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Characterization of the ribosomal DNA units in two related *Prunus* species (*P. Cerasifera* and *P. Spinosa*)

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Summary. The genetic relationships between two *Prunus* species, involved in rootstock breeding, were examined at the level of the ribosomal RNA genes. Twenty clones of *P. cerasifera*, a diploid species, and 12 clones of *P. spinosa*, a tetraploid wild species, were studied. The use of three heterologous ribosomal DNA probes covering different regions of the ribosomal tandem repeats enabled us to construct restriction maps for *Eco*RI and *Bam*HI. We identified two unit types (unit I and unit II) in *P. cerasifera*. In *P. spinosa*, *P. cerasifera* units were present in addition to a third ribosomal unit type (unit III). These results appeared to confirm previous cytological studies (Salesses 1973) indicating that one of the genomes in *P.spinosa* has homology with the one from *P. cerasifera*.

Introduction

The *Prunus* species constitute a polyploid series in which the basic number of chromosomes is x = 8. The genus is very large and economically important as it includes stone fruits (i.e., peaches, cherries, apricots, and almonds). Within the genus *Prunus*, the subgenus *prunophora* is of particular interest since it includes the domesticated plums (*i.e.*, forms of *P. domestica* such as greengages, french prunes, etc.) and species of value as rootstocks (*i.e., P. cerasifera*). *Prunus spinosa*, with its wide range of environmental adaptibility, may be a useful source of resistance to cold, calcarious soils and drought. *Prunus cerasifera* and *P. spinosa* are diploid and tetraploid species respectively and represent possible ancestors of the cultivated hexaploid plums (*Prunus domestica*). Despite several biosystematic studies (Salesses 1970, Salesses 1973, Eremin et al. 1985, Eremin 1990) based on morphological or isoenzymatic characters, and on the cytogenetic behaviour of different hybrids, much remains to be learnt concerning the phylogeny of the *Prunus*. This is of particular interest in the improvement of varieties or rootstocks through interspecific hybridization. For such improvement, knowledge of the degree of homology between the different genomes involved is required. To date, only one study on *Prunus* has been carried out at the molecular level. Kaneko et al. (1986) surveyed 11 species and three subgenera of *Prunus* for chloroplastic DNA variation. The authors constructed dendrograms showing the genetic relationships between the species and could identify the cytoplasmic donor in an interspecific cross between two related species. However in this study the subgenus *Prunophora* was not examined.

The tandemly repeated multigene family encoding the 18S, 5.8S and 25S rRNA has proven to be a useful tool for molecular evolutionary studies in plants. It has been used previously in *Helianthus* (Choumane and Heizmann 1988), *Ferula communis*, and in the *Umbelliferae* (Oldmedilla et al. 1985). Variations in this multigene family have been surveyed between species (Appels and Dvorak 1982a, 1982b), populations within a species (Appels and Dvorak 1982a) and cultivars (May and Appels 1987) within the *Triticeae*. Variations in the rRNA genes of plants are detectable by repeat length heterogeneity, restriction site location polymorphism, copy number differences and variations in the intergenic spacers.

In this study, we constructed restriction maps of the ribosomal units for both *P. cerasifera* and *P. spinosa*, in order to evaluate the degree of polymorphism within and between these two species. Previous cytogenetic studies (Salesses 1970, 1973) have shown that *P. spinosa* is an allotetraploid species and that some

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homology exists in *P. cerasifera*. These species are of particular interest since they represent possible ancestors of *P. domestica*.

Materials and methods

Plant material. All the clones examined in this study come from a collection at the INRA Bordeaux and were checked previously for their ploidy number (Salesses, personal communication). We tested 19 diploid clones of *P. cerasifera* and 11 tetraploid clones of *P. spinosa* (Table 1).

