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Structure and level of genetic diversity in various bean types evidenced with microsatellite markers isolated from a genomic enriched library

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Abstract We have constructed a common bean genomic library enriched for microsatellite motifs (ATA), (CA), (CAC) and (GA). After screening, 60% of the clones selected from the library enriched for the (ATA) repeat contained microsatellites versus 21% of the clones from the library enriched for (GA) (CA) and (CAC) repeats. Fifteen primer pairs have been developed allowing for the amplification of SSR loci. We have evaluated the genetic diversity of these loci between 45 different bean lines belonging to nine various quality types. A total of 81 alleles were detected at the 15 microsatellite loci with an average of 5.3 alleles per locus. We have investigated the origin of allelic size polymorphism at the locus PvATA20 in which the number of repeats ranges from 24 to 85. We have related these large differences in repeat number to unequal crossing-over between repeated DNA regions. The diversity analysis revealed contrasted levels of variability according to the bean type. The lower level was evidenced for the very fine French bean, showing the effect of breeders intensive selection.

Keywords Genetic diversity · SSR-enriched libraries · Common bean · Germplasm · *Phaseolus*

Introduction

Simple sequence repeats (SSRs) or microsatellites were first described by Hamada et al. (1982) as short tandemly repeated DNA sequences (2–5 bp in length) widely spread throughout the nuclear genome of eucaryotes (Tautz and Renz 1984). In plant genomes, the AT/TA repeats are predominant but present at relatively low frequency compared to AC/TG repeats in humans (Powell et al. 1996). The repeats are divided into perfect repeats (without interruption), imperfect repeats (interrupted by non-repeated bases) and compound repeats (two or more repeat runs adjacent to each other). Their isolation is difficult due to the palindromic nature of these sequences. The variation in the number of repeats for a defined locus among different genotypes is easily detected by PCR, leading to highly informative DNA markers. Because of their abundance, high polymorphism between individuals within populations or closely related genotypes and their multiallelic and codominant nature allowing the genotype establishment of individuals, microsatellite markers have been used for individual identification, diversity analysis (Powell et al. 1996) and evolutionary and population structure studies of related species. In plants, they have been successfully used to map genomes of cereal species such as rice (Davierwala et al. 2000) or soybean (Rongwen et al. 1995), to fingerprint cultivars and breeding lines of barley (Pillen et al. 2000), wheat (Prasad et al. 2000), rapeseed, chickpea and sugar beet, to detect genetic diversity and carry out phylogenetic analysis on *Cucumis* (Danin-Poleg et al. 2001).

Previous studies have shown that common bean lines obtained from a particular breeder have a tendency to cluster. This clustering was related to the origin of genetic diversity of each breeder's germplasm (Métais et al. 2000). This work was carried out using RAPD markers. The number of allelic states, revealed with this kind of present/absent markers, sets the problem of homoplasy as the absence could be related to various molecular events. This could also lead to misinterpretation concerning the genetic structure and the origin of germplasm. Microsatellite markers exhibiting higher allelic polymorphism should give more accurate data, although the problem of homoplasy is not completely avoided. Stockton and Gepts (1994) have shown that the microsatellite sequence $(GACA)_4$ used as a probe was useful to ascertain relationships at the species and subspecies levels of *Phaseolus* lines. More recently, Yu et al. (1999) integrated 15 SSR loci into the bean molecular map.

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In the present work, using an adaptation of the Fischer and Bachmann (1998) protocol, we constructed a highly enriched bean-SSR genomic library. To-date 15 primer pairs have been developed allowing us to amplify STMS markers. We analysed the level of diversity on 45 commercial bean lines split up into nine different quality types. This allowed us to point out the major variations in term of diversity in the different groups. Moreover, we investigated the origin of allelic polymorphism at a locus exhibiting very high repeat number variations.

