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Research Article

SSR Markers Assessment in Estimation of Genetic Polymorphism in Sunflower

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Abstract

There were screened 42 sunflower genotypes for polymorphism using 10 simple sequence repeats (SSRs/microsatellites) primer pairs. It was identified a total number of 179 alleles. The number of alleles per locus ranged from 4 (ORS240) to 27 (ORS328), with a mean of 17.9 alleles per locus. All SSRs tested in the present study displayedhigh level of polymorphism and are suitable for genetic diversity estimation. PIC value varied form 0.67 (ORS240) to 0.95 (ORS653), with a mean of 0.89. It was performed clustering ofinvestigated genotypes into heterotic groups/clusters, which is especially of great value for hybrid breeding.

Keywords: Genetic polymorphism; molecular markers; oilseed crops; phylogenetic relationships.

Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important crops worldwide and it is grown on area of $25.59 \cdot 10^4$million m^2 with an annual production of $44.75 \cdot 10^3$million kg. In Republic of Moldova in 2013 annual production of this oilseed crop was 504.5millionkgand it was placed at third place after wheat and maize by cultivated area ($275 \cdot 10^7$ m^2). (FAOSTAT,2014).

Establishment of germplasm diversity and relationships among different genotypes and especially elite lines is of great value in crop breeding. For this purpousedifferent types of molecular markers such as RAPD (Arias& Rieseberg, 1995), AFLP (Hongtrakulet al., 1997; Gedil et al., 2001), RFLP (Berry et al., 1994; Gentzbittel et al., 1994), TRAP (Yue et al., 2009), SSR (Dehmer & Friedt, 1998; Yu et al., 2002; Burke et al., 2002; Tang et al., 2002, 2003; Tang and Knapp, 2003) etc. are widely used. Marker-based approaches could facilitate sunflower breeding programms, saving time and workforce.

Placement of molecular markers to the linkage groups and construction of genetic maps is a crucial step toward genomic analysis of crop species. Such maps ensure a better understanding of genome organization, molecular evolution of crops and relationships between related species. From the breeding point of view, genetic maps are useful for identification and cloning of genes encoding economically important traits, marker assisted selection and gene pyramiding (Kapoor et al.,2009).The most widely used PCR-based markers are microsatellites which are characterized by high level of polymorphism, reproducibility, codominant inheritance, low cost and easy implementation (Paniego et al., 2002).

The first saturated genetic linkage map was constructed by Tang et al. (2002). For construction of this were used 94 RHA280 × RHA801 F_7 recombinant inbred lines (RILs) and 408 polymorphic SSR markers (462 SSR marker loci segregated in the mapping population). 459 of SSR marker loci were mapped and assigned into 17 linkage groups corresponding to haploid set of chromosomes in the sunflower genome (c = 17). The length of the map was 1368.3-cM with density of 3.1 cM per locus. (Tang et al., 2002)

A total number of 2968 SSR markers, including 1707 mapped markers, are available for molecular breeding and genomics research in sunflower (Sujatha et al.,2008).

The aim of the present study was to assess the genetic diversity of germplasm and to identify divergent lines for use in sunflower breeding programmes.

Materials and Methods

Biological material

Investigations were carried out on 42 genotypes of sunflower used in breeding programs ofAMG-Agroselect Company, Soroca, Republic of Moldova. Set of studied genotypes included 22 Rf lines, 12 CMS lines and 8 commercial hybrids F_1 (Fig. 2 and 3).

Growing conditions

The experiments were conducted in laboratory conditions. Sunflower seeds were grown in pots with sand till two cotyledon leaves stage. Obtained seedlings were used for DNA extraction.

DNA extraction and SSR amplification

DNA extraction was performed from three seedlings of each studied sunflower genotype (bulk samples) with GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scientific).

SSR analysis was realized using 10 primer pairs from ORS series (ORS31, ORS203, ORS204, ORS240, ORS254, ORS328, ORS653, ORS805, ORS1035 and ORS1242)(Tang et al.,2002; Yu et al., 2003). Some features of the primers are shown in Table 1.