Table 1: The clones studied for each species

Prunus species	genotype or clone number
P. cerasifera (2x)[a]	P18, P2032, P1079, P2646, P2794, P2795, P103, P3104, P3105, P3106, P3188, P3189, P3194, P3195, P3196, P3201,P3206, P3254, PU188D
P. spinosa (4x)[a]	P51, P55, P106, P2790, P2919, P3190, P3197, P3198, P3200, P3204, P3212

a: ploidy number

DNA isolation. DNA was isolated from fresh or frozen leaves. Leaf material was collected in different trials located in southwest France. Collection was made soon after budbreak, in order to obtain young material. Leaves were frozen rapidly in liquid nitrogen and then maintained at -80°C. DNA extraction was carried out according to the method of Doyle and Doyle (1990), with the following modification. One gram of fresh leaves was ground rapidly with pestle and mortar in the presence of liquid nitrogen and alumina. Doyle's buffer was added with 1% (w/v) soluble PVP. The rest of the procedure was as previously described (Doyle and Doyle 1990) with the exception that chloroform /octanol was used instead of chloroform/isoamylalcohol, and generally two extractions were necessary to obtain a clear interphase. Following the isopropanol precipitation, a further precipitation step with ethanol was carried out.

DNA restriction and agarose gel electrophoresis. DNA was digested with the restriction enzymes using the incubation conditions described by the suppliers (Gibco BRL). Complete DNA digests were obtained by using an excess of enzyme and a long incubation (2-6 h). Partial digests were carried out by using a larger amount of DNA (up to 15µg) and a limited amount of enzyme. In this case, aliquots were taken after 1, 5, 15 and 30 min and then mixed together. The restriction fragments were separated by horizontal 0.8% (w/v) agarose gel electrophoresis overnight at 50V in TBE buffer (Sambrook et al. 1989). Following electrophoresis, gels were stained with ethidium bromide and photographed under U.V.- illumination. They were then tranferred onto a Hybond N+ membrane (Amersham) by alkali blotting following the manufacturer's specifications.

Southern blot hybridization. Probes were labelled by nick translation (Sambrook et al. 1989) using [^{32}P] dCTP or [^{32}P] dATP at specific activities of 10^7 to 10^8 cpm/µg. A 1kb ladder was labelled with [^{32}P] dCTP by end filling. Hybridizations were carried out overnight at 62°C in 5x SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 5x Denhardt's solution and 0.1% (w/v) SDS using a roller-bottle hybridization oven (Hybaid). Following hybridization, filters were washed at 65°C, three times in 3x SSC, 0.1% (w/v) SDS and three times in 0.3x SSC, 0.1% (w/v) SDS, then exposed to AGFA X-Ray films with intensifying screens (Dupont Chronex Lightning Plus) for 5 to 48 h at -70°C. Radioactive probes were removed from the nylon membrane by immersion in a boiling 0.1% (w/v) SDS solution.

Characteristics of the probes. The conserved rDNA coding regions allowed us to use heterologous probes isolated from flax and radish (see Fig.4C). PBG35 (Goldsbrough and Cullis 1981) is a *Bam*HI fragment covering an entire flax ribosomal unit cloned in pAT153. PRE12 (Delseny et al. 1983) is a *Bam*HI fragment covering the 3' end of the 18S rRNA coding region, the small spacer, and the 5' end of the 25S rRNA coding region cloned in pAT153. PRG3 (Grellet et al. 1989) is an *Eco*RI fragment covering almost the entire 18S rRNA coding region cloned in pUC8.

Results

Size of the ribosomal units.

The size of the ribosomal units has been determined by different approaches. *Pst*I and *Sal*I restriction enzymes cut the ribosomal units at only one site and generated a fragment, hybridizing with PBG35, of approximately 10 Kb. Partial digestions with *Eco*RI also generated a fragment of the same length. The exact size of the ribosomal unit has been determined from a complete digestion by addition of the two fragment lengths hybridized with PBG35 which covers the entire ribosomal unit (6400 + 3800 = 10.2 Kb in *Eco*RI digestions), and by taking into account the approximate unit length of 10 Kb. We set the length of the rDNA repeat for both *Prunus* species at 10.2 Kb.

Restriction maps for P. cerasifera.

