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DEVELOPMENT OF MARKER SYSTEM FOR IDENTIFICATION AND CERTIFICATION OF SUNFLOWER LINES AND HYBRIDS ON THE BASIS OF SSR-ANALYSIS

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SUMMARY

An analysis of DNA microsatellite sequences of 17 sunflower inbred lines and hybrid combinations developed at VNIIMK has been conducted. Nine of the10 studied microsatellite loci were polymorphic, with the average number of alleles per locus of 2.2. One locus was monomorphic. Dominant type of inheritance was found in two loci, codominant in seven.

At the level of similarity between lines from 0.19 to 0.97, individuality of each line and hybrid combination is revealed. Their molecular genetic passports were made on the basis of the nine loci. Suitability of the 9 loci for genetic purity tests of sunflower seeds in commercial lots has been shown. The seven codominant loci are suitable for definition of hybrid vigor.

Key words: PCR, microsatellites, alleles, polymorphism, identification, markers, sunflower inbred lines, level of similarity

INTRODUCTION

Sunflower is the main oil crop in Russia. Along with traditional cultivars, sunflower hybrids are cultivated widely. Hybrid development requires control over the genetic diversity of an initial material, for effective choice of parental pairs, tests of seed similarity in commercial parties and identification of genotypes for protection of breeders' rights.

The analysis of polymorphism of DNA amplified microsatellite sequences is one of the most convenient methods used in recent years for this purpose. It is known that microsatellite loci, on the whole, are not coding sites for DNA, and, hence, are not liable for natural selection. Mutations may accumulate in these sites, causing a high level of polymorphism. They frequently have the codominant type of inherit-

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ance. Such simple repeats are widely spread in plant genomes including genes loci (Brown *et al.*, 1996; Chesnokov, 2005).

Recent studies of genome mapping (Tang *et al.*, 2002; Paniego *et al.*, 2002) have allowed to establish the frequency of various SSR loci in sunflower genome and use them for identification of genotypes. Solodenko *et al.* (2004, 2005) investigated the polymorphism of 13 known SSR loci of some wild species and 20 lines of sunflower from three Ukrainian breeding centers. The study showed that the analysis of microsatellite loci in breeding material may be used to define genetic purity, hybrid vigor and DNA characteristics of the lines.

The purpose of this study was to define the quantity and inheritance type of allelic variants of 10 known microsatellite loci, to estimate their usefulness for identification and certification of sunflower lines and hybrids and to calculate genetic relationships between inbred lines in domestic breeding programs.

MATERIALS AND METHODS

Seventeen inbred lines and two hybrid combinations developed at All-Russian Research Institute of Oil Crops (VNIIMK) were used as plant material for genetic investigation: VK 639, VK 464, VK 541, VK 571, VK 157, VK 678, VK 276, VK 580, VK 653, VK 910, VK 937, VK 680, VK 789, VK 551, VK 585, VK 876, VA 93, F_1 (VK 678 × VK 464), F_1 (VK 678 × VK 580).

DNA was isolated from 5- to 7-day seedlings according to the CTAB-protocol as modified by Saghai-Maroof (Saghai-Maroof *et al.*, 1984). PCR-amplifications were performed with 10 ng of genomic DNA in 25 μl reaction vials. Each vial contained 50 mM KCl, 67 mM Tris-HCl pH 8.8, 16.6 mM ammonium sulphate, 2.5 mM $MgCl_2$, 0.01% Tween 20, 0.2 mM of each dNTP, 10 pM of each primers and 1 unit Tag-polymerase. PCR reactions were carried out using a Tercik thermocycler (DNA-Technology, Russia). The optimum thermal conditions for PCR reactions were: denaturation at 96°C for 2 min, followed by 30 cycles of amplification; denaturation at 94°C for 30 s, annealing at 60°C for 40 s, strand synthesis at 70°C for 60 s and final terminal extension at 70°C for 2 min. Only the primer HNCA-2 was used at the annealing temperature of 55°C. Amplification products were resolved by gel electrophoresis on 2% agarose gel with subsequent staining by ethidium bromide and were registered under UV illumination by a video system (DNA-Technology, Russia) using "Gel-Imager 2" software. SSR-analysis was performed with 10 pairs of primers that were developed by Tang *et al.* (2002) and Paniego *et al.* (2002). Table 1 gives a description of the investigated microsatellite loci.

