Aspects of sunflower genotyping based on SSR markers

Received for publication, March 25, 2008
Accepted, April 20, 2008

DUCA MARIA*, CĂPĂŢÂNĂ ANA*, LEVITSKY ALEXEI*

Plant Biology Department, Moldova State University, Kogălniceanu 65[a] street, Chisinau, MD 2009, Republic of Moldova, e-mail: mduca2000@yahoo.com, ana_c27@yahoo.com

Abstract

The paper addresses the problem of revealing the varieties of crops and definition of their purity. The commonly accepted technique of estimation of genetic diversity of crops through observation of phenotypic traits observation can be succeeded by SSR technique. The work considers the possibility of application of SSR technique in investigation of commercial lines and hybrids. The results demonstrate the possibility of its usage in genotype differentiation and evaluation of the level of hybridization based on co-dominantly inherited loci. In addition, new alleles for several SSR loci are revealed.

Key words: plant breeding, selection, simple sequence repeats, sunflower

Introduction

One of the major problems met in breeding is to clearly distinguish the varieties of crops and to define their purity. The most common technique applied for the estimation of the genetic diversity of the crops is based on pedigree records and phenotypic trait observation. Morphological traits are traditionally used for an individual evaluation of the uniformity and stability of the genotype. Breeding of the cultivars for obtaining a new homozygous line or high productive hybrid based on traditional methods and technologies needs time, money expenses and human effort, though their efficiency is often low.

Therefore, implementation of the molecular biology techniques in plant breeding is of great importance. Molecular markers have a great number of advantages in comparison to traditional breeding methods, since the level of DNA is invariable to environment action [25]. Their implementation ensures exact and quick results, being more profitable for plant breeder, facilitating breeding strategies. Evaluation of allelic diversity in vegetal populations is an important stage during the elaboration of the techniques and systems for genotype identification. It must correspond to the following criteria: the possibility to reveal an optimal number of loci with the maximal number of alleles and their amplification products have to be clear and simple [20]. These conditions are indispensable during the author's rights confirmation for different plant varieties, seed quality certification and the conditions of parental germplasma protection.

One of the most effective methods of genetic polymorphism identification is SSR technique [29]. SSR markers possess a locus-specific position in the genome and are represented by simple repetitive sequences of di-, tri- and tetranucleotides. Microsatellites commonly are valuable multiallelic dominant and co-dominant markers [14], with high level of variability. The polymorphism is determined by the high level of insert/delete mutations, being dispersed in the majority of eukaryote genomes [5, 11, 14, 16, 17, 26].

Comparative analysis of different marker systems revealed that microsatellites show a higher level of polymorphism [11, 14, 26], being easier in handling and with a higher reproducibility. SSR markers possess a better determination of genetic polymorphism with a higher probability, in comparison with RFLP and RAPD techniques [14].

The capacity of SSR markers to discover high allele diversity is used to put in evidence the differences between genotypes. This type of markers were successfully applied at several crops as barley *(Hordeum vulgare)* [17, 18], rice *(Oryza sativa)* [27], grain *(Triticum aestivum)* [17], apple *(Malus domestica)* [23] and sunflower (*Helianthus annuus*) [7]. The level of polymorphism was detected to be high, being described up to 37 of different alleles for a single SSR locus at barley (Saghai Maroof et al., 1994). Several studies showed that the identification of the differences between 71 lines of barley there were sufficient just six SSR markers [12]. Microsatellite based genotyping at grapes [2, 9] allowed to determine the complexity of relations between a set of European cultivars [19].

Materials and Methods

Plant material. Parental lines (maternal and paternal) and their first generation hybrid Valentino represent annual diploid genotypes of sunflower (2n=34), offered by Research and Production Association "Magroselect" SRL. Maternal homozygous line is characterized by cytoplasmic male sterility. It has vegetation period of 110-115 days, height of 145-160 cm, inflorescence diameter 13-17 cm. Paternal homozygous line is male fertile. It is characterized by a vegetation period of 98-105 days, height of 112-130 cm, inflorescence size 12-16 cm. Hybrid has vegetation period of 118-123 days, height of 165-185 cm, inflorescence diameter 20-23 cm, oil content 48-50%. In addition, it is characterized by a high plasticity to various cultivation conditions. It manifests resistance to such pests as *Plasmopara helianthi*, *Puccinia helianthi*, *Orobanche cumana*, *Homoesoma electellum* and *Phomopsis helianthi*. Vegetal material was grown in laboratory conditions in vegetation vessels until the stage of two real leaves.

DNA isolation. Genomic DNA was isolated from the germs of two homozygous and one hybrid genotype. From the start, the vegetal material was grounded with liquid nitrogen. DNA extraction was realized with buffer solution 2xCTAB (500 ml 2x CTAB: 1 g CTAB, 4.09 g NaCl, 2.5 ml Tris stock (1M, pH 8.0), 1 ml Na-EDTA stock (0.5 M, pH 8.0)), then the samples were incubated at 65°C for 60 min. at the next step the samples were extracted with the mix of chloroform-isoamyl alcohol in proportion of 24:1, following DNA precipitation with isopropyl alcohol. Obtained DNA was washed with chilled ethanol of 70%. The sediment of ADN was solved in TE solution and stored at -20°C.

Primer design. Sunflower genotype screening was performed with 48 SSR primers. The sequences were arbitrary selected from Compositae Genome Database [28] and previously elaborated sequence maps. The primers, which amplify selected sequences, were elaborated using Primer3 soft and received from *Integrated DNA Technologies, INC*.

SSR analysis. For the analysis of sunflower genotypes, the screening of 48 randomly selected SSR primers was performed. PCR reaction was realized in 15 µl volume on a 96-well microplate in GenAmper 9700 thermal cycler (Applied Biosystems). Reaction mix had the following composition: 2 µl of DNA, 1.5 µl of 10X buffer solution, 1 µl of 25 mM $MgCl_2$, 1 µl dNTP 5 mM, 10 pmol of primers, and 1.5 units of *Taq* DNA polymerase (QIAGEN, Valencia, CA). The reaction of amplification was realized according to the next Touch Down PCR program: 94°C for 3 min; 10 cycles at 94°C - 45 s, 61°C - 45 s, - 0.5°C/cycle and 72°C - 1 min; following 35 cycles at 94°C - 45 s, 56°C - 45 s and 72°C - 1 min, with the final extension at 72°C - 20 min. PAGE analysis was realized in 6.5% gel in Li-Cor Global DNA Sequencer (Li-Cor Biosciences, Lincoln, NE), at 1500 V voltage for 2,2 h. Amplycon size was estimated in base of GelPilot 100pb Plus Ladder with the interval of 100 – 1500 bp.

Data analysis. Obtained gels were scanned at Typhoon 9420 and quantified by ImageQuant software (Amersham Pharmacia Biotech/GE Healthcare). Amplified fragments were considered as 1 in case of the presence of the band and 0 in case of its absence, and by "-" as no amplification. Polymorphism level was estimated by PIC value (*P*olymorphic *I*nformation *C*ontent): $PIC = 1 - \sum P_{ij}$, where P_{ij} is the frequency of ***j*** sample for ***i*** marker [15].

Results and Discussions

A SSR analysis with 48 primers pairs with exact localization was performed. Only 19 from 48 pairs of primers were selected for the microsatellite variations study at sunflower genotypes due to their polymorphism /Table 1, Figure 1/. Both lines and their first generation hybrid manifested polymorphism for the analyzed loci.

Localization on the chromosome map of the regions corresponding to the selected 19 primer pairs was performed according to their linkage groups (LG, *L*inkage *G*roup) from sunflower [24]. So, there was identified 1 locus for LG1, LG3, LG7, LG12, LG14 and LG16, 2 loci for LG5, LG8, LG15 and LG17, but LG9 was characterized by the amplification of the 5 loci /Table 2/.

The highest rate of identification possess dinucleotide motifs $(GA)_n$, $(GT)_n$, $(AT)_n$, more presented being $(GA)_n$ motifs then $(GT)_n$ [3]. Identified SSR loci represent 5 types of nucleotide repetitions: 1 mono-, 7 di-, 4 tri-, 1 tetranucleotide and 6 combined. 17 from 19 loci (89.5%) were polymorphic. Almost 83 SSR alleles were identified, but the number of alleles per locus varied between 1 (ORS158 and ORS166) and 12 (ORS70), with the average 4.3 allele per locus. The biggest number of alleles was shown for LG 9 with the average of 5.2 alleles.

The results put into evidence the presence of new loci, which were not revealed previously in literature, for markers ORS70, ORS78, ORS123, ORS135, ORS176, ORS188, and ORS261.

Table 1. The characteristics of studied polymorphic primers

Marker	Sequence name	Type of repetition and length	3'-5' primer sequence	5'-3' primer sequence
ORS 31	ORS-31	$(AAG)_{10}$	AAT TCA TGC CCC AAG AGA TG	CAC AAT TCA TGC ATT TCT
ORS 53	ORS-53	$(T)_{30}$	GCT GGC AAT TTC TGA TAC ACG AT	CAT CTA GAC AAC GAC AGA
ORS 70	ORS-70	$(CTT)_9$	GAC CCT GGT CAC CGA AGT TA	ATC TGA AAT CGG ACA AG
ORS 78	ORS-78	$(AAG)_{10}$	GTT CGT CGA GTA CAT GTT CTG C	TTT CCC TCT GGA AAG TT
ORS 123	1B7	$(TC)_{17}$	GAA AAC CCA TGC AGG CAT AC	ACATCCATCACAGTCCAT
ORS 134	3A1	$(AC)_{16}$	TAAAGATGTTGAGGTCCTGAATCG	TACGTGTACGTATGCATA
ORS 135	3A6-A	$(AC)_4\ N_1\ (CA)_7$	CAAAATGGAGAACAAAGCTCCC	TATTGCCGGTCCGACAT
ORS 158	7E9	$(CA)_{12}$	TTTGGTTTTAGCCAGAATTTCA	GCACGCCCGATGATACC
ORS 163	8A11	$(CA)_4\ N_2\ (CA)_{20}$	ATGGTTAAGTTACACACCCGCT	GCTATCAACGCCACCAC
ORS 166	8D3	$(AC)_{17}$	CAGCCACATGCCCTCTGAC	TGTTAAGAACCGCGACAA
ORS 176	9D7	$(TG)_{16}$	CCCTAACTGGTTTTCTGACCC	AACTTTTGTTTGTTTGTC
ORS 188	10F6	$(GT)_{18}$	CTTCGTAGCCAACTCCCACC	CAATGGTTGACAATGGGT
ORS 203	14C8	$(AC)_4\ N_{11}\ (CA)_5\ N_2\ (CA)_5$	GCCCAAGATGTGAAGCGAATG	GTCAGAACAGGACCGAAC
ORS 204	14D3	$(GT)_{17}$	CGTCTGGCATTATGAAATCGTC	CCGCATAACAGCAATGGT
ORS 216	16B5	$(CT)_{12}\ N_7\ (TC)_4\ N_2\ (TC)_6$	CTTCCTCCACCCTCAAGCG	TCCCTAATGTACCACCAC
ORS 240	14B5	$(GCG)_6$	GGTGATGATGGAGGAGCAACTG	CACTCAACCATTGTTCTC
ORS 254	10G9	$(TACA)_{25}$	AAATCCCACTTCATACAAACGT	CCTTCAGTGCTCATGCA

