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Description and analysis of genetic diversity between commercial bean lines (*Phaseolus vulgaris* L.)

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Abstract The effectiveness of RFLP, DAMD-PCR, ISSR and RAPD markers in assessing polymorphism and relationships between 24 commercial lines of *Phaseolus vulgaris* L.was evaluated. We have used a *Phaseolus*-specific minisatellite sequence as a probe, which enabled 23 of the bean lines tested to be fingerprinted. Based on the sequence information obtained, primers corresponding to the bean-specific minisatellite core sequence were used in subsequent PCR amplifications. Our observations indicated that while the DAMD-PCR was sensitive in detecting genetic variation between bean species and between accessions of *P. vulgaris*, when used alone it may be limited in its ability to detect genetic variation among cultivated bean lines due to the low number of loci amplified. Only one out of the five ISSR primers tested was efficient in generating multiple band profiles, which was insufficient to distinguish all the different bean lines. Reproducible RAPD profiles were obtained, and these allowed us to differentiate all the genotypes tested with seven primers. We ultimately used only results from RFLP and RAPD markers to explore the genetic diversity among commercial bean lines. Both analyses led to the same clustering of the bean lines according to their geographical origins (United States or Europe). With respect to the European lines, the results obtained from RAPD data also enable the lines to be clustered according to their creators.

Keywords Genetic diversity · Molecular markers · Common bean · Germplasm

Introduction

Common bean (*Phaseolus vulgaris* L.) is a widely cultivated crop plant used for both human comsumption and as a fodder crop. With the recent development of molecular markers, a number of studies have been carried out to assess germplasm diversity and genetic structure in *Phaseolus vulgaris*. Skroch et al. (1998) used random amplified polymorphism DNA (RAPD) markers (Williams et al. 1990) to compare core and reserve samples of the CIAT (Centro Internacional de Agricultura Tropical Cali Colombia) *Phaseolus vulgaris* germplasm collection. Their results indicated that the genetic structure of the core collection is representative of the reserve collection. Most studies based on biochemical and restriction fragment length polymorphism (RFLP) markers (Botstein et al. 1980) support the concept of two distinct domestication events for *Phaseolus vulgaris* (Brown et al. 1982; Gepts and Bliss 1986; Gepts 1988; Kairallah et al. 1990, 1992; Chase et al. 1991). Zink et al. (1994) obtained RFLP-based cladograms of the *Pha* genes for 21 *Phaseolus* and *Vigna* genotypes. For their study they used hypervariable sequences as a new means of providing RFLP probes, thereby allowing the detection of many polymorphisms due to insertion/deletion or single base pair changes, followed by fingerprinting of the bean lines. Stockton and Gepts (1994) also used the protein III tandem repeat of the M13 phage and the human 33.15 minisatellite sequences as RFLP probes on DNA of wild forms, landraces and cultivars of *Phaseolus vulgaris*. They showed that the banding patterns obtained using these probes were constant irrespective of the environment and remained constant in spite of several generations of selfing. Moreover, both probes were useful in detecting variability within a population of common bean. In the same studies, they showed that the microsatellite $(GACA)_4$ was also useful in ascertaining relationships at the species and subspecies level. Garber et al. (1999) derived probes from the bean retrotransposon Tpv2 and from flanking genomic sequences able to classify *Phaseolus* cultivars.

In recent years there has been a rapid increase in the number of molecular techniques available for genotyping and fingerprinting plant lines and for studying genetic diversity. Polymerase chain reaction (PCR)-based methods: RAPD (Williams et al. 1990; Welsh

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Table 1 List of *Phaseolus vulgaris* lines used in molecular analysis: American (US) lines and European (EU) lines