The investigated inbred lines were tested for genetic uniformity, using seedlings typical for a given genotype for the subsequent DNA identification. DNA extracted from seedlings of lines VK 580 and VK 678 was used in all PCR along with a marker of molecular weight as the control for overestimation of amplified fragments length.

RESULTS AND DISCUSSION

For authentic identification of genotypes it is necessary to use optimum quantity of loci with maximum number of alleles and well readable marker spectra. The use of 1-3 markers per chromosome is an optimum for the molecular genetic characteristic of cultivated varieties. However, as a rule, an increase in the number of microsatellite markers for one variety leads to a more detailed molecular genetic description of the investigated sample, but it does not influence much the efficiency of identification (Hlestkina *et al.*, 2004). Four microsatellite loci are necessary for definition of genetic purity of inbred lines and only one for hybridization level definition in hybrids (Solodenko, Sivolap 2004). We have estimated the allelic diversity of 10 microsatellite loci in 17 sunflower inbred lines developed at VNIIMK.

Table 1: Characteristics of the investigated microsatellite loci

Locus	Repeat	Sequence 5'-3'	No. of alleles
Ha 432	GT	CTT TAT CCC CCA CCC CCT CC GGG TTT AGT GGC CAG TAG TTG TC	2
Ha 514	GA	GGT CAA CGG ATT TAG AGT C GTA TTG ATT CCA ACA TCC AG	2
Ha 1327	ATT	CCG TTA GGT AGT TTA CTT GCG AC GGT GGG GGG AAT ATT CTG AGG TG	2
Ha 1442	ATT	GCT TAT GTG CTT ACG TGT TCC TG CTA AAC AGT TCG GCG AGT GTA GG	2
Ha 1608	ATT	GAT CTT AGG TCC GCC AC GAT GGC ATT TGG CTA GAC	3
ORS-6	AGG	GTG GAG AGA GGT GTA GAG AGC CAC CCC TCA CCC TGA CAC	2
ORS-5	AAC	ATC TGG AGC AGC AAA TTC AG CTG CTG CCC ACC ATA CTG	3
IUB-6	GT	TCG GTA TCG TTT GCT AAT GG GGT AAC TCT AAA GCT CTG TC	2
HNCA-2	GT	TGA GAC AAG CAT AAG CAC TAG ACA AGA CAA GGG ACT	2
IUB-3	TTTTTTTG	GCA TTA GGT AGA TAG CCC CAG GTG GTA CCC TCA CTA GTC CTC T	1

The quantity of alleles varied from 1 to 3 per locus (Table 1). Average alleles per locus were 2.2 (without taking into account the monomorphic locus). The obtained average value corresponded to those reported by other authors for other sets of microsatellite markers. Solodenko *et al.* (2003) analyzed 20 sunflower inbred lines from three Ukrainian breeding centers. They have revealed an average of 3 alleles per microsatellite locus. A study of a collection of inbred lines from three world breeding centers showed that 170 polymorphic microsatellites produced on average 3.5 alleles per locus within the reference group consisting of 16 inbred lines (Paniego *et al.*, 2002).

We designated the revealed alleles with letters of the Latin alphabet. Heterozygous spectra, *i.e.,* the spectra that showed fractions of two different alleles, were designated with two letters. The informative value of microsatellite loci appeared to be various. The locus IUB 3 appeared to be monomorphic in the studied material. The loci Ha 1608 and ORS 5 had the greatest allelic diversity. Two allelic states characterized the following loci: Ha 432, Ha 514, Ha1327, Ha 1442 and IUB-6, ORS 6, HNCA2 (Figure 1).