Materials and methods

Plant material and DNA preparation

The *Phaseolus vulgaris* L. lines used in this work are listed in Table 1. Young leaves of each line were harvested and DNA was extracted according to a method adapted from Dellaporta et al. (1983).

Construction of an enriched microsatellite library

A bean enriched microsatellite library was constructed according to the Fischer and Bachmann (1998) procedure with slight modifications. One microgram of genomic DNA was hydrolysed with ten units of *Mse*I. The restriction fragments were adapted with a *Mse*I adapter (hybrid of a 16-mer 5′-GACGATTGATCATGAG-3′ and a 18-mer 5′-TACTCATGATCAATCGTC-3′) containing a *Bcl*I restriction site. Enrichment for microsatellites was carried out by hybridisation to biotinylated microsatellite oligonucleotides using, on one hand, $(ATA)_8$ and, on the other hand, a mix of $(CAC)_8$, $(CA)_{12}$ and $(GA)_{12}$. The hybrids were then bound to streptavidin-coated magnetic beads (Promega). Beads were washed twice at low- stringency conditions (2 × SSC), twice at higher-stringency conditions (1 × SSC) and the last two washes at Tm-5 °C [37 °C for $(ATA)_8$, 57 °C for the mix of $(CAC)_8$, $(CA)_{12}$ and $(GA)_{12}$]. The eluted fragments were amplified by PCR using the 16-mer adapter primer. A second round of enrichment was carried out using the amplification products obtained from the PCR reaction. The enriched DNA was then hydrolysed with *Bcl*I and ligated into a *Bam*HI-linearized and de-phosphorylated pUC 18 vector (Appligene Oncor France). Competent *Escherichia coli* DH5α cells were

Table 1 Description of *P. vulgaris* lines used in the determination of allelic variation at 15 microsatellite loci

No	Variety	Breeder	Type
1	ANDROS	Nunhems	
2	BACCARA	Bakker	
3	BOOSTER	Vilmorin	
4	CABRI	Clause	
5	CAPITOLE	Clause	
6	INTER	Vilmorin	French bean (for processing) (1)
7	LABEL	Vilmorin	very fine (sieve 5/6.5 mm)
8	LONGIO	Sluis Groot	
9	MASTER	Vilmorin	
10	PANAMA	Royal Sluis	
11	SKIPPER	Vilmorin	
12	TWIGGY	Pop Vriend	
13	ARRAS	Vilmorin/Nickerson	
14	CASTEL	Vilmorin	
15	FORUM	Caillard	
16	LAKE SUPERIOR	Keystone	
17	MASAI	Sluis Groot	
18	POLDER	Vilmorin	French bean (for processing) (2)
19	ROMEO	Novartis	(sieve 6.5/12 mm)
20	SONATE	Pop Vriend	
21	STRIKE	Asgrow	
22	TENDERETTE	Charter	
23	TUF	Royal Sluis	
24	GOLDEN SAND	Ferry morse	Wax bean (3)
25	PI 165426		Black bean mexican accession (A)
26	FIN DE BAGNOLS	Public ownership	
27	MORGANE	Clause	Stringy French bean (4)
28	TRIOMPHE DE FARCY	Public ownership	
29	ARAMIS	Sluis Groot	
30	ROCBRUN	Sluis Groot	
31	RUDY	Tezier	Snap bean (5)
32	TALISMAN	Caillard	
33	TOTEM	Sluis Groot	
34	ROCQUENCOURT	Vilmorin	
35	CLOVIS	Tezier	Market snap bean (6)
36	SUNRAY	Yates	
37	ROMA 2	Sluis Groot	Flat bush bean (7)
38	FLAGRANO	Holland select	
39	FLAMATA	Royal Sluis	Kidney bean (8)
40	VERNEL	Vilmorin	
41	BIG BORLOTTO	Varietal type	
42	EXTASY	Sluis Groot	
43	MOGEX	Vilmorin	Butter bean (9)
44	PB 6246	Vilmorin	
45	RED KIDNEY	Varietal type	

transformed with recombinant plasmids and plated onto LB-agar containing ampicillin and X-gal. White colonies were cultivated in LB-liquid medium in 96-well microplates and stored at –80 °C in 50% glycerol.

