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AFLP-analysis of genetic diversity in soybean [*Glycine max (l.) Merr.*] cultivars Russian and foreign selection

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Abstract. Genetic diversity of 11 cultivars of soybean (Glycine max) from a VIR (N.I. Vavilov Institute of Plant Genetic Resources) collection was analyzed by the AFLP (amplified fragment length polymorphism) technique. From 18 tested primer combinations, both of them were selected for further analysis. From these two primer combinations (E32/M60 and E32/M59), 90 DNA fragments were obtained, 39 (43%) of them were polymorphic. Unique DNA fragments were found in accessions SibNIISHoz 6, Soer-4, Determinant. The Jaccard's similarity indices varied from 0.79 (between Sonata and Ugra) to 0.94 (between Ugra and Garmoniya) with an average of 0.775. Cluster analysis placed the cultivars into the several groups but separation in groups was not related to their origin or biological characteristics, even though cultivars Nordik and Soer-4 (cultivars recommended for Central Black Earth region of Russia) clustered together on the dendrogram. The obtained high values of Jaccard genetic similarity index and low level of polymorphism and differentiation illustrated a relatively low genetic diversity in our studied cultivars, which correlated with different other studies on soybean genetic diversity with AFLP-analysis.

Key words: Soybean, Amplified Fragment Length Polymorphism (AFLP), Genetic diversity, Molecular markers.

INTRODUCTION

Soybean (Glycine max (L.) Merr.) is the most important crop as the source of high-protein and oil for human consumption and animal feed. Soybean is known as the first crop among the oilseed crops in all over the world, soybean variety selection is the first step in producing a high-yielding soybean crop. Despite soybean plasticity, varieties have different level of adaptability to various environments, therefore, each country has its own cultivars genbanks. By increased demand on varieties (high yield, environmental factors), countries organize different breeding programs to create new improved cultivars, hence the requirement to introduce new genotypes to select breeding materials.

Knowledge of soybean genetic diversity is a fundamental importance for efficient breeding programs as it helps breeders and geneticists to understand the structure of germplasm, predict which combinations would produce the best offspring and facilitate to widen the genetic basis of breeding material for selection (Zargar et al., 2011; Bisen et al., 2015). Mentioned information is useful to organize a working collection, identify heterotic groups, and select parents for crosses. Genetic diversity between individuals can be estimated by using both morphological, biochemical and molecular markers, although the use of morphological and biochemical markers has its constraints, given that they are limited in number, stage specific and highly influenced by the environmental conditions (Cox et al., 1985). This problem has been overcome by using molecular markers, RFLP technique has also been used to study exotic soybean germplasm and it has allowed the identification of different gene pools (Kisha et al., 1998). Similar studies have been carried out using other types of molecular markers, such as RAPD markers (Abdelnoor et al., 1995; Brown-Guedira et al., 2000), simple sequence repeat (SSR) markers (microsatellites) (Diwan & Cregan, 1997) and amplified fragment length polymorphism (AFLP) markers (Zhu et al., 1999; Ude et al., 2003).

AFLP analysis is one of the most popular fast and highly reproducible methods that can detect very large number of DNA bands, thus enabling identification of many polymorphic markers. AFLP is employed for a variety of applications, such as: analysis of population polymorphism, phylogenetic relations, genetic diversity assessment within species or among closely related species, identification of loci linked to economically valuable traits, deduction of population-level phylogenies and biogeographic patterns, genetic maps design and determination of relatedness among cultivars (Renganayaki et al., 2001; Soleimani et al., 2002; Kim et al., 2010; Ovidiu & Schönswetter, 2012). This method also can be used by breeders to preliminary assess the initial genetic material to plan the strategy of crosses, to identify the best combinations of genotypes, and for general selection (Kim et al., 2010; Sensi et al., 2003; Portis et al., 2004; Zargar & Pakina, 2014).

Molecular markers have also been used in Russia to analyze genetic diversity in soybeans with RAPD (Seitova et al., 2004), SSR (Ramazanova et al., 2008) and ISSR (Kozyrenko et al., 2007) being the more prominent. However, studies of the genetic diversity on Russian soybean cultivars using AFLP markers are very few.

Keeping in view that fact, the objective of the present study was to assess the genetic diversity of 11 Glycine max cultivars from VIR-collection using the AFLP analysis. The 11 cultivars were chosen from a big collection of cultivars introduced in the central European part of Russia (Moscow region) from different regions of Russia and other countries. Some of mentioned cultivars were developed by different institutes for various regions of Russia, such as Mid-Volga, East-Siberian, Far East, Southeast Central Black Earth, lower Volga, mid-Volga, Volga-Vyatka, mid-Volga, Ural regions, and two cultivars were from other countries as Poland and Sweden. Some of these cultivars were developed from the same parental lines or, are from the same sub-species – spp. *Manshurica*. They were selected for additional studies, after showing good results in Moscow region, thus making them interesting for different breeding programs (Romanova et al., 2013; Shafigullin et al., 2016).

