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Cloning, quantification and characterization of a minisatellite DNA sequence from common bean *Phaseolus vulgaris* L.

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Abstract We describe the cloning and the characterization of a 130-bp DNA fragment, called OPG9-130, amplified from bean (*Phaseolus vulgaris* L.) genomic DNA. This fragment corresponds to a minisatellite DNA sequence containing seven repeats of 15 bp which differ slightly from each other in their sequence. Southern analysis showed that the core sequence of 15 bp is repeated in clusters dispersed throughout the genome. The use of this fragment as a probe allowed us to identify common bean lines by their DNA fingerprints. We suggest that OPG9-130 will be useful for line identification as well as for the analysis of genetic relatedness between bean species and lines.

Key words Minisatellite · DNA fingerprinting · DNA polymorphism · *Phaseolus*

Introduction

Minisatellites are relatively short DNA sequences (15–35 bp) repeated in tandem, dispersed throughout the genome, and associated with extensive allelic variations based on the number of core units they contain (Jeffreys et al. 1985). Many studies have demonstrated that the polymorphic nature of repeated sequences, such as micro- or mini-satellites, allows their use as highly informative genetic markers in individual identification, parentage testing, genetic relatedness and linkage mapping (Nybom et al. 1989; Santoni et al. 1992; Poulsen et al. 1993; Wolff et al. 1994; Rongwen et al. 1995). These multiple loci revealed by DNA fingerprints represent a set of polymorphic markers that are usually dispersed throughout the genome (Epplen et al. 1991). The origin and function of these repeated sequences remain unclear. Tandemly repeated satellite DNAs are usually transcriptionally inactive and preferentially located in constitutive heterochromatin. The mapping of tandemly repeated sequences by in situ hybridization techniques led Harrison and Heslop-Harrison (1995) to locate them at centromeric and telomeric regions of the chromosomes.

In contrast to the well-characterized repeated sequences of cereals (Mao et al. 1994; Devos et al. 1995), there is a lack of information concerning the repetitive sequences of bean. However, minisatellite sequences, such as tandem repeats of the M13 protein-III gene and human minisatellites have been reported to hybridize to plant sequences and particularly to common bean DNA (Dallas 1988; Weising et al. 1989; Stockton et al. 1992; Hamann et al. 1995). A probe corresponding to the synthetic microsatellite $(GACA)_4$ allowed Stockton and Gepts (1994) to ascertain relationships at the bean species and lines level. Furthermore, the markers revealed were used for mapping experiments.

In order to locate suitable repetitive sequences, synthetic oligonucleotides can be used as multiloci probes. Cultivar-specific DNA fingerprinting has been obtained with synthetic oligonucleotide probes from *Nicotiana tabacum* and *Brassica napus*, whereas individual specific DNA fingerprinting has been obtained with *Cicer arietinum* (Weising et al. 1991, 1992). Another approach is to randomly test DNA sequences as RFLP (restriction fragment length polymorphism) probes. The RAPD technique (Welsh and McClelland, 1990; Williams et al. 1990), based on the PCR amplification of random sequences from genomic DNA, has provided numerous markers useful to complete genetic linkage maps and to screen genotypes (Hu and Quiros, 1991; Vierling and Nguyen 1992). This strategy can provide markers in genomic regions not accessible to classical RFLP analysis due, in particular, to the presence of repetitive DNA sequences. Williams et al. (1990)

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applied the RAPD technique to various species and used the RAPD products as RFLP probes. It was shown that RAPD fragments corresponding to repetitive DNA were not always suitable for RFLP probes, leading sometimes to uniform hybridization patterns (Williams et al. 1990; Devos and Gale 1992), but can become useful markers when they lead to hybridization patterns with numerous discrete and polymorphic bands (Zhao and Kochert 1992; Francis et al. 1995).

In the present paper, we describe the cloning and characterization of a RAPD fragment containing a minisatellite sequence that allows the specific fingerprinting of species and lines from the genus *Phaseolus*.

Materials and methods

Plant materials

Nine common bean (*Phaseolus vulgaris* L.) lines were grown in a greenhouse for DNA isolation : PI165426, Fin de Bagnols, Masaï, Forum, Gabriella, Tuf, Marona, Strike and Lasso. One *Phaseolus acutifolius* genotype was also used.