Selection of clones containing microsatellite sequences and primer design

PCR amplifications were carried out on DNA from a 1-µl culture of each recombinant clone, using universal primers flanking the cloning site. Amplification products were separated by electrophoresis on 1.4% agarose in TBE buffer, stained with ethidium bromide and transferred according to Southern (1975) onto a nylon membrane. Hybridisation experiments were conducted using end-labelled γ-[^{32}P]-ATP (111 TBq/mmol; Isotopchim) oligonucleotides as probes. Positive clones were further sequenced using ABI Prism BigDye terminator cycle sequencing ready reaction kits (PE Biosystems) on a ABI Prism 310 DNA Sequencer. PCR primers were designed from the sequences flanking the microsatellite and synthesised by MWG Biotech.

PCR amplification of the microsatellite loci

Each amplification reaction contained 25 µl consisting of 1 × reaction buffer (Promega), 2 mM of $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each primer, 0.75 U of *Taq* DNA polymerase (Promega) and 25 ng of genomic DNA. The reaction mixtures were overlayed with 30 µl of mineral oil. Amplifications were performed in a PTC-100 MJ Research Thermal Cycler programmed for 30 cycles of 30 s at 94 °C, 30 s at Tm and 30 s at 72 °C.

The PCR products were separated onto 6% polyacrylamide denaturing gels run at 100 W for 2.5 h in 1 × TBE buffer and visualised by silver staining (Briard et al. 2000).

Data analysis

Microsatellite data of the 15 specific loci were scored for all genotypes, on the basis of the presence (1) or absence (0) of specific alleles. We computed a Jaccard dissimilarity index (Jaccard 1908) matrix. These data were subjected to cluster analysis with the UPGMA algorithm using PHYLIP 3.5 (Felsenstein 1989).

Allelic polymorphic information content (PIC) was calculated using the following formula: $PIC = 1 - \Sigma(P_i)^2$ where P_i is the proportion of the population carrying the i^{th} allele, calculated for each microsatellite locus (Botstein et al. 1980).

We computed the mean allelic diversity per type by dividing the number of different alleles among loci in a type by the number of lines studied in this type.

Cloning of microsatellite alleles

The silver-stained amplified bands were overlayed with 50 µl of TE10/0.1 (Tris-HCl 10 mM, EDTA 0.1 mM pH 8) then scratched from the polyacrylamide gel and transferred to a microtube. After 15 min at 37 °C and a brief centrifugation (5 min at 14,000 rpm), 5 µl of supernatant were used to further amplify the alleles of interest employing the same PCR conditions as described above. The amplified products were then cloned using the pGEM-T vector system (Promega). After selection of the recombinant clones, the DNA fragments were sequenced as described above.

Results

Library construction and screening

We used the method of Fischer and Bachmann (1998) with slight modifications concerning adapter design. We have constructed a bean DNA library enriched for four microsatellite motifs, $(ATA)_8$ $(CA)_{12}$ $(GA)_{12}$ and $(CAC)_8$. Experiments were carried out separately for the ATA motif and the CA, GA and CAC motifs; 1,200 recombinant clones (half of the whole library) were individualised from both enrichments.

Out of 336 recombinant clones coming from the ATA-enriched library, 201 (60%) hybridised with labelled $(ATA)_8$ oligonucleotides. Out of the 35 sequenced clones, 29 (83%) contained microsatellite sequences, 3 (8.5%) contained minisatellite sequences and 3 (8.5%) contained no repeated sequences. Six out of the 29 inserts containing microsatellites were redundant. Out of the 23 microsatellite sequences isolated, 19 (83%) were perfect repeats, 4 (17%) were compound repeats. Finally 16 inserts had suitable flanking regions for primer design.

