

GENOTYPING OF *Helianthus* BASED ON MICROSATELLITE SEQUENCES

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SUMMARY

Analysis of microsatellite sequences was used for estimation of genetic diversity of cultivated and wild sunflower and usefulness for genotype identification, genetic purity and "hybridity range" definition. In total 13 SSRs were tested. Unique alleles for some wild sunflower species were found. Eight out of 13 SSR loci were polymorphic for the investigated inbred lines. Four of the investigated microsatellites are suitable and sufficient for genome identification and determination of parentage of hybrids. A procedure is proposed for sunflower genotype registration based on DNA-typing.

Key words: genotyping, *Helianthus*, microsatellites, PCR, identification

INTRODUCTION

The sunflower (*Helianthus annuus* L), an annual, diploid ($\times=17$) species, is an important source of oil, feed and an universally known and widely grown ornamental. *H.annuus* is one of the 48 *Helianthus* species belonging to the family *Compositae*. Sunflower breeding programs are focused basically on yield, oil content and disease resistance. Use of germplasm of *Helianthus* species for hybrids development requires detailed genotype control.

Molecular-genetic polymorphism investigations that analyze the variability of certain DNA sites are becoming one of the most efficient approaches in plant genetics. One of the most effective systems of DNA-analysis is amplification of microsatellite sequences (SSR). Microsatellites, or simple sequence repeats, are short-tandem sequence elements arranged in simple internal repeat structures that are densely and randomly distributed throughout the eukaryotic genomes. The number of some microsatellites has been shown to be highly variable within and between species.

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Locus specific, individually typeable, typically multiallelic, codominant inheritance, genome-widespread, relative simplicity in use and reproducibility are principal attributes of SSR markers. These characteristics have encouraged microsatellite application in different plant species such as soybean (Maughan *et al.*, 1995), rice (McCouch *et al.*, 1997), maize (Senior and Heun, 1993), wheat (Roder *et al.*, 1995), common bean (Yu *et al.*, 1999) and others.

In sunflower SSR markers are not as polymorphic as RAPD (Sivolap and Solodenko, 1998) but they are significantly more polymorphic than AFLPs (Hongtracul *et al.*, 1997) or allozymes (Cronn *et al.*, 1997). PCR amplification of sunflower microsatellites was used to uncover a genetic diversity among elite inbred lines (Yu *et al.*, 2000; Paniego *et al.*, 2002).

This work describes the use of SSR marker system for investigation of allelic diversity in *Helianthus*, identification of inbred lines and genotypes and determination of parentage of hybrids.

MATERIALS AND METHODS

The materials under study included 21 species and subspecies of *Helianthus*, some populations of *Helianthus tuberosus*, and 6 Ukrainian inbred lines of *Helianthus annuus*: Od 1036, Od 3369, Od BO, 4B, RHA CD and Orange, and their hybrids: Od 122 - a cross Od 1036 × 4B, Od 123 - (Od 3369 × 4B), Zgoda - (Od 3369 × RHA CD), BOO - (Od BO × Orange), Odor - (Od 1036 × Orange). The inbreds and hybrids were kindly provided by Dr. R. Serby.

DNA was extracted from the leaves and seedlings according to the CTAB-protocol. PCR mix in 20 μ l consisted of 50 mM KCl, 20 mM Tris-HCl pH 8.0, 1.5 mM MgCl₂, 0.01% Tween-20, 0.2 mM of each dNTP, 0.2 μ M of each primers, 20 ng DNA and 1 unit Taq-polymerase. PCR reactions were performed using "Tercik" thermocycler that was programmed for one initial denaturation step for 1 min. at 92°C, then 30 cycles of amplification: 1 min. at 92°C, 30 s at 60°C, 30 s at 72°C, and finally terminal extension step of 3 min. at 72°C. The amplification products were analyzed by electrophoresis on denatured polyacrylamide gels (10% acrylamide/bisacrylamide, 8 M urea in TBE). Bands were revealed by silver staining followed by the evaluation of bands size using VDS (Pharmacia Biotech) and computer program "Image Master 1D Elite".

RESULTS AND DISCUSSION

In this work we applied primers to sunflower microsatellite loci that have been developed by Tang *et al.* (2002), and Paniego *et al.* (2002). Thirteen SSR loci were investigated in a set of 21 species and subspecies of wild *Helianthus*, 5 populations of *Helianthus tuberosus* and 6 inbred lines and 5 hybrids of cultivated *Helianthus*

annuus. The treated microsatellite loci produced from 1 to 16 alleles per locus (Table 1).

Table 1: Characteristics of investigated microsatellite loci

Locus (code)	Repeat	Size range (b.p.)		Alleles no.	
		In species	In lines	In species	In lines
ORS 5	(AAC)	275-350	338	16	1
ORS 16 (A)	(AAT)	-	131-143	-	2
ORS 32	(AAT)(AAT)	155-176	155	6	1
ORS 78 (B)	(AAG)	152-165	156-168	3	4
ORS 509 (C)	(AT)(GT)	175-195	184-202	5	3
ORS 815 (D)	(CTT)	178-199	181-193	7	3
ORS1030	(GGT)	422	422	1	1
ORS1238	(AG)(TG)(ATT)	337	337	1	1
ORS 533 (E)	(CT)	136-169	166-174	4	5
ORS 595 (F)	(AG)	87-147	105-144	4	4
ORS1144 (G)	(CT)(CA)	120-145	126-142	7	2
Ha 1209 (H)	(ATT)	-	149-164	-	4
Ha 1796 (I)	(ATT)	-	164-236	-	2

Note: "-" - not analyzed

Samples of wild *Helianthus* are characterized by larger allelic diversity than cultivated sunflower. However, in some cases (ORS 78, ORS 533 loci) inbred lines are more polymorphic. While monoallelic SSR patterns were typical for inbred lines, varieties of *H. tuberosus* and *Helianthus* species in the majority of cases were heterozygotic (Figure 1).