American lines: Code	Breeder
US1	USA
US2	USB
US3	USC
US4	USD
US5	USE
US6	USF
US7	USG
US8	USG
US9	USH
US10	USI
European lines: Code	**Breeder**
EU1	EUA
EU2	EUA
EU3	EUB
EU4	EUF
EU5	EUC
EU6	EUB
EU7	EUD
EU8	EUE
EU9	EUD
EU10	EUA
EU11	EUE
EU12	EUB
EU13	EUB
EU14	EUE

and McClelland 1990), AFLP (amplified fragment length polymorphism, Vos et al. 1995), SSR (simple-sequence repeat polymorphism) or microsatellites (Tautz 1989), ISSR (inter-simple sequence repeat, Zietkiewicz et al. 1994) and IRAP and REMAP (inter-retrotransposon-amplified polymorphism and retrotransposon-microsatellite-amplified polymorphism, Kalendar et al. 1999) have been successfully improved to generate polymorphisms. These polymorphic markers have been used for the estimation of genetic diversity among maize inbred lines (Ajmone-Marsan et al. 1998; Pejic et al. 1998), pea (Lu et al. 1996), barley (Russel et al. 1997) and *Brassica oleracea* L. genotypes (Dos Santos et al. 1994).

In the study presented here, RFLPs revealed by a specific minisatellite locus (Métais et al. 1998), DAMD-PCR (direct amplification of minisatellite region DNA, Heath et al. 1993), ISSRs and RAPDs were determined among 24 genotypes of *Phaseolus vulgaris* of related industrial types. Our first objective was to explore the ability to individualise two genetic pools (North American and European) with results obtained from molecular marker analysis. At a secondary level, we analysed the relationships in a set of lines coming from six European breeders.

Materials and methods

Plant materials

The genotypes of *Phaseolus* are listed in Table 1. Two sets of lines were used. One coming from nine North American breeders and the others from six European breeders. All the lines are commercial industrial types differing at least for the level of pod quality. Seeds were grown in a greenhouse on wet sand under a light bench. After 15 days, the primary leaves were harvested and DNA was extracted.

Due to commercial confidentiality, we do not provide the names of the lines and breeders. This information is available form Vilmorin S.A. upon request.

DNA isolation

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

Restriction fragment length polymorphism

Restriction and Southern transfer

Five-microgram aliquots of DNA from the different bean lines were hydrolysed 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 by electrophoresis on a 1% agarose gel run in 0.5 × TBE (Tris Borate EDTA), stained with ethidium bromide and visualised under UV light. They were then blotted to Positive Membrane (Appligene) according to Southern (1975).

Probe

The probe used in this study corresponds to a 130-bp PCR fragment (OPG9–130) containing seven repeats of a 15-bp minisatellite sequence (Métais et al. 1998). A 100-ng aliquot of OPG9–130 DNA was radiolabeled by random priming with α-[^{32}P]-dCTP (111000 GBq/mmol; Isotopchim) using the kit Ready-to-go labeling system (Pharmacia). Unincorporated triphosphate nucleotides were removed by column chromatography using Sephadex G-50.

Southern hybridisation

Filters were hydrated in 2 × SSPE and prehybridised in 5 ml 5 × SSPE, 0.5% SDS, 5 × Denhardt's in an oven (Hybaid) at 65°C for 4 h. The probe was denaturated and added to 3 ml hybridisation solution (5 × SSPE, 0.5% SDS). Blots were hybridised 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 few hours.

RAPD amplification

Random decamer oligonucleotides were purchased from Operon Technologies and used as single primers for the amplifications. The reactions were performed according to Williams et al. (1990) with slight modifications: each reaction contained 25 µl consisting of 10 m*M* TRIS-HCl pH 9.0, 50 m*M* KCl, 2 m*M* $MgCl_2$, 100 *µM* each dNTP (Eurogentec), 20 n*M* primer, 50 ng of genomic DNA and 0.5 U *Taq* DNA polymerase (Eurogentec). 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 at 94°C, 1 min at 36°C and 2 min at 72°C, with a precycle of 4 min at 94°C, 1 min at 36°C, 2 min at 72°C and a final cycle of 1 min at 94°C, 1 min at 36°C and 20 min at 72°C, using the fastest available transitions between each temperature.

The total amplification products were analysed by electrophoresis on 1.4% agarose gels and visualised after ethidium bromide staining (Sambrook et al. 1989). The sequence of the seven primers used in this study are reported in Table 2.