ORS 258	2E8	$(CT)_4\ N_{52}\ (CA)_4$	GGCCCGATTACAAGATAACG	TTGCGTCCGATGCTGT
ORS 261	7D7	$(GGT)_3\ N_{18}\ (CG)_4$	CTGTTCCGTTCGTCAGAAACTC	AGCGAAAGGATCGAGAAT

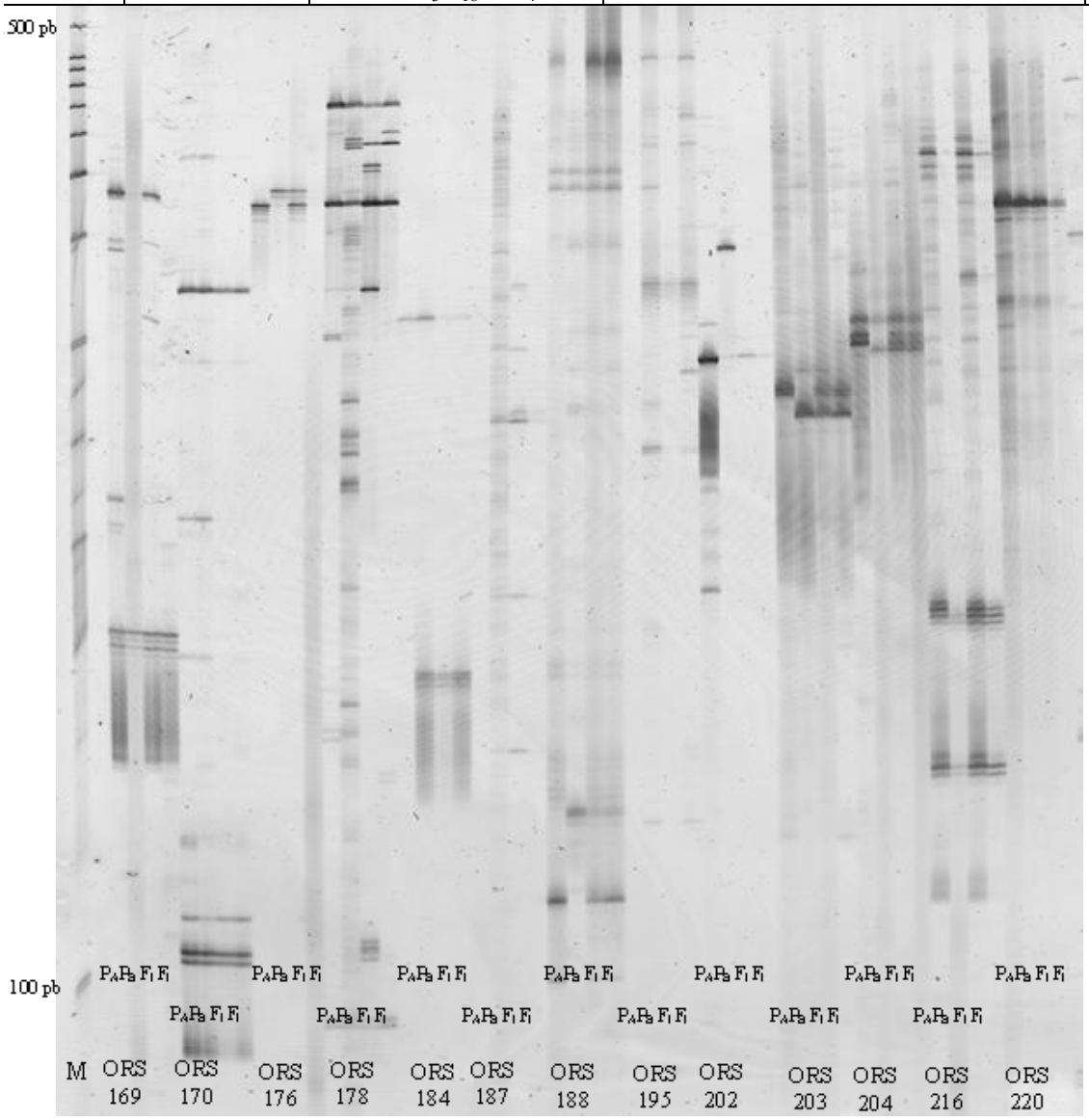

Figure 1. Polymorphism detection of paternal genotype (P_A), maternal line (P_B) and first generation hybrid (F_1) using SSR primers.

