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Inter simple sequence repeat (ISSR) markers as a tool for the assessment of both genetic diversity and gene pool origin in common bean (*Phaseolus vulgaris* L.)

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Summary

In this study, we report the use of ISSR to assess genetic diversity and to determine the relationships among ten cultivars of common bean developed in Argentina and three materials from France. ISSR markers resolved two major groups corresponding to the Andean and Mesoamerican gene pools of common bean. We compared the results of previous analysis, performed with RAPD markers (Galván et al., 2001), with the results generated by means of ISSR. It appears that ISSR are better tools than RAPDs to identify beans by gene pool of origin though they did not revealed as many differences between individuals as RAPDs.

Introduction

Common bean (*Phaseolus vulgaris* L., Fabaceae) is an annual, diploid (2n=2x=22) species derived from wild ancestors distributed from northern Mexico to northwestern Argentina. Gepts et al. (1986) described two distinct gene pools of cultivated beans as the result of different domestication events that occurred in the Andes (Peru and Argentina) and in Middle America (Mexico, Central America and Colombia).

Genetic variability in domesticated common bean is low. This is due in part to the fact that most cultivars within each gene pool were developed from a relatively narrow genetic base (Voysest et al., 1994). The low variability might also be due to the existence of genetic incompatibility factors that prevent the combination between germplasms of the two centers of origin (Singh & Gutiérrez, 1984; Gepts & Bliss, 1985). Therefore, one of the goals of actual breeding programs in *Phaseolus* is to use less related genetic resources like representatives from the primary, secondary, tertiary and quaternary gene pools of *Phaseolus* (Singh, 2001).

Among molecular markers RAPDs are the most widely applied most probably because they do not require the knowledge of genomic sequences and also because the protocol is relatively simple, rapid and cost effective (Fofana et al., 1997, Johns et al., 1997; Duarte et al., 1999; Maciel et al., 2001). RAPD markers have been used to study diversity among common beans and also to discriminate accessions based on their gene pool of origin with different levels of success (Fofana et al., 1997; Johns et al., 1997; Briand et al., 1998; Galván et al., 2001; Maciel et al., 2001).

Simple sequence repeats (SSR), also known as microsatellites, are tandem repeat motifs composed of one to six nucleotides, which are ubiquitous, abundant and highly polymorphic in all eukaryotic genomes (Tautz & Renz, 1984). Hamman et al. (1995) fingerprinted the genome of beans by means of SSR suggesting that these sequences are also ubiquitous among the genome of common beans. Inter-simple

Table 1. Characteristics and pedigree of 13 accessions of common bean (*Phaseolus vulgaris* L.) from France and Argentina

Seed type	Name	Status	Pedigree	Line (origin)[a]
Black	**Cambá**	Cultivar	BAT 304 × XAN 196	Cegro 1/17 (Salta, Argentina)
Black	**Camilo**	Cultivar	XAN 19 × ICA PIJAO	XAN 201 (CIAT)
Black	**Nag12**	Cultivar	[(G03664 × G02045) × (G04792 × G05694)] × [(G04495 × G05431) × (G03645 × G05481)]	NAG12 (CIAT)
White	**Perla**	Cultivar	Not available*	L-190 (Leales, Argentina)
White	**Paloma**	Cultivar	Not available*	Alule 91 (INTA- Salta)
Light Red Kidney	**Coral**	Cultivar	G4615 × XAN 43	AFR 285 (CIAT)
Carioca	**Paulina**	Cultivar	Unknown	CNF 5542 (EMBRAPA)
Navy	**Canela**	Cultivar	G18402 × (IN00017 × sel72)	PAN 194 (CIAT)
Cranberry	**Overito**	Cultivar	G5167 × BAT 1387	SUG 18 (CIAT)
White	**Alubia**	Cultivar	Individual selection in local population	L–7 (INTA- Salta)
White	**Pactol**	Cultivar	Not available	France (OBS)
White	**PF10**	Landrace	Not available	France (OBS)
White	**PF13**	Landrace	Not available	France (OBS)

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* Cultivars that present Alubia (Andean gene pool) in their background (S. Medina, personal communication).
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sequence repeat (ISSR) is a novel PCR technique that uses repeat-anchored or non-anchored primers to amplify DNA sequences between two inverted SSR (Zietkiewicz et al., 1994). ISSR markers do not require a prior knowledge of the SSR targets sequences, are highly reproducible due to their primer length and to the high stringency achieved by the annealing temperature and were found to provide highly polymorphic fingerprints (Zietkiewicz et al., 1994; Kojima et al., 1998; Bornet & Branchard, 2001). ISSR markers have been successfully used for the assessment of genetic diversity in corn (Kantety et al., 1995), for cultivar identification in oilseed rape and potatoes (Charters et al., 1996, Bornet et al., 2002), for mapping of plant chromosomes (Kojima et al., 1998) and for linkage to a specific gene (Akagi et al., 1996a).

In this study, we describe the use of ISSR to assess genetic diversity among common beans, in doing so we found that ISSR clustered them based on their gene pool of origin more accurately than RAPD markers.

Materials and methods

Thirteen accessions of *Phaseolus vulgaris*, French cultivar (Pactol), two landraces (PF10, PF13) from France (Organisation Bretonne de Sélection, Plougoulm – France) and the ten most important commercial cultivars developed in the northern region of Argentina were analyzed. Pedigree and other characteristics are provided in Table 1.

Genomic DNA was isolated by a CTAB (Cetyl-TrimethylAmmonium Bromide) protocol Bornet & Branchard (2001). PCR was performed by means of twenty-three anchored or non-anchored primers. Nine

Table 2. ISSR polymorphic primer sequences used for analysis of 13 accessions of *Phaseolus vulgaris* with primer annealing temperatures, number of bands amplified and number of polymorphic bands amplified

N°	Primer sequence (5'–3')	Annealing temp. (°C)	Number of bands amplified	Number of polymorphic bands
1	$GAG(CAA)_5$	52	10	5
4	$CTC(GT)_8$	54	9	3
6	$(AG)_8CG$	54	7	2
7	$(AG)_8TG$	52	12	5
11	$(AG)_8$	49	6	2
13	$(CCA)_5$	55	6	4
16	$(AC)_8$	57	11	5
19	$(GCC)_5$	66	10	5
23	$(GAA)_5$	54	4	2

Table 3. Results of experiments performed among accessions of *Phaseolus vulgaris* with RAPD and ISSR markers

	RAPD*	ISSR
Total number of primers	16	23
Number of polymorphic primers	4	9
Total number of bands amplified from polymorphic primers	27	75
Size range of amplified products	250–800 bp	300–2400 bp
Average number of bands per polymorphic primer	7	8
Total number of polymorphic bands identified	17	33
Average number of polymorphic bands per polymorphic primer	4	4
Percentage of polymorphic primers	25%	40%

* From Galván et al. (2001).

oligonucleotides were selected based on the number and level of polymorphism of the amplified bands. Sequences and specific annealing temperatures of each primer (Eurogentec, Belgium) are shown in Table 2. The PCR mixture was made up by: an amount close to 12 ng of template DNA, 100 pM of primer, 200 μm of each dNTP, 10X buffer 750 mM Tris-HCl pH8.8 (at 25 °C), 200 mM $(NH_4)_2SO_4$0.1% (v/v) Tween 20, and 1.25 unit of Goldstar red DNA polymerase (Eurogentec, Belgium) in a total volume of 25 μl. The thermocycler RoboCycler (Stratagene) was programmed as follows: one min at 94 °C and then 30 cycles, each of 94 °C, 1 min, hybridization temperature 1 min and 72 °C, 4 min. A final extension at 72 °C for 7 min ended the reaction. Each reaction was repeated at least twice and three plants of each accession were analyzed as replicates of the cultivars tested. PCR products were electrophoresed in 2% agarose gels.

DNA fragment size was estimated by comparing the DNA bands with a Smart Ladder marker (Eurogentec, Belgium). The bands were recorded as present (1) or absent (0) and assembled in a data matrix table. Pairwise comparisons were calculated using the Jaccard coefficient (Sneath & Sokal, 1973). The similarity values found were utilized to generate a dendrogram via the Unweighted Pair Group Method (UPGMA). The correlation between the similarity and the cophenetic matrices for the clusters was computed. NTSYSpc version 2.0 (Rohlf, 1998) was used to perform all the analysis.

Results

Clearly detectable amplified ISSR ranged from 300 to 2400 bp in size. The average number of clear bands generated per polymorphic primer were 8, with a maximum of 12 for primer 7. Among the primers selected, the 5'-anchored and the 3'-anchored were, based on the number of amplified bands, the most informative since the fingerprints of common beans had the higher number of amplified bands which were between 7 and 12. The average of polymorphic markers generated by non-anchored, 5'-anchored, and 3'-anchored primers were 3.6, 4 and 3.5, respectively. A summary of all the amplification reactions is presented in Table 3. Primers 1, 4, 7, 16 and 19 provided the clearest banding pattern including a large number of scorable bands that were among those selected to generate the dendrogram. Although, most of the bands generated with primer 7 were monomorphic, some diversity between and within cultivars was observed. A band of 1500 bp was present only in cultivars Camba, Canela, Camilo, Nag12 and Paulina. Furthermore, a second band of 800 bp was amplified by the same primer in the presence of template DNA from several cultivars like Perla, Coral, Paloma, Overito, Pactol and landraces PF10 and PF13. Although it appeared that both bands efficiently discriminate between gene pools of origin, the 800 bp one was present only in some individuals of cultivars Perla and Paloma and was absent in PCR reactions containing genomic DNA from cultivar Alubia.