ISSR

The sequence of the ISSR primers used are shown in Table 2 (Gupta et al. 1994; Niemann et al. 1997). The amplification mixture is the same as that described for RAPD amplification except for the quantity of ISSR primers (0.4 µ*M*). Amplification was performed in a PTC-100 MJ Research Thermal Cycler programmed for 40 cycles of 30 s at 94°C, 45 s at 45°C, 2 min at 72°C, with a precycle of 4 min at 94°C and an elongation cycle of 7 min at 72°C. The ISSR products were analysed by electrophoresis on 1.4% agarose gels as previously described.

Data analysis

Band profiles generated by RFLP or RAPD were compiled onto a data matrix on the basis of the presence (1) or absence (0) of selected bands. Dendrograms were constructed by UPGMA cluster analysis from a Jaccard dissimilarity (Jaccard 1908) using PHYLIP 3.5 (Felsenstein 1989).

Table 2 ISSR, DAMD and RAPD primers used in the study

Primer	5′-3′ Sequence
ISSR3	ACTGACTGACTGACTG
ISSR4	GACAGACAGACAGACA
ISSR5	VHVCTCTCTCTCTCTCTCT
ISSR6	VDVGTGTGTGTGTGTGTGT
ISSR7	VHVCACACACACACACACA
REP1	GTGAGGTTATCGTGG
REP2	CCACGATAACCTCAC
OPH03	AGACGTCCAC
OPG05	CTGAGACGGA
OPG06	GTGCCTAACC
OPG07	GAACCTGCGG
OPG08	TCACGTCCAC
OPG10	AGGGCCGTCT
OPG14	GGATGAGACC

Results

Levels of polymorphisms

RFLP

The RFLP profiles were generated with a probe containing seven minisatellite repeats, and the same patterns were obtained using only the core sequence repeat (REP1 oligonucleotide) as a probe (data not shown).

The hybridisation of 23 bean line DNAs, hydrolysed by the restriction enzyme *EcoRV,* with the minisatellite probe OPG9-130 led to highly variable banding patterns. The hybridisation signals ranged from 200 bp to 12 kbp. There were 35 clearly distinguishable and scoring hybridisation signals (Fig.1 A). The hybridisation patterns differed between 22 of the cultivars studied, with lines US5 and US6 sharing the same banding pattern. Only two hybridization signals were shared by all lines.

DAMD-PCR

We derived two primers called REP1 and REP2 from the 15-bp core sequence of the minisatellite to amplify regions rich in minisatellite repeats (Table 2). The DAMD-PCR profiles showed RAPD-like results: there were 13 clearly distinguishable bands amplified by the DAMD-PCR REP1 primer in the 24 different lines with 6 polymorphic bands (Fig.1B). Seven amplification products were amplified using primer REP2 with 2 polymorphic bands (data not shown).

ISSR

Five ISSR primers were tested using the 24 bean DNAs but only one, called ISSR3, led to 15 clearly amplified products (Fig.1 C) with 6 polymorphic bands. The others produced smear patterns. We tried to adjust the temperature of annealing or modify the DNA quantities without significant improvement in the patterns obtained with these five primers.

RAPD

Seven RAPD primers were used to amplify the 24 line DNAs and led to score 120 amplification products (Fig. 1D). Of these, 42 were monomorphic, leading to 66.6% of polymorphic bands. Four primers amplified polymorphic fragments able to differentiate US5 from US6 DNA.

Table 3 compares the results obtained with each of the tested molecular techniques. The number of bands scored after each pattern ranged from 15 to 120. RFLPs showed the highest level of polymorphic bands. In contrast, RAPD and ISSR markers showed an almost equal percentage of polymorphism – 66.6% and 60%, respectively – while DAMD-PCR markers were the least informative.

Genetic relatedness

The 24 lines studied are runner beans or French beans and belong formally to two groups coming from North American or European breeders. We have at our disposal accurate data concerning phenotypes and industrial quality for lines of European origin. Moreover, we know some elements concerning the genealogy or at least the origin of some genitors used in the corresponding breeding programmes.

The banding patterns obtained with RFLP and RAPD techniques were compared using the Jaccard dissimilarity index and clustered with UPGMA.