The average value of PIC parameter was 0.59, with the highest value 0.88 for ORS204 and the lowest 0.34 for ORS123. These results are similar to the studies of a big number of genotypes, where PIC value varied between 0 and 0,93, on average 0,57 according to one source [24], and 0,3-0,6 , with the average 0,55 according to others [13].

Table 2. The characteristics of studied microsatellite loci

Locus	Repetition	Linkage group	Length, bp		Allele number		PIC
			In species	In analyzed genotypes	In species	In analyzed genotypes	
ORS 31	(AAG)	16	282-307	250-300	7	5	0,79
ORS 53	(T)	1	438-483	450-510	6	4	0,82
ORS 70	(CTT)	8	125-185	125-185	5	12	0,57
ORS 78	(AAG)	9	162-174	160-172	4	8	0,72
ORS 123	(TC)	12	158-184	150-180	3	4	0,34
ORS 134	(AC)	3	299-301	209-301	2	2	0,48
ORS 135	(AC) (CA)	14	183-189	185-190	2	5	0,42
ORS 158	(CA)	9	253-259	260	3	1	0,46
ORS 163	(CA) (CA)	7	380-395	380-395	4	3	0,58
ORS 166	(AC)	8	333-375	340	7	1	0,72
ORS 176	(TG)	9	453-479	430-480	4	6	0,76
ORS 188	(GT)	9	119-167	115-160	3	8	0,62
ORS 203	(AC) (CA) (CA)	17	188-271	255-275	5	5	0,64

ORS 204	(GT)	17	294-320	280-300	8	4	0,88
ORS 216	(CT)(TC)(TC)	15	145-184	145-170	4	3	0,54
ORS 240	(GCG)	5	253-267	250-270	3	2	0,70
ORS 254	(TACA)	15	543-599	550-580	17	2	0,76
ORS 258	(CT)(CA)	9	319-340	315-340	6	4	0,46
ORS 261	(GGT)(CG)	5	201-207	190-200	2	4	—

The lines and the hybrid were polymorph for the analyzed loci. But some of the primers put into evidence the absence of the bands during electrophoresis, null alleles being revealed, and the other showed the presence of the bands which cannot be explained by the heterozygous or heterogeneity phenomenon for the analyzed genotypes. Probably, these new bands appear due to the duplication of microsatellite loci [1] for sunflower.

The investigation of the microsatellite sequences at parental lines and its hybrid determined the character of inheritance of these markers being dominant and co-dominant /Table 3/.

Thus, as dominant inherited were ORS123 and ORS163 loci. Furthermore, there we observed the possibility to evaluate the level of hybridization for the F_1 Valentino hybrid, because some of the loci were inherited as co-dominant: ORS53, ORS176, ORS188, ORS203, ORS204, ORS240, and ORS254. The rest of the loci showed an intermediate type of inheritance.

Table 3. Variability of SSR loci determined for the Valentino hybrid and it's parental lines

Primer	Allele	Genotype ♀	♂	F_1	Primer	Allele	Genotype ♀	♂	F_1	Primer	Allele	Genotype ♀	♂	F_1	Primer	Allele	Genotype ♀	♂	F_1
ORS31	**1**	+	+	+	*ORS135*	**1**		+	+	*ORS203*	**1**		+	+	*ORS176*	**1**		+	+
	2		+	+		**2**		+	+		**2**	+		+		**2**		+	+
	3		+	+		**3**		+	+		**3**	+		+		**3**	+		+
	4		+	+		**4**		+	+		**4**		+	+		**4**	+		+
	5		+	+		**5**	+				**5**		+	+		**5**	+		+
																6	+		+