*Eco*RI restriction patterns: Hybridization with the PRG3 probe produced two main restriction fragments at 6400 and 2600 bp (Fig.1A). After hybridization with PRE12 (Fig.1B) and PBG35 (Fig.1C) another fragment at 3800 bp appeared. A faint band also appeared with all the probes for some individuals at 6200 bp as well as a faint band at 1900 bp for PBG35 only. The fragment at 6400 bp was always present even after very long digestions with an excess of enzyme. PRG3 covers only the 18S part of the ribosomal gene, therefore both 6400 and 2600 bp fragments were localised in this region, whereas the 3800 bp fragment is outside of the 18S region. Taking into account the length of the ribosomal unit at 10.2 Kb, the fragments at 6400 and 3800 bp could not exist together with that of 2600 bp. For this reason we hypothesized that there were two types of ribosomal unit. In the type I unit (Fig.4A) there were only two *Eco*RI restriction sites: E_2 at the 3' end of the 18S rRNA gene, and E_3 at the 3' end of the 25S rRNA gene generating the 6400 and 3800 bp fragments. In the type II unit (Fig.4A) there were two additional *Eco*RI sites: E_1 and E_4 located in the intergenic spacer generating the 2600 bp fragment, and the faint band at 1900 bp which had a reduced homology with the flax probe PBG35 as it was located at the 3' end of the 25S. The E_4E_1 1900 bp fragment

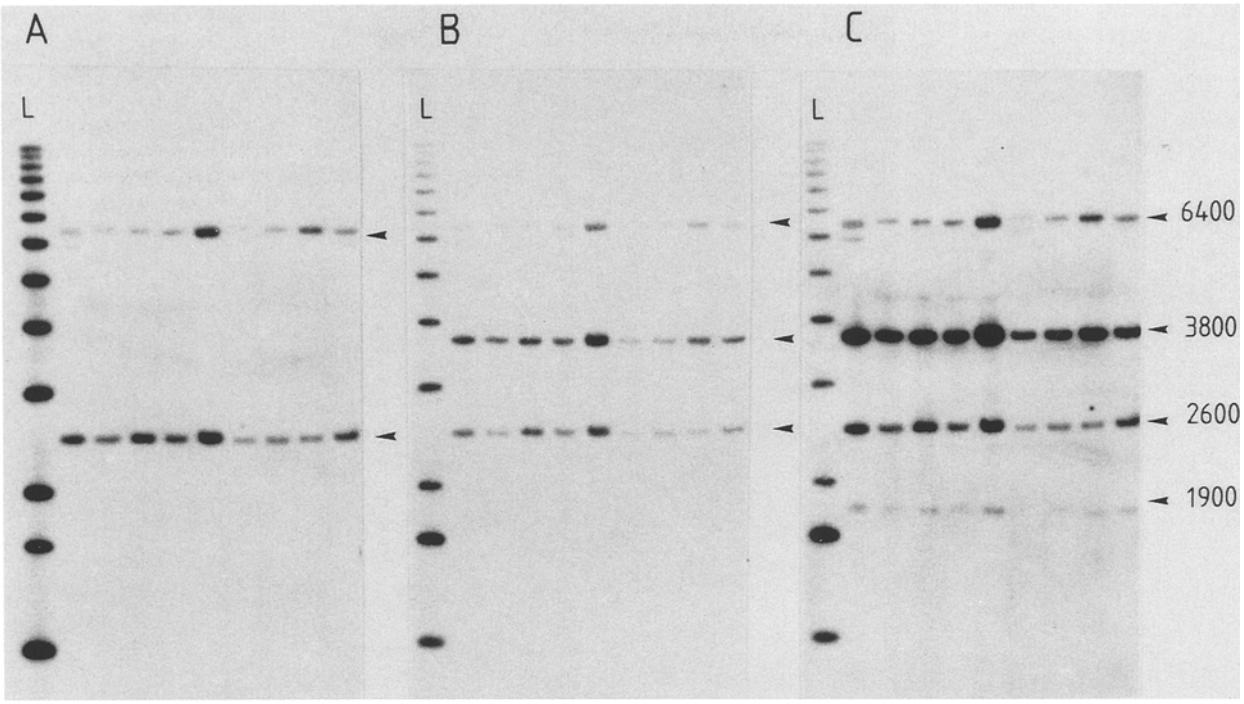

Fig.1A, B and C. *Eco*RI restriction patterns for 9 clones of *Prunus cerasifera* hybridized with PRG3 (Fig.1A), PRE12 (Fig.1B), PBG35 (Fig.1C). The fragments of 6400 and 2600 bp are present in all cases and are located in the 18S RNA coding region. The fragment at 3800 bp is located in the 25S RNA coding region. The faint band at 1900 bp appears only with PBG35. L : 1 Kb ladder DNA