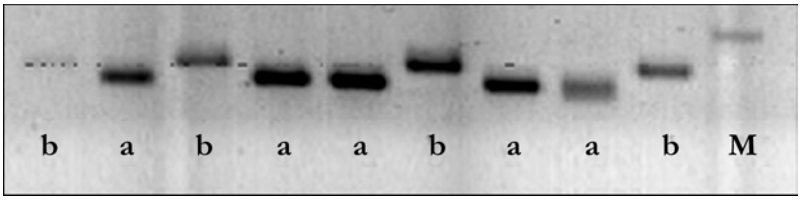

Figure 1: Amplification patterns of SSR locus of sunflower lines Ha 514 developed at VNIIMK: a, b - alleles, M - molecular weight marker 1 kb (fraction 250 bp).

It is interesting that, according to Solodenko and Sivolap (2005,) the locus ORS 5 was monomorphic in the lines of the Ukrainian origin, but it had three alleles in the lines from VNIIMK. The locus Ha 1608 had 2 and 4 alleles in the Ukrainian lines, and 3 alleles in the VNIIMK lines. It confirms the specificity of allelic polymorphism of the microsatellite locus in breeding lines of different origin.

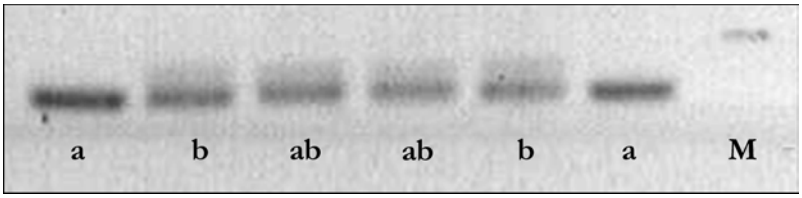

Figure 2: Amplification patterns of SSR locus of sunflower line Ha 432 and its hybrids developed at VNIIMK: a, b - alleles, M - molecular weight marker 1 kb (fraction 250 bp).

In locus **b** of the genotype Ha 432, there were two fractions of different intensity, of which the more intensive one was equal to that in allele **a** (Figure 2). We assume that this genotype is homozygous since on self-pollination the given character did not segregate. Using the primers pair Ha 1442, two microsatellite loci were amplified. Table 2 shows data for one of them (Ha 1442-1). The locus inheritance in the amplicon occurred as presence - absence of the fraction, *i.e.*, one allele was represented by zero-variant designated as **0** (Figure 3).

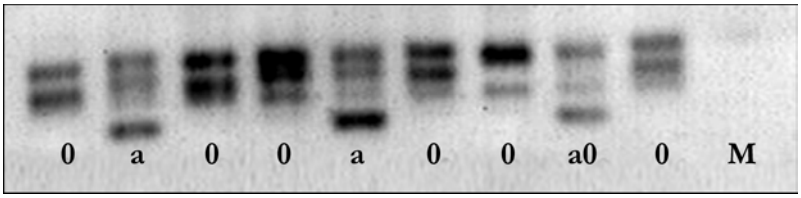

Figure 3: Amplification patterns of SSR locus of sunflower line Ha 1442-1 and its hybrids developed at VNIIMK: a, 0 - alleles, M - molecular weight marker 1 kb (fraction 250 bp).

All of the 10 SSR-loci showed clear electrophoretic spectra, typical for microsatellites. They were well reproduced in repeated analyses. The size of amplicons was 100 to 250 bp.

Table 2: Identification of sunflower lines by microsatellite loci

Line	Locus								
	Ha 432	Ha 514	Ha 1327	Ha 1442-1	Ha 1608	ORS6	ORS5	IUB6	HNCA2
VK 639	a	b	a	0	c	-	c	a	a
VK 464	b	a	b	0	b	a	a	a	a
VK 541	a	a	a	a	c	a	a	a	a
VK 571	b	b	b	0	b	a	a	a	a
VK 157	b	b	b	0	b	a	c	a	b
VK 678	a	b	a	0	b	a	b	a	a
VK 276	b	a	b	a	b	a	c	b	b
VK 580	b	a	a	a	c	a	a	a	b
VK 653	-	a	b	0	a	a	a	a	b
VK 910	a	b	b	a	b	a	b	a	b
VK 937	a	a	a	0	c	a	c	b	b
VK 680	b	b	a	0	a	b	c	a	a
VK 789	b	a	b	a	b	a	-	a	-
VK 551	b	b	b	a	a	a	b	a	a
VK 585	a	ab	a	a	b	a	b	a	a
VK 876	a	b	b	0	b	b	b	a	b
VA 93	a	ab	b	-	c	b	c	a	b

a, b, c, 0 - alleles

The analysis of electrophoretic spectra of 17 lines revealed that all of them were specific regarding the allelic structure. Nine primer pairs revealed polymorphism in all investigated lines. Thus, the specified microsatellite loci can be used as markers for identification of lines developed in VNIIMK, for drawing up their molecular genetic passports.

