

SSR Analysis of Maternal and Paternal Lines Selected in the Don Region (Russia)

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Abstract: Evaluation of DNA polymorphisms of breeding material of sunflower from L.A. Zhdanov Don Experimental Station of oil Crops of V.S. Pustovoit All-Russian Research Institute of Oil Crops represented by 17 maternal (CMS) lines and 12 paternal (Rf) lines was conducted. There were identified 35 allelic variants of CMS lines and 42 allelic variants of Rf lines with use of 11 SSR markers. It is shown that the level of genetic diversity of microsatellite loci of CMS lines is 1.2 times lower than that of Rf lines. The average number of alleles per SSR locus for maternal lines also lowers than for paternal lines. In addition, CMS lines and Rf lines differ in the frequency of alleles of SSR loci. Thus, the discriminatory capacity of the studied marker system for identification of cultivated sunflower lines was quite high and is suitable for certification of the lines.

Keywords: CMS Lines, Rf Lines, SSR Markers, Polymorphism, Sunflower

Introduction

The main objective of heterosis breeding of crops is creating hybrids with high productivity and complex resistance to biotic and abiotic environmental factors. The success of the breeding for heterosis in the first place depends on the genetic potential of parental lines of F1 hybrids.

There are new opportunities to study genetic diversity of organisms after introduction of DNA markers in the practice of biological research (Goodfellow *et al.*, 2005; Dong *et al.*, 2007; Okumus, 2007; Wong *et al.*, 2009; Acharya and Sharma, 2009; Nagaty *et al.*, 2011; Pandey *et al.*, 2011; El-Assal and Gaber, 2012; El-Awady *et al.*, 2012; Dakah *et al.*, 2015).

In order to intensify and to reduce the complexity of the selection process for promising hybrid combinations, analyses of DNA polymorphism of inbred lines of different crops are actually conducted. In particular, SSR or microsatellite analysis is effective for solving such problems (Goodfellow, 1993; Solodenko and Yu, 2005; Usatov *et al.*, 2014a; 2014b; Bhavsar *et al.*, 2015). Microsatellite loci are mainly presented in non-coding regions of DNA and thus they do not fall under the direct action of natural selection. They accumulate mutations,

which causes a high level of polymorphism. The most commonly microsatellite loci have dominant inheritance. Identification of sunflower varieties using microsatellites, first carried out by Brunel (1994), showed that the microsatellite loci represented by multiple alleles and are characterized by a relatively high heterogeneity. They are convenient and promising tool for the analysis of genomic DNA polymorphism.

The aim of this work was to study using microsatellite analysis the genomic DNA polymorphism of the CMS lines and Rf lines of sunflower selected in L.A. Zhdanov Don Experimental Station of oil Crops of V.S. Pustovoit All-Russian Research Institute of Oil Crops (DOS VNIIMK).

Materials and Methods

Plant material: The object of the study was the breeding material from DOS VNIIMK represented by 17 maternal (CMS) lines and 12 paternal (Rf) lines of sunflower. Ten to 15 10-15 plants of each line were analyzed.

Isolation of Plant DNA from fresh tissue: The total DNA was isolated from the first pair of true leaves of sunflower seedlings according to Boom *et al.* (1990)

with our modifications. Leaf tissue was intensively homogenized by a Teflon pestle in 300 μ l of a solution containing 10 mM Tris-HCl, pH 8.0, 5.5 M Guanidinium thiocyanate, 10 mM EDTA, 1% Triton X-100. Additionally, the homogenate was incubated for 60 min in a solid-state thermostat TT-1 (DNA Technology, Russia) at 65°C, stirring occasionally by vortex FV-2400 (BioSan, Latvia). After that the tubes were centrifuged at 12000 rpm for 5 min in a tabletop centrifuge MiniSpin (Eppendorf AG, Germany) at room temperature. The supernatant was collected into a new tube, added 25 μ L of sorbent SiO₂ (Helicon, Russia) in 32% HCl, pH 2.0. The mixture was vortexed and held into a support for 3 min, shaken again and held into a support for 5 min. Then the sorbent was pelleted by centrifugation at 5000 rpm for 30 sec, the supernatant was removed and the adsorbent was washed twice with a solution containing 50% ethanol, 50 mM NaCl. After the second washing, the tubes were centrifuged at 10000 rpm for 30 sec. The maximum of liquid was removed and the tubes with open lids were placed in an oven (65°C, 8-10 min) for drying the sorbent. DNA was eluted in 100 μ L of TE buffer (0.01 mM Tris-HCl pH 8.0, 0.001 mM EDTA) at 65°C for 5-8 min, occasionally vortexing. Sorbent was pelleted at 12000 rpm for 3 min and the resulting DNA solution was used in the polymerase chain reaction. The concentration and purity of the obtained DNA solution was determined with use of 1% agarose gel and by spectrophotometry (SmartSpec, USA).

PCR amplification and gel electrophoresis: We selected 12 pairs of primers according to the literature and as a result of preliminary studies were used in the SSR analysis (Table 1).

The polymerase chain reaction was done using 25 μ L of the reaction mixture with the following composition: 67 mM Tris-HCl, pH 8.8, 16 mM (NH₄)₂SO₄, 2.5 mM MgSO₄, 0.1 mM mercaptoethanol, 0.25 mM of each dNTP (dATP, dCTP, dTTP, DSTF), 10 pmol of each primer, 2.5 U of Taq-polymerase, 30 ng of isolated DNA. Amplification was performed in a thermocycler Palm Cycler (Corbett Research, Australia).

Thermal reactions mode was selected for each pair of primers based on their nucleotide composition. For the majority of conducted reactions optimum temperature mode was under initial denaturation at 95°C for 4 min, then 35 cycles: Denaturation at 94°C for 30 sec, annealing at 55-60°C for 40 sec, elongation for 1 min at 72°C, final elongation for 10 min at 72°C.

Products of amplification reaction were separated by electrophoresis on 2% agarose gel with ethidium bromide (1 μ g mL⁻¹) using tris-borate buffer. After finishing electrophoresis gels were transferred to a transilluminator and photographed by GelDoc 2000 (BioRad, USA). GeneRuler 100 bp DNA Ladder (ThermoScientific, Lithuania) was used as molecular markers.

Evaluation of polymorphism SSR-loci: To evaluate polymorphism of SSR loci was used PIC (Polymorphism Information Content): $PIC = 1 - \sum p_i^2$, where p_i is frequency of i -th allele detected in the analyzed sampling (Nei, 1973).

To determine the genetic similarity data of SSR analysis using Phoretix ID software were represented as a state matrix of binary features. Assisted by state matrix with WinBoot software (Yap and Nelson, 1996) was formed divergence matrix by use of Jacquard index and using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), constructed dendrogram showing the degree of differences between the SSR patterns of the investigated samples (Tamura *et al.*, 2011).

Results and Discussion

As a result of SSR analysis of 17 CMS lines and 12 Rf lines of sunflower were obtained specific and reproducible DNA fragments. Using 11 microsatellite loci were detected 35 alleles in the genotypes of CMS lines and 42 alleles in the Rf lines. The number of detectable alleles with use of the primers system ranged from 2 to 5 and averaged 3.2 for CMS lines and from 2 to 6, an average of 3.8 for Rf lines. Sizes of amplified fragments ranged from 100 to 690 bp (Table 1).

Allelic differences were observed both in the number of amplified fragments and their mobility on an agarose gel. As an example, Fig. 1 show the SSR profiles of CMS and Rf lines of sunflower obtained using primers for one of informative loci *Ha* 1442. Primers of this locus initiate amplification of 4 polymorphic fragments for maternal lines and 5 ones for paternal lines. Thus, some Rf lines have a unique fragment of 190 bp, which is not observed in the CMS sunflower lines.

Ha 1287 also proved to be polymorphic; it has size of 130-510 bp. However, we found a unique, peculiar only to certain lines bands. Thus, for a CMS line VD 255 is characterized by 2 amplicons with sizes 370 and 510 bp. Rf line VD 33509 has a fragment of about 130 bp. These amplicons allow distinguishing these lines from the rest of the lines. In some cases, for example, for CMS lines: VD 1448, 344 VD, VD 22, ED 95, ED 73 with primers for this locus failed to initiate amplification.

Locus *Ha* 432 is characterized by high heterogeneity, which is confirmed by the high values of PIC. Most lines have their own unique patterns that different from each other.

Locus *Ha* 514 less polymorphic than the previous one, but, nevertheless, it has clear and reproducible bands specific to a particular line. Thus, fragment of about 180 bp are more typical for CMS lines, while for Rf lines are 190 bp and 200 bp. At the same time, these sequences were found both in CMS and Rf lines.

Table 1. Primers used in the SSR analysis

Locus name	Repeat	Sequence of flanking primers 5'-3'	Number of alleles ♀/♂	PIC*♀	PIC*♂	Size of amplified fragment, bp
<i>Ha</i> 432	GT	CTT TAT CCC CCA CCC CCT CC GGG TTT AGT GGC CAG TAG TTG TC	4/6	0.73	0.77	180-690
<i>Ha</i> 1442	ATT	GCT TAT GTG CTT ACG TGT TCC TG CTA AAC AGT TCG GCG AGT GTA GG	4/5	0.67	0.79	170-240
<i>Ha</i> 1608	ATT	GAT CTT AGG TCC GCC AC GAT GGC ATT TGG CTA GAC	3/3	0.52	0.63	220-250
<i>IUB</i> 4	AT	GGC CAT GAT TTA TTC ACT CAG ACA GAT GAG AGG CGT TCT CAC	2/4	0.50	0.69	130-190
<i>ORS</i> 509	AT GT	CAA CGA AAA GAC AGA ATC GAA A CCG GGA ATT TTA CAA GGT GA	3/4	0.43	0.58	190-210
<i>Ha</i> 514	GA	GGT CAA CGG ATT TAG AGT C GTA TTG ATT CCA ACA TCC AG	4/4	0.63	0.65	180-200
<i>IUB</i> 6	GT	TCG GTA TCG TTT GCT AAT GG GGT AAC TCT AAA GCT CTG TC	2/2	0.36	0.15	350-370
<i>ORS</i> 6	AGG	GTG GAG AGA GGT GTA GAG AGC CAC CCC TCA CCC TGA CAC	2/2	0.21	0.50	250-260
<i>Ha</i> 1287	GA	GAT ATG AGC CCA TCA CTC ATC GAA GAT ATG TCA GGT CAC ACC C	5/6	0.72	0.73	130-510
<i>HNCA</i> 2	GT	TGA GAC AAG CAT AAG CAC TAG ACA AGA CAA GGG ACT	2/2	0.36	0.50	210-340
<i>OSU</i> 1	GGG	ACA AGT CGG CTG GTG AGC ACA TGA AAC ACG AGC TAA ACC A	4/4	0.70	0.71	100-160
The average value			3.2/3.8	0.53	0.61	

*PIC-Polymorphism Information Content

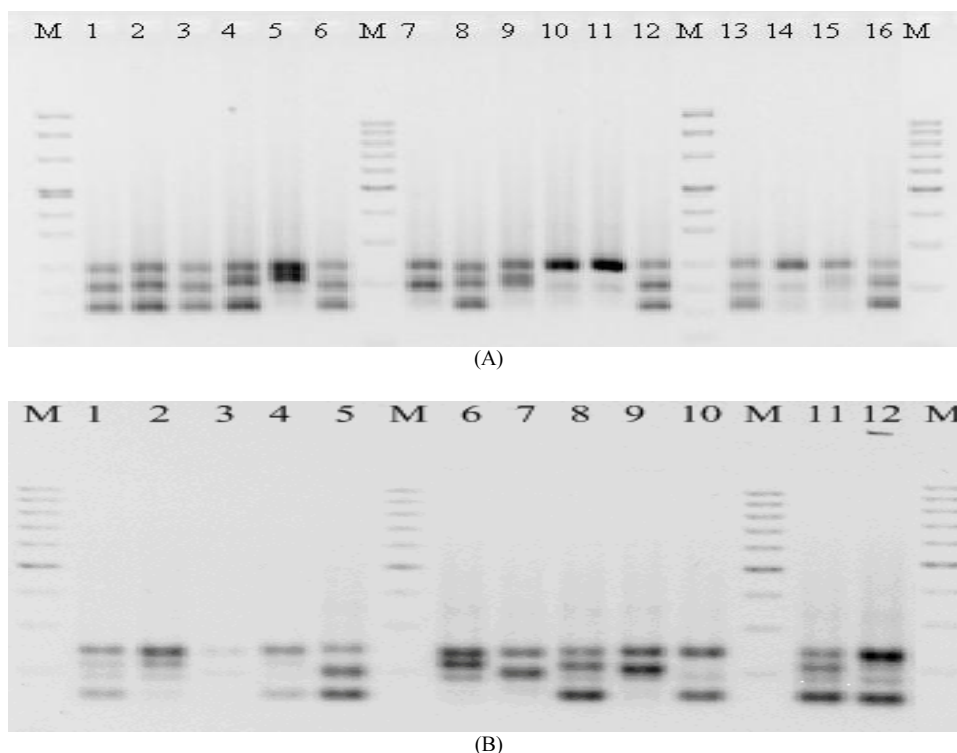


Fig. 1. SSR profiles of CMS lines (A) and Rf lines (B) of sunflower using primers for Ha 1442 locus

A - 1 - VD 255, 2 - VD 22, 3 - VD 149, 4 - VD 356, 5 - VD 350, 6 - VD 354, 7 - ED 869, 8 - VD 151, 9 - VD 344, 10 - ED 95, 11 - ED 77, 12 - VD 1448, 13 - ED 73, 14 - ED 236, 15 - ED 169, 16 - ED 931. M - molecular markers.
 B - 1 - VD 0211, 2 - VD 0306, 3 - VD 361, 4 - VD 33509, 5 - VD 7307, 6 - VD 515, 7 - VD 545, 8 - VD 1228, 9 - VD 1671, 10 - VD 541, 11 - VD 62, 12 - VD 110.

Locus *Ha* 1608 is suitable for rapid identification of the line VD 0306, which does not have fragments typical for the rest of the parental forms.

Using primers for locus *IUB* 4 commonly two amplicons were obtained for each line. Only for Rf lines we observed polymorphism of the marker's size (130-190 bp). Interestingly, some Rf lines have no 130 bp fragment. Fragment of 180-190 bp is present in all studied lines.

A characteristic feature of the primers for locus *IUB* 6 is the induction of 350-370 bp amplicon. In this case only 5 lines are characterized by the presence of 370 bp fragment, while the others have 350 bp fragment. Virtually all Rf lines, except VD 1671 does not have the polymorphism of this marker.

Primers for SSR loci *ORS* 6 and *ORS* 509 have induced amplicons ranging in size from 250 to 260 bp and from 190 to 210 bp, respectively. They are presented in the studied lines differently.

Locus *OSU* 1 is represented by two fragments of 150-160 bp and 750 bp. However, the line VD 0306 is characterized by the appearance of the amplicon about 100 bp in size.

Primers for locus *HNCA* 2 initiate the amplification of two loci with sizes of about 210 and 340 bp. The lines can be differentiated by the presence or absence of a fragment of 340 bp.

When evaluating the DNA polymorphism of CMS and Rf lines values of PIC varied not only between different microsatellite markers, but depending on the

belonging genotyped lines to paternal or maternal lines (Table 1). Thus, the Rf lines characteristic by higher PIC values of investigated SSR markers, except *IUB* 6. The average values of the Polymorphic Information Content of SSR markers for maternal and paternal lines of sunflower are 0.53 and 0.61, respectively (Table 1).

Study of DNA polymorphism of CMS lines revealed PIC values for the loci we selected ranged from 0.21 for *ORS* 6 primers to 0.73 for *Ha* 432 primers (Table 1). PIC values for loci *ORS* 6, *ORS* 509, *IUB* 6 and *HNCA* 2 were in the range 0.21-0.43, which is sufficient for the identification and certification of studied lines. Seven loci with PIC values above 0.5 are most effective for the differentiation of the studied genotypes.

Based on the obtained data was constructed UPGMA-dendrogram showing the genetic similarity between the studied genotypes of CMS lines of sunflower (Fig. 2). All the lines clustered by the set of alleles in varying degrees with coefficients of similarity from 0.95 to 0.40.

PIC values of Rf lines ranged from 0.15 (primers for *IUB* 6) to 0.79 (primers for *Ha* 1442). Loci *Ha* 432, *Ha* 1442, *Ha* 1608, *IUB* 4, *ORS* 509, *Ha* 514, *Ha* 1287, *OSU* 1 with PIC values above 0.5 proved to be the most effective for genotyping of studied samples. The average PIC value in this case was 0.61, which is higher than PIC value obtained in the analysis of CMS lines (Table 1).

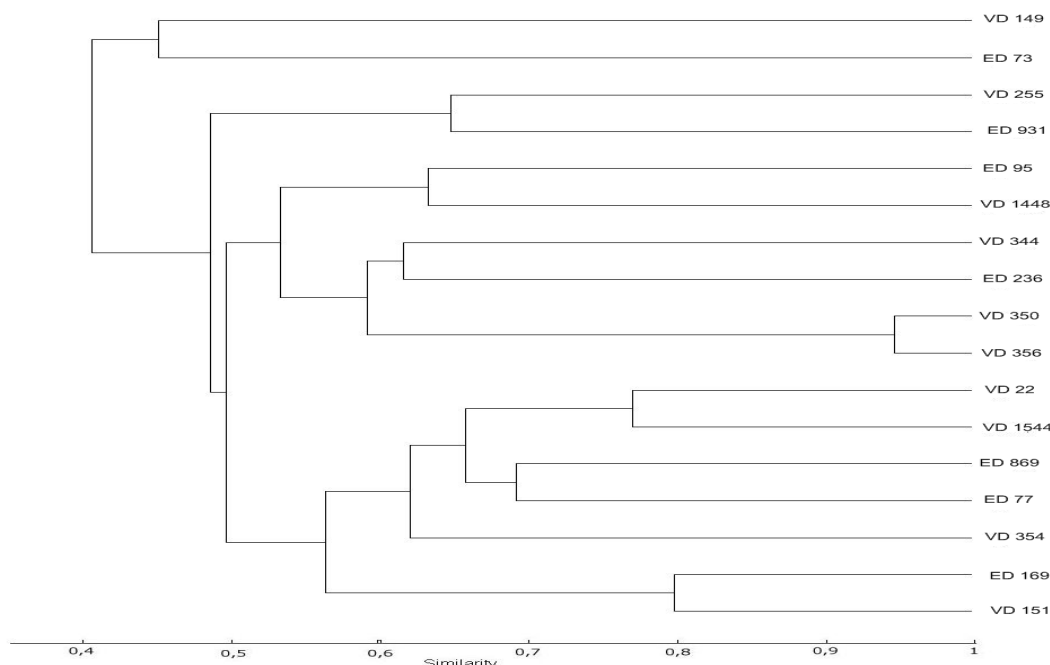


Fig. 2. The dendrogram of genetic similarity of CMS lines of cultivated sunflower, based on the results of SSR-analysis

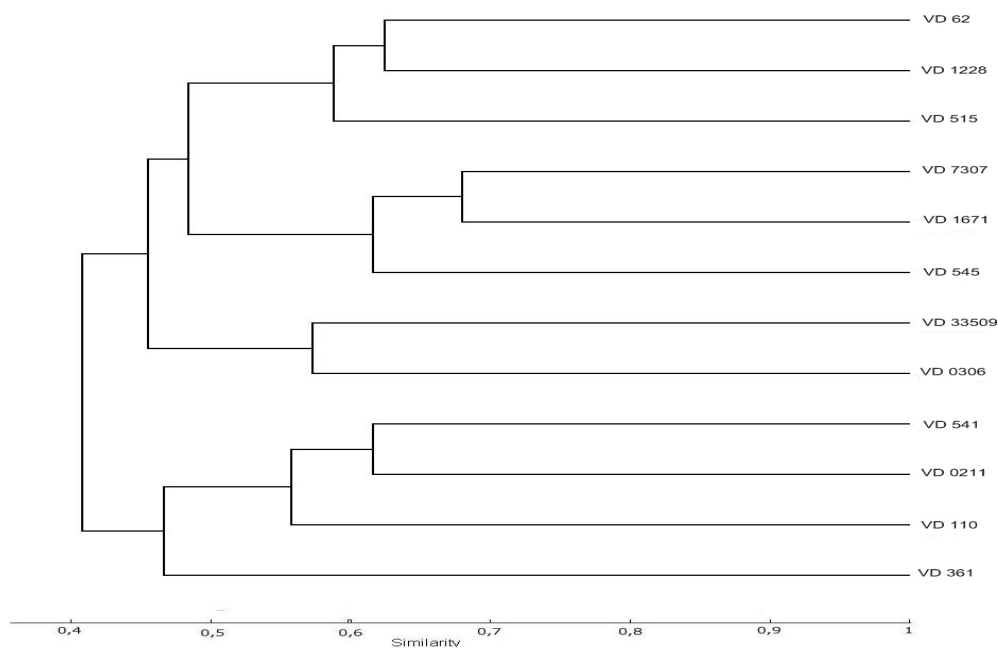


Fig. 3. The dendrogram showing the genetic similarity of Rf lines of sunflower, based on the results of SSR-analysis

Figure 3 shows the dendrogram of genetic similarity of Rf lines of sunflower, based on the results of SSR analysis of genomic DNA.

As shown in the dendrogram with use of the marker system were detected genetic differences between the lines at different degrees. Value of genetic similarity is less than 0.7, indicating higher degree of genetic differences between Rf lines in comparison with studied CMS lines of sunflower.

Despite in the SSR analyses conducted in this study was defined a small number of unique amplicons specific to individual genotypes, identified patterns allow together the identification of all the investigated lines. At the same time a high degree of pattern similarity in SSR analysis of genomic DNA of CMS and Rf lines can be explained in terms of the origin of Rf lines from the CMS lines using backcrossing.

Conclusion

Here, 35 and 42 allelic variants resulted from analysis of polymorphism of 11 microsatellite loci of 17 genotypes of CMS lines and 12 genotypes of Rf lines, respectively (breeding material from DOS VNIIMK). Assessment of the allelic distribution of studied microsatellites identified differences among all examined sunflower lines and also among the CMS and Rf lines, indicating high efficiency of the SSR analysis for genotyping of linear material of this crop. As a result of this work, it was found that the level of genetic diversity of the microsatellite loci of CMS lines was 1.2 times

lower than that of Rf lines. The average numbers of alleles per SSR loci for maternal lines also lowered than for paternal lines. Furthermore, the CMS and Rf lines differ in the frequency of alleles of SSR loci. The discriminatory capacity of the studied marker system for identifying lines of cultivated sunflower was quite high and is suitable for certification of a linear material. Data on the distribution of alleles of SSR loci in different lines of sunflower represents a database that can be used to plan effective crossings ensuring the highest level of genetic polymorphism of F_1 hybrids.

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Author's Contributions

Markin N.V. and A.V. Usatov: Designed and performed experiments and wrote the paper.

Gorbachenko, O.F., V.N. Vasilenko and A.I. Klimenko: Designed and performed experiments.

Maidanyuk D.N. and L.V. Getmantseva: Developed analytical tools and analyzed data.

Ethics

This article is original and contains unpublished materials. The corresponding author confirms that all of

the other authors have read and approved the manuscript and no ethical issues involved.

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