Table 3. Variability of SSR loci determined for the Valentino hybrid and it's parental lines (continue)

Primer	Allele	Genotype ♀	♂	F_1	Primer	Allele	Genotype ♀	♂	F_1	Primer	Allele	Genotype ♀	♂	F_1	Primer	Allele	Genotype ♀	♂	F_1
ORS70	**1**	+			*ORS78*	**1**	+	+	+	*ORS188*	**1**	+		+	*ORS204*	**1**	+	+	+
	2	+				**2**	+	+	+		**2**	+		+		**2**	+		+
	3	+				**3**		+	+		**3**	+		+		**3**	+		+
	4	+				**4**		+	+		**4**	+		+		**4**		+	+
	5	+				**5**		+	+		**5**		+	+	*ORS258*	**1**		+	+
	6	+				**6**	+	+	+		**6**		+	+		**2**	+	+	+
	7	+				**7**	+	+	+		**7**	+		+		**3**	+	+	+
	8	+				**8**		+	+		**8**	+		+		**4**	+	+	+
	9		+	+	*ORS53*	**1**	+		+	*ORS123*	**1**	+		+	*ORS261*	**1**		+	+
	10		+	+		**2**	+		+		**2**	+		+		**2**		+	+
	11		+	+		**3**		+	+		**3**	+		+		**3**			+
	12		+	+		**4**		+	+		**4**	+		+		**4**			+
ORS134	**1**	+			*ORS163*	**1**		+	+	*ORS216*	**1**	+		+	*ORS240*	**1**	+		+
	2		+	+		**2**		+	+		**2**	+	+	+		**2**		+	+
ORS158	**1**	+	+	+		**3**		+	+		**3**	+		+	*ORS254*	**1**	+		+
					ORS166	**1**	+	+	+							**2**		+	+

Maternal genotype was characterized by 46 alleles /Figure 2/. The most polymorphic were ORS70 and ORS188 loci, which showed 8 and, correspondingly, 6 alleles. Two markers manifested the presence of null alleles, ORS163 and ORS261, with a 4% frequency. Seven loci showed just a single allele, but none of them was genotype specific.

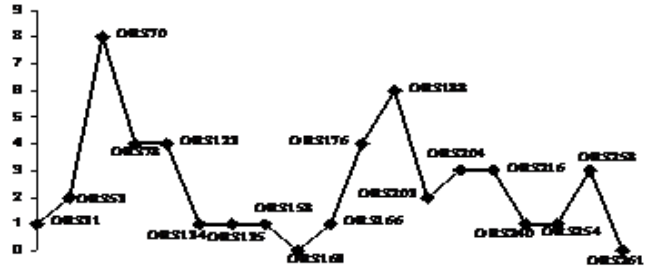

Figure 2. The level of polymorphism of SSR loci at maternal genotype of Valentino

Paternal line characterized by 47 alleles of SSR loci /Figure 3/. Just a single locus ORS123 was not determined (2% frequency). The biggest number of alleles was put in evidenced for ORS 78 locus. In comparison with maternal form, paternal genotype was characterized by six single allele loci, also without genotype specificity.

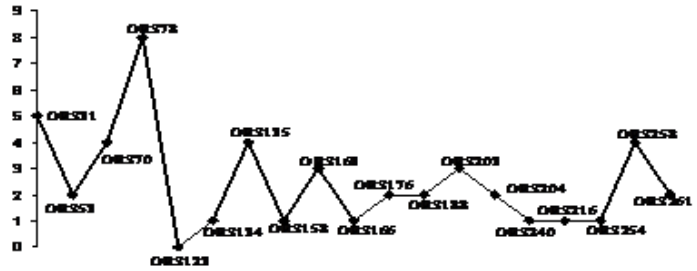

Figure 3. The level of polymorphism of SSR loci at paternal genotype of Valentino hybrid

At hybrid genotype, 73 loci were put in evidence. Some of the loci showed a single allele: ORS134, ORS158, and ORS166. The number of single allele loci is less then in parental lines. The most polymorphic were the ORS 78 and ORS188 loci, each with 8 alleles.