The dendrogram obtained from the RFLP results shows two main groups (Fig. 2A). The first group was formed by 12 European lines, the second by eight American lines. The American lines US4 and US10 are separated from the main American group. The line EU10 also does not cluster with the other European lines. Two genotypes, US5 and US6, were impossible to differentiate. In contrast, all the other lines could be

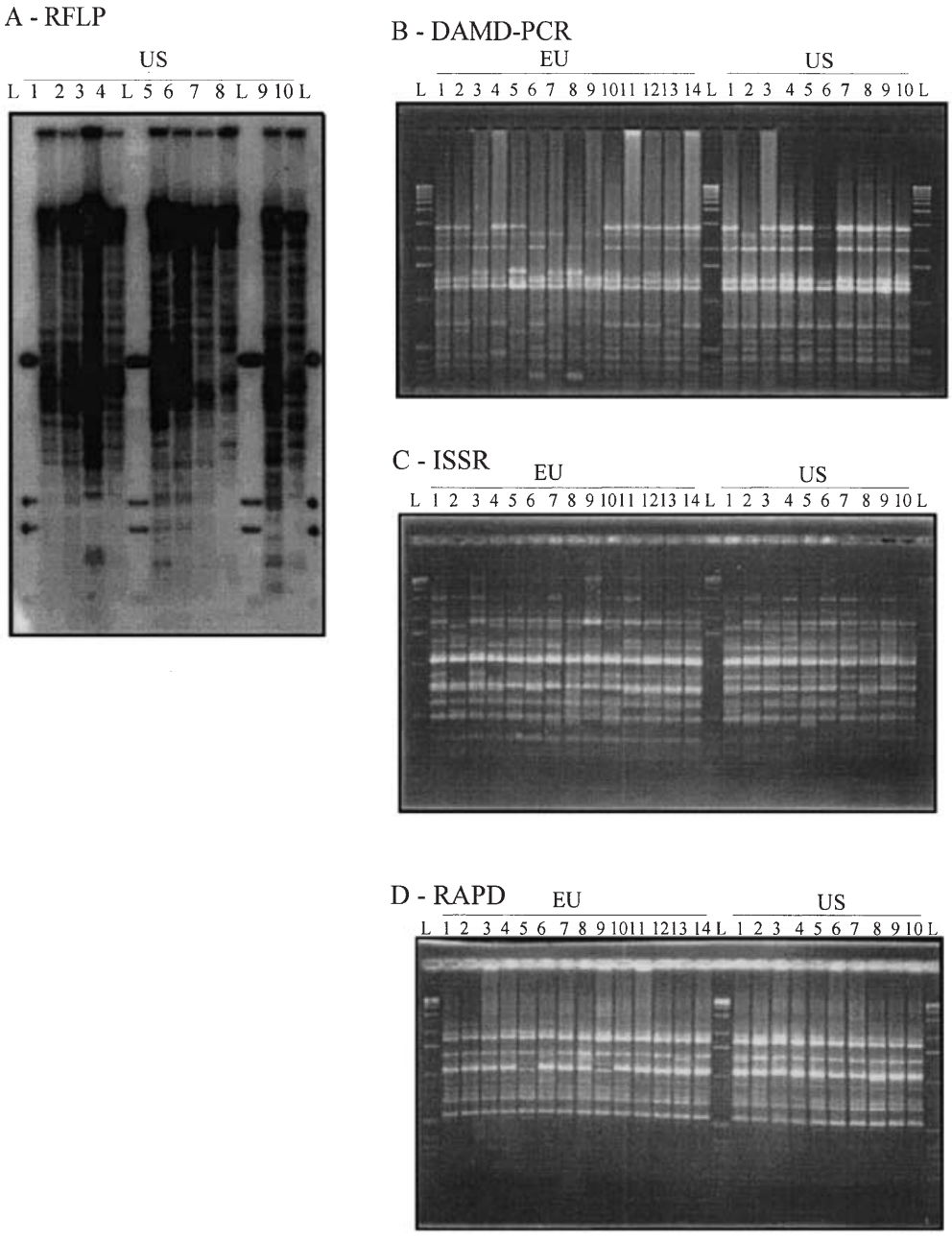

Fig 1A-D An example of the different information content observed with: **A** RFLPs on ten American (US) lines using a *Phaseolus*-specific minisatellite sequence as a probe, **B** DAMD-PCR profiles of the 14 European (EU) and ten US bean lines using the REP1 primer; **C** ISSR banding patterns of the 14 EU and ten US bean lines using ISSR3 primer, and **D** RAPD profiles obtained with primer OPG10. *L* 1-kb ladder (BRL)

Table 3 Analysis of the RFLP-, RAPD-, DAMD- and ISSR-generated banding patterns

Marker	Number of assay units	Total number of bands	Number of polymorphic bands	Percentage polymorphic bands
RFLP	1 (one probe, one enzyme)	35	33	94.2
DAMD	2 primers	20	8	40.0
ISSR	5 primers	15	9	60.0
RAPD	7 primers	120	80	66.6

distinguished on the basis of their band profile. Nevertheless, the clustering obtained could not be related to our knowledge concerning the structuration of European lines.

The dendrogram obtained from the RAPD profiles analysis (Fig. 2B) also enabled us to separate both groups: the first with 12 European lines, the second with the ten American lines. A third group was formed by the Euro-

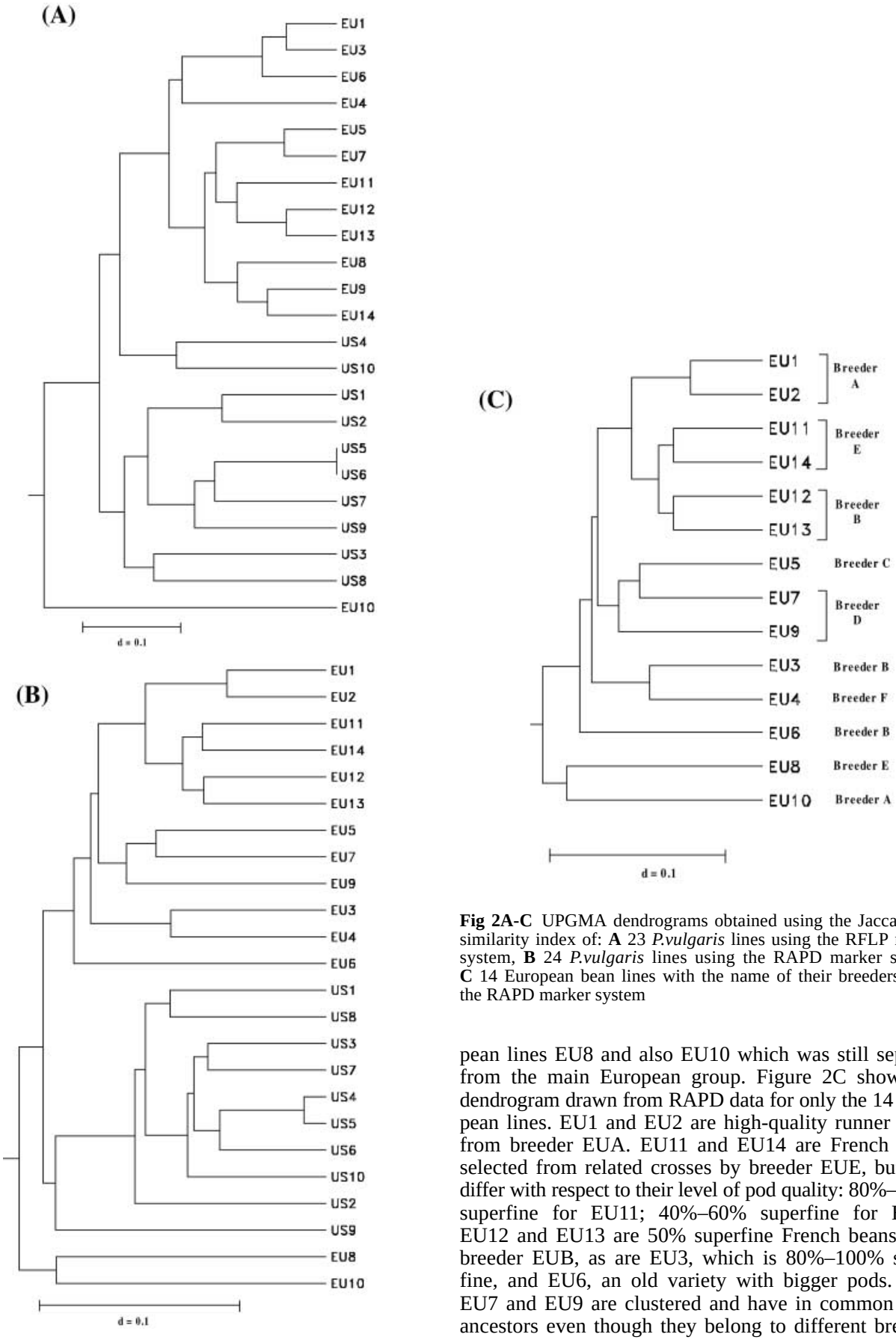

Fig 2A-C UPGMA dendrograms obtained using the Jaccard dissimilarity index of: **A** 23 *P.vulgaris* lines using the RFLP marker system, **B** 24 *P.vulgaris* lines using the RAPD marker system, **C** 14 European bean lines with the name of their breeders using the RAPD marker system

pean lines EU8 and also EU10 which was still separate from the main European group. Figure 2C shows the dendrogram drawn from RAPD data for only the 14 European lines. EU1 and EU2 are high-quality runner beans from breeder EUA. EU11 and EU14 are French beans selected from related crosses by breeder EUE, but they differ with respect to their level of pod quality: 80%–100% superfine for EU11; 40%–60% superfine for EU14. EU12 and EU13 are 50% superfine French beans from breeder EUB, as are EU3, which is 80%–100% superfine, and EU6, an old variety with bigger pods. EU5, EU7 and EU9 are clustered and have in common some ancestors even though they belong to different breeders

(EUC and EUD). EU4 from breeder EUF belongs to the same industrial type as EU3 from breeder EUB. On this dendrogram, EU8 from breeder EUE is clustered with EU10 from breeder EUA but excluded from the main EU group as on Fig. 2B.

Discussion

The first purpose of the study was to compare the genetic diversity within and among European and North American bean cultivars. Estimation of the genetic distance between genotypes is important information for breeders in order to select the genotypes to cross and to control genetic diversity and germplasm resources. Moreover, the defence of breeder rights necessitates an accurate description of germplasm in order to identify essentially derived varieties or fraudulently multiplicated lines.

