Genetic Diversity in Southeast European Soybean Germplasm Revealed by SSR Markers

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Summary

Breeding material and registered soybean cultivars in Southeast European countries are strongly linked to Western breeding programs, primarily in the USA and Canada. There is little reliable information regarding the source of germplasm introduction, its pedigree and breeding schemes applied. Consequently, use of these genotypes in making crosses to develop further breeding cycles can result in an insufficient level of genetic variability.

The objective of this study was to assess genetic diversity and relationships of 23 soybean genotypes representing several independent breeding sources from Southeastern Europe and five plant introductions from Western Europe and Canada using 20 SSR markers.

In total 80 alleles were detected among 28 genotypes with an average of four alleles per locus and an average marker diversity of 0.585. Allele frequency distribution was characterised with a high proportion of alleles at very low frequencies with 11 % of unique alleles. Cluster analysis clearly separated all genotypes from each other assigning them into three major clusters, which largely corresponded to their origin. Results of clustering were mainly in accordance with the known pedigrees.

Key words

Glycine max, genetic similarity, microsatellites

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Introduction

Soybean provides a major source of protein for animal and human consumption and is among the most important crops in the world. As in other major crops, genetic diversity of soybean commercially grown cultivars has been decreasing at an alarming rate. The narrowness of North American (Gizlice et al., 1994; Sneller, 1994) as well as Brazilian (Vello et al., 1988) soybean germplasm has been well documented by pedigree analyses. Gizlice et al. (1994) determined that only 35 ancestors contributed more than 95 % of all alleles and only five lines account for more than 55 % of the genetic background of public cultivars in North America. Similarly, Gai et al. (1998, according to Wang et al., 2008) reported that 651 soybean cultivars released from 1923 to 1995 in China could be traced back to only 308 ancestors or about 1.5% of the germplasm resources available in China.

To efficiently broaden the genetic base of modern soybean cultivars a detailed insight into genetic diversity of soybean resources is required. Such insight could be achieved through molecular characterization using DNA markers, which are more informative, stable and reliable, compared to pedigree analysis and traditionally used morphological markers. Microsatellites or simple sequence repeats (SSRs) act as molecular markers, which have been widely applied in the genetic diversity studies of the soybean germplasm (Brown-Guedira et al., 2000; Narvel et al., 2000; Abe et al., 2003; Fu et al., 2007; Li et al., 2008a; Li et al., 2008b; Wang & Takahata, 2007; Wang et al., 2006a; Wang et al., 2006b; Wang et al., 2008; Yoon et al., 2009). Their advantages over other types of molecular markers are that they are abundant, have a high level of polymorphism, are codominant, can be easily detected with PCR and typically have a known position in the genome. High levels of polymorphism at SSR loci have been reported for both the number of alleles per locus and the gene diversity (Akkaya et al., 1992; Morgante & Olivieri, 1993; Rongwen et al., 1995; Powell et al., 1996; Diwan & Cregan, 1997; Abe et al., 2003; Wang et al. 2006b; Fu et al., 2007; Wang et al., 2008).

Most diversity studies on cultivated soybean published by now have focused on North American (Brown-Guedira et al., 2000; Narvel et al., 2000; Fu et al., 2007) Asian (Abe et al., 2003; Xie et al., 2005; Wang et al., 2006b; Li et al., 2008b; Wang et al., 2008; Yoon et al., 2009) as well as South American (Bonato et al., 2006) soybean germplasm. In several studies only a few genotypes of European origin have been represented among germplasm studied (Brown-Guedira et al., 2000; Narvel et al., 2000; Fu et al., 2007; Hwang et al., 2008). Baranek et al. (2002) evaluated genetic diversity of 19 G*lycine max* accessions from the Czech National Collection using RAPD markers. Recently, Tavaud-Pirra et al. (2009) evaluated SSR diversity of 350 cultivated soybean genotypes including 185 accessions from INRA soybean collection originating from various European countries and 32 cultivars and recent breeding lines representing the genetic improvement of soybean in Western Europe from 1950 to 2000. They found the genetic diversity of European accessions to be comparable with those of the Asian accessions from the INRA collection, whereas the genetic diversity observed in European breeding lines was significantly lower. Breeding material and registered soybean cultivars in Southeast European countries have mostly originated from the USA and Canada. The path of introduction and breeding schemes are mostly unknown, as well as the pedigree of most genotypes. Consequently, use of these materials in making crosses to develop further breeding cycles may not be optimized to increase genetic variability in progeny. Assessing the genetic diversity of this germplasm at genomic DNA level would complement the knowledge on the European soybean gene pool (germplasm) and facilitate the utilization of the resources from Southeastern Europe by soybean breeders.

The objective of this study was to assess genetic diversity and relationships of 23 soybean genotypes representing several independent breeding sources from Southeastern Europe and five plant introductions from Western Europe and Canada using 20 SSR markers.

Materials and methods

Soybean plant material. Twenty-eight soybean genotypes (cultivars and breeding lines) that were selected for characterization in this study (Table 1) included 23 cultivars and breeding lines from four Southeast European countries (Croatia, Serbia, Macedonia and Bulgaria), two cultivars of German origin released in Croatia, one French cultivar and two cultivars from Canada.

The genetic material was obtained from the Faculty of Agriculture of the University of Zagreb, Croatia and from the Institute of Southern Crops, Strumica, Macedonia.

DNA extraction. Thirty seeds of each soybean genotype were germinated in an incubator at 25°C. Fresh young leaves from 10 two week old seedlings of each genotype were bulked and freeze dried for three days. Dry leaves from each bulked sample were ground to a fine powder in a 2-ml Eppendorf tube with 3-mm metal globes in a multidirectional shaker. Samples were stored at -20°C until DNA extraction. Genomic DNA was extracted from 100 mg of bulked leaf tissue of each genotype using DNeasy plant mini kit (Qiagen, Valencia, CA).

SSR marker selection. Twenty SSR loci used in the present study (Table 2) had been previously mapped on the integrated genetic linkage map of soybean (Cregan et al., 1999). These SSR loci were selected from each of 17 out of 20 genetic linkage groups to provide a uniform coverage of the entire soybean genome. Selected primer sets have proven to be highly polymorphic and effective for the assessment of soybean diversity in several recent studies (Wang et. al. 2008; Yoon et al. 2007; Fu et al. 2007; Abe et al. 2003; Narvel et al. 2000). Nineteen of the SSRs had a core motif ATT, and one (HSP176) had AT motif (Table 2).

Polymerase chain reaction (PCR) amplification was performed in 13 µl reaction mixture containing 30 ng of soybean genomic DNA, 2 mM magnesium chloride, 0.2 µM of forward and reverse primers, 0.2 mM of each of the four dNTPs, 1.3 µl 1 x PCR buffer, and one unit of *Taq* DNA polymerase. For primers SOYPRP, Satt373 and Satt294, the concentration of magnesium chloride was changed to 1.5 mM for better amplification. PCR was conducted according to standard procedures (Morgante and Olivieri, 1993) with the slight modifications regarding the annealing temperature. Three different PCR touch-down thermal profiles regarding the annealing temperature were performed for the set of used microsatellite markers (Table 2).

PCR products were separated on 6 % polyacrylamide gels, 8M urea on S2 vertical electrophoresis unit (Gibco-BRL Life Technologies, Paisley, UK) at a constant current of 45W and

Table 1. The name and origin of 28 soybean genotypes used in the study

Cultivar/Line	Country[a]	Program[b]
Buga	HRV	FAZ
Hrvatica	HRV	FAZ
Ružica	HRV	FAZ
Dubravka	HRV	FAZ
Zagrebčanka	HRV	FAZ
Sanja	HRV	FAZ
XL-3	HRV	FAZ
XL-7	HRV	FAZ
XL-9	HRV	FAZ
S00-130	HRV	FAZ
S00-181	HRV	FAZ
Anica	HRV	AIOS
Tisa	HRV	AIOS
Maja	HRV	AIOS
Bosa	SRB	MRIZP
ZPS015	SRB	MRIZP
L-8	SRB	MRIZP
L-111	SRB	MRIZP
Balkan	SRB	IVFCNS
Ilindenka	MKD	ISC
Pela	MKD	ISC
Daniela 97	BUL	ISP
Pavlikeni 121	BUL	ISP
Diana	HRV[G]	SCHL
Sabina	HRV[G]	TPS
Essor	FRA	LGI
OAC Vision	CAN	UG
Delta	CAN	SPI

[a] Country of origin: HRV, Croatia; SRB, Serbia; MKD, Macedonia; BUL, Bulgaria; HRV[G], Cultivar originating in Germany and released in Croatia; FRA, France; CAN, Canada

[b] The code for origin or breeding program from which a cultivar or line was developed. FAZ, Faculty of Agriculture of the University of Zagreb; AIOS, Agriculture Institute Osijek; MRIZP, Maize Research Institute Zemun Polje; IFVCNS, Institute of Vegetable and Field Crops Novi Sad; ISC, Institute of Southern Crops (UGD) Strumica; ISP, Institute of Soya-bean Pavlikeni; SCHL, Dr. V. Schlesinger Saatzucht-Beratung; TPS, Thomas Pehl Satzucht; LGI, Limagrain Genetics Inc; UG, University of Guelph; SPI, Semmences Prograin Inc

silver stained according to Bassam et al. (1991). The size of each band was approximately estimated by M3 marker (75 - 622 bp). Amplified SSR fragments of different sizes were considered as different alleles.

Statistical analysis. Bands detected by each primer pair were interpreted as alleles at a genetic locus. In cases where no bands were detected (5.35% cases) data was excluded from the analysis, because electrophoresis was not repeated and thus, it was not possible to distinguish between null alleles and the failure of PCR. The number of alleles per locus was determined for the entire set of 28 genotypes. Marker diversity (D) at a single locus (j) was estimated according to Nei (1987) as

$$D = n\,(1 - \Sigma x^2_{ij})\,/\,(n - 1)$$

where n was the number of individuals sampled, and x_{ij} was the observed frequency of the i_{th} SSR allele at the j_{th} SSR locus. Marker diversity corresponds to the Polymorphic Information Content used in some other studies (Smith et al. 1997; Fu et al. 2007; Wang et al. 2008) and gives an estimate of the discriminative power of a marker taking into account not only the number of alleles but also their relative frequency. To generate a binary data matrix, the presence and absence of an allele per locus for each genotype was coded into 1 and 0, respectively. Genetic similarity (*GS*) among genotypes was estimated with simple matching coefficients (SMC = m / (m + n)),

where m was the number of matches and n was the number of mismatches. To visualize the relationship among genotypes an UPGMA cluster analysis based on Simple matching coefficient distance matrix was performed using TREECON for Windows ver. 1.3 b (Van de Peer and De Wachter, 1994). Bootstrap analysis was performed on 1,000 bootstrap samples to test the reliability of branches (Felsenstein, 1985). The cophenetic correlation coefficient was calculated, and Mantel's test (Mantel, 1967) was performed to check the goodness of fit of a cluster analysis for the matrix on which it was based using NTSYS-pc v. 2.1 (Rohlf, 2000).

Results

A total of 80 alleles were detected at 20 SSR loci across 28 soybean genotypes (Table 2). The number of alleles per SSR locus varied from two (Satt358 and HSP176) to six (Satt414), with an average of 4.0 alleles per locus. The marker diversity values ranged from 0.138 for Satt358 to 0.808 for Satt100, with an average of 0.585 per locus. The number of alleles per locus and marker diversity value were positively correlated (r=0.73**). The four most informative loci (marker diversity higher than 0.740) were Satt100, Satt185, Satt414, and Satt173 on linkage groups C2, E, J, and O, respectively.

