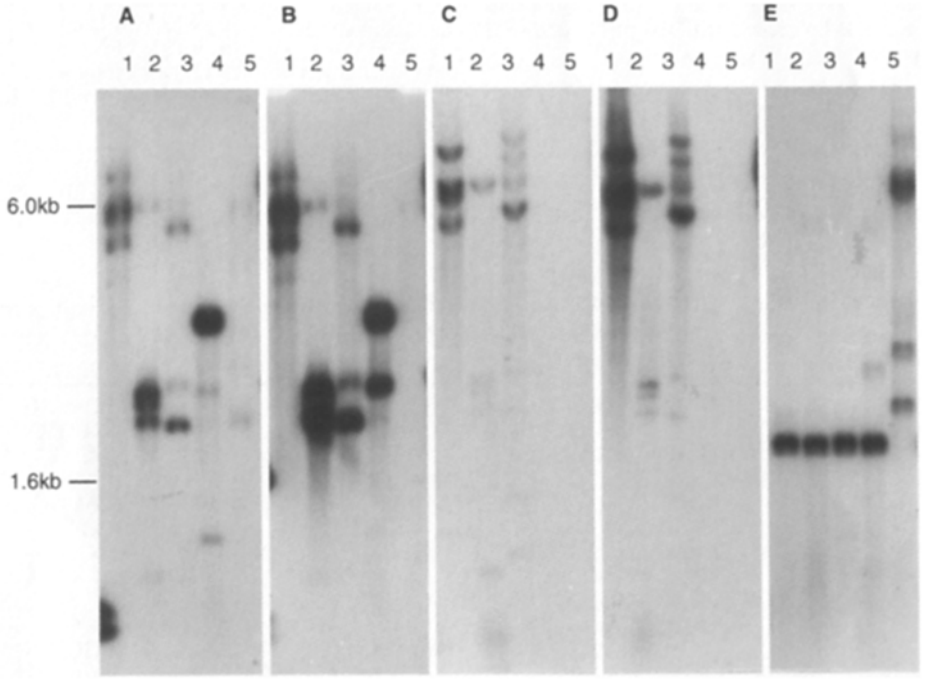

Fig. 5A–E Evidence for differential hybridization of the DNA from four *Fraxinus* species, hybridized with the 450-bp and 650-bp *Hae*III fragments and the short subrepeats. DNAs from the four species were restricted with *Eco*RI, electrophoresed in a 0.6% agarose gel, Southern blot-transferred onto Nylon filter and hybridized with: **A** the pE1G12/*Hae*III 450-bp fragment, **B** the pE1G12/*Hae*III 650-bp fragment, **C** the 120-bp *Hae*III fragment (tetramer of 32-bp subrepeat), **D** the 41-bp subrepeat, **E** pE1C9 (18 S rRNA gene). *Lanes 1 F. excelsior, 2 F. oxyphylla, 3 F. oxyphylla* (063) *4 F. ornus, 5 F. americana*

numerous species (Appels and Dvorak 1982; Baldridge et al. 1992). The length of the repeat motif in *F. excelsior* is among the shortest so far reported, and it is comparable to the 22- to 45-bp repeat motif found in most crucifers (Lakshmikumaran and Negi 1994) or to the 32-bp one in *Pharbitis nil* (Katayama et al. 1992), although it is shorter than its counterparts in tomato (54 bp) (Levesque et al. 1990), barley (128 bp) (Procunier and Kasha 1990), radish (150 bp) (Tremousaygue et al. 1988), and rice (150 bp) (Cordesse et al. 1993).