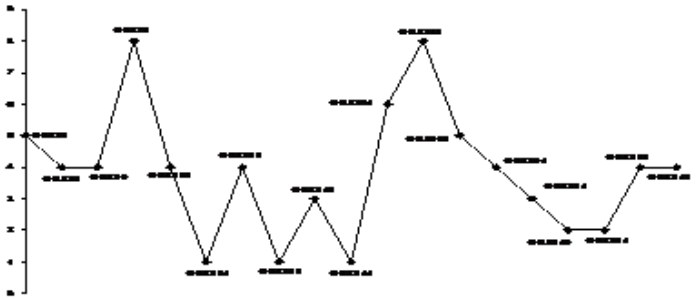

Figure 4. The polymorphism level of SSR loci at hybrid Valentino

The bigger frequency of alleles evidenced at hybrid genotype is determined by the combined manifestation of dominant, co-dominant and mixed inheritance.

SSR markers became efficient tools in appreciation of genetic polymorphism, DNA fingerprinting, genetic mapping and molecular amelioration at various crops [11, 14]. The advantage of these markers consists in their utility and multiple functionalities. SSR markers are highly informative, Mendelian or non-Mendelian inheritance, relative reduced expenses for analyses, quick genotyping and the possibility of automatization of the technique [4].

Acknowledgments

The authors thank to Dr. CC. Jan and Dr. J. Feng for the precious information about microsatellite primers of sunflower.

Reference

1. ACAGI, H., YOKZEKI, Y., INAGAKI, A., FUJIMURA, T. 1998. Origin and evolution of twin microsatellites in the genus *Oryza*. Heredity. **81**:187-197
2. CIPRIANI, G., FRAZZA, G., PETERLUNGER, E., TESTOLLIN, R. 1994. Grapevine fingerprinting using microsatellite repeats. Vitis. **33**:211-215;
3. DEHMER, K.J., FRIEDT, W. 1998. Evaluation of different microsatellite motifs for analyzing genetic relationship in cultivated sunflower (*Helianthus annuus* L.) Plant Breed. **117**:45-48
4. DIWAN, N., CREGAN, P.B. 1997. Automated sizing of fluorescent labeled simple sequence repeat markers to assay genetic variation in soybean. Theor. Appl. Genet. **95**:723-733
5. HAMADA, H., PETRINO, M.G., KAKUNAGA, T. 1982. A novel repeated element with Z-DNA-forming potential is widely found in evolutionarily diverse genomes. Proc. Natl. Acad. Sci. **79**:6465-6469
6. HONGTRACUL, V., HUESTIS, G., KNAPP, S. 1997. Amplified fragment length polymorphisms as a tool for DNA fingerprinting sunflower germplasm: genetic diversity among oilseed inbred lines. Theor. Appl. Genet. **95**:400-407
7. KNAPP, S.J. 1998. Marker-assisted selection as a strategy for increasing the probability of selecting superior genotypes. Crop Science. **38**(5):1164-1174
8. KORZUN, V., KARTEL, N., MALYSHEV, S. et al. 1998. A genetic linkage map of rye (Secale cereale L.). Theor. Appl. Genet. **96**:203–208
9. LAMBOY, W.F., ALPHA, C. 1998. The utility of simple sequence repeats (SSRs) for DNA fingerprinting germplasm accessions of grape (*Vitis* L.) species. Journal of the American Society for Horticultural Science. **123**:182-188
10. LIU, Z.-W., BIYASHEV, R.M., SAGHAI MAROOF, M.A. 1996. Development of simple sequence repeat markers and their integration into a barley linkage map. Theor. Appl. Genet. **93**:869-876
11. MORGANTE, M., OLIVIERI, A.M. 1993. PCR-amplified microsatellites as markers in plant genetics. Plant J. **3**(1):175-182
12. OLUFOWOTE, J.O., XU, Y., CHEN, X., et. al. 1997. Comparative evaluation of within-cultivar variation of rice (Oryza sativa L.) using micro-satellite and RFLP markers. Genome. **40**:370–378
13. PANIEGO, N., ECHAIDE, M., MUNOZ, M., et.al. 2002. Microsatellite isolation and characterization in sunflower (Helianthus annuus L.). Genome. **45**:34-43
14. POWELL, W., MACHRAY, G., PROVAN, J. 1996. Polymorphism revealed by simple sequence repeats. Trends Plant Sci. **1**:215-222
15. PREVOST, A., WILKINSON, M.J. 1999. A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theor. Appl. Genet. **98**:107-112