Table 1. Primers used in investigation

Marker	L, bp	Repeat	Forward primer	Revers primer	Tm
ORS31	286	$(AAG)_{10}$	AAT TCA TGC CCC AAG AGA TG	CAC AAT TCA TGC ATT TCT CTG G	52
ORS203	264	$(AC)_4N_{11}(CA)_5N_2(CA)_5$	GCCCAAGATGTG AAGCGAATG	GTCAGAACAGGA CCGAACCACT	52
ORS204	312	$(GT)_{17}$	CGTCTGGCATTAT GAAATCGTC	CCGCATAACAGC AATGGTCAAC	52
ORS240	259	$(GCG)_6$	GGTGATGATGGA GGAGCAACTG	CACTCAACCATTG TTCTCCCAC	52
ORS254	386	$(TACA)_{25}$	AAATCCCACTTCA TACAAACGT	CCTTCAGTGCTCA TGCAGTG	51
ORS328	271	$(ACAAC)_{34}$	GACCTGTAGGCC AATATGAGACTT	TTATACCGGTGTT GTATCGTATCC	57
ORS653	312	$(CT)_{15}$	CACCCACCAAGA ACCCTAGA	CCGATACATACCA TAGCCGATT	60
ORS805	276	$(AG)_{20}$	CATGGATTATAAG AACGGGTGTT	AATCCCAGGGGT AAAATTGC	57
ORS1035	321	$(CT)_{13}$	CAACCCAACTTCT CCTCATAACC	AGGGCTGATATTC ACTTCACACA	59
ORS1242	269	$(CT)_{14}$	GCAATCGTTTCAC TCTTCCATTC	TGGTCGTAGAATT GTCGGTCAT	59

Amplification was carried out using following reaction mixture composition: 200 µM dNTP, 2.5 mM $MgCl_2$, 0.75 units of DreamTaq Green DNA Polymerase (Thermo Scientific), 0.4 µM of each primer, 50 ng DNA. Reaction volume was 15 µl.

The Touch Down PCR amplificationprogram included the following steps: 95°C - 3 minutes; 8 cycles of 95°C - 30 sec, 62°C↓55°C - 30 sec (-1°C/cycle), 72°C - 45 sec; 30 cycles - 95°C - 30 sec, 54°C - 30 sec, 72°C - 45 sec; 72°C - 5 minutes. Amplicons were visualized on a 6 % polyacrylamide gel (PAA) using TBE buffer under non-denaturing conditions (Green and Sambrook, 2012).

In conducted research, polyacrylamide-gel electrophoresis was used for the visualization of amplicons. In comparition with agarose-gel electrophoresis it was more accurate technology and relatively to automated analysis it was the most-accessible technology for routine analysis of these kinds of markers. (Sancez-Perez et al., 2006).

Cluster analysis and Polymorphic Information Content (PIC) estimation

Obtained profiles were scored for the presence [1] or absence [0] of alleles. Dendrograms were constructed based on data obtained from the SSR analysis using Treecon software. Estimation of genetic distance was performed according to Nei and Li (1979). Clustering was performed using the UPGMA method (Unweighted Pairwise Group Method with Arithmetic Mean)**.**

Polymorphism of each marker was determined using value of PIC (Polymorphic Information Content), calculated according to Anderson et al. (1993):

$PIC = 1 - \sum_{i=1}^{n} p_i^2$, where pi is the frequency of the i-th band and n is the number of bands observed.

Results and Discussion

SSR analysis

42 sunflower genotypes, cultivated in Moldova, were screened using ten pairs of SSR primers including five dinucleotide repeats, two - trinucleotide, one of each complex, tetra- and pentanucleotide repeats.

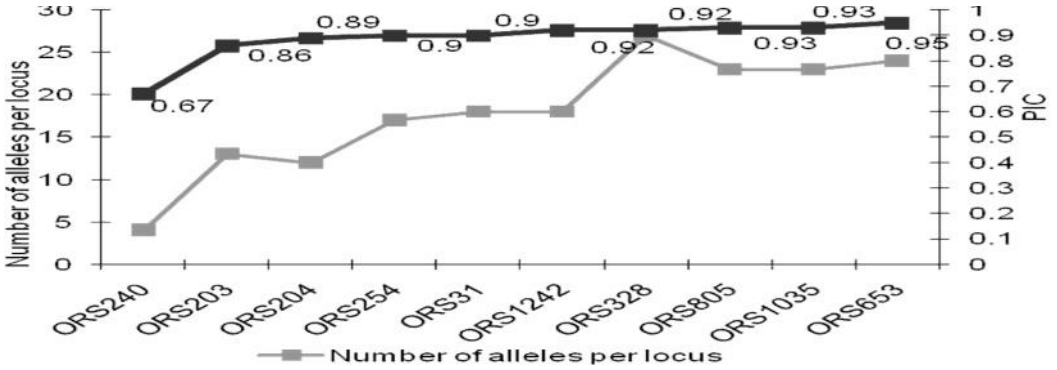
Primers showed a different level of polymorphism. A total number of 179 SSR alleles have been identified. The number of alleles per locus ranged from 4 (ORS240) to 27 (ORS328), with a mean of 17.9 alleles per locus. SSR primer pairs belonged to different linkage groups (LG, Linkage Group) described in sunflower (Tang et al.,2002) (Table 2).