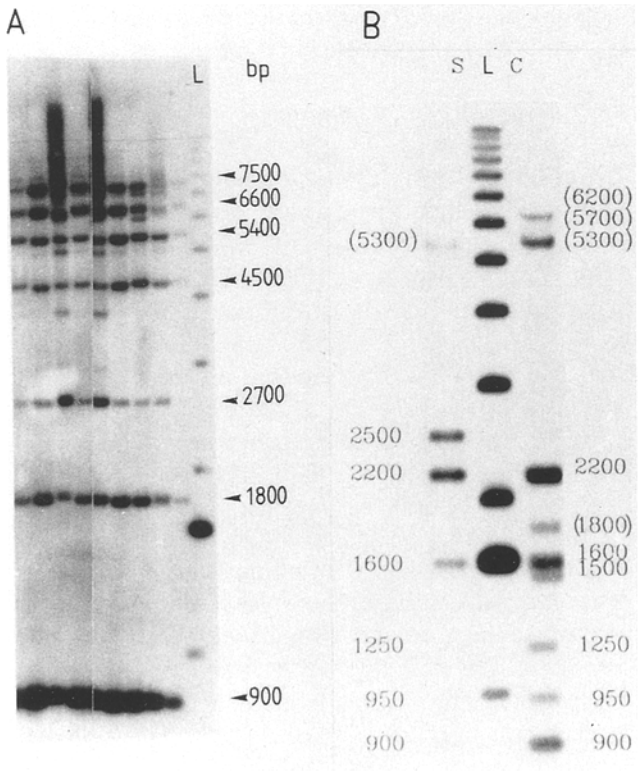

Fig.2A and B. *Bam*HI restriction patterns (Fig.2A) for 9 clones of *Prunus cerasifera* hybridized with PRG3. The main fragments are located at 900, 1800, 2700, 4500, 5400, 6600 and 7500 bp. All the fragments above 1800 bp correspond to partial digestion. Comparison of *Eco*RI & *Bam*HI double digestion patterns (Fig.2B) between *Prunus cerasifera* (C) and *P. spinosa* (S). Double digestion restriction fragments can be located at 1500, 1600 and 2200 bp for *P. cerasifera*. A new fragment of 2500 bp appears for *P. spinosa* but the 1500 bp one disappears. This confirms the existence of a third ribosomal unit in *P. spinosa*. C : *P. cerasifera;* S : *P. spinosa;* L : 1 Kb ladder DNA

located entirely in the intergenic spacer did not hybridize. In both cases, the ribosomal unit was 10.2 Kb long i.e., 6400 + 3800 = 10.2 Kb, or 2600 + 3800 + 1900 + 1900 = 10.2 Kb.

*Bam*HI restriction patterns: Figure 2A shows the result of partial digestions with *Bam*HI and hybridization with PRG3. The major fragments were 900, 1800, 2700, 4500, 5400, 6600, and 7500 bp long (additional fragments were also present at 3800, 5100, and 7150 bp). All these fragments corresponded to partially digested DNA apart from those of 900 and 1800 bp. Another fragment, resulting from complete digestion, appeared at 1250 bp with PBG35 (data not shown) and was therefore located in the 25S region of the rRNA gene. The double *Eco*RI & *Bam*HI digestion patterns (Fig.2B) revealed six fragments at 900, 950, 1250, 1500, 1600, and 2200 bp. The 1500 bp fragment appeared only with PBG35 and PRG3: it was located at the 3' end of the 18S rRNA coding region. The 1600 bp fragment appeared only with PBG35 and PRE12: it was located at the 5' end of the 25S rRNA coding region. The 950 and 2200 bp fragments hybridized only with PBG35: they were therefore situated at the 3' end of the 25S rRNA gene. The final location of the *Bam*HI restriction sites was determined by analysing both single and double digestions hybridized with all