DNA isolation

Bean genomic DNA was isolated from fresh leaves according to the method of Dellaporta et al. (1983). DNA concentration was measured by fluorimetry (Hoefer Scientific).

RAPD analysis

Random decamer oligonucleotides (Operon Technologies Inc., Alameda, USA), were used as single primers for the amplifications. Reactions were performed according to Williams et al. (1990) with slight modifications : each reaction was carried out in 25 µl consisting of 10 mM Tris-HCl pH 9.0, 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100 (Promega), 100 µM of each dNTP (Eurogentec), 0.2 µM primer, 50 ng genomic DNA and 0.75 units of *Taq* DNA polymerase (Promega). The reaction mixture was overlayed with 30 µl of mineral oil. Amplification was performed in a PTC-100 MJ Research Thermal Cycler programmed for 45 cycles of 1 min/94°C, 1 min/36°C and 2 min/72°C, with a pre-cycle 4 min/94°C, 1 min/36°C, 2 min/72°C and a final cycle of 1 min/94°C, 1 min/36°C and 20 min/72°C, using the fastest available transitions between temperatures. Amplification products were analysed after electrophoresis on 1.4% agarose gels in 0.5 × TBE (Tris borate EDTA) and staining with ethidium bromide. A specific amplified fragment is named by reference to the primer, followed by the size of the fragment.

Cloning and sequencing of DNA fragments

After ethidium bromide staining, a slice of agarose containing the RAPD marker to be cloned was excised from the gel and frozen at − 20°C. Three microliters of the de-frosted solution were used to amplify the DNA fragment using RAPD-PCR conditions, except that the primer concentration was reduced by two-fold (Adam-Blondon et al. 1994). The PCR fragment was then cloned using the pGEM-T Easy cloning kit (Promega) and the Rec$^-$ *Escherichia coli* strain DH5α was transformed. Sequencing data were obtained from the Euro Sequence Gene Service using the M13 T3 and T7 primers. The nucleotide sequences determined for both strands were analyzed with DNA Strider and PCGene programs.

Southern-blot hybridization with OPG9-130 as a probe

Five micrograms of DNA from the different bean lines were hydrolyzed with restriction endonucleases according to the manufacturer's recommandations (Eurogentec) except that 0.01% bovine serum albumin was added to the reaction mixtures. DNA fragments were separated after electrophoresis on a 1% agarose gel run in 0.5 × TBE (Tris-borate EDTA), stained with ethidium bromide and visualized under UV light. They were then blotted to Positive Membranes (Appligene) according to Southern (1975).

Filters were hydrated in 2 × SSPE and pre-hybridized in 5 ml of 5 × SSPE, 0.5% SDS, 5 × Denhardt's in an oven (Hybaid) at 65°C for 4 h. Fifty nanograms of OPG9-130 DNA were labelled with (α-^{32}P)-dCTP (> 3000 Ci/mmol; Isotopchim) using the Ready to go kit labelling system (Pharmacia), denaturated and added to 3 ml of hybridization solution (5 × SSPE, 0.5% SDS). Blots were hybridized at 65°C for at least 18 h, washed twice with 2 × SSPE, 0.1% SDS at room temperature for 10 min, then once with 1 × SSPE, 0.1% SDS at 65°C for 15 min, and finally with 0.1 × SSPE, 0.1% SDS for 10 min at 65°C. They were air-dried and then autoradiographed on X-OMAT AR films (KODAK) with intensifying screens at –80°C for a few hours.

Dot-blot hybridization

Five micrograms of genomic DNA were denaturated in 5 × SSPE and spotted onto Positive Membranes (Appligene) using the dot system (Minifold I, Schleicher and Schuell). The blot was then hybridized as described above.

Colony blotting

Fifty microliters of overnight 2-ml bacterial cultures were blotted onto Positive Membranes (Appligene) using the dot system (Minifold I, Schleicher and Schuell). The filter was washed with 1.5 M NaCl, 0.5 M NaOH for 10 min, 1.5 M NaCl, 0.5 M NaOH, 0.2% Triton X-100 for 5 min, twice with Tris-HCl 10 mM, NaCl 50 mM for 5 min, 4 × SSPE for 5 min, 96% ethanol for 5 min, and 4 × SSPE for 5 min. The blot was air-dried and then hybridized as described above.