Use of ISSR

Only one out of the five ISSR primers tested was efficient in generating multiple band profiles, but it did not permit all the different bean lines to be distinguished. This may be due to the abundance of priming sites in the bean genome or to the characteristics of the primers. Moreover, a level of polymorphism of 60% was obtained with only one primer. Prevost and Wilkinson (1999) showed that the combined use of only two ISSR primers out of the four tested allowed the diagnosis of 34 potato cultivars. This strategy applied to bean did not distinguish all of the lines. Nevertheless, the number of ISSR primers tested here is too low to conclude that the ISSR technique is inefficient.

Use of DAMD-PCR

Our observations suggest that the DAMD-PCR was sensitive in detecting genetic variation between the *P.vulgaris* lines. In 1997, Zhou et al. showed that the DAMD-PCR produced RFLP probes that could be useful in breeding programmes to identify rice cultivars. We show here that DAMD-PCR primers can be derived from minisatellite sequences and used to generate polymorphic amplification profiles between bean varieties. However, the use of these primers did not enable us to characterise each genotype tested. The level of polymorphism was lower (40%) than that revealed by RFLP profiles. The direct amplification of minisatellite-region DNA led in fact to the amplification of the inter-minisatellite region; the sequencing of 3 amplified products showed that for 2 of them, there was no minisatellite repeat in their sequences except for the minisatellite core sequence present in opposite orientations at both ends of the fragment (data not shown). Only 1 of the sequenced products contained 37 minisatellite repeats, confirming that the minisatellite sequence is repeated in clusters in the bean genome. The low number of amplified products is probably due to the distances between the clusters being too high for the DNA polymerase to achieve amplification between the opposite primers. Zhou et al. (1997) have already shown that the DAMD-PCR alone may be limited in its ability to detect genetic variation among cultivated rice varieties due to the low number of loci amplified and to the narrow genetic base among rice cultivars coming from a single breeding programme.