Table 3: Allelic conditions of microsatellite loci of sunflower parent lines and hybrids

Line, hybrid	Locus								
	Ha 432	Ha 514	Ha 1327	Ha 1442-1	Ha 1608	ORS6	ORS5	IUB6	HNCA2
VK 678	a	b	a	0	b	a	b	a	a
VK 580	b	a	a	a	c	a	a	a	b
VK 464	b	a	b	0	b	a	a	a	a
VK 678 × VK 580	b	ab	a	a	bc	a	ab	a	a
VK 678 × VK 464	b	ab	ab	0	b	a	ab	a	a

When developing sunflower hybrids, it is necessary to define the hybrid vigor in order to maintain the level of seed production. In the case of codominant inheritance, molecular genetic markers are a suitable tool for the estimation of this parameter during breeding and tests of genetic purity of commercial lots of hybrids

seeds. To define the inheritance type of the studied loci, individual plants of the lines VK 678 and VK 580 were crossed and the crosses were analyzed for allelic condition of the loci Ha 432, Ha 514, Ha 1442, Ha 1608 and ORS-5. Also, crosses between the lines VK 678 and VK 464 were analyzed for allelic condition of the loci Ha 432, Ha 514, Ha 1327, Ha 1442 and ORS-5. F_1 seeds were used for the analysis of DNA in hybrid plants. The segregating F_2 progenies were analyzed.

The spectra of amplified DNA of lines VK 678, VK 580, VK 464 and their hybrids confirmed the codominant inheritance of the loci Ha 514, Ha 1608, Ha 1327 and ORS-5 (Table 3). The hybrids contained fractions of both parents (Figure 4). It is necessary to note that among the products of amplification of the loci Ha 514 and Ha 1327, fraction **b** of the male form VK 580 was more intensively coloured. F_2 progenies were analyzed in 17 seedlings. Regarding the character of amplification products of seedlings, both combinations were divided into three groups: the two parents' spectra and one hybrid spectrum.

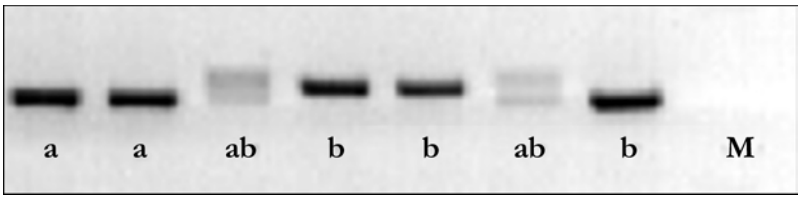

Figure 4: Amplification patterns of SSR locus of sunflower line ORS 5 and its hybrids developed at VNIIMK: a, b - alleles, M - molecular weight marker 1 kb (fraction 250 bp).

This confirms the codominant type of inheritance of these microsatellite markers. Hence, the analysis of allelic structure of the listed loci can be used for definition of hybrid vigor of F_1 seed. Dominant inheritance was revealed for the loci Ha 432 and Ha 1442. Since only one of the parental fractions was amplified, they cannot be used for hybrid vigor definition (Figures 2, 3).

Although the markers in the homozygous state are not suitable for definition of hybrid vigor, they can be used for both, identification of hybrids and definition of impurity level in seed lots. The impurities found in hybrid seed lots either as mechanical admixtures or as a result of pollination by alien pollen have another genotype which can be revealed by comparing their molecular genetic passports. The markers considered in the present work can be used not only for identification and certification of lines, but also for definition of hybrid seed uniformity and the impurity level resulting from self-pollination of parental forms.