MATERIALS AND METHODS

Plant material: In this study, 11 soybean cultivars of Russian and foreign selection from a VIR (N.I. Vavilov Institute of Plant Genetic Resources) (Table 1) collection were analyzed using AFLP markers.

Table 1. A list of soybean cultivars used in AFLP-analysis

Accession name	Developers (Institute)	Region, Country
Sonata	All Russian Research Institute of Soya; Far East State Agrarian University	Russia
Determinant	All-Russia Research and Development Institute of Vegetable Crop Selection and Seed Growing	Russia
Altom	Altai scientific-research institute of agriculture	Russia
Soer-4	Ershov Experimental Station of Irrigated Agriculture, Research Institute of Agriculture of the Southeast	Russia
Nordik	Poland	Poland
SibNIIK 315	Siberian Federal Research Center of Agricultural Biotechnology of the Russian Academy of Science	Russia
SibNIISHoz 6	Siberian Research Institute of Agriculture of Russian Academy of Agricultural Sciences	Russia
Okskaya	Ryazan scientific research institute of agriculture of the Russian Academy of Agricultural Sciences	Russia
Garmoniya	All Russian Research Institute of Soya	Russia
Lidia	All Russian Research Institute of Soya	Russia
Ugra	Sweden Svalöf AB in Malmöhus	Sweden

(http://www.gossort.com)

DNA extraction: DNA was isolated from 5-day-old seedlings in accordance with the protocol of Doyle & Doyle (1987) by some modifications. The concentration of isolated DNA was measured in comparison to phage DNA of known concentration (Fermentas, Lithuania) with gel electrophoresis. DNA samples were stored at -20 °C.

AFLP-analysis: AFLP analysis was carried out in standard form (Vos et al., 1995).

For the *Restriction/Ligation,* 300 ng of genomic DNA of each accession was double-digested with 5 U *Eco*RI and 5 U *Mse*I restriction enzymes (NEB) in a final volume of 40 µl at 65 °C for 3 hours using Gene Amp PCR system 9700. The genomic DNA fragments were ligated using 1 unit of T4 DNA Ligase (Invitrogen) and 10 µl of Ligation solution (ligase buffer, 10 mM ATP, 10mM *Eco*RI, 10mM *Mse*I) at 36 °C for 6 hours. Then T4 DNA Ligase was inactivated at 65 °C for one hour. (Gene Amp PCR system 9700) ligated to 10 *Eco*RI and *Mse*I adapters overnight at 15 °C to generate template DNA for amplification.

For *Pre-amplification,* the ligation mixture was diluted to 4-fold with deionized water. The template DNA generated was first pre-amplified using the primer pair combination each having one selective nucleotide, 10 mM *Eco*RI+A and mM *Mse*I+C. The selective amplification PCR reaction was performed with a final volume of 15 µl containing 10 × PCR buffer, 25 mM $MgCl_2$, 2 mM dNTP, 10 mM each of *Eco*RI and *Mse*I primers, 0.5 U of BioTaq polymerase. The thermal profile: denaturation at 95 °C

for 5 min, 12 cycles of 30 sec at 94 °C, 30 sec at 65 °C with 0.7 °C lowering for each cycle and 1 min at 72 °C, followed by 27 cycles of 30 sec at 94 °C, 30 sec at 56 °C and 1 min at 72 °C and final elongation at 72 °C for 10 min. PCR-reactions were carried out in the Gene Amp PCR system 9700. For quality control, the products of pre-amplification were visualized on a 1.2% agarose gel.

For *Selective amplification,* which's aim is to restrict the level of polymorphism and to label the DNA, we added three more nucleotides at the 3' end of the *Eco*RI and four nucleotides at the 3' end of the *Mse*I primers (Table 2). These additional nucleotides make the amplification more selective and will decrease the number of restriction fragments amplified (polymorphism). The selective amplification PCR reaction was performed with a PCR solution volume of 10 µl containing 10x PCR buffer, 25 mM MgCl2, 2 mM dNTP, 10 mM each of *Eco*RI and *Mse*I primers, 0.5 U of BioTaq polymerase. The thermal profile: denaturation at 95 °C for 5 min, 12 cycles of 30 sec at 94 °C, 30 sec at 65 °C with 0.7 °C lowering for each cycle and 1 min at 72 °C, followed by 32 cycles of 30 sec at 94 °C, 30 sec at 56 °C and 1 min at 72 °C and final elongation at 72 °C for 10 min. PCR-reactions were carried out in the Gene Amp PCR system 9700. The amplification products were separated in 6% polyacrylamide gel (PAAG) and stained with silver nitrate as described in (Benbouza et al., 2006). The length of the amplification fragments was assessed using the 100 bp DNA ladder (Invitrogen, United States) (0.05 g L^{-1}).

Table 2. Primers for selective amplification used in the study

Adapter/primer	Code	Nucleotide sequence
EcoRI-primer + A + AGC	E40	5′-GAC TGC GTA CCA ATT C + AGC-3'
EcoRI- primer + A + ACT	E38	5′-GAC TGC GTA CCA ATT C + ACT-3'
EcoRI- primer + A + AAC	E32	5′-GAC TGC GTA CCA ATT C + AAC-3'
EcoRI- primer + A + ATG	E45	5′-GAC TGC GTA CCA ATT C + ATG-3'
MseI- primer + C + CTC	M60	5′-GAT GAG TCC TGA GTA A + CTC-3'
MseI- primer + C + CAT	M50	5′-GAT GAG TCC TGA GTA A + CAT-3'
MseI- primer + C + CGA	M55	5′-GAT GAG TCC TGA GTA A + CGA-3'
MseI- primer + C + CTA	M59	5′-GAT GAG TCC TGA GTA A + CTA-3'
MseI- primer + C + CCC	M52	5′-GAT GAG TCC TGA GTA A + CCC-3'
MseI- primer + C + CTG	M61	5′-GAT GAG TCC TGA GTA A + CTG-3'
MseI- primer + C + CTC + C	M60C	5′-GAT GAG TCC TGA GTA A + CTC+C-3'
MseI- primer + C + CTC + T	M60T	5′-GAT GAG TCC TGA GTA A + CTC+T-3'
MseI- primer + C + CAT + C	M50C	5′-GAT GAG TCC TGA GTA A + CAT+C-3'
MseI- primer + C + CAT + G	M50G	5′-GAT GAG TCC TGA GTA A + CAT+G-3'
MseI- primer + C + CCC + A	M52A	5′-GAT GAG TCC TGA GTA A + CCC+A-3'
MseI- primer + C + CCC + C	M52C	5′-GAT GAG TCC TGA GTA A + CCC+C-3'

Both DNA isolation and AFLP analysis were carried out in the Laboratory of Plant Genetics of the Vavilov Institute of General Genetics, Russian Academy of Sciences (Moscow, Russia).

Data Analysis: The presence or absence of the amplification products was registered visually in the gel. Obtained data were loaded into a binary matrix, in which I corresponded to the presence of a fragment and 0 corresponded to its absence. For each combination of primers, the number of monomorphic and polymorphic fragments was estimated. The percentage of polymorphic fragments was assessed as the ratio of the

number of polymorphic fragments to the total number of fragments. The Jaccard genetic similarity index was calculated using the PAST 3.09 program (Hammer et al., 2001). Dendrogram was also constructed with PAST 3.09 programs. Clusterization of the samples was performed using the UPGMA method.

RESULTS AND DISCUSSION

18 primer combinations were initially tested, but only two of them detected a high level of polymorphism, and were further used to analyse the genetic diversity in illustrated soybean cultivars (Table 3). The other 16 combinations showed a low level of polymorphism or were monomorphic, therefore were not used for further analysis.

The AFLP analysis of 11 soybean samples allowed us to identify 90 fragments, 39 (43.3%) of which appeared to be polymorphic (Table 3). The high numbers of fragments were obtained with the primer combination E32/M59. The number of polymorphic fragments varied from 18 to 21 per primer combination with the average of 19.5, and, the average level of polymorphism was 43.5%. In AFLP spectra of SibNIISKHoz 6, Soer-4, Determinant samples were found unique fragments, which can be used as molecular markers of these varieties.

Table 3. Characteristics of polymorphism in *Glycine max* cultivars using the selected AFLP primers

Primer combination	Amplifed fragments (a)	Polymorphic fragments (b)	Percentage polymorphism (b x 100/a)
E32/M60	40	18	45
E32M59	50	21	42
Total	90	39	43.3

Bonato et al. (2006) investigated the genetic similarity among 317 soybean cultivars released to Brazil by applying (AFLP) technique and achieved lower values of polymorphism level. The six primer combinations used to analyze generated 394 bands, but 78 (19.8%) of those were found to be polymorphic among genotypes. The average number of polymorphic markers per primer combination was 13 (Bonato et al., 2006).

Analysis of the 12 *G. max* and 11 *G. soja* accessions with 15 AFLP primers pair identified a total of 759 fragments, of which 274 (36%) were polymorphic, average number of polymorphic fragments for each primer pair was 18.3 (Maughan et al., 1996). Among the 274 polymorphic fragments detected, 37 were observed only in *G. max* and 147 were observed about *G. soja*. Satyavathi et al. (2006) used 12 AFLP primer pairs to assess genetic diversity in 72 soybean cultivars under Indian cropping systems, they produced 1,319 products, which 1,257 of them (95%) were polymorphic (Satyavathi et al., 2006).

Based on the AFLP spectra obtained in our study, the levels of Jaccard genetic similarity index in *G. max* samples were calculated. The maximum level of genetic similarity (0.94) was detected between the Ugra and Garmoniya samples, whereas t minimum level of similarity (0.79) was obtained between Sonata and Ugra samples.

Regarding to the mentioned AFLP analysis above, the values of the genetic similarity index achieved in our study show a significant level of similarity in our cultivars compared to other studies, although those studies analyzed a significant high

number of cultivars. For example, the genetic similarity coefficients between Brazil soybean cultivars varied from 0.17 to 0.97 (Bonato et al., 2006). Jaccard's similarity coefficients among the Indian cultivars varied from 0.787 to 0.118 (Satyavathi et al., 2006). On the other hand, Maughan et al. (1996) found that in their study high similarity coefficients from 0.74 to 1.00 within the Glycine max varieties, and the examined cultivars were from different parts of the world (Maughan et al., 1996).

The clusterization of the samples was further perfomed (Fig. l).

On the dendrogram (Fig. 1) constructed according to AFLP-analysis the eleven accessions of *G. max* formed two clusters.

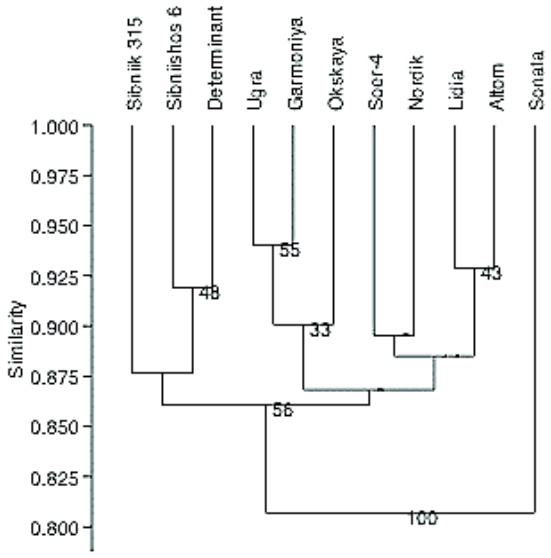

Figure 1. Dendrogram constructed on the basis of AFLP analysis of 11 soybeans samples using UPGMA method.

The first cluster includes one variety Sonata and the second cluster involved the rest of the samples. Within the second cluster, examined samples were formed two sub-clusters with a similarity index of 0.86. The first subcluster was formed by the varieties SibNIIK 315, SibNlISHoz 6, Determinant. Cultivars Ugra, Garmoniya, Okskaya, Lidia, Altom, Nordik, Soer-4 formed the second subcluster.

Generally, separation of samples in groups is not related to their geographical origin although Nordik and Soer-4 (varieties recommended for cultivation in the Central Black Earth region of Russia) clustered together also on the dendrogram.

The high values of Jaccard genetic similarity index, the low level of polymorphism and differentiation illustrated the relatively low genetic diversity present in the studied soybean varieties. The obtained results of low genetic diversity was similar to that obtained by Nimnual et al. (2014), which also showed that cultivars didn't cluster according to their origin (Nimnual et al., 2014). The explanation given to the high genetic diversity obtained by Yan et al. (2014) compared to our results, was the bulking and mixing of cultivated gene pool from geographically distant populations by crossbreeding under condition of artificial domestication (Yan et al., 2014), but in our case studied

cultivars were developed by collaborating organizations, from probably the same parental forms. The other explanation of the low genetic diversity can be the fact that some cultivars are from the same subspecies *manshurica*, some varieties were created by the same organization or from the same parental form and were developed for the same ecological zone.

CONCLUSIONS

AFLP markers used in present study to assess the genetic diversity in 11 cultivars revealed a relatively low level of genetic diversity. From the 18 primer combinations initially chosen for the study only two primer combinations (E32/M60 and E32/M59) allowed to detect polymorphic fragments. However, even with them we obtained a low level of polymorphism – of the 90 DNA fragments obtained, 39 (43%) were polymorphic. Unique DNA fragments were found for varieties SibNIISKHoz 6, Soer-4, Determinant. Jaccard genetic similarity index between varieties was high. Cluster analysis separated varieties into several groups but that separation was not related to their origin or biological characteristics. Such low genetic diversity level can probably be explained by the fact that all examined cultivars belong to subspecies *manshurica*, some varieties were created by the same organization or from the same parental form and can grow in the same ecological zone.

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