We characterised 112 recombinant clones coming from a (CA), (GA) and (CAC) enriched library. Hybridisation experiments were performed on the clones successively with CA, GA and CAC labelled oligonucleotides. Twenty four (21%) inserts were revealed. Out of the 24 clones sequenced, 13 (54%) contained microsatellite sequences, 1 (4%) contained minisatellite sequences

Table 2 Polymorphic microsatellite markers identified in this study. (1) *P. vulgaris* hydroxyproline-rich glycoprotein precurseur gene EMBL: PV18791; (2) *P. vulgaris* NADP-dependent malic enzyme EMBL: PVME1G; (3) Bng91/R common bean genomic clone EMBL AZ301561; (4) *P. vulgaris* phaseolin G-box binding protein PG2 EMBL PV18349

Locus	Repeated motif	Tm (°C)	Expected size (bp)	No. of alleles	PIC
PvATA2	(TAA)20 (GAA)10	56	129	3	0.20
PvATA3	(TAA)9	56	133	3	0.27
PvATA4	(TTA)8	52	154	2	0.12
PvATA5	(TTN)42 N=A, G or T	58	191	2	0.16
PvATA6	(TAT)19	54	143	10	0.66
PvATA7	(ATA)11	50	136	6	0.38
PvATA9	(AAT)12	62	191	2	0.43
PvATA10	(ATT)11	48	106	4	0.54
PvATA13	(TTA)68	52	315	9	0.65
PvATA16	(TAA)14	52	155	5	0.64
PvATA20	(TAA)72	48	293	9	0.69
PvHRG(1)	(AT)22	56	124	11	0.57
PvME1G(2)	(AT)12	56	169	9	0.72
PvBN(3)	(TAT)9	56	158	4	0.31
PvPG2(4)	(CGG)8	58	138	2	0.32