The proposed restriction map of the 6.8-kb *Eco*RI fragment indicates that approximately 70% (4.7 kb) of the IGS region is composed of subrepeats. The two types of subrepeats found in *F. excelsior* may have a common origin because they share a 23-bp common sequence. These two subrepeats could have occurred either by a deletion in the 41-bp subrepeats or an insertion in the one of 32 bp. It has been demonstrated that unequal crossing-over at the subrepeat level leads to the homogenization of their sequences within the IGS of a rDNA unit (Dover et al. 1993). In our case, such a mechanism would favor the fixation of one or the other types of subrepeats. We could not determine whether or not the two families of subrepeats are randomly distributed in the IGS. Using MVP-PCR (polynerase chain reaction) (Jeffreys et al. 1991) in *Drosophila melanogaster*, Linares et al. (1994) have demonstrated that variant subrepeats within tandem arrays have a strong tendency to cluster on one or the other half of the array.

For each species in which the IGS has been investigated, there are distinct repeat sequences. However, we found a TGAAAAT motif present in *F. excelsior* that is also found in most crucifers (Lakshmikumaran and Negi 1994). A degree of similarity has also been demonstrated between the IGS subrepeats of wheat (Appels and Dvorak 1982) and maize (McMullen et al. 1986). The TGAAAAT motif has been shown to bind to the homologous nuclear protein fraction of radish, (Echeverria et al. 1992), and this could be interpreted as evidence that in plants the subrepeats have functions and may act as enhancers of transcription, as has been suggested by Reeder (1984) for animals. The presence of common motifs between these species indicate that the subrepeats may have evolved from an ancestral sequence common to woody and annual plants. These observations are further supported by the strong secondary structure similarities observed between the subrepeats of 17 phylogenetically diverse eukaryotes (Baldridge et al. 1992).

Genome specificity of the *F. excelsior* rDNA spacer

In order to determine if the subrepeats are unique of *F. excelsior*, we examined related species for the presence of these types of sequences. *F. excelsior* subrepeats shared some homology with the IGS of *F. oxyphylla* but displayed no homology with the spacers of *F. ornus* or the *F. americana*. Mechanisms of concerted evolution, like unequal crossing-over at the whole unit level or gene conversion, are responsible for the fixation of sequence variants throughout the rDNA family of a species. This process was identified in our case by the presence of a family of subrepeats in *F. excelsior* that are absent in *F. ornus* and *F. americana*. In contrast, we did not observe the fixation of species-specific subrepeats within *F. excelsior* or *F. oxyphylla*. We were not able to characterize a rDNA sequence that would be specific to *F. excelsior*. *F. excelsior* and *F. oxyphylla* are closely related species with numerous natural hybrid forms, and in certain areas they are sympatric. *F. ornus* and the American ash are phylogenetically distant and geographically separated in eastern America and eastern Europe, respectively. In other species such as radish, the short repeats are specific for the *Raphanus* genus, and they do not hybridize to the closely related *Brassica* genus (Delcasso-Tremousaygue et al. 1988). In *Pharbitis nil* the subrepeats are specific for the genus (Katayama et al. 1992). In wild tomato (*Lycopersicum hirsutum*) a 54-bp subrepeat has allowed the differentiation between lines (Levesque et al. 1990).

Our results also confirm that the diverse regions of the rDNA spacer are not evolving at the same rate: the 450-bp probe hybridized with the IGS of all the species assayed, but the *Hae*III 41-bp and 120-bp probes did not hybridize with *F. ornus* and *F. americana*.

All these results indicate that, depending upon the analyzed regions, the rDNA intergenic spacer provides a good marker in *Fraxinus* for studying evolutionary processes at different levels: in the hybridization complex *F. excelsior* and *F. oxyphylla*, IGS subrepeats could be used to generate fingerprints or restriction fragment length polymorphism (RFLP) patterns to differentiate species and individuals. Between more distant species, like *F. excelsior* and *F. americana*, IGS subrepeats could be used as specific probes to detect the presence of *F. excelsior* genome in artificial hybrids.

Acknowledgements This work was supported by the grant INRA-Région Franche-Comté 1991–1993. We thank Albert Abbott (Clemson University, S.C.), Jean Bousquet and Ken Dewar (Université Laval, Québec) for their helpful comments.