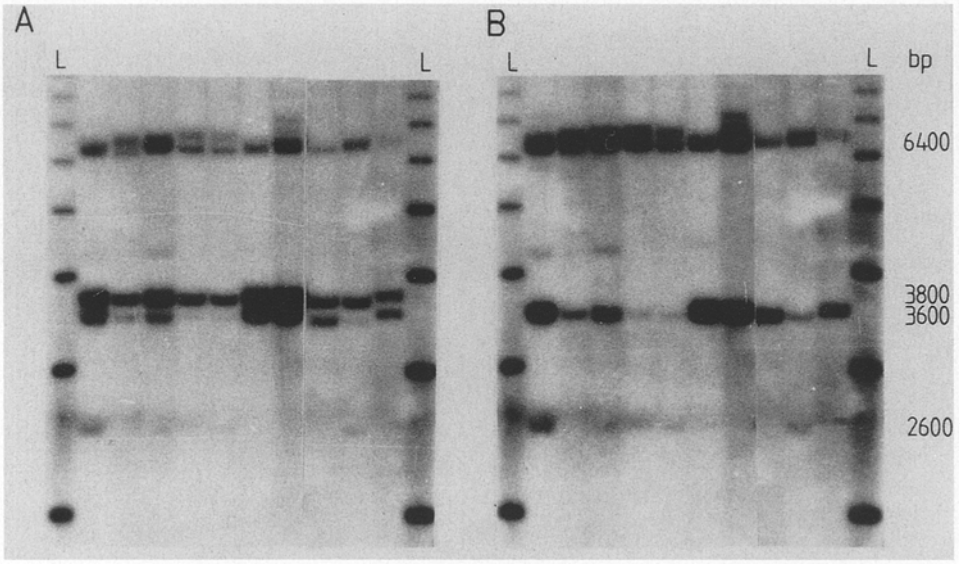

Fig.3A and B. *Eco*RI restriction patterns for 10 clones of *Prunus spinosa* hybridized with probe PRE12 (Fig.3A.) and PRG3 (Fig.3B.). The 6400, 6200, 3800 and 2600 bp fragments are present in *P. spinosa* as in *P. cerasifera*. A new fragment at 3600 bp appears for all the clones and corresponds to a third ribosomal unit. L: 1 Kb ladder DNA

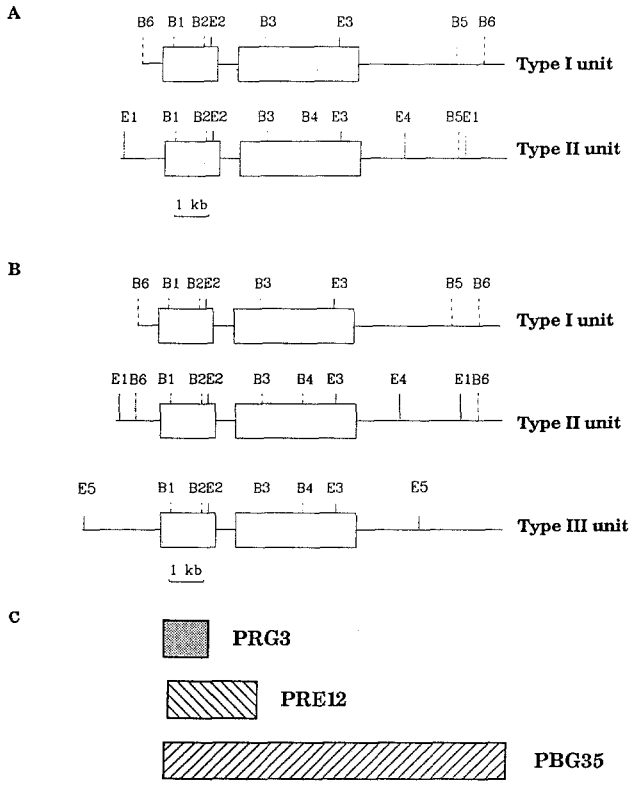

Fig.4A, B and C. Complete restriction maps for *Eco*RI and *Bam*HI, for the 2 units present in *Prunus cerasifera* (Fig.4A) and for the 3 units present in *P. spinosa* (Fig.4B). The ribosomal DNA probes used in this study are presented in figure 4C. Probes PBG35, PRE12 and PRG3 correspond to fragments of 8300, 2700 and 1600 bp respectively, covering different parts of the ribosomal unit in flax and radish.

probes. Consequently, six restriction sites were identified on unit I and II (Fig.4A). The 2200 bp B_3 E_3 fragment could only exist if B_4 was absent while the 1500 bp E_1 B_1 fragment could only exist if B_6 was absent and E_1 present. These observations confirmed the existence of two rDNA units.

The complete restriction maps for the two ribosomal units present in *P. cerasifera* for *Eco*RI and *Bam*HI restriction sites are presented in figure 4A.

Restriction maps for P. spinosa.

The position of the restriction sites in *P. spinosa* was determined by comparison with *P. cerasifera.*

*Eco*RI restriction patterns: Figure 3A shows that the major fragments of 6400, 3800 and 2600 bp were present as in *P. cerasifera* with PRE12. A new fragment of 3600 bp appeared for all the clones and could not be explained by *P. cerasifera* maps. This fragment was present also upon hybridization with PRG3 (Fig.3B) while the 3800 bp fragment disappeared. The 3600 bp fragment must be located in the 18S coding region and could only be accounted for by a site E_5 located 3600 bp upstream of E_2. This led us to the conclusion that there was a third ribosomal unit in addition to the two already present in *P. cerasifera*. This type III ribosomal unit lacked the E_1 site though had the E_5 site. In all the *P. spinosa* clones checked, the amount of *Eco*RI 2600 bp fragment appeared generally weak, some clones completely lacking this fragment. Most of the clones had both the 6400 and the 6200 bp fragments.

*Bam*HI restriction patterns: Single digestions with *Bam*HI gave the same results as those observed for *P. cerasifera* (data not shown): all clones exhibited the same pattern with the main fragments at 900 and 1800 bp for PRG3, and at 900, 1250, and 1800 bp for PBG35. In figure 2B, the patterns obtained using the

*Eco*RI & *Bam*HI double digestion for *P. spinosa* and *P. cerasifera* showed that a new fragment of 2500 bp appeared in *P. spinosa* (corresponding to E_5B_1 of unit III) and the 1500 bp fragment disappeared. We concluded that B_5 and B_6 were absent from unit III but present in both unit I and II, although in *P. cerasifera* B_6 was absent from unit II (Fig.4B).

The complete restriction maps for the 3 units present in *P. spinosa* are described in figure 4B.

The ribosomal type for each species was characterised by an *Eco*RI digestion fragment on hybridization with PRG3, PRE12 or PBG35 (6400 bp for unit I, 2600 bp for unit II, and 3600 bp for unit III). Similarly for the *Eco*RI & *Bam*HI double digestion, a 2500 bp appeared only when the type II unit was present.

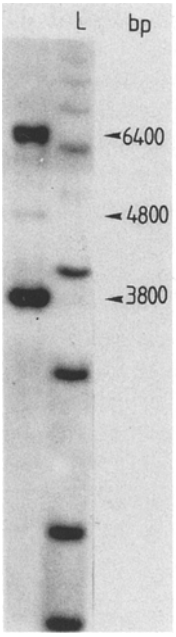

Fig.5.: ***Eco*RI restriction pattern of *Prunus microcarpa* hybridized with probe PRG3 reveals three main fragments at 6400, 4800 and 3800 bp. L : 1 Kb ladder DNA**