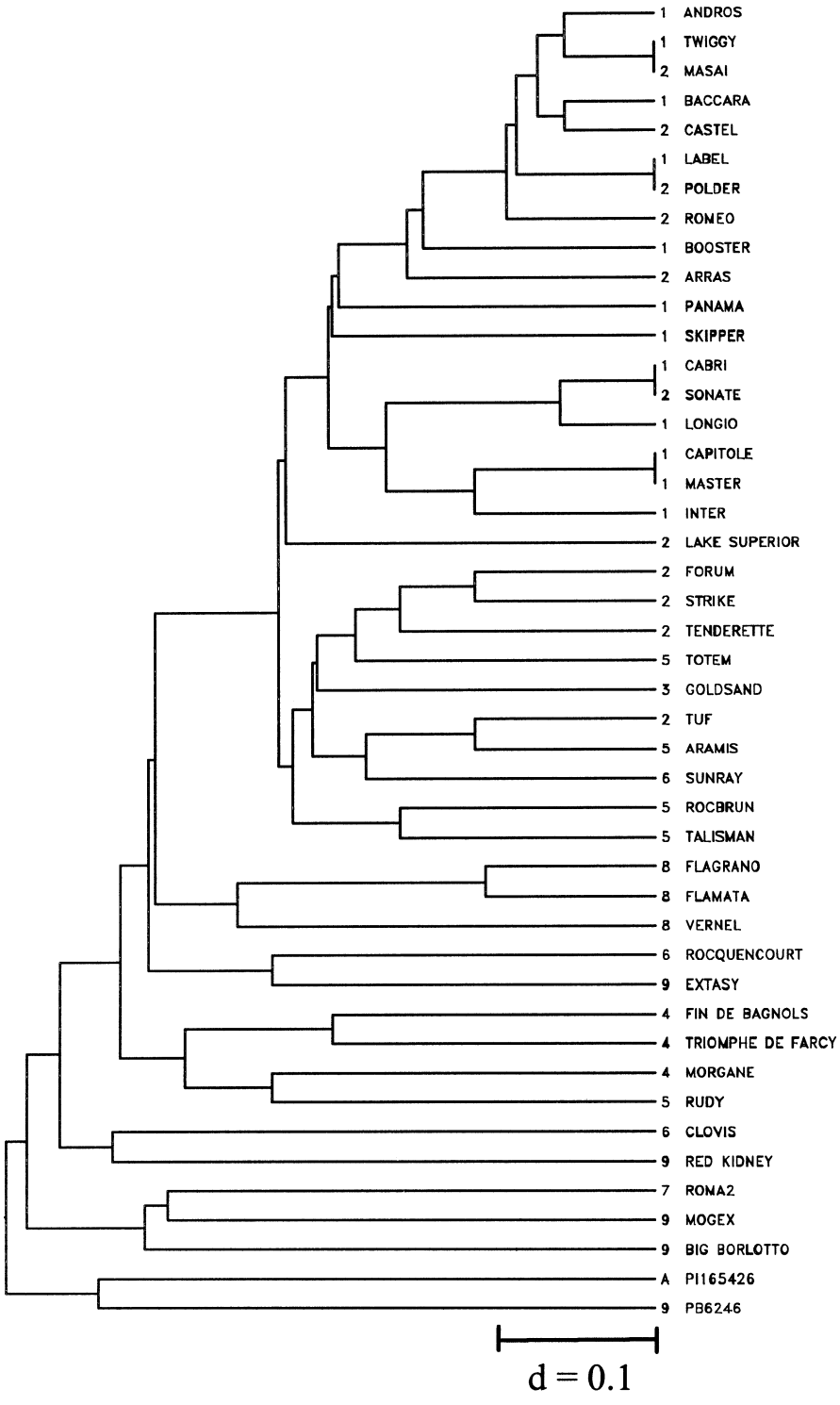

Fig. 1 UPGMA dendrogram obtained using the Jaccard dissimilarity index of 45 bean lines with data from 15 microsatellite loci. The *number* at the left indicates the quality type of each line

and 10 (42%) contained no repeated sequences. Four out of the 13 inserts containing microsatellites were redundant. Out of the nine microsatellite sequences isolated, seven (77.5%) were perfect repeats, two (22.5%) were compound repeats. Finally nine inserts had suitable flanking regions for primer design.

Ultimately we used 25 primer pairs to amplify DNA from bean lines. Eleven led to polymorphic readable electrophoresis patterns and corresponded to TTA and ATA repeats. To-date none of the microsatellite primer pairs issuing from the (CA) (GA) and (CAC) enriched library led to clearly readable patterns.

Beside this sequencing work, all EMBL published bean DNA sequences (June 2000) were screened for microsatellite repeats. Four primers pairs were selected to amplify two dinucleotide and two trinucleotide repeats.

Characterisation of selected microsatellite sequences

Fifteen primer pairs were selected to assess polymorphism. All the amplified products obtained from the line Fin de Bagnols were in the expected size range compared to products amplified from DNA insert sequences issuing from our library. Among the 45 commercial bean lines characterised, the repeat lengths ranged from 24 to 216 bp and the size of alleles ranged from 103 bp to 320 bp. The size variation among the alleles of a single locus tested on the 45 line DNAs ranged between 1 and 170 bp. PvATA20 amplified alleles showed the higher size polymorphism with a difference of 170 bp between the extreme alleles.

A total of 81 alleles were detected at the 15 microsatellite loci (Table 2). Each primer pair amplifies one main product. Therefore all the bean lines are homozygous with a single allele length at each studied locus. In two cases (PvATA9 and PvATA13) no product was amplified for several lines, and were scored as null-alleles. The number of alleles per microsatellite locus ranged from 2 to 11. On average, 5.3 alleles were detected per locus. The PIC values were also estimated ranging from 0.12 to 0.72 with an average of 0.44.