Estimation of copy number per genome of sequences which hybridized with the probe OPG9-130

Known quantities of bean genomic DNA from the Fin de Bagnols line ranging from 0.1 pg to 2 µg and from OPG9-130 ranging from 0.1 pg to 2 ng were spotted onto nylon membranes (Appligene) and hybridized as described above with OPG9-130 DNA.

Results

While screening for RAPD markers from different bean lines, we selected an amplified DNA band displaying polymorphism between the lines. This DNA band, called OPG9-520, was amplified from PI165426 genomic DNA by PCR with a 10-mer RAPD primer

(OPG9 : 5′CTGACGTCAC3′). Further study using Southern-blot hybridization with this DNA fragment as a probe led to complex patterns for all the bean lines analysed. We related these profiles to those obtained with minisatellite sequences. We then attempted to isolate, clone, and sequence this fragment for further characterization.

The DNA of band OPG9-520 was excised from an agarose gel and amplified once more with the OPG9 primer. Cloning experiments from the resulting amplification product led us to recover 500 recombinant colonies. In order to identify clones containing a highly repeated genomic sequence, all the colonies were blotted and hybridized with genomic DNA as a probe. This experiment led us to recover one clone carrying a recombinant fragment of 130 bp, called OPG9-130. Used as a probe, this 130-bp fragment provided the expected complex patterns after hybridization with bean-line hydrolyzed DNAs. The random analysis of other clones led us to recover four recombinant plasmids containing different fragments of 520 bp. When tested as a probe, these sequences did not reveal the expected complex patterns and shared no sequence homology with OPG9-130.

In order to search for homologies with other amplified fragments, OPG9-130 DNA was tested as a probe on a blot carrying the amplification products obtained from ten bean-line genomic DNAs with the OPG9 primer, after separation by electrophoresis. The results are shown on Fig. 1. The probe hybridized with a 520-bp band amplified from the ten bean-line DNAs. This result showed that the OPG9-130 fragment is homologous to a sequence contained in the 520-bp fragment. The OPG9-130 fragment hybridized also strongly to a 130-bp fragment amplified from five of the nine *P. vulgaris* lines tested, PI 165426, Gabriella, Tuf, Masaï and Strike, and more weakly with a *P. acutifolius* 130-bp fragment.

After sequencing of OPG9-130, the nucleotide sequence obtained showed that this DNA fragment was

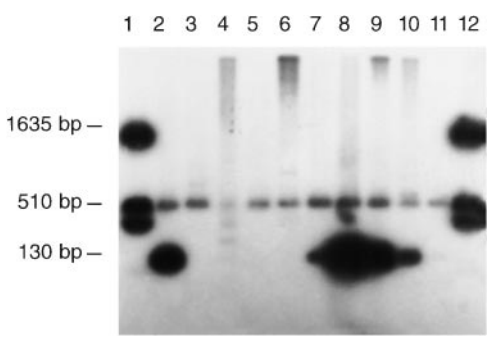

Fig. 1 Hybridization patterns obtained using the OPG9-130 fragment as a probe on a blot carrying the PCR products amplified from ten lines of bean DNA using primer OPG9. *Lane 1* 1-kb ladder (BRL), *lane 2* Pl165426, *lane 3* Fin de Bagnols, *lane 4* Forum, *lane 5* Marona, *lane 6* Lasso, *lane 7* Gabriella, *lane 8* Tuf, *lane 9* Masaï, *lane 10* Strike, *lane 11 P. acutifolius* and *lane 12* 1-kb ladder (BRL)

a

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CTGACGTCAC GATGAGAGGT TACTTTGTGA GTGAGGTTAG CGCGGGTGAG 50

GTTATCGGGG GTGAGGTTAT CGTGGGTGAG TTTATCGTGG TGAAGTTATC 100

GTGGGTGACT TTACCGTGGG TGACGTCAGA 130
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b

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5′CTGACGTCACGAT
  GAGAGGTTACTTTGTGA
  GTGAGGTTAGCGCGG
  GTGAGGTTATCGGGG
  GTGAGGTTATCGTGG
  GTGAGTTTATCGTG
  GTGAAGTTATCGTGG
  GTGACTTTACCGTGG
  GTGACGTCAGA 3′
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c