We had little data dealing with the molecular characterisation of bean at our disposal and therefore chose to use molecular techniques which do not involve intensive preliminary work, such as screening probes or microsatellites sequences. The minisatellite RFLP probe and its sequence was available in our laboratory from previous work, thereby facilitating the design of primers for DAMD-PCR. RAPD and ISSR use PCR primers which can be synthesised without specific information concerning the genome studied. Ultimately, only the RFLP and RAPD markers provided us with enough information to deal with the analyses of bean lines relationships.

Analysis of the dendrograms

RFLP and RAPD results were used to analyse the genetic distances and to draw dendrograms. In Fig. 2A and 2B, dendograms obtained from cluster analysis, all lines could be assigned to their specific main clusters, in agreement with the available data. Both analyses resulted in the separation of the 24 bean lines into two main pools corresponding to both sources of germplasm: on the first hand, European bean accessions and on the other hand, North American creation lines. Only line EU10, a European creation, was not related to the European germplasm whatever analysis technique used. On the basis of breeder data this European line is related to American US1, US7 and US8. Differences between marker techniques in grouping geneticall-y closed related lines could be due to the lower number of markers generated here by RFLP than by RAPD.

The minisatellite/*Eco*RV combination detected the highest level of polymorphism. A great advantage of the minisatellite probe tested in our work is that only one enzyme/probe combination was able to fingerprint all the bean lines tested, except for US5 and US6. This tool should be useful for making a fast comparison of many different lines, for example to determine the most related lines.

On dendrogram 2C, RAPD clustered EU1 and EU2, both of which belong to breeder A; EU11 and EU14 come from breeder E programme; EU12 and EU 13 from breeder B (Table 1). In the other cases (cluster EU5, EU7, EU9 or cluster EU3, EU4), the varieties belong to different breeders but some data (personal communication) allowed us to determine that they share some ancestors in their close or distant genealogy. According to the breeder data, EU8 belongs to European germplasm but is clustered with EU10. Nevertheless, from the standpoint of the breeder, EU10 is closely tied to US1 and in a more distant manner to US8. With respect to EU8, no explanation could be found for its clustering, but it is possible to suppose that an introduction of materials of U.S. origin occurred during breeding. The same correlation cannot be realised for the

American lines as there are nine breeders for ten lines and we lack information concerning their ancestral origins.

The data associated with the European lines showed that it is possible to relate the clustering to germplasm origin since there was a tendency for accessions obtained from a particular breeder to cluster. Cattan-Toupance et al. (1998) showed that structuration using molecular markers may provide information on the history and biology of plant populations, but it does not necessarily reflect what may be observed with respect to agronomic traits. The selection process leads to an accumulation of the best alleles for the traits under selection. In this way, one could expect a high similarity between genotypes of equivalent value, such as industrial bean types. However, RAPDs are dispersed throughout the genome and the genetic structure depends on their association, which is influenced by the breeder only in the region under selection pressure. In the other loci, the organisation is subjected to random genetic drift. The recombination which occurs during meiosis should concern blocks of markers, as has been proposed in the case of *Petunia* (Peltier et al. 1994). In such a scheme, a breeder has at his disposal some arrangements of alleles which could recombine but this will lead mostly to limited block rearrangements as the number of recombinations at the chromosome level is limited. Therefore, one should consider that each breeder starting a breeding programme should create his own ''signature'' in term of organisation of allelic blocks. In the case of EU5 EU7 EU9, and EU3 EU4, some exchanges of material between breeders from different countries have occurred. Most of the time, these facts are difficult to prove due to competition between breeders and confidentiality of data practised by the same breeders. The individuals resulting from such crosses will exhibit the signature in term of allelic blocks of each breeder from his genitors, and this organisation will persist more or less depending on the kind of subsequent crosses. For example, backcrosses will rapidly eliminate the genetic background of a parent when a point introgression is wished. In order to assess this hypothesis, it would be necessary to study diversity with genetically mapped markers. Such a work will be difficult to manage using markers with only two allelic forms. In such a way, microsatellite markers exhibiting a high allelic diversity should be well adapted as they allow the breeder to compare the allelic organisation of blocks in different lines coming from different germplasms. These studies should allow the detection of derived varieties.

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