Genetic relatedness

The 45 lines studied belong to ten different groups based on their quality types, as shown in Table 1. We have at our disposal accurate data concerning phenotype and quality for all lines. SSR data were used for phenetic analysis among the 45 bean lines. The lines were then clustered with UPGMA. Genetic distances among the lines ranged from 0 for the similar lines (Twiggy-Masaï) to 1.00 for the most diverse ones (Fin de Bagnols-PB 6246). The dendrogram (Fig. 1) obtained from data using the 15 loci first clustered 29 lines belonging to four types 1, 2, 3 and 5 which contained two main industrial types, type 1 (French bean, very fine) and type 2 (French bean). Belonging to those groups, eight lines were clustered by pairs: Twiggy-Masaï, Label-Polder, Cabri-Sonate and Capitole-Master. The second part of the dendrogram clustered three main groups: three lines belonging to type 4 (Stringy French bean), three lines belonging to type 8 (Kidney bean) and Roma 2, a flat bush bean (type 7) which is clustered with the butter bean lines (type 9). The three lines of type 6 were dispersed and seemed genetically distant from each other. One of the type-5 lines (Snap bean), Rudy, was excluded from the first group and clustered with the second one. The most-distant lines from all the others were PI165426, a Mexican accession, and a butter bean, PB 6246, both excluded from the others.

The mean allelic diversity per type, shown in Table 3, was 2.16 and 3.09 respectively for type 1 (12 lines) and 2 (11 lines). For the five other types (3 or 5 lines per type), the mean allelic diversity per type ranged from 6 (Snap bean) to 10.33 (Market snap bean).

Table 3 Number of alleles and lines per type, and mean allelic diversity per type

Type	Allele number	Line number per type	Mean allelic diversity per type
1	26	12	2.16
2	34	11	3.09
3	15	1	*
A	14	1	*
4	26	3	8.66
5	30	5	6
6	31	3	10.33
7	15	1	*
8	23	3	7.66
9	46	5	9.2

Origin of allelic polymorphism

Amplification products obtained from the PvATA20 primer pair were excised from the gel, cloned and then sequenced to examine their flanking regions since their sizes surprisingly ranged from 150 to 320 bp. Sequence alignments showed that the allelic polymorphism was due to the repeat number, as the sequences flanking the repeats were homologous to each other. The repeat number ranged from 24 for the shorter allele of 150 bp to 85 for the longer one of 320 bp. Two alleles of intermediate size were also sequenced: the 240-bp allele presented 53 repeats and the original allele sequence from Fin de Bagnols line DNA contained 72 repeats.

Discussion

SSR screening

We have successfully constructed a microsatellite-enriched DNA library from common bean. Previous dot-blot hybridisation studies (data not shown) showed that (ATA) repeats seemed to be more abundant in the bean genome than the (GA), (CAC) and (CA) motifs. The strategy we have used allowed us to recover 60% of positive clones for the ATA-enriched library. This value is consistent with the result obtained by Fischer and Bachmann (1998) (60% positive clones) or Maguire et al. (2000) (85.8%). The most abundant motifs found in the sequences were TAA/ATT repeats. This is in accordance with the results of Yu et al. (1999) who identified bean microsatellites from searches on sequence databases. The low level of positive clones in the (CA), (GA) and (CAC)-enriched library could then be related to the low abundance of such motifs in the bean genome, as previously shown by dot-blot hybridisation. Both perfect and compound microsatellite repeats were useful for detecting genetic variation between lines. Less than 28% of the ATA-enriched library was exploited since only 336 recombinant clones were analysed from the 1,200 clones plated out of the entire library (2,500 clones). This shows the high efficiency of the enrichment techniques for developing microsatellite DNA markers.