16. RAMSAY, L., MACAULAY, M., DEGLI IVANISSEVICH, S., et.al. 2000. A simple sequence repeat-based linkage map of barley. Genetics. **156**(4):1997-2005
17. RÖDER, M.S., PLASCHKE, J., KONIG, S.U. et al. 1995. Abundance, variability and chromosomal location of microsatellites in wheat. Mol. Gen. Genet. **246**:327–333
18. SAGHAI MAROOF, M.A., BIYASHEV, R.M., YANG, G.P., et. al. 1994. Extraordinarily polymorphic microsatellite DNA in barley: species diversity, chromosomal locations, and population dynamics. p. 5466-5470 In: Proceedings of the National Academy of Science, USA, nr. 91
19. SEFC, M.K., REGNER, F., TURETSCHEK, E., et. al. 1999. Identification of microsatellite sequences in *Vitis riparia* and their applicability for genotyping of different Vitis species. Genome **42**:367-373
20. SOLODENKO, A., SIVOLAP, YU. 2005. Genotyping of *Helianthus* based on microsatellite sequences. Helia. **28**(42):19-26
21. SMITH, J.S.C., CHIN, E.C.L., SHU, H., et. al. 1997. An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): comparisons with data from RFLPs and pedigree. Theoretical and Applied Genetics. **92**(1-2):163-173
22. STEPHENSON, P., BRYAN, G., KIRBY, J., et. al. 1998. Fifty new microsatellite loci for the wheat genetic map. Theoretical and Applied Genetics. **97**:946–949
23. SZEWC-MCFADDEN, A.K., KRESOVICH, S., BLIEK, S.M., et. al. 1996. Identification of polymorphic, conserved simple sequence repeats (SSRs) in cultivated Brassica species. Theoretical and Applied Genetics. **93**:534-538
24. TANG, S., YU, J.-K., SLABAUGH, M.B., SHINTANI, D.K., KNAPP, S.J. 2002. Simple sequence repeat map of the sunflower genome. Theor. Appl. Genet. **105**:1124–1136
25. TANKSLEY, S.D., GANAL, M.W., MARTIN, G.B. 1995. Chromosome landing: a paradigm for map-based gene cloning in plants with large genomes. Trends Genet. **11**:63-68
26. TAUTZ, D., RENZ, M. 1984. Simple sequences are ubiquitous repetitive components of eukaryotic genomes. Nucleic Acids Research. **12**:4127-4138
27. WU, K.S., TANKSLEY, S.D. 1993. Abundance, polymorphism and genetic mapping of microsatellites in rice. Mol. Gen. Genet. **241**:225-235
28. www.compositdb.ucdavis.edu
29. YANG, G.P., MAROOF, M.A.S., XU, C.G., et. al. 1994. Comparative analysis of microsatellite DNA polymorphism in landraces and cultivars of rice. Mol. Gen. Genet. **245**:187-194