Table 2. Polymorphism of studied loci in investigated genotypes

Locus	Linkage group	Allele number	Detected alleles	PIC
ORS31	5/16/17	18	291/296/300/306/312/314/321/324/333/ 338/343/351/376/398/412/416/427/459	0,90
ORS203	2/17	13	226/245/252/254/265/291/297/305/313/ 512/540/773/809	0.86
ORS204	17	12	277/286/301/313/327/331/345/368/376/ 389/406/427	0.89
ORS240	5	4	239/242/258/268	0.67
ORS254	15	17	507/543/547/552/553/556/564/573/579/ 582/591/603/615/626/632/647/668	0.90
ORS328	7/8	27	96/102/115/185/194/198/200/202/205/ 215/223/230/246/260/280/292/298/312/ 339/352/371/395/408/416/523/540/647	0.92
ORS653	2	24	190/224/243/258/272/277/279/282/294/ 296/299/314/319/333/334/338/353/382/ 398/416/420/454/462/484	0.95
ORS805	9	23	195/246/254/268/276/283/286/302/346/ 351/356/365/370/380/402/410/417/444/ 455/468/486/601/621	0.93
ORS1035	2	23	296/303/320/328/334/343/355/358/376/ 380/393/405/407/409/412/421/424/434/ 440/452/462/473/484	0.93
ORS1242	15	18	239/253/261/266/275/285/311/322/328/ 336/353/359/362/378/398/402/421/426	0.92

The PIC value ranged from 0.67 (ORS240) to 0.95 (ORS653) with a mean 0.89. It was observed strong correlation between PIC value and number of alleles per locus (Spearman`s rank correlation coefficient 0.88) (Figure 1).

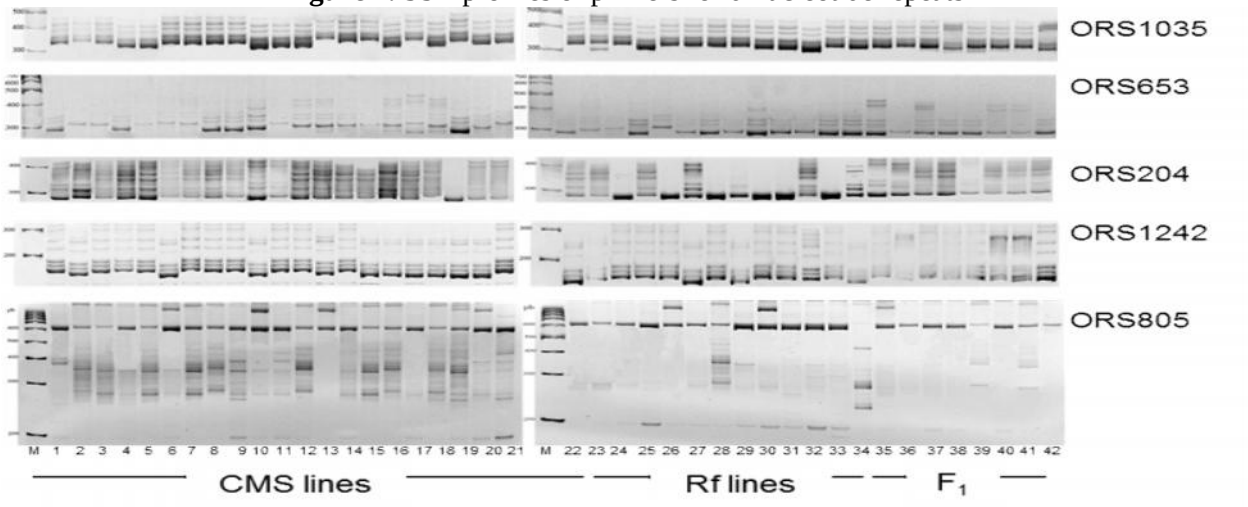
Figure 1. Correlation between PIC value and number of alleles per locus



Dinucleotide repeats were the most polymorphic. Two of them – ORS653 and ORS1242 created overloaded profiles with stutter bands, that are difficult to analyse (Figure 2). A great interest for mapping and fingerprinting purposes presents markers for trinucleotide repeats ORS240, because they mostly amplify low copy number loci and produce PCR products almost free of band artifacts with low number of bands, which could be easy analyzed. It was observed no association between stuttering and specific dinucleotide motifs or repeat types. These data are in concordance with results of other authors Paniego et al. (2002), who determined no association between stuttering (multiple bands in a microsatellite allele) and specific dinucleotide motifs. Also it was mentionedby them that amplificationproducts of (ATT)n, (TGG)n, and (GA)n repeats usuallyproduced few stutter bands, whereas (GT)n, (CATA)n, and(ATC)n repeats had a lower quality patterns, but stillproduced scorable bands (Paniegoet al., 2002).

Comparision into profiles generated on F_1 hybrids and their parental lines revealed that F_1 hybrids patterns are mostly similar with these of maternal CMS lines (especially in case of ORS1035 and ORS240).Some of investigated markers generated bands specific for CMS, Rf lines or F_1 hybrids. For example, 120 bp and 647 bp fragments obtained with ORS328 were characteristic only for Rf lines and F_1 hybrids respectively. Generally, Rf lines and F_1 hybrids generated more abundant profiles in comparison with CMS lines. This is obviously observed in patterns produced by ORS31, ORS254, ORS805 etc. (Figure 3).

Figure 2. SSR profiles of primers for dinucleotide repeats



M – DNA ladder, 1 – MS-2440C, 2 – MS-2064C, 3 – MS-1924C, 4 – MS-1944C, 5 – MS-1950C, 6 – MS-2080C, 7 – MS-1985C, 8 – MS-1995C, 9 – MS-2570C, 10 – MS-2275C, 11 – MS-3470C, 12 – MS-1920C, 13 – MS-2555C, 14 – MS-2540C, 15 – MS-2203C, 16 – MS-2583C, 17 – MS-2400C, 18 – MS-2565C, 19 – MS-2005C, 20 – MS-2020C, 21 – MS-2090C, 22 – MS-2550C, 23 – MS-2077A, 24 – MS-2067A, 25 – MS-2091A, 26 – MS-1589A, 27 – MS-2039A, 28 – MS-2098A, 29 – MS-2161A, 30 – MS-2073A, 31 – MS-2185A, 32 – MS-2075A, 33 – MS-2036A, 34 – MS-2026A, 35 – Codru, 36 – Dacia, 37 – Nistru, 38 – Zimbru, 39 – Talmaz, 40 – Doina, 41 – Cezar, 42 – Oscar

Markers with high PIC values and patterns with distinct bands and opportunity of easy analysis such as ORS203 (1-4 bands per profile), ORS240 (null/1/3 bands per profile), ORS254 (null/3 bands per profile) etc., could be effectively used in genetic diversity studies of sunflower, fingerprinting of valuable genotypes and estimation of lines genetic purity.

ORS240 generated four alleles of 239, 242, 258 and 268 bp. Four genotypes (MS-2064C, MS-1950C, MS-2039A and MS-2185A) from 42 investigated is characterized by the presence of null allele. With the exception of maternal MS-2077A line and three hybrids - Codru, Cezar and Oscar, which showed three bands, other genotypes were characterized by a single amplification fragment (Figure 3). Such markers with simple profiles could be usefull for genotyping, development of genetic passports of varieties and hybrids and finally estimation of the authenticity of valuable genotypes.

Primer pair ORS203 produced 13 alleles between 226 and 809 bp on studied genotypes and in 512-809 bp region were generated four alleles: 512, 540, 773 and 809 bp only in seven genotypes –MS-2090C, MS-2550C, MS-2091A, MS-2098A, MS-2161A, MS-2185A and MS-2075A. Patterns generated for each genotype contained from one to four bands. The presence of four bands were mainly observed in hybrids with exception of Dacia and Talmaz hybrids, in which were identified only one amplified fragment (Figure3).