Evolution of allele size

In plants, the conservation of microsatellite loci has been observed between cultivars, sub-species and between related species. The same microsatellite loci can be amplified from different-line DNAs depending on the sequence conservation of the flanking regions and on the stability of the microsatellite loci over time. Consequently, we have cloned several amplified products from different lines at the locus PvATA20 in order to investigate the origin of their great difference in allelic size (170 bp). The allelic size variation could indeed be explained either by an increase or a decrease in repeat number, or by variations in the flanking non-repetitive regions. Davierwala et al. (2000) have demonstrated that both events occurred in cereal-related genomes. The existence of null alleles at two loci in bean could be related to the second type of event.

Replication slippage could lead to insertion or deletion of dinucleotide or trinucleotide repeats, but sometimes only a single nucleotide could be inserted or deleted. In the case of PvATA20 alleles, sequence alignments have shown that the size variations were due to large differences in repeat number as the longest sequenced allele had 61 more repeats than the shortest one. Although mutations are well known to occur on a rapid time scale in microsatellite sequences, it is not very likely that so high an increase in repeat number would have occurred due only to replication slippage. In this case, the probability of size increase could be considered equal to the probability of size decrease. The size of the allele should then be distributed around a mean value according to the binomial law. We evidenced two groups of allelic sizes (23 to 31 repeats, 53 to 85 repeats). Thus we propose that such variability in allelic sizes would rather be caused by an unequal crossing-over between repeated DNA regions than by replication slippage. Unequal crossing-over occurs when two arrays of tandem repeats match and recombine out of phase. This results both in recombination between repeats and variation in copy number. Such kind of events are highly informative in terms of diversity structure. Microsatellite evolution in the single stepwise model (Shriver et al. 1993) introduces the problem of homoplasy as two alleles of the same size could have a different history. Unequal crossing-over at a particular locus could be considered as unique in time, then followed by replication slippage. Finally, two groups of alleles different in average size would be defined. Clustering of lines on this basis will reflect the evolution process and will be useful for phylogenetic reconstruction.

Polymorphism detected by SSRs among bean lines

The object of our study was to estimate the genetic diversity of 45 bean lines including nine quality types. Forty one out of the 45 lines were differentiated with 81 alleles detected at 15 loci. The mean allelic diversity per type for two industrial bean types, 1 and 2 was evaluated as 2 and 3 respectively. For types 4, 5, 6, 8 and 9, the mean allelic diversity per type ranged from 6 to 10.3 whereas the number of lines per type was lower than for types 1 and 2. The allelic diversity is lower between the lines of the two first types belonging to the French bean, very fine, and the French bean, showing that the breeders work with low genetic diversity created new lines of these types. This point of view is supported by the clustering of lines not being differentiated with the 81 alleles used in this study. All of them belong to phenotypic types 1 or 2. The likelihood of clustering two lines randomly was estimated as 1.23×10^{-8}. Hence, the clustering of four pairs of lines should be related to their genotype identity for the studied loci. But, based on the breeder's knowledge, only one pair out of the four shared a common progenitor. Moreover, the other pairs are unambiguously distinguished in the field. These results should be related to bias according to the limited number of loci studied. Nevertheless, one could accept that the phenotypic variations are due to genetic drift for the loci which are not under selection pressure in the different breeder's germplasms. In the regions subject to active selection, reduction of genetic diversity occurred leading to a reduction of the information given by the microsatellite markers.

The allelic diversity is higher for lines belonging to the other types. For example, a mean allelic diversity per type of 10.33 is estimated for type 6 whereas only three market snap bean lines were used for the study. The fact that these lines are spread in the different groups could reflect the fact that most of the genetic diversity comes from (or stays in) this type. The breeders carried out a stabilising selection in order to improve the traits of interest. This led to a reduction of phenotypic diversity as a consequence of the reduction of allelic diversity. Bean breeding is a very active field. We found that the diversity in bean industrial types 1 and 2 is roughly 2–5-times lower than in the other types. This could be regarded as a success of the selection process but introduces an asymptote to the genetic progress. At this step it should be necessary either to introduce some new material with the hope to recover an increase of progress or to adopt new selection goals.

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