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5′GTGAGGTTATCGTGG 3′
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Fig. 2 **a** DNA sequence of OPG9-130. **b** Sequence comparisons of the seven 15-bp repeats units of the OPG9-130 fragment with the consensus sequence (**c**)

exactly 130 bp in length (Fig. 2 a). The average G + C content was 52.30%. Analysis of the sequence showed the presence of seven direct repeats with minor sequence variations (Fig. 2 b). From this result, we were able to define a 15-bp consensus sequence (Fig. 2 c). Computer-based sequence-similarity searches performed with the BLAST network service revealed that the 130-bp fragment, as well as the consensus repeat, present no homology with any sequences in the GenBank database.

To determine the copy number of the sequence in the bean genome, OPG9-130 was used both as a probe and as a standard for a dot-blot hybridization experiment on DNA from the *P. vulgaris* line Fin de Bagnols. Quantification of the hybridization signals estimated the copy number of the 15-bp core repeat as 650 000 per haploid genome.

To determine the chromosomal organization of this repetitive element, the OPG9-130 fragment was used to probe Southern blots of two bean lines (PI165426 and Fin de Bagnols) in which the genomic DNA was hydrolyzed with several restriction endonucleases. Variable complex patterns with numerous hybridization signals were obtained after hydrolysis of bean DNA with *Eco*RV, *Hin*cII, *Bsp*143I and *Alu*I. The profiles obtained with these four restriction enzymes differed in the number and the size of the fragments revealed after hybridization, ranging from 200 bp to more than 12 kbp. All the other enzymes tested : *Eco*RI, *Hin*dIII, *Pvu*II, *Sac*I, *Bgl*II, *Pst*I, *Sma*I, *Aat*II, *Ava*I, *Bam*HI, *Bgl*I, *Bsp*106I, *Kpn*I and *Nsi*I, led to a strong hybridization signal with high-molecular-weight DNA fragments. The typical results obtained after hydrolysis of PI165426 and Fin de Bagnols DNA with *Eco*RI, *Eco*RV, *Hin*dIII, *Pvu*II and *Sac*I are shown in Fig. 3.

The probe OPG9-130 was tested on nine bean-cultivar DNAs hydrolyzed with *Eco*RV leading to highly variable hybridization signals and fingerprint patterns (Fig. 4 a). OPG9-130 was then hybridized with *P. acutifolius* DNA hydrolyzed with *Eco*RV. The hybridization pattern obtained is shown in Fig. 4 b.

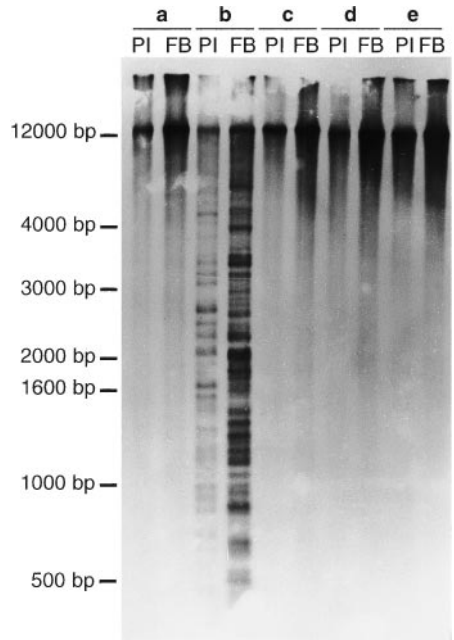

Fig. 3 Southern-blot hybridization of labelled OPG9-130 with *P. vulgaris* P1165426 (*PI*) and Fin de Bagnols (*FB*) DNAs hydrolyzed with various restriction enzymes. *a Eco*RI, *b Eco*RV, *c Hin*dIII, *d Pvu*II and *e Sac*I. The sizes were determined according to the 1-kb DNA ladder (BRL)