Marker ORS254 generated 17 alleles and three polymorphic bands per profile with the exception of two genotypes (MS-2064C and MS-2026A), which are characterized by the presence of null alele(Figure 3).

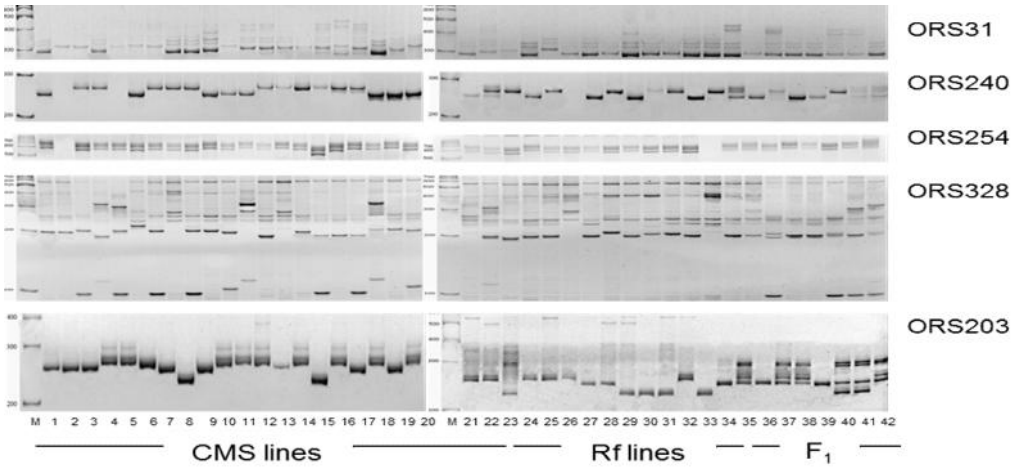


Figure 3. SSR profiles of primers for tri-, tetra-, pentanucleotide and complex repeats

M – DNA ladder, 1 – MS-2440C, 2 – MS-2064C, 3 – MS-1924C, 4 – MS-1944C, 5 – MS-1950C, 6 – MS-2080C, 7 – MS-1985C, 8 – MS-1995C, 9 – MS-2570C, 10 – MS-2275C, 11 – MS-3470C, 12 – MS-1920C, 13 – MS-2555C, 14 – MS-2540C, 15 – MS-2203C, 16 – MS-2583C, 17 – MS-2400C, 18 – MS-2565C, 19 – MS-2005C, 20 – MS-2020C, 21 – MS-2090C, 22 – MS-2550C, 23 – MS-2077A, 24 – MS-2067A, 25 – MS-2091A, 26 – MS-1589A, 27 – MS-2039A, 28 – MS-2098A, 29 – MS-2161A, 30 – MS-2073A, 31 – MS-2185A, 32 – MS-2075A, 33 – MS-2036A, 34 – MS-2026A, 35 – Codru, 36 – Dacia, 37 – Nistru, 38 – Zimbru, 39 – Talmaz, 40 – Doina, 41 – Cezar, 42 – Oscar

ORS240 and ORS254 markers are characterized by the presence of null allele on investigated genotypes.

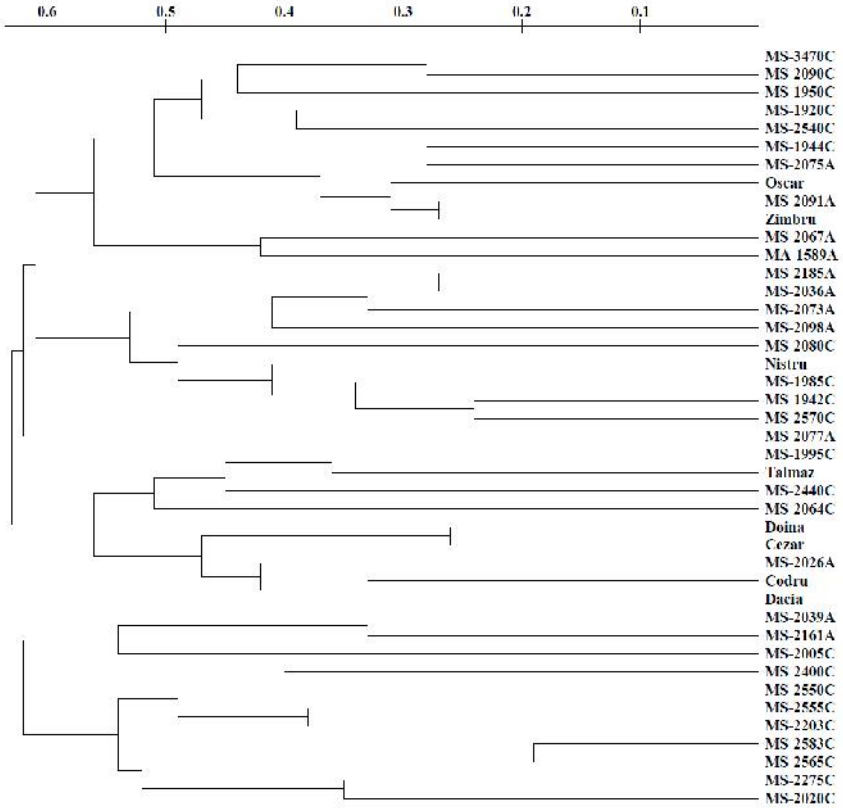
Clustering analysis based on SSR markers

Investigated genotypes were classified into five clusters based on polymorphism revealed using SSR markers (Figure 4):

Cluster 1contains ten Rf lines (MS-3470C, MS-2090C, MS-1950C, MS-1920C, MS-2540C, MS-1944C, MS-2080C, MS-1985C, MS-1942C and MS-2570C), eight CMS lines (MS-2075A, MS-2091A, MS-2067A, MS-1589A, MS-2185A, MS-2036A, MS-2073A and MS-2098A) and three hybrid forms (Zimbru, Oscar and Nistru). Hybrids were grouped togheter with parental forms (eg., hybrids Zimbru

and Oscar with maternal line MS-2091A but Nistru hybrid with paternal form MS-2570C). From studied parental forms only parental line of Zimbru hybrid – MS-2440C was not included in the first cluster, being in cluster three (Figure 4).

Figure 4. Dendrogram of distribution of sunflower genotypes based on profiles obtained with SSR markers



Cluster 2 is formed by a single CMS genotype - MS-2077A.

Cluster 3 included nine genotypes: five hybrids (Talmaz, Doina, Cezar, Codru and Dacia), three Rf lines (MS-1995C, MS-2440C and MS-2064C) and CMS line – MS-2026A. The four hybrid forms from that cluster were grouped together with paternal line MS-2440C. Exception present Doina hybrid parental forms which have been classified in the first and second cluster.

Cluster 4 includes two genotypes with CMS (MS-2039A; MS-2161A) and Rf genotype (MS-2005C).

Cluster 5was formed from eight Rf lines: MS-2400C, MS-2550C, MS-2555C, MS-2203C, MS-2583C, MS-2565C, MS-2275C and MS-2020C.

Information about genetic polymorphism offered by SSR markers can serve for the classification of germplasm into heterotic groups/clusters, which is especially have great value for hybrid breeding. Actually the genetic mechanisms of heterosis are not fully highlighted, but it is demonstrated that hybrids obtained from crosses between parents with higher genetic distance, show performant characters than crosses between closely related parents (Stuber, 1994). Thus, crosses between parental lines assigned in different clusters would give a better results than crosses between genotypes from same cluster (e.g. Rf lines from cluster five with CMS lines from first cluster).

Data obtained about the genetic polymorphism and relationships among hybrids and their parental lines is not only useful for germplasm conservation and identification of lines, but also for the selection of parental line pairs for hybrid productivity breeding programs in crops, including sunlfower (Usatov et al., 2014; Gvozdenovic et al., 2009).

Conclusion

SSR analysis with 10 pairs of primers allowed the identification of a total number of 179 SSR alleles. The number of alleles per locus ranged from 4 (ORS240) to 27 (ORS328), with an average of 17.9 alleles per locus. Markers used showed a different level of polymorphism. The PIC value ranged from 0.67 (ORS240) to 0.95 (ORS653) with a mean 0.89.

Rf lines and F_1 hybrids used in study generated more abundant profiles in comparison with CMS lines. This is obviously observed in patterns produced by ORS31, ORS254, ORS805 etc.

Investigated microsatellite markers can be useful for: fingerprinting (ORS240), highlighting the authenticity of valuable genotypes, determination of intraspecific genetic polymorphism (ORS203, ORS254 etc.), seed hybridization degree and genetic purity of seeds.

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