Although eighteen oligonucleotides generated amplification products, only 9 generated polymorphic markers (Table 2). A total of 75 bands were generated by 9 primers and 33 (44%) were found to be polymorphic among all the plants tested. Dinucleotide motif primers resulted in the amplification of a total of 45 bands among them only 17 were polymorphic (38%). Trinucleotide motif primers primed the amplification of 30 bands being the polymorphic ones only 16 (53%).

Cluster analysis generated a dendrogram (available upon request) with two main branches that clustered individuals that share the same gene pool of origin. Branch 'A' included all those cultivars whose genomic background is mainly from parent plants collected from Mesoamerica (cultivars Cambá, Canela, Nag12, Camilo and Paulina) and cluster 'B' included cultivars with parents from the Andean gene pool (cultivars Perla, Overito, Coral, Paloma, Alubia and French cultivar Pactol and landraces PF10 and PF13).

The Jaccard's similarity coefficient ranged from 0.23 to 1.00 with a mean of 0.8. The goodness of fit of the UPGMA dendrogram was highly significant ($r = 0.96$; $p < 0.0001$). At the 0.23 phenon level, two distinct clusters (labeled 'A' and 'B') were apparent. 'A' included 38% of the accessions and encompassed similarity values ranging from 0.67 to 1.00. Cluster 'B' included 62% of the accessions and corresponded to a similarity coefficient range of 0.84 to 1.00.

Cultivars Camilo and Cambá have Mesoamerican cultivars in their pedigrees (XAN19 × ICA PIJAO and BAT 304 × XAN 196, respectively) and this was reflected by their position in the dendrogram that was generated by the genetic data. Similarly, both cv. Perla and cv. Paloma have parents with Andean pedigree and they also were grouped together. In order to compare this analysis with previous clustering characterization performed with RAPD markers (Galván et al., 2001), we generated another dendrogram that included only American cultivars (data not shown) and the association between accessions was similar to the relationships shown in the dendrogram generated by means of ISSR.

The French accessions were clustered in group B among the Andean gene pool beans and they appeared to have more than 72% genetic similarity with the American Andean beans suggesting that they had most probably an Andean origin. According to phaseolin patterns, only part of the American germplasms is represented in Europe and most are of Andean origin (Gepts & Bliss, 1988). The ISSR markers showed that French bean accessions have a lower level of genetic variability than the Latin-American beans analyzed.

Discussion

Although a higher number of primers need to be analyzed our results suggest that dinucleotide and trinucleotide SSR occur at high frequencies along common bean genomes. In previous surveys, in rice, maize and soybean, it also was found that di and trinucleotide SSR occur along the genomes at higher frequencies than tetranucleotide repeats (Rongwen et al., 1995; Akagi et al., 1996b; Chin et al., 1996). Yu et al. (1999) reported that dinucleotide SSRs are more important sources for developing polymorphic SSR markers in common bean. However, in our experiments trinucleotide motif primers amplified a higher percentage of polymorphic bands in comparison with dinucleotide motifs. Fifty-three percent of the ISSR markers generated with trinucleotide motif primers were polymorphic, while only 38 percent of the bands amplified by dinucleotide motif primers were polymorphic. Kantety et al. (1995) found similar results in maize. Therefore, ISSR-PCR products generated with trinucleotide motif primers, should be useful markers for analyses of common bean germplasms.

A previous study has considered the utility of ISSR markers for the analysis of *Phaseolus* germplasm (Métais et al., 2000). Only one out of the five ISSR primers tested was efficient in generating multiple band profiles, which was not enough to distinguish the commercial lines analyzed. However, the similarity coefficient obtained in this work, by means of ISSR markers, was lower between the Mesoamerican and Andean beans. As a consequence of this, the consistency with which accessions were classified indicated that ISSR analysis was very robust for identifying beans by their gene pool of origin. The Andean and Mesoamerican gene pools seem to have specific SSR-regions and because of this they are useful markers to discriminate between different gene pools of origin. Ecogeographical and cultural isolation, coupled with the inbreeding nature of the species, would be conducive to the maintenance of distinct genes and morphologies on each gene pool (Mumba & Galwey, 1998). Therefore ISSR might be useful markers to provide accurate and reliable evidences of the earlier divergence of the Mesoamerican and Andean gene pools.

French bean accessions have a lower level of genetic variability than the Latin-American beans analyzed. This might be due to the low number of French cultivars included in our study or to the low genetic base from which most of them were generated. Since a wider genetic base may provide more useful genes to be used by breeders, research programs aimed at transferring desirable traits between gene pools should be encouraged.

The fact that the association of accessions on the basis of these ISSR data fitted with pedigree information suggests that this set of ISSRs is providing reliable

information of the genome for the identification of germplasms. We conclude that ISSR may constitute appropriate genetic markers to identify beans by gene pool of origin.

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