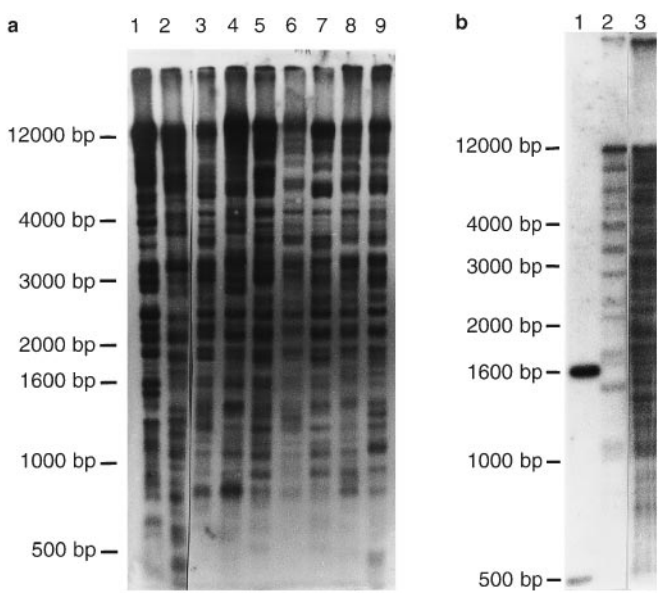

Fig. 4 **a** Southern-blot hybridization obtained using OPG9-130 as a probe with different *P. vulgaris* line DNAs hydrolyzed with *Eco*RV. (*1*) PI165426, (*2*) Fin de Bagnols, (*3*) Forum, (*4*) Masaï, (*5*) Lasso, (*6*) Gabriella, (*7*) Tuf, (*8*) Marona, (*9*) Strike. The sizes were determined according to the 1-kb DNA ladder (BRL). **b** Southern-blot hybridization of labelled OPG9-130 with *P. acutifolius* (*lane 2*) and *P. vulgaris* Strike (*lane 3*) DNA hydrolyzed with *Eco*RV. The sizes were determined according to the 1-kb DNA ladder (BRL), *lane 1*

The presence of this repetitive sequence in other genera was tested on dot blots of 5 µg of genomic DNAs from *P. vulgaris* (PI165426 and Fin de Bagnols), *P. acutifolius*, *Pisum sativum*, *Spinacea oleracea*, *Nicotiana tabacum*, *Pelargonium hortorum*, *Arabidopsis thaliana*, *Medicago sativa*, *Melilotus*, *Lolium perenne* and *Lolium multiflorum* using the OPG9-130 fragment as a probe. The results, shown in Fig. 5, revealed hybridization signals only with genomic DNA from the genus *Phaseolus*. These results were confirmed by further testing *Phaseolus coccineus*, *Vigna radiata* and *Vigna unguiculata* DNA : a hybridization signal appeared only for the *P. coccineus* sample (data not shown).

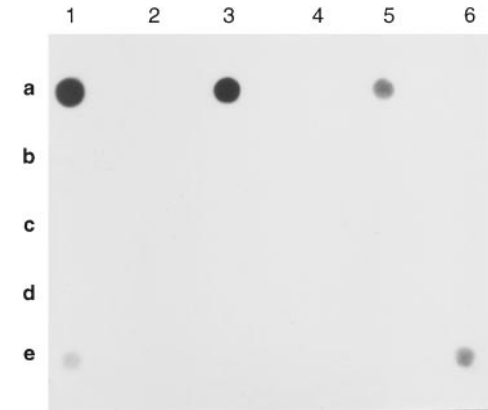

Fig. 5 Dot-blot hybridization of labelled OPG9-130 with 5 µg of DNA from different genera (*a*/*1*) *P. vulgaris* PI165426, (*a*/*3*) *P. vulgaris* Fin de Bagnols, (*a*/*5*) *P. acutifolius*, (*b*/*2*) *P. sativum*, (*b*/*4*) *S. oleracea*, (*b*/*6*) *N. tabacum*, (*c*/*1*) *P. hortorum*, (*c*/*3*) *A. thaliana*, (*c*/*5*) *M. sativa*, (*d*/*2*) *M. sp* (*d*/*4*) *L. perenne* and (*d*/*6*) *L. multiflorum*, (*e*/1) 5 pg OPG9-130, (*e*/*6*) 10 pg OPG9-130

We checked the Mendelian transmission of the markers revealed with OPG9-130. DNA was isolated from F_1 and individual F_2 plants obtained from a cross between PI165426 and Fin de Bagnols. Each DNA was hydrolysed with *Eco*RV and hybridized with the OPG9-130 probe after gel electrophoresis and blotting on a filter (data not shown).