A dendrogram for the 17 studied lines was made on the basis of the polymorphism in nine SSR loci (Figure 5). The nearest neighbor algorithm was used for construction of the triangular matrix of standard Euclidean distances. The level of similarity ($S_{1.2}$) between two genotypes was calculated by the formula $S_{1.2}=1-E_{1.2}$, where $E_{1.2}$ is standard Euclidean distance between genotypes 1 and 2. Calculations were made with the computer program "Graph-analyses" (Vorobyov, 2005). Accept-

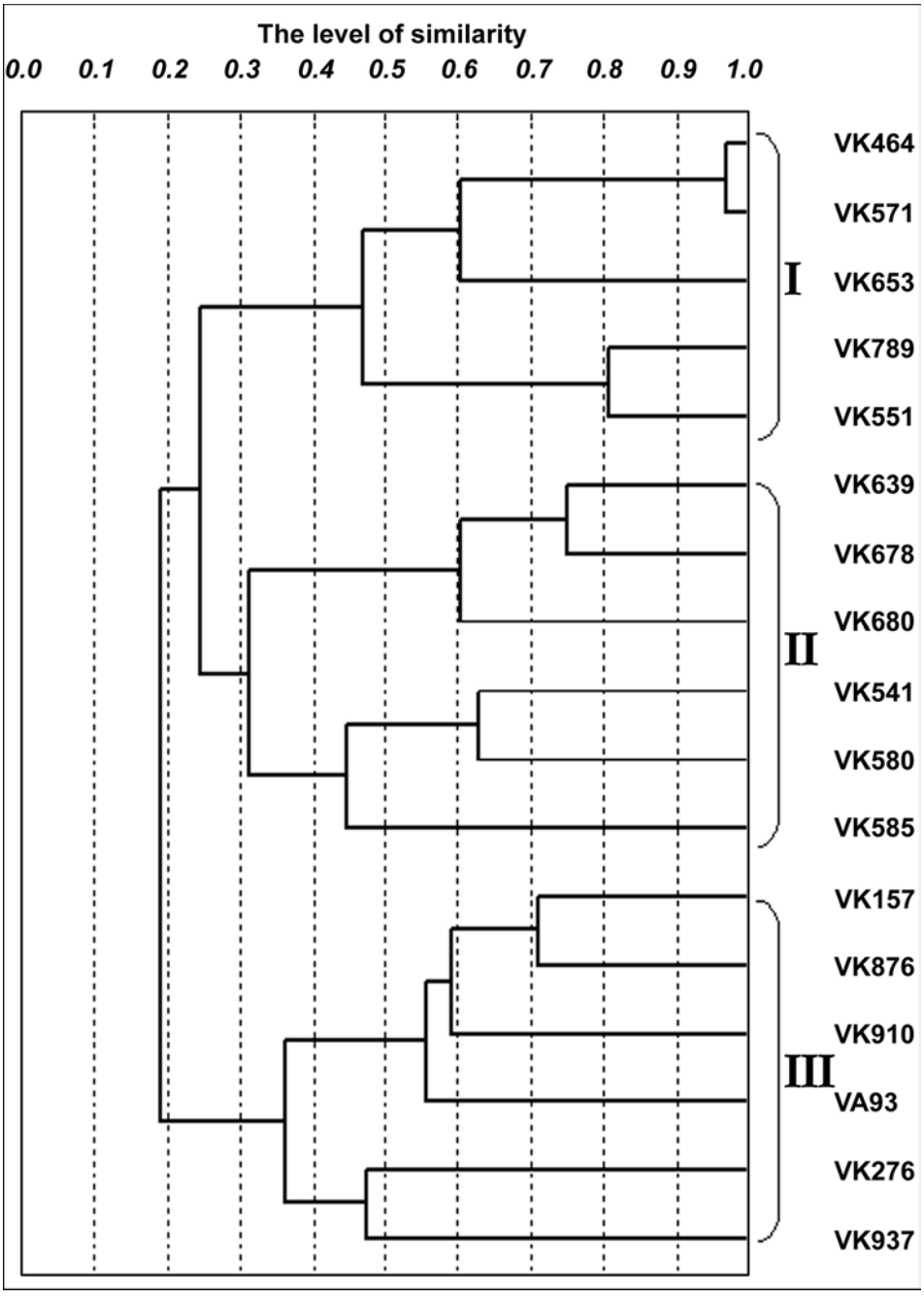

Figure 5: Dendrogram of sunflower lines developed in VNIIMK, constructed on the basis of allelic diversity between 9 SSR loci

ing for authentic distinctions at 0.5 level of similarity in the dendrogram, it was possible to allocate three clusters:

I - VK 464, VK 571, VK 653, VK 789, VK 551;

II - VK 639, VK 678, VK 680, VK 541, VK 580, VK 585;

III - VK 157, VK 876, VK 910, VA-93, VK 276, VK 937.

The maximum level of similarity between the genotypes was 0.97, the minimum 0.19.

It is interesting that the lines VK 571 (cluster I) and VK 157 (cluster III) and VK 678 (cluster II) and VK 876 (cluster III) were grouped in different clusters although these pairs were analogues that differed in 1-2 economically valuable characters. It is probable that some loci used by us (ORS 5 for the pair VK 571 and VK 175; Ha 1327, ORS 6 and HNCA 2 for the pair VK 678 and VK 876) can be in one group regarding the genes controlling the given characters.

CONCLUSION

The 17 sunflower lines developed at VNIIMK had nine polymorphic microsatellite loci, with the average number of alleles per locus of 2.2, and one monomorphic locus. Two loci out of nine had the dominant type of inheritance, and seven had codominant.

At the level of similarity between lines from 0.19 up to 0.97, individuality of each line and hybrid combination is revealed. Their molecular genetic passports were made on the basis of the nine loci. Suitability of the nine loci for genetic purity tests of commercial sunflower seed was confirmed. The seven codominant loci were suitable for defining hybrid vigor.

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DESARROLLO DE SISTEMAS DE MARCADORES PARA LA IDENTIFICACIÓN Y CERTIFICACIÓN DE LÍNEAS E HÍBRIDOS DE GIRASOL SOBRE EL ANÁLISIS DE LOS FRAGMENTOS SSR

RESUMEN

Fueron analizadas las secuencias microsatélites de DNA en 17 líneas consanguíneas (inbred) y las combinaciones híbridas de girasol, engendradas en el Instituto VNIIMK. Nueve de 10 loci microsatélite investigados fueron polimorfos, con el número promedio de alelos por locus de 2.2. Un locus fue monomorfo. El tipo de herencia dominante fue determinado en dos loci, y el tipo codominante, en siete.

En el grado de similitud entre las líneas de 0.19 a 0.97, fue confirmada la individualidad de cada línea y combinación híbrida. Los D.N.I. genéticos moleculares del material investigado, se hicieron sobre la base de nueve loci. En la investigación fue confirmado que el test de pureza genética de la semilla del girasol, sobre la base de estos nueve, era favorable para los lotes de semilla comerciales. Los siete loci codominantes determinados, son favorables para determinación de la potencia híbrida.

DÉVELOPPEMENT D'UN SYSTÈME DE MARQUEURS POUR L'IDENTIFICATION ET LA CERTIFICATION DE LIGNÉES ET D'HYBRIDES DE TOURNESOL À PARTIR DE L'ANALYSE DES FRAGMENTS SSR

RÉSUMÉ

Une analyse des séquences microsatellites de l'ADN de 17 sources pures de tournesol et de combinaisons hybrides développée à l'institut VNIIMK a été effectuée. Neuf des dix locus microsatellites étaient polymorphes avec un nombre moyen d'allèles par locus de 2.2. Un locus était monomorphe. Le type dominant hérité a été confirmé dans deux locus, un type codominant dans sept.

L'individualité de chaque lignée et de la combinaison hybride a été établie au niveau de similarité entre les lignées de 0.19 à 0.97. La carte d'identité génétique moléculaire a été faite à partir de neuf locus. Il a été établi que les tests de pureté génétique des akènes de tournesol sur la base de ces neuf locus étaient appropriés pour les lots d'akènes commerciaux. Les sept locus codominants sont appropriés pour la définition de la vigueur hybride.

Presented at: