Distribution and chromosomal organization of 18S-5.8S-25S and 5S rDNA in Petunia species

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agronomie (1997) 17, 349-360

Original article

Distribution and chromosomal organization of 18S-5.8S-25S and 5S rDNA in *Petunia* species

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(Received 10 september 1997; accepted 22 october 1997)

Summary — We have analyzed the interspecific variation of the 5S rDNA spacer by the polymerase chain reaction in seven *Petunia* species. The species studied could be separated into two groups with regard to the size of the amplified 5S rDNA variants: one group, with a 460-bp repeat unit, including *P linearis* (2n = 18) and *P integrifolia* (2n = 14) and the second group, with a 350-bp repeat unit, including all the other wild species (2n = 14) studied and the *P hybrida* lines. The amplified fragments have been cloned, and used in FISH experiments to determine the number and the location of the 5S rDNA units. The chromosomal organization of the 5S rDNA enabled us to distinguish a group of coloured flowered species with one locus adjacent to the major 18S-5.8S-25S rDNA locus on chromosome II, and a group of white flowered species with an additional locus in the centromeric region of a metacentric chromosome (IV/VII). FISH analysis also revealed four hybridization sites of 18S-5.8S-25S rDNA in the majority of the *Petunia* species studied. Only *P linearis* and *P parviflora* (2n = 18) showed two hybridization sites. The four sites of 18S-5.8S-25S rDNA are transcriptionally active as shown by their expression pattern in interphase nuclei. Our FISH results combined with PCR amplification and RFLP studies of the rDNA clusters give a new insight into the phylogenetic relationships between wild species and the *P hybrida* lines.

***Petunia* species / PCR / 5S and 18S-5.8S-25S rDNA / FISH**

Résumé — Distribution et organisation chromosomique des gènes ribosomiques 18S-5.8S-25S et 5S chez *Petunia*. Nous avons analysé la variation interspécifique de l'espaceur 5S ADNr par la tehnique d'amplification génique (PCR) dans sept espèces de *Petunia*. Les espèce étudiées peuvent être séparées en deux groupes suivant la taille des variants 5S ADNr amplifiés : un groupe avec une unité de répétition de 460 pb comprenant *P linearis* (2 *n* = 18) et *P integrifolia* (2 *n* = 14), et un deuxième groupe avec une unité de répétition de 350 pb comprenant toutes les autres espèces sauvages (2 *n* = 14) étudiées et les lignées de *P hybrida*. Les fragments amplifiés ont été clonés et ont servi de sondes pour l'hybridation in situ fluorescente afin de déterminer le nombre et la localisation des unités 5S. L'organisation chromosomique du 5S ADNr nous a permis de distinguer un groupe d'espèces à fleurs colorées avec un locus adjacent au locus majeur 18S-5.8S-25S ADNr du chromosome II et un groupe d'espèces à fleurs blanches avec un locus supplémentaire situé dans la région centromérique d'un chromosome métacentrique (IV/VII). L'hybridation in situ fluorescente a également révélé quatre sites d'hybridation pour le 18S-5.8S-25S ADNr dans la majorité des espèces de *Petunia* étudiées. Seuls *P linearis et P parviflora* (2 *n* = 18) ont montré deux sites d'hybridation. L'observation des pattern d'expression dans les noyaux interphasiques nous a permis de montrer que les quatre sites d'ADNr18S-5.8S-25S sont en activité de transcription. Les résultats obtenus en hybridation in situ fluorescente ainsi que les amplifications par PCR et les études RFLP des ADNr nous ont conduits à une nouvelle approche des relations phylogéniques entre les espèces sauvages et les lignées de *P hybrida*.

***Petunia* / PCR / ADNr 5S / ADNr 18s-5.8S-25S / hybridation in situ**

Article communicated by Hervé Thiellement (Versailles)

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INTRODUCTION

The structure, organization and evolution of the 18S-5.8S-25S and 5S rDNA multigene families have been studied in detail in several plant species. The 18S-5.8S-25S ribosomal RNA genes (rDNA) are present in several hundreds of tandemly repeated units of the three genes with intergenic spacers, organized in one or more clusters within the genome (Appels et al, 1980; Saghai-Maroof et al, 1984; Flavel, 1986; Rogers and Bendich, 1987). The genes coding for the 5S ribosomal RNA (5S rDNA) are also present in multiple copies per genome. They are arranged head to tail and are separated from each other by simple spacers (Appels and Honeycut, 1986). As in all higher eukaryotes, the 5S and 18S-5.8S-25S rDNA are organized in separate loci. The 18S-5.8S-25S rDNA repeat unit and the 5S rDNA unit of *Petunia hybrida* have both been characterized (Waldron et al, 1983; Frasch et al, 1989). In situ hybridization revealed two 18S-5.8S-25S rDNA clusters and one 5S rDNA cluster localized on the short arm of chromosome II (Montijn et al, 1994; Montijn, 1997).

RFLP studies of rDNA have shown variation between *P hybrida* lines and wild species by using a heterologous probe. They revealed three length variants in the 5S rDNA (Zeboudj et al, 1994; Kabbaj et al, 1995).

We aimed to study the polymorphism in the chromosomal organization of the rDNA repeat units in *P hybrida* and its putative parents. The number of 5S rDNA loci and their chromosomal localization with respect to the 18S-5.8S-25S rDNA loci were analyzed, by fluorescent in situ hybridization (FISH), in six *Petunia* wild species, two F1 hybrids and four *P hybrida* lines. The homologous 5S rDNA probes were generated by PCR amplification using 'universal' primers. The amplification profiles obtained and the 5S rDNA restriction polymorphism in the different genotypes were also analyzed.

Our ultimate aim was to establish the phylogenetic relationships inside the genus *Petunia* based on the PCR amplified 5S rRNA genes, their restriction polymorphism and their physical distribution in the putative parental species of the interspecific hybrid *P hybrida* and in some *P hybrida* lines.

MATERIALS AND METHODS

Plant materials

The *Petunia* species and the *P hybrida* lines used in this study, as well as their characteristics, are listed in table I. Plant growth occurred in a greenhouse at 20 °C for a 16-h day and 15 °C at night.

Table I. List of the main traits and origins of the plant material.

Species	*Code*	*2n* =	*Corolla colour*	*Origin*
P linearis	S11	18	violet pink	IGA S11
P parviflora		18	purple	USA Michigan
P integrifolia	S13	14	violet pink	Dijon S13
P inflata	D1	14	purple	Paris, Museum
P axillaris	M1	14	white	USA Cornell
P parodii		14	white	USA Michigan
P hybrida	Lines	14		
	ST40		clear pink	R40 (IGA)
	TB1-3		purple	'Velours rouges'
	TLH7		violet-white	'Rose du ciel' × *P axillaris*
	TLV1		clear violet	T1 × g
F1 hybrid	code	14		
	M1 × S13		clear violet	*P axillaris* × *P integrifolia*
	D1 × M1		clear violet	*P inflata* × *P axillaris*

USA Michigan: Sink, KC, East Lansing (USA). USA Cornell: M Hanson, Cornell University. IGA: Institute of Genetics, Amsterdam (the Netherlands).

Chromosome preparation

Synchronization of mitosis

Root-tips of about 2 cm in length were synchronized by the method of Pan et al (1993) with minor modifications. The roots were placed in Hoagland solution (Gamborg et al, 1968) supplemented with 1.25 mM hydroxyurea (HU) for 18 h. After this period, they were rinsed three times and then transferred to fresh medium without HU. After 8 h, excised root-tips were treated with 0.05% colchicine for 90 min to accumulate metaphases. All treatments were performed at 27 °C in the dark. After rinsing with distilled water, the root-tips were fixed in 3:1 (v/v) 100% ethanol/acetic acid and stored at –20 °C until use.

Preparation of metaphase spreads

The fixed root-tips were rinsed with distilled water and then incubated at 37 °C in an enzyme mixture (0.25% pectolyase Y-23, 3% cellulase onozuka R-10, 0.4 M sorbitol, pH 5.5) for 90 min. After incubation the root-tips were transferred to fresh cold 45% acetic acid for 15 min. A single root-tip was transferred in a drop of 45% acetic acid onto a cleaned slide before gentle squashing. The cover slips were removed by immersing slides in liquid nitrogen and the preparations were dehydrated for 10 min each in a graded series of 70%, 95% and absolute ethanol at room temperature. The air-dried slides were stored at room temperature, up to 1 month, in sealed containers until use.

PCR amplification of the 5S rDNA and cloning of the amplification products

Amplification of the 5S rDNA was obtained by the method of Cox et al (1992) with some modifications. Total plant DNA was extracted from young growing leaves according to the method of Bernatzky and Tanksley (1986). Templates for PCR consisted of total genomic DNA. The standard amplification mixture (50 µL) contained 60 ng genomic template DNA, 10 ng of each primer, 500 µM each of dATP, dCTP, dGTP and dTTP (Pharmacia), 5 µL reaction buffer (170 mM $(NH_4)_2 SO_4$, 30 mM $MgCl_2$, 670 mM Tris-HCl, pH 8.8) and 2.5 units Taq polymerase (BRL). Thirty amplification cycles were performed on a PTC 200 thermal-cycler (MJ Research). Each cycle consisted of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and primer extension at 72 °C for 2 min. The primers used were PIII and PIV established by Cox et al (1992). The sequences of these two primers were as follows:

PIII: 5'-GAGAGTAGTACATCGATGGG-3' (20-mer);

PIV: 5'-GGAGTTCTGACGGGATCCGG-3' (20-mer).

Amplification products were separated by electrophoresis on 3% agarose gels made up in 1 × TAE (40 mM Tris-acetate, 1 mM EDTA). The gels were loaded with 15 µL amplification mixture and electrophorezed for 2 h at 10 V cm^{-1}. They were then stained with ethidium bromide and visualized using an ultraviolet transilluminator.

The PCR gels were transferred to nylon membranes (Amersham, N^+) according to Southern (1975) and the filters hybridized with the pBG13 probe which contains ten copies of the 5S rDNA unit from flax (Goldsbrough et al, 1981; Goldsbrough et al, 1983). This clone was kindly provided by N Ellis. The pBG13 probe was labelled with ^{32}P-dCTP (3000 mCi/mL) using a random primed DNA labelling kit (Boehringer) and hybridized to the membranes in hybridization buffer (6 × SSC, 5 × Denhardt's, 0.1% SDS) at 65 °C. After overnight hybridization, the filters were washed for 2 × 15 min at 65 °C in (2 × SSC, 0.1% SDS), 2 × 15 min at 65 °C in (0.5 × SSC, 0.1% SDS), and then 2 × 15 min at 65 °C in (0.1 × SSC, 0.1% SDS). Autoradiograms were obtained after overnight to 96-h exposure at –70 °C.

Each amplification product (amplified using the primers PIII/PIV under the conditions described above) was extracted with phenol chloroform and ethanol precipitated. The pellet was washed twice in 70% ethanol and dried under vacuum before being resuspended in 10 µL TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0). Each amplification product was cloned, without further digestion, in pT7 Blue T-Vector (Novagen) following the manufacturer's instructions and transformed into *E coli* XL1-blue. Recombinant clones were plated onto selective media and screened by the blue/white method. Minipreparations of positive clones were prepared according to Maniatis et al (1982) and insert sizes were checked by PCR using the PIII/PIV primer combination before being sequenced using the improved dideoxy chain terminating method described by Murphy and Kavanagh (1988). The obtained sequences were compared between themselves and with the published *P hybrida* 5S rDNA sequence (Frasch et al, 1989) using the Sequence Analysis Software of the Genetics Group of the University of Wisconsin.

Southern hybridization analysis

Total genomic DNA from the chosen *Petunia* species, *P hybrida* lines and the F1 hybrid *P inflata* D1 × *P axillaris* M1 were digested overnight with EcoRI, BamHI and DraI (BRL). Ten units of enzymes per microgram DNA at 37 °C were used according to the manufacturer's instructions. Agarose (1%) electrophoresis took place in TAE (Maniatis et al, 1982). The digested DNAs were transferred to nylon membranes (Amersham, hybond N^+) and hybridized with the cloned 5S rDNA. Labelling of the probes, hybridization and post hybridization washes were carried out as described above.

FISH analysis

DNA probes

For the 18S-5.8S-25S rDNA probes we used either the 6.1-kb EcoRI fragment from sunflower kindly provided by P Heizmann (Choumane and Heizman, 1988) or the 2.8-kb NS3/NLD22 amplification product from Pea [NS3 primer: White et al (1990); NLD22 primer: Van Tuinen et al (1997)]. The 6.1-kb EcoRI fragment from sunflower carries an almost complete 18S fragment, the IGS and a small part of the 25S fragment. The 2.8-kb NS3/NLD22 amplification product from pea carries a part of 18S fragment, the complete ITS1, ITS2 and 5.8S fragments and a large part of the 25S fragment. Both probes were labelled by means of nick translation with digoxigenin-11-dUTP following the supplier's instructions. The *Petunia* 5S rDNA probes were from the cloned PCR product obtained by using the PIII/PIV primer combination. These probes were labelled by digoxigenin-11-dUTP either by means of nick translation (BRL) or by PCR amplification using the same primers according to the supplier's instructions.

In situ hybridization

The in situ hybridization reaction was modified from Montijn et al (1994). The hybridization mixture was prepared to a final concentration of 10 ng/μL digoxigenin labelled probe, 600 ng/μL of autoclaved herring sperm DNA, 2 × SSC, 10% dextran sulfate, 0.1% SDS, 1 mM EDTA, 1 × Denhardt's and 50% deionized formamide.

Slides were incubated with 100 μL of Rnase A (100 μg/mL in 2 × SSC) for 60 min at 37 °C under a plastic coverslip in a humid chamber. After 3 × 5 min washes in 2 × SSC and 5 min in proteinase K buffer (20 mM Tris-HCl, pH 7.5, 2 mM $CaCl_2$) at 37 °C, slides were incubated with 100 μL of proteinase K solution (1 μg/mL) for 10 min at 37 °C and 5 min in proteinase K stop-buffer (20 mM Tris-HCl, pH 7.5, 2 mM $CaCl_2$, 50 mM $MgCl_2$). Chromosome preparations were then fixed in ethanol/acetic acid 3/1-V/V for 10 min at room temperature, rinsed 3 × 5 min in 2 × SSC, dehydrated in 70, 95 and 100% (v/v) ethanol and air dried. The hybridization mixture was denatured at 70 °C for 10 min and immediately quenched in ice for 5 min, and 20 μL was then applied on each slide. A plastic cover slip was applied on the preparation and sealed with rubber solution.

The chromosomes and the hybridization mixture were denatured at 80 °C for 10 min, and incubated overnight at 37 °C in a humid chamber. After hybridization, the rubber solution was peeled off and cover slips were removed by dipping the slides into 2 × SSC at 37 °C. The slides were then washed twice in 50% formamide, 2 × SSC for 10 min each at 42 °C, twice in 2 × SSC for 10 min each at 37 °C, twice in 0.1 × SSC for 10 min each at 37 °C and in 2 × SSC for 10 min at room temperature.

Hybridization sites were detected using a three-step immunodetection procedure (Boehringer). The slides were first washed in the detection buffer (PBS, 0.2% Tween 20) for 5 min at room temperature, and blocked with 200 μL 5% BSA (Serum Bovine Albumin-Fraction V, Sigma) in detection buffer for 45 min at 37 °C. The blocking solution was drained off and the slides incubated with 0.5 μg/mL mouse monoclonal anti-Dig for 45 min at 37 °C in a moist chamber. After 3 × 5 min washes at room temperature, the slides were incubated with 2 μg/mL Dig-conjugated sheep anti-mouse antibody for 45 min at 37 °C. After 3 × 5 min washes, the slides were incubated with 2 μg/mL fluorescein-conjugated sheep anti-Dig antibody for 45 min at 37 °C. The final washes were performed with 4 × SSC for 2 × 3 min at room temperature in the dark.

Chromosomes were counter-stained with 5 μg/mL in 2 × SSC propidium iodide for 10 min at room temperature followed by washing in 2 × SSC. The drained slides were mounted in an antifade solution (90% glycerol in PBS, 1 mg/mL *p*-phenylene diamine), covered by a 24 × 34 mm cover slips and stored at 4 °C for 1 day to stabilize the fluorescence.

Chromosome preparations were analyzed using a Leica DMRB epifluorescent microscope with an appropriate filter (I3: excitation 488, emission 520). Photographs were taken on Fujicolor 400 colour slide film. Some observations were made on a Leica confocal scanning microscope (Leica CLMS, Heidelberg, Germany) with appropriate filters. For each sample, eight to ten metaphases were analyzed.

RESULTS

Physical localization of 18S-5.8S-25S

The number of rDNA clusters for each species has been determined on metaphase chromosomes and interphase nuclei by in situ hybridization with the 18S-5.8S-25S rDNA probes. Four fluorescent hybridization sites were observed in the *P hybrida* lines and in the wild species with 2n = 14 chromosomes. The two strongest signals indicating the sites of the major rDNA cluster were located at the NOR region of chromosome II, and the second pair of hybridization signals corresponded to the minor NOR region of chromosome III with less visible secondary constriction (fig 1a–c, e–j). In the interphase nuclei the transcribed rDNA was visualized as a track of dispersed punctuate hybridization spots which connected the homologous sites (fig 1h, j). In the two *Petunia* species with 2n = 18 chromosomes, *P linearis* and *P parviflora*, only two hybridization signals were observed at the distal part of a non-identified chromosome pair (fig 1d).

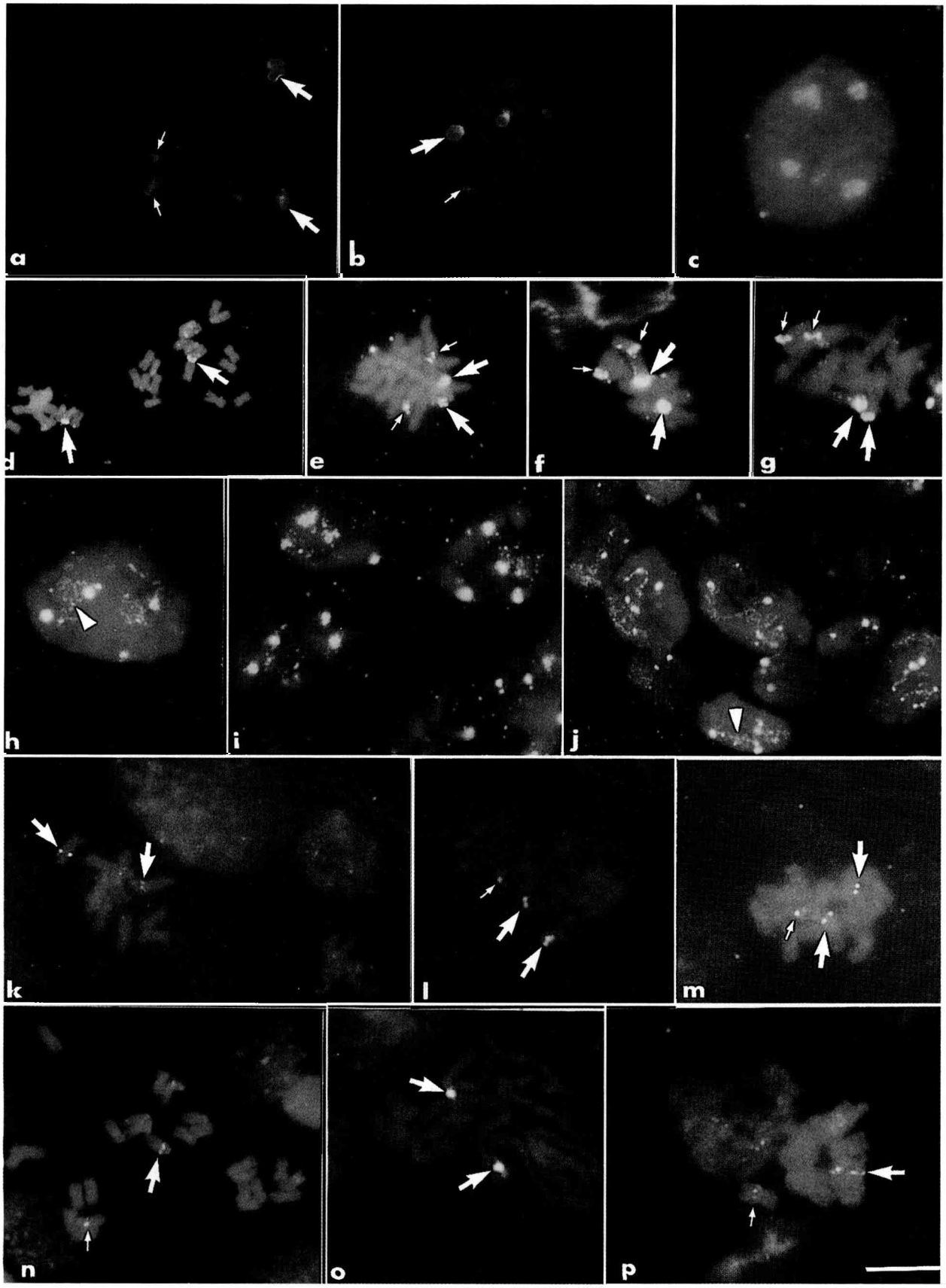

Fig 1. Fluorescent in situ hybridization of rDNA probes to metaphase chromosomes and interphase nuclei in *Petunia* species. Micrographs (a–j) show the 18S-5.8S-25S rDNA hybridization sites (green and yellow spots) in *P axillaris* (a), *P inflata* × *P axillaris* (b and h), *P parodii* (c), *P linearis* (d), *P integrifolia* (e), *P hybrida* ST40 (f), *P hybrida* TLV1 (g), *P hybrida* TLH7 (i) and *P hybrida* TB1-3 (j). On the metaphase plates, small arrows indicate the minor sites carried by chromosome III and large arrows indicate the major sites carried by chromosome II. In the interphase nuclei (h, j) four hybridization signals were detected with a variable number of small hybridization spots corresponding to the transcriptionally active rDNA gene clusters (arrowheads).
Micrographs (k–p) show the 5S rDNA hybridization sites (yellow spots) revealed by the PIII/PIV amplified products in *P inflata* (k), *P inflata* × *P axillaris* (l and m) *P parodii* (n), *P hybrida* ST40 (o) and *P hybrida* TLH7 (p). Large arrows indicate the 5S rDNA locus carried by chromosome II while the small arrows indicate the second locus carried by the meta to sub-metacentric chromosomes (IV/VIII). Bar equals 6 μm in (a–c); 7 μm in (h, p); and 8 μm in (d–g, i–o).

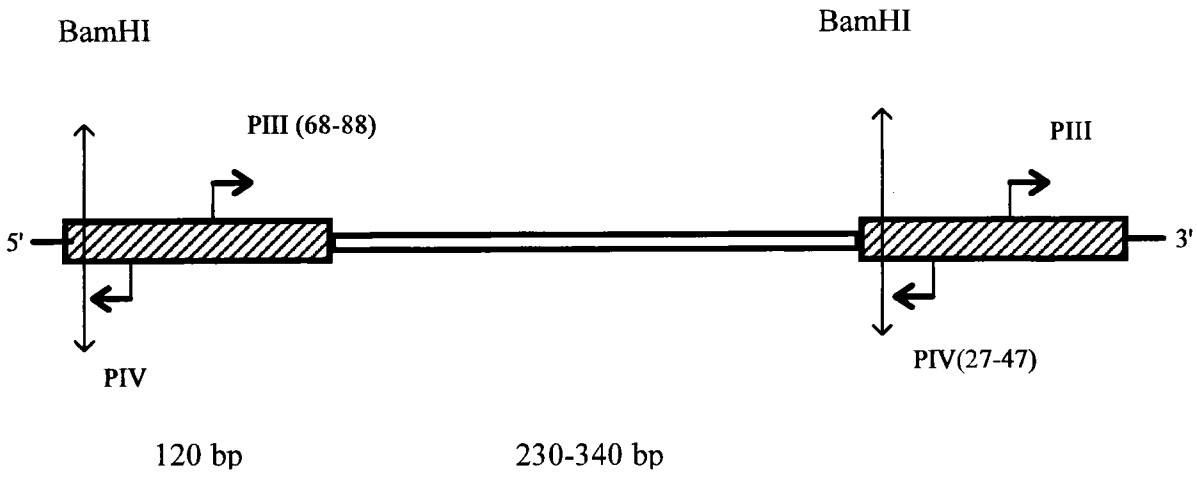

Fig 2. The position from which the primers initiate DNA amplification in the 5S rDNA repeating unit is shown. Shaded squares represent the coding region and open squares the non-transcribed spacer region.

Physical localization of 5S rDNA

Characteristics of PCR amplified 5S rDNA

The positions of the primers PIII and PIV (fig 2) were established by comparison of the published *P hybrida* 5S rDNA sequence and the two primer sequences using the Research program (Amersham). As expected, the two primer positions found in the *Petunia* 5S rDNA were identical to those found in pea by Cox et al (1992), namely from nucleiotide 27 to 47 for PIV and from nucleotide 68 to 88 for PIII.