Discussion

During the search for RAPD markers from different bean lines, a 130-bp DNA sequence containing seven repeats of 15-bp was identified. When used as a probe, the OPG9-130 fragment hybridized strongly with the genomic DNA of the *P. vulgaris* lines and *P. acutifolius*, but not with genomic DNA of the nine other plant species tested.

Analysis of the 130-bp sequence showed a minisatellite organization with seven tandemly repeated sequences of 15-bp. The repeat family was heterogenous since no repeat unit sequence was identical to any other. An average of 12% divergence was established for the seven repeats based on a sequence comparison. The mismatches in the sequence come from point mutations. These mutations create or destroy restriction-enzyme recognition sites in the minisatellite sequence. For example, the recognition site for *Eco*RV,

GATATC, or for *Hin*cII, GTPyPuAC, appeared from a base substitution of the sequence GTTATC in the consensus sequence GTGAGGTTATCGTGG. These mutational events could explain the fact that complex hybridization patterns were obtained with only a few enzymes. Copy number estimation revealed that the repeat occurred in approximately 6.5×10^5 copies in the bean genome, which put it in the highly repetitive DNA category. We estimated that the 15-bp repeat represents 1.5% of the bean genome.

Using OPG9-130 as a probe, Southern-blot analysis on bean DNA hydrolysed with *Eco*RV, *Hin*cII, *Bsp*143I and *Alu*I revealed polymorphic patterns, allowing a distinction between the *Phaseolus* lines tested. A clear-cut banding pattern appeared, lacking any background smear. Hybridization signals were also detected in the high-molecular-weight portion of each hydrolysis, even when the total DNA had been extensively hydrolysed with the restriction enzymes. Consequently, these signals are probably not caused by partial hydrolysis of the total DNA but by sequence alteration in the repetitive DNAs (Irifune et al. 1995). The reason for the hybridization of a large fragment could be the consequence of the clustering of different simple repetitive motifs in regions that exhibit a limited assortment of restriction sites. None of the restriction enzymes used in these experiments has a recognition site in the OPG9-130 DNA fragment.

The lack of a "ladder" in the hybridization patterns indicates that OPG9-130 sequences are not organized as simple tandem arrays in *P. vulgaris* lines, but rather in clusters dispersed throughout the genome. The length of the restriction fragment produced by sequences repeated in tandem is proportional to the number of core units it contains. The organization of the sequence seems to be different in *P. acutifolius*. Here, the hybridization profile obtained after *Eco*RV hydrolysis of the genomic DNA showed a simpler organization of the sequence.

The results, shown in Fig. 5, indicated that there was no significant homology between the cloned sequence and the genome of the different genera tested, even in other leguminous genera such as *Pisum* or *Vigna*. Thus, this result suggests that this repeated sequence was expanded only in the genus *Phaseolus*. The repeat unit shares no homology to any published repetitive sequence in *Phaseolus* or the other genera. Santoni and Bervillé (1992) have already characterized two different satellite DNAs from *Beta vulgaris* L. which are not present in all species of the *Beta* genus. Comparisons between the repeated sequences of closely related species which have diverged from a common ancestor have shown that many of the repeats found in more than one species differ by their copy number and by their organization according to the species (Flavell 1986). In our case, the repetitive sequence allowed us to differentiate *P. vulgaris* bean lines from *P. acutifolius*. The simpler pattern revealed in *P. acutifolius* indicates that the sequence is less degenerate than in *P. vulgaris*. One hypothesis could be that by accumulating numerous variants the breeding process has led to complex profiles. Nevertheless, PI 165426 is an accession recovered from Mexico and has not been subject to either crosses or selection. Hence, it confirms that the divergence between *P. acutifolius* and its common ancestor with *P. vulgaris* arose earlier than the divergence between *P. vulgaris* and its ancestor, as reported by Zink et al. (1994) using RFLP of *Pha* genes. Consequently, in further study we will investigate other species from the genus *Phaseolus* for the presence of this minisatellite sequence. We aim also to determine the localization of this minisatellite sequence at the chromosomal level by in situ hybridization. Moreover, the molecular taxonomy of the bean might well be complemented by the fingerprint similarity derived from the OPG9-130 probe, which has the potential both for identifying bean species and lines and for the evaluation of linkage data in defined crosses.

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