Discussion

Through the use of heterologous probes from radish and flax, this study has confirmed that the coding sequences of ribosomal genes are highly conserved. We calculated the position of the restriction sites by taking into account the length of the coding regions determined in other species. One finds the *Eco*RI and *Bam*HI sites of *Prunus* present in other species (E_2, E_3, B_2, B_3, B_4), notably within the genus *Brassica* (Delcasso-Tremousaygue 1987, Delseny et al. 1990). The differences between genus and species are mainly in sequence divergence in the intergenic spacer with or without length heterogeneity. We characterized the length of *Prunus* ribosomal genes at 10.2 Kb and have shown no length heterogeneity, neither within nor between species. In addition, this is particularly noticeable since we have shown the existence of different units within a single plant. We characterized in effect three types of ribosomal units with different restriction maps for two enzymes (*Eco*RI, *Bam*HI). The differences between these units are mainly located in the intergenic spacer and *Eco*RI restriction patterns which appear to be the most relevant in distinguishing the different units. Units I and II are found in both *P. cerasifera* and *P. spinosa*. The only difference is that for unit II, site B_5 is present while B_6 is absent for *P.cerasifera* and it is the contrary for *P.spinosa*. There is no within species variation for *P. cerasifera* since units I and II are present in all the clones. There is some variability in *P. spinosa* in that unit II is either less represented or even absent in some clones. Unit III, absent from all *P. cerasifera* clones, is present in all *P. spinosa* clones examined. This third unit characterizes *P. spinosa*.

Concerning the genetic relationships between *P. cerasifera* and *P. spinosa*, this result is in good agreement with previous cytological studies. *Prunus spinosa* is in effect suspected to be an allotetraploid and Salesses (1973) have shown that there is some homology between *P. cerasifera* and one of the two *P. spinosa* genomes. We suggest that units I and II may originate from *P. cerasifera* and unit III from another ancestor. This would thereby support the allotetraploid nature of *P. spinosa*. Eremin (1990) has suggested *P. microcarpa* to be the second parent of *P. spinosa*. *EcoRI* restriction patterns (Fig.5) for P3187 (the only clone available of *P. microcarpa*) shows three major fragments at 6400, 4800, and 3800 bp. This implies the existence of another type IV ribosomal unit with an *EcoRI* restriction site 4800 bp upstream of E_2. Eremin's hypothesis is therefore questioned by these results and the origin of the genome which contains the type III unit remains to be determined.

Variability in the copy number of the different units between clones in *P. spinosa* and the greater homogeneity found in *P. cerasifera* may be explained by two phenomena. First, *P. spinosa* is widely spread over Europe and West Asia and the clones studied have not been selected for any particular use. In contrast, *P. cerasifera* has a smaller area of origin (the Balkans) and the clones studied are selected clones used as rootstocks thus explaining the reduced variability. In addition, the rDNA sequences show a concerted evolution (Vedel and Delseny 1987, Seperack et al. 1988) and exchanges within a locus are more frequent than between loci (Saghai et al. 1984). Different mechanisms like mitotic recombination (Keil and Roeder 1984) tend to maintain sequence homogeneity particularly among nearly identical repeat units in a

rDNA family. Besides, the phenomena of nucleolar competition has been reported in wheat and rye (Appels et al. 1986) where the rye NOR locus is under expressed in the wheat genomic background. In *P. spinosa*, it is unclear whether all the ribosomal units characterized are functional or not. During evolution certain *P. spinosa* may have preferentially lost a certain number of copies of I-II units. One can imagine that in the presence of allele III, allele I-II may be under-expressed and that evolution has a parallel tendency to reduce the number of copies of an inactive allele by mitotic recombination mechanisms (Nelson et al. 1989, Russell et al. 1988, Petes and Botstein 1977).

Our present work on ribosomal DNA represents a novel study of the molecular variability at the level of the genome in *Prunus*. It has allowed us to confirm that the different clones observed belong to *P. cerasifera* and *P. spinosa* and has indicated a weak intraspecific variability. The difference between these two species is made up of a type III ribosomal unit specific for the tetraploid species *P. spinosa*. The other two types of ribosomal unit indicated are present in both species. These results appear to confirm the allotetraploid nature of *P. spinosa* and the involvement of *P. cerasifera* in the origin of *P. spinosa*. Eremin's hypothesis stating *P. microcarpa* as the second parent should be rejected on analysis of the *Eco*RI profiles. The observed differences in copy number of the various ribosomal units between *P. spinosa* clones suggest the existence of reorganization phenomena of mitotic recombination which have evolved in the same direction as nucleolar dominance phenomena. In the presence of the type III unit, the type I and II units shall have a tendency to disappear.

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