The results of PCR amplification of the 5S rDNA in four wild *Petunia* species, four *P hybrida* lines and two F1 hybrids are shown (fig 3). Two size classes of repeat units corresponding to two groups of genotypes were observed. The first group including *P linearis* (2n = 18) and *P integrifolia* (2n = 14), was characterized by a repeat unit of approximately 460 bp and its multimers (fig 3, lanes 2, 3). The 350-bp repeat unit and its multimers characterized the second group which included *P axillaris*, *P parodii*, *P inflata* (2n = 14) and the four *P hybrida* lines (2n = 14) analysed (fig 3, lanes 4–6, 9–12). As expected, in the F1 hybrids the amplification profiles corresponded to those obtained for each parent. In the F1 hybrid *P inflata* × *P axillaris* (fig 3, lane 7) a single 350-bp unit and its multimers were amplified but in the F1 hybrid *P axillaris* × *P integrifolia* (fig 3, lane 8) two bands of 350 and 460 bp and their respective multimers were obtained.

In order to verify the 5S rDNA origin of the PIII/PIV amplified fragments, they were hybridized with the pBG13 probe, which revealed a banding pattern corresponding exactly to the pattern obtained with the PCR amplification (fig 4). These results confirmed the specificity of the PIII/PIV amplification and the 5S rDNA origin of the amplified bands. The amplified 460-bp and 350-bp repeat units were sequenced. As shown in figure 5, the 460-bp sequence, which revealed high homology with the published *P hybrida* 5S rDNA sequence, differed from the 350-bp sequence by an extra DNA of 110 bp.

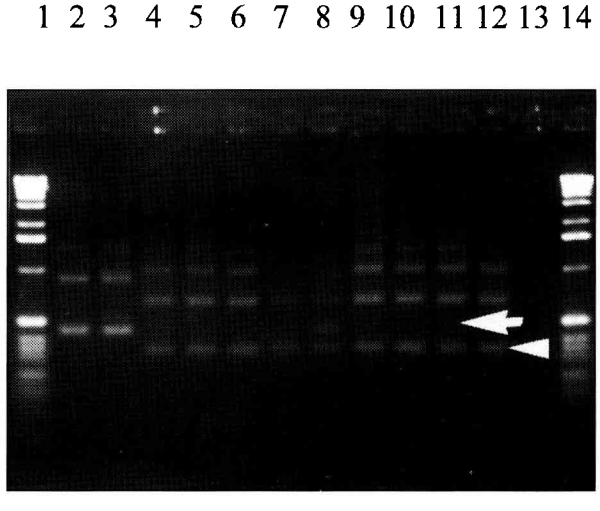

Fig 3. Gel-fractionated products of the PCR reaction using the PIII/PIV primers. The 350- and 460-bp units are marked with an arrowhead and an arrow, respectively. Lane 1 and 14 represent the 1-kb ladder (BRL); lane 2, *P linearis*; lane 3, *P integrifolia*; lane 4, *P axillaris*; lane 5, *P parodii*; lane 6, *P inflata*; lane 7, *P inflata* × *P axillaris*; lane 8, *P axillaris* × *P integrifolia*; lane 9, ST40; lane 10, TB1-3; lane 11, TLH7; lane 12, TLV1; and lane 13, negative control.

1 2 3 4 5 6 7 8 9 10 11 12 13 14

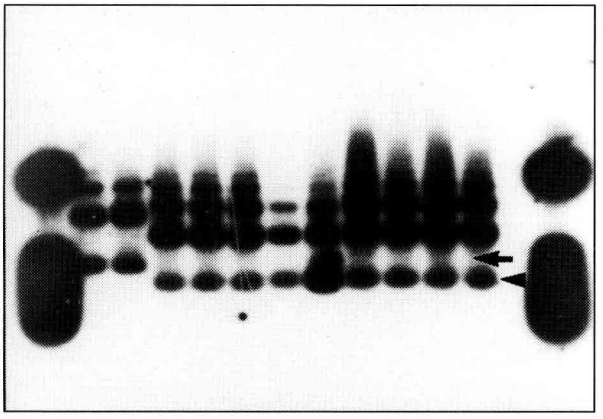

Fig 4. Hybridization profile of the transferred gel shown on figure 3 with the probe pBG13. The 350- and 460-bp revealed bands are marked with an arrowhead and an arrow, respectively.

The hybridization patterns obtained with the two cloned 5S rDNA amplified units (350 bp and 460 bp) against total digested genomic DNA are shown in figure 6. For each genotype, the two amplified 5S rDNA units produced the same ladders of restriction fragments on the three DNA digestions. These ladders appeared clearly in the BamHI digestion (fig 6B) and more weakly with DraI (fig 6C). The EcoRI digest (fig 6A) showed a ladder only for *P linearis*. The periodicity of the ladders was approximately 500 bp in *P linearis* (2n = 18), 460 bp in *P integrifolia* (2n = 14) and 350 bp in *P axillaris*, *P inflata*, *P parodii* and in the *P hybrida* lines (ST40, TB1-3, TLH7 and TLV1).

5S rDNA in situ hybridization

In the 2n = 14 *Petunia* species including *P integrifolia*, *P inflata* and the *P hybrida* lines ST40, TB1-3 and TLV1, two 5S rDNA hybridization sites were detected on the short arm of chromosome pair II between the NOR region and the centromere (fig 1k, o). Two 5S rDNA hybridization sites were also detected in a sub-terminal position on a chromosome pair in *P linearis* and *P parviflora* with 2n = 18. However, in *P axillaris*, *P parodii* (2n = 14) and the *P hybrida* line TLH7 two additional signals were localized near the centromere on a pair of meta to submetacentric chromosomes probably corresponding to chromosome pair IV or VII (fig 1n, p).

In the F1 hybrid *P inflata* × *P axillaris*, as expected, three hybridization sites were observed in both interphase nuclei and metaphase chromosomes (fig 1l, m). Two sites were carried by the satellited chromosome pair II, and the third pericentromeric site was carried by the meta to submetacentric chromosome IV or VII. Figure 7 summarizes the distribution of 5S rDNA hybridization sites in the *Petunia* species (2n = 14).

DISCUSSION

This study reports the first data on 5S rDNA PCR amplification in *Petunia* species. The sequenced amplification products were used to analyse the restriction length polymorphism of the 5S rDNA and to detect by FISH the number of clusters as well as their chromosomal localization. Our FISH results also revealed a variable number of 18S-5.8S-25S rDNA loci in *P linearis* and *P parviflora* (2n = 18) in comparison with the other studied *Petunia* species and *P hybrida* lines (2n = 14).

The PCR amplifications using 'universal' primers PIII and PIV combined with the hybridization results with the heterologous 5S rDNA probe from flax and the sequencing analysis, clearly showed the 5S rDNA origin of the amplified products and their size polymorphism. The species studied here could be separated into two groups according to the size of the amplified 5S rDNA variants: one group, with a 460-bp repeat unit included *P linearis* (2n = 18) and *P integrifolia* (2n = 14) and the second group, with a 350-bp repeat unit, included all the other (2n = 14) *Petunia* species (*P axillaris*, *P inflata*, *P parodii* and the *P hybrida* lines ST40, TB1-3, TLH7 and TLV1). Interspecific DNA variation was also found between wheat and different *Brachypodium* species using the 5S rDNA amplification protocol (Cox et al, 1992). Comparison of the amplified 460-bp repeat unit, 350-bp repeat unit and the published *P hybrida* 5S rDNA sequences (phrn5s1, Frash et al, 1989) revealed a high homology between them. It showed that the 460-bp repeat unit corresponded to phrn5s1 with a coding sequence of 120 bp and a spacer sequence of 340 bp. This 460-bp repeat unit differed from the 350-bp repeat unit mainly by an extra DNA of 110 bp in the spacer region. As mentioned by Cox et al (1992) it seems more probable that the shorter spacers were derived from the longer ones, as a result of deletions, rather than vice-versa by insertions or duplications.

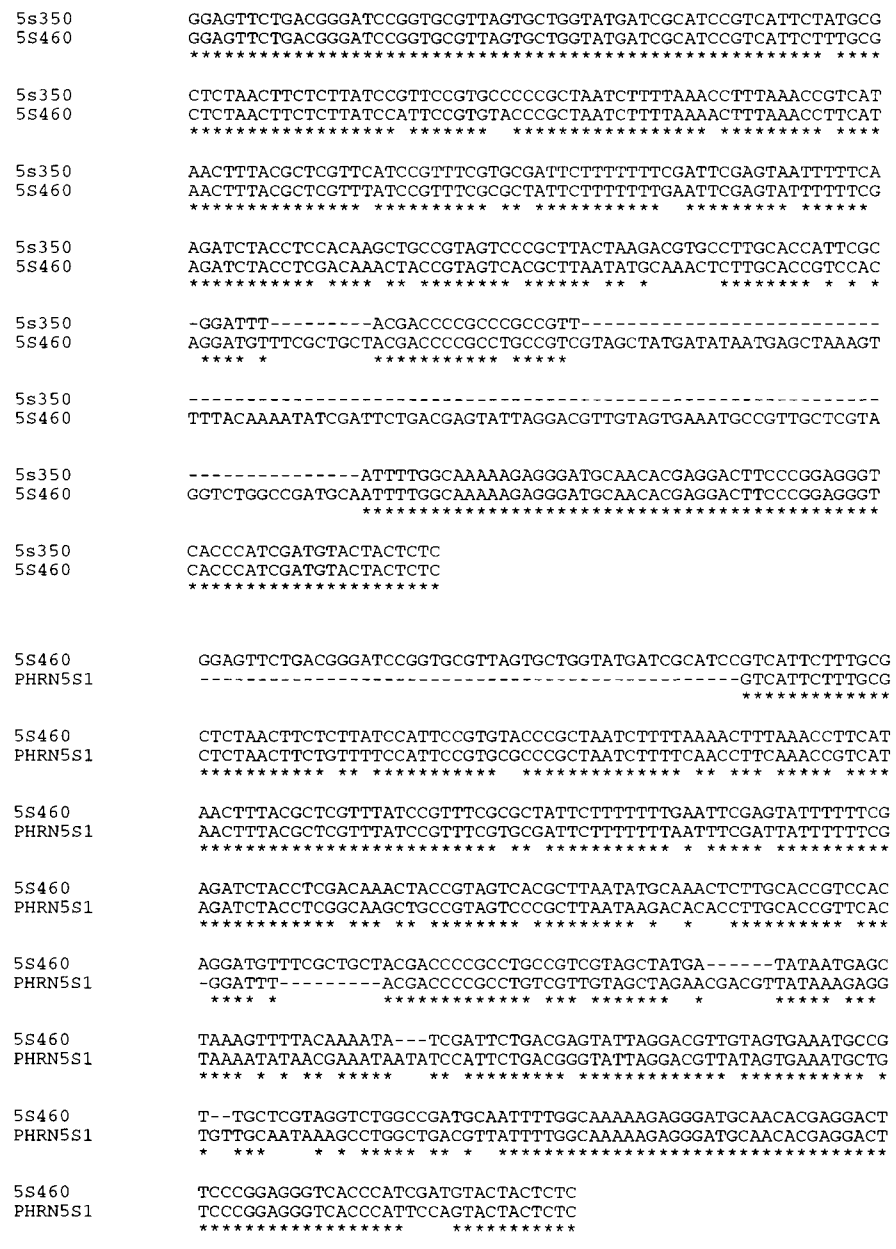

Fig 5. Sequence comparison between the published 5S rDNA (phrn5s1, Frash et al, 1989) and the two amplified 350- and 460-bp repeat units.

The Southern analysis with the two amplified 5S rDNA probes was consistent with the results found by Kabbaj et al (1995) using the heterologous probe pBG13 from flax. Our results after a BamHI digestion revealed three variants of band patterns: 500 bp for *P linearis*, 460 bp for *P integrifolia* and 350 bp for the other *Petunia* species. These results indicated that the common ancestor unit type probably corresponded to the BamHI/500 bp fragment found in *P linearis*, which by successive deletions could have produced the 460- and 350-bp units. In all the *Petunia* species studied, the 5S rDNA region was especially polymorphic when cut by BamHI which produced a ladder of restriction fragments. This ladder in the BamHI-digested DNAs is probably due to C-methylation of the restriction sites as discussed for the diploid species of wheat (Dvorak et al, 1989). C-methylation in rDNA also affects EcoRI restriction sites (Ellis et al, 1989), which probably occurred in the *Petunia* species studied with the exception of *P linearis*. Thus, a 500-bp EcoRI band was only revealed in *P linearis*. The size discrepancy observed between the amplified fragments and the restriction bands in *P linearis* (460 bp versus 500 bp) could probably be due to a variation in the binding sites of the universal primers in comparison with the other *Petunia* species.

In all the *Petunia* wild species (2n = 14) and *P hybrida* lines, the 18S-5.8S-25S rDNA units

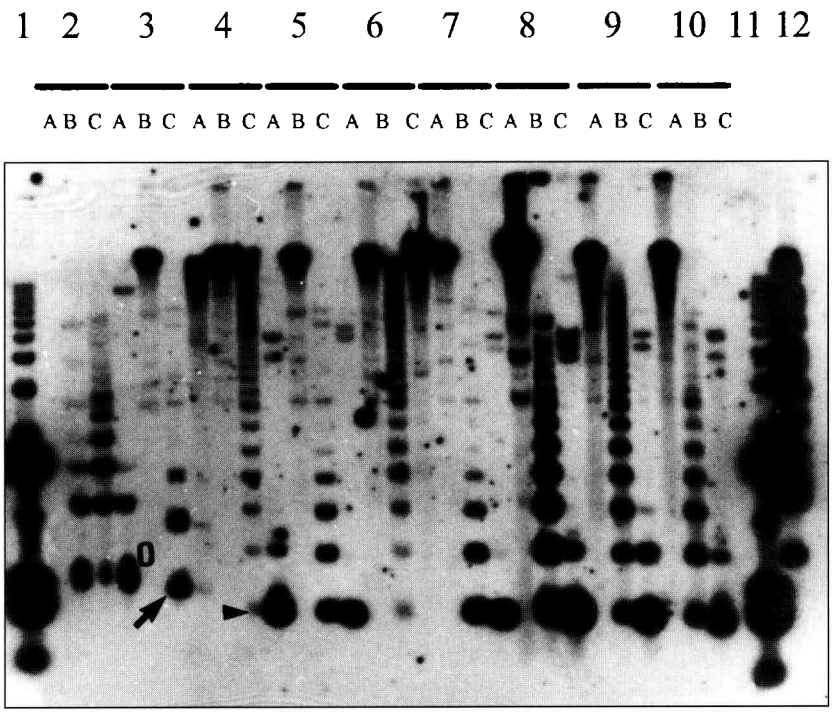

Fig 6. Genomic DNAs (5 µg per lane) of *Petunia* species digested with EcoRI (A), BamHI (B) and DraI (C) and probed with the 350-bp 5S rDNA fragment. Lane 1 and 11, 1-kb ladder (BRL); lane 2, *P linearis*; lane 3, *P integrifolia*; lane, 4 *P axillaris*; lane 5, *P inflata*; lane 6, *P inflata* × *P axillaris*; lane 7, ST40; lane 8, TB1-3; lane 9, TLH7; lane 10, TLV1; lane 12, Raoul ladder (Appligene). The ladders of restriction bands with a periodicity of 500 bp, 460 bp and 350 bp are marked with a circle, an arrow, and an arrowhead respectively. The 460-bp 5S rDNA fragment revealed the same pattern (not shown).

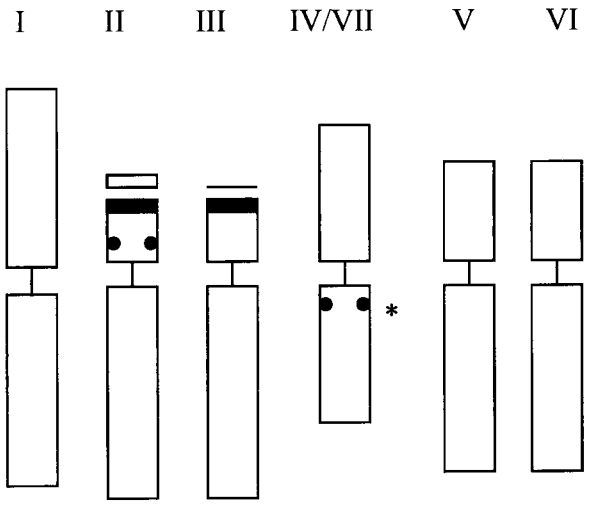

Fig 7. Ideogram of the *Petunia* species with 2n = 14 chromosomes as defined by Maizonnier (1971). The positions of the 5S (shaded circles) and 18S-5.8S-25S (shaded squares) rDNA genes are shown. ✶ : corresponds to the second 5S rDNA locus found in the white flowered species.

were organized in two loci, one major and one minor. They were mapped, respectively, to chromosome II and to chromosome III. FISH analysis of interphase nuclei showed that the four sites seemed to be involved in transcription. Similar results were obtained with silver staining and 3-D studies of the gene position in the interphase nuclei (Montjin et al, 1997). In the *Petunia* species with 2n = 18 (*P linearis* and *P parviflora*) only one locus for the 18S-5.8S-25S rDNA genes was revealed. As the *P linearis* and *P parviflora* chromosomes cannot be discriminated by their length nor by their centromeric index, we suggest using the 18S-5.8S-25S rDNA gene clusters as a marker to identify this chromosome pair.

FISH results revealed that all the *Petunia* species studied have in common one 5S rDNA locus which was associated with the major 18S-5.8S-25S locus on the short arm of chromosome II. The adjacent localization of the 5S and 18S-5.8S-25S loci was also shown in some members of *Triticeae* (Castilho and Heslop-Harrison, 1995), in *Hordeum* species (De Bustos et al, 1996) and in *Lolium* species (Thomas et al, 1996). An additional 5S rDNA locus appeared in *P axillaris* and *P parodii*, and has also been revealed in the *P hybrida* line TLH7, which was derived from back-crosses with *P axillaris* (table I). This locus was carried, in a centromeric position, by chromosome IV or VII [as defined by Maizonnier (1971)]. A similar centromeric position of the 5S rDNA genes was also reported in tomato (Lapitan et al, 1991), in cultivated beet (Schmidt et al, 1994) and in cotton (Hanson et al, 1996).