Allele frequency distribution in the present study was characterised with a high proportion of alleles at very low frequencies (Fig. 1). About 11% of alleles were unique, detected in only one genotype, 55% of alleles were at frequencies below 0.2 and 90% of alleles were at frequencies below 0.5. One unique allele was found in each of the six genotypes Sanja, Maja, Ilindenka,

Table 2. Linkage groups, the range of allele sizes, number of alleles and marker diversity (D) values of 28 soybean genotypes across 20 SSR loci

SSR primer	Linkage group	Number of alleles	Marker diversity (D)
Satt042	A1	3	0.657
Satt409	A2	4	0.457
Satt534	B2	5	0.484
Satt294	C1	5	0.543
Satt100	C2	5	0.808
Satt179	D1a	3	0.585
Satt005	D1b	5	0.720
Satt002	D2	3	0.419
Satt185	E	5	0.741
Satt146	F	3	0.415
HSP176	F	2	0.389
Satt012	G	3	0.362
Satt414	J	6	0.785
Satt001	K	5	0.710
SOYPRP	K	3	0.659
Satt373	L	4	0.677
Satt463	M	5	0.711
Satt009	N	4	0.700
Satt173	O	5	0.749
Satt358	O	2	0.138
Total		80	
Average		4.0	0.585

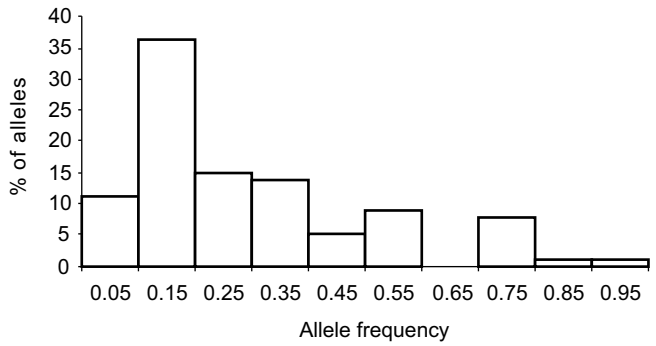

Figure 1.
The distribution of allele frequencies of 80 alleles found at 20 SSR loci within 28 soybean genotypes

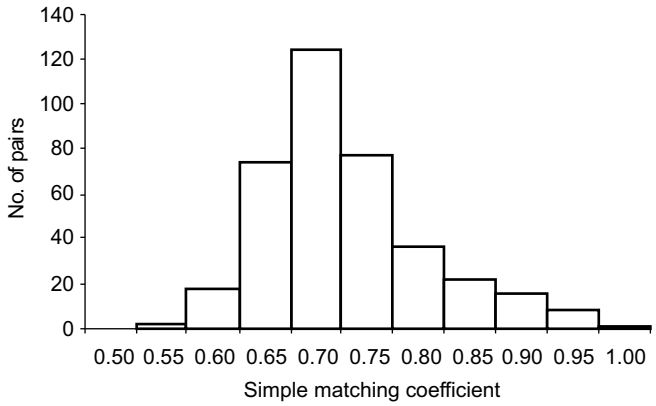

Figure 2.
Frequency distribution of 378 genetic similarity coefficients among 28 soybean genotypes

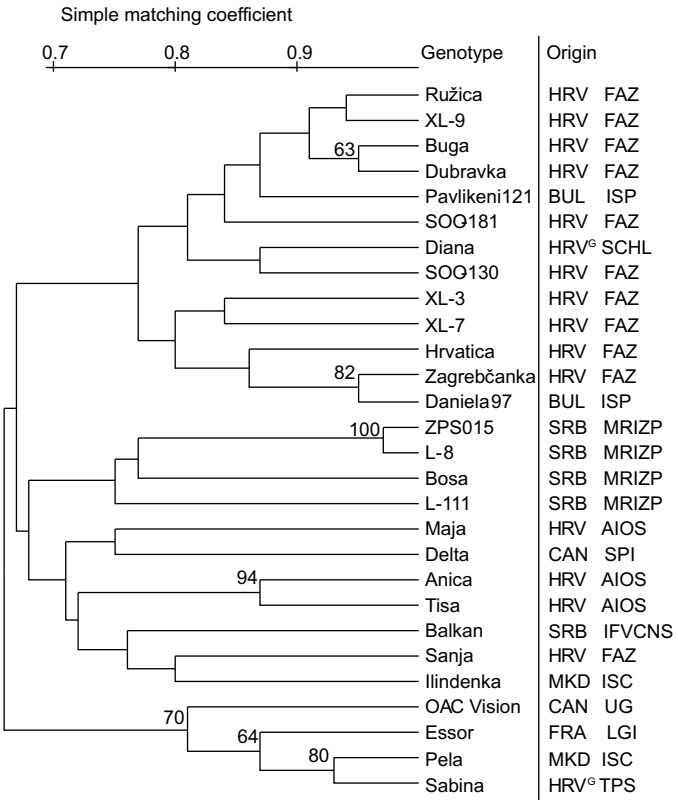

Figure 3.
UPGMA dendrogram showing genetic relationships among 28 soybean genotypes based on SSR similarity coefficients. Numbers at nodes indicate the bootstrap values (%) out of 1000 pseudoreplicates; only the values above 50 % are shown

Bosa, L-111 and OAC Vision, whereas three unique alleles were found in cultivar Balkan. Alleles with frequencies of 0.70 or larger were found at seven loci. The most frequent allele (frequency 0.93) was found at the locus Satt358.

A similarity coefficient matrix has been constructed from the genetic similarity calculations for the 28 genotypes. Figure 2 shows the frequency distribution of the genetic similarity coefficients. The average similarity coefficient among all genotypes was 0.70, ranging from 0.53 for two pairs (XL-7 and L-8 and XL-7 and L-111) to 0.98 for the pair ZPS015 and L-8. About 42 % of the estimated similarity coefficients had values higher than 0.70 and only 5.3 % had values equal to or lower than 0.60.

The average GS of a genotype with all other genotypes ranged from 0.66 to 0.74 with a group average of 0.70. The five most distinct genotypes with average GS ranging from 0.66 to 0.67, were Pela, L-8, ZPS-015, Maja and Sabina, whereas the five genotypes with the highest average GS of 0.74 were Croatian genotypes originating from the soybean breeding program of the University of Zagreb (Dubravka, Hrvatica, Buga, Ružica and XL-9).

The dendrogram based on genetic similarities between genotypes showed that the 28 genotypes formed three major clusters, and that the clusters largely corresponded to their origin (Fig. 3).

The cophenetic correlation coefficient of 0.83 was significant at $P<0.01$. Cluster I contained 13 genotypes grouped in the two subclusters. In the first subcluster, seven Croatian genotypes (six from the University of Zagreb and one plant introduction from Germany) were grouped together with one Bulgarian cultivar, whereas in the second subcluster two lines and two cultivars from the University of Zagreb were grouped together with another Bulgarian cultivar. Cluster II included eleven cultivars grouped in two subclusters. The first subcluster comprised four Serbian genotypes, whereas the second subcluster included four Croatian cultivars (three from the Agricultural Institute Osijek and one from University of Zagreb) and one cultivar from each: Canada, Macedonia and Serbia. Cluster III contained three plant introductions (from Germany, France and Canada) and one Macedonian cultivar.

Discussion

In the present study all 20 SSR loci were polymorphic and produced unique allelic profiles for the 23 soybean genotypes from Southeastern Europe and five plant introductions. In total 80 alleles were detected among 28 genotypes with an average of four alleles per locus and an average marker diversity of 0.585. The SSR diversity observed in the present study is comparable to those reported by Narvel et al. (2000) who detected 4.9 alle-

les per locus and an average marker diversity of 0.56 among 40 plant introductions from several Asian and European countries analysed at 74 SSR loci. Similar results were reported by Tavud-Pirra et al. (2009) who observed five alleles per locus and an average marker diversity of 0.65 among 32 breeding lines representing genetic improvement of soybean in Western Europe from 1950 to 2000. A slightly higher SSR diversity was reported by Fu et al. (2007), who found 6.3 alleles per locus (included null alleles) and an average polymorphic information content of 0.63 among 45 Canadian soybean cultivars and 37 exotic germplasm accessions analysed at 37 SSR loci. In the present study null alleles were excluded from the analysis, which probably led to an underestimation of both the average number of alleles per locus as well as average marker diversity. On the other hand, Diwan & Cregan (1997) reported as many as 10.1 alleles per locus with a mean gene diversity of 0.80 in 35 North American cultivars examined at 20 SSR loci. A high average number of alleles per locus (over 10) have also been reported in several other studies (Abe et al. 2003; Wang et al. 2006b; Wang & Takahata, 2007; Wang et al. 2008; Tavaud-Pirra et al., 2009; Yoon et al., 2009), but in those studies much higher numbers of genotypes were analysed compared to our study.

The average GS among all genotypes in the present study was 0.70 and 42% of them having values higher than 0.75, reflecting relatively low degree of genetic diversity among the genotypes used in this study. The levels of average GS observed in this study were much higher compared to those reported by Narvel et al. (2000) who found the average genetic similarities of 0.44 and 0.50 for 40 plant introductions from several Asian and European countries and 39 North American elite cultivars, respectively. On the other hand Fu et al. (2007) reported an average similarity of 0.815 (dissimilarity of 0.185) for 45 Canadian soybean cultivars and 37 exotic germplasm accessions, which was higher than GS reported in the present study.

The number of alleles per locus measures genetic diversity at the gene level. In contrast, the average genetic distance (similarity) among a set of genotypes measures genetic diversity at the population level (Lu & Bernardo, 2001). Compared to results reported by Fu et al. (2007) we found a higher level of genetic diversity among 28 soybean genotypes expressed in terms of genetic similarities than measured in terms of diversity statistics (average number of alleles per locus and marker diversity). This could be the consequence of using a relatively low number of elite genotypes as the source of desirable alleles for soybean breeding programs in Southeast Europe with rearrangements of these alleles into new developed cultivars being different among breeding programs.

Cluster analysis separated all genotypes from each other assigning them into groups, which largely corresponded to their origin and pedigree (Fig 3). Tightly groupings of genotypes according to their respective breeding program are especially emphasized for breeding programs represented with higher number of genotypes (HRV FAZ, HRV AIOS and SRB MRIZP). Grouping of genotypes revealed congruency of some clusters with the known pedigrees. For example Macedonian cultivar Pela (Sabina/ZPS015//OAC Vision) clustered together with two of its parents, whereas another Macedonian cultivar Ilindenka (L-111/L-8//Balkan) clustered together with all of its three parents. Furthermore, the breeding lines XL-3 and XL-4, which are progeny of the same cross (Hrvatica/Sabina), were clustered together with one of their parents, cultivar Hrvatica. Similarly, breeding lines S00-181 and S00-130 developed from the cross Ružica/Essor clustered together with cultivar Ružica. Tight clustering of cultivars Daniela 97 and Zagrebčanka explains their pedigree that is basically the same. Both cultivars are reselections out of an old cultivar Hodgson (USA).

Molecular evidence of genetic diversity and existing relationships in the Southeast European germplasm analysed in the present study could help soybean breeders in selecting diverse materials for use in their breeding programs. Relatively low average number of alleles per locus and allelic diversity observed in the set of genotypes analysed as well as their tightly groupings according to the origin of breeding programs, indicate that the genetic base of the material used in the Southeast European breeding programs is relatively narrow. However, a similar level of genetic diversity observed in elite material from Western European breeding programs (Tavud-Pirra et al., 2009) compared to those from Southeast European ones suggests they are not essentially different. To ensure sustaining breeding progress in the future, the introduction of new germplasm into these breeding programs, especially by the aid of molecular markers, is recommended.

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