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 intergenic spacers? J Mol Evol 35:514–523

Choumane W, Heizmann P (1988) Structure and variability of nuclear ribosomal genus in the genus *Helianthus*. Theor Appl Genet 76: 481–489

Cordesse F, Second G, Delseny M (1990) Ribosomal gene spacer length variability in cultivated and wild rice species. Theor Appl Genet 79:81–88

Cordesse F, Grellet F, Reddy AS, Delseny M (1992) Genome specificity of rDNA spacer fragments from *Oryza sativa* L. Theor Appl Genet 83:864–870

Cordesse F, Cooke R, Tremousaygue D, Grellet F, Delseny M (1993) Fine structure and evolution of the rDNA intergenic spacer in rice and other cereals. J Mol Evol 36:369–379

Delcasso-Tremousaygue D, Grellet F, Panabieres F, Ananiev E, Delseny M (1988) Structural and transcriptional characterization of the external spacer of a ribosomal RNA nuclear gene from a higher plant. Eur J Biochem 721:767–776

Dover GA, Linares AR, Bowen T, Hancock TM (1993) Detection and quantification of concerted evolution and molecular drive. In: Zimmer EA, Zimmer TJ, Cann RL, Wilson AC (eds) Methods in enzymology, vol. 224: molecular evolution: producing the biochemical data. Academic Press, San Diego, pp 525–541

Echeverria M, Penon P, Delseny M (1994) Plant ribosomal DNA external spacer binding-factors. A novel protein binds specifically to a sequence close to the primary pre-ribosomal-RNA processing site. Mol Gen Genet 243:442–452

Faivre-Rampant P, Jeandroz S, Lefèvre F, Lemoine M, Villar M, Bervillé A (1992) Ribosomal DNA studies in poplars: *Populus deltoides* Bartr. and Marsh., *P. nigra* L., and *P. trichocarpa* Torr. and Gray, *P. maximowiczii* Henry, *P. alba* L. Genome 35: 733–740

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

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

Hemleben V, Grierson D, Dertmann H (1977) The use of equilibrium centrifugation in actinomycin-cesium chloride for the purification of ribosomal DNA. Plant Sci Lett 9:129–135

Higgins DG, Fuch R, Blesby A (1992) CLUSTAL: a new multiple sequence alignment program. Comput Appl Biosci 8:189–191

Jeandroz S, Faivre-Rampant P, Pugin A, Bousquet J, Bervillé A (1995) Organization of nuclear ribosomal DNA and species-specific polymorphism in closely related *Fraxinus excelsior* L. and *F. oxyphylla*. Theor Appl Genet 91:885–892

Jeffreys AJ, Mc Leod A, Tamaki K, Neil DL, Monkton DG (1991) Minisatellite repeat coding as a digital approach to DNA typing. Nature 354:204–209

Katayama S, Hirano HY, Tsukaya H, Naito S, Komeda Y (1992) Analysis of intergenic spacer in the nuclear rDNA of *Pharbitis nil*. Genome 35:92–97

Lakshmikumaran M, Negi MS (1994) Structural analysis of two length variants of the rDNA intergenic spacer from *Eruca sativa*. Plant Mol Biol 24:915–927

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

Linares AR, Bowen T, Dover GA (1994) Aspects of non-random turnover involved in the concerted evolution of intergenicspacers within the rDNA of *Drosophila melanogaster*. J Mol Evol 39: 151–159

McMullen MD, Hunter B, Phillips RL, Rubenstein I (1986) The structure of the maize ribosomal DNA spacer region. Nucleic Acids Res 14:4953–4968

Procunier JD, Kasha KJ (1990) The rDNA intergenic spacer region nucleotide sequence of *Hordeum bulbosum* L. Plant Mol Biol 15: 661–663

Reeder RH (1984) Enhancers and ribosomal genes spacers. Cell 38: 349–351

Rocheford TR, Osterman JC, Gardner CO (1990) Variation in the ribosomal DNA intergenic spacer of maize population mass-selected for high grain yield. Theor Appl Genet 79:793–800

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

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

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