The chromosomal localization of the 5S rDNA allowed us to distinguish two *Petunia* groups which corresponded to the separation according to corolla pigmentation. *P hybrida* is believed to be an interspecific hybrid derived from crosses

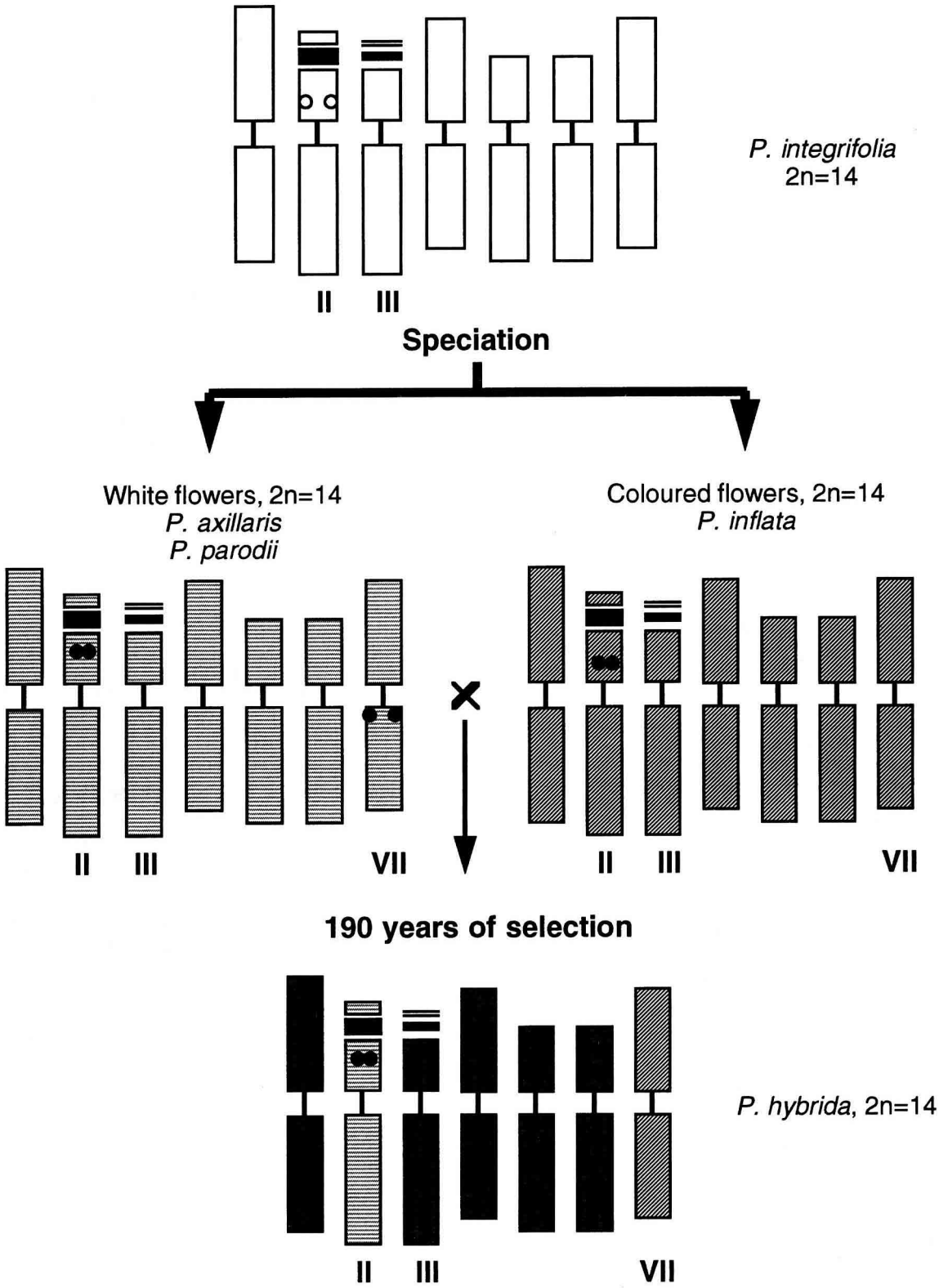

Fig 8. A schematic representation of the possible origin and genome organization of the *P hybrida* lines studied as revealed by our results on the ribosomal RNA genes. *P integrifolia* is separated from *P inflata*.

■: 18S-5.8S-25S rDNA sites), ○ 460-bp 5S rDNA (*P integrifolia*) and ● the 350-bp 5S rDNA (*Petunia* species with 2n = 14). ▨ chromosomal regions of white flowered group origin, ▩: chromosomal regions of coloured flowered group origin. In *P hybrida* (except for line TLH7), chromosome II is believed to be inherited from the white flowered *Petunia* species, while chromosome VII is believed to be inherited from the coloured flowered *Petunia* species.

performed 190 years ago between two genetic pools of white and coloured flowered *Petunia* species (2n = 14). Restriction polymorphism in rDNA genes (Kabbaj et al, 1995), RAPD and DAF studies (Peltier et al, 1994; Cerny et al, 1996) indicated that the most probable parents of *P hybrida* were *P parodii* and *P axillaris* for the white flowered parent and *P inflata* and *P integrifolia* for the coloured flowered parent.

Our FISH results on the number and chromosome organization of rDNA clusters combined with PCR amplification and RFLP studies of the 5S rDNA lead us to suggest a possible model for the genome organization in *P hybrida* (fig 8). The two BamHI- and EcoRI/500-bp 5S rDNA fragments specific to *P linearis* as well as the presence of a single 18S-5.8S-25S rDNA locus allowed us to separate *P linearis* from the other *Petunia* species. The question of whether *P linearis* could be an ancestor of the other species remains open since some homology between *P linearis* and the *Petunia* species with 2n = 14 was shown by genomic in situ hybridization (Benabdelmouna and Darmency, 1997), but taxonomists have proposed to transfer it to a different genus (Wisjman, 1990). In the *Petunia* species (2n = 14) with coloured flowers, *P integrifolia* differs from *P inflata* by the size of its 5S rDNA repeat unit (460 bp versus 350 bp) while the number and localization of the rDNA loci are similar. In the *P hybrida* lines, the exclusive presence of a 350-bp 5S rDNA repeat unit on chromosome II leads us to assume that chromosome II of *P integrifolia* probably did not contribute to the genome of *P hybrida*. This chromosome, which carries RAPD markers exclusively found in the white flowered *Petunia* species (Peltier et al, 1994), could be inherited from the white flowered species (*P axillaris* or *P parodii*). All the *P hybrida* lines studied, with the exception of the line TLH7, have lost the second 5S rDNA locus carried by the chromosome pair IV or VII, which indicates that this chromosome pair may be inherited from the coloured flowered species carrying only one 5S rDNA locus. The RAPD markers mapped on chromosome VII were essentially inherited from the coloured species, chromosome IV being without any associated RAPD marker (Peltier et al, 1994). We propose that chromosome VII is the most probable candidate to carry the second 5S rDNA locus in the white flowered *Petunia* species.

Acknowledgements: We would like to thank D Van Tuinen and D Zickler for helpful comments, J Delbut for technical assistance and C Humbert from the CMAB, université de Bourgogne, Dijon for his expertise in confocal microscopy observations.

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