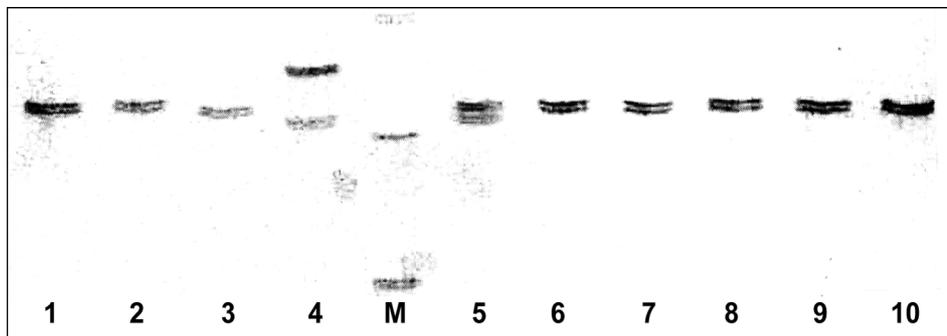


Figure 1: Patterns of amplification of SSR locus ORS 5: 1-5, 10 - inbred lines, 6 - *H. maximiliani*, 7 - *H. hirsutus*, 8 - *H. rigidus*, 9 - *H. microcephalus*, M - molecular weight marker (pUC 19/ MspI).

Individual patterns were obtained for representatives of the same species but different origin. It was revealed that some alleles were specific for genotypes from different taxonomic subgenera groups. High allelic diversity was a specific feature of *H. tuberosus*, some loci allowing unique identification of individual varieties. Alleles

that were revealed only in wild sunflower may be considered as potential markers in large-scale interspecific crossing programs.

Allelic diversity estimation is an important step in the development of genotypes identification system. Such systems have to comply with two rules: 1) optimum quantity of loci with maximum alleles and 2) amplification products of such loci should be simple and unambiguous.

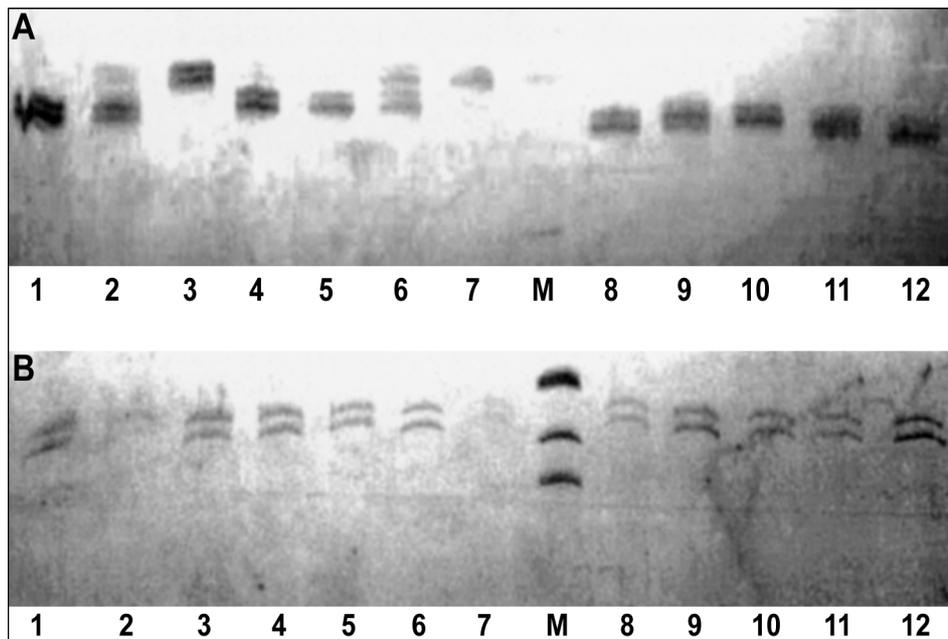


Figure 2: Amplification patterns of SSR loci ORS 815 (A) and ORS 1030 (B) of: 1 - Od 1036, 2 - Od 122, 3 - 4B, 4 - Od 123, 5 - Od 3369, 6 - Zgoda, 7 - RHA CD, 8 - BO, 9 - BOO, 10 - Orange, 11 - Odor, 12 - Od 1036, M - molecular weight marker (pUC 19/ MspI).

Some primer combinations evidenced the amplification of multiple band patterns (Figure 2). In the majority of cases this kind of amplification profile could not be explained by heterozygosity or heterogeneity of the investigated genotypes. A possible reason is the presence of two (or more) duplicated microsatellite loci in the sunflower genome. This amplification pattern was described previously by Acagi *et al.* (1998) in different species of the genus *Oryza*.

Nine out of the 13 SSR loci were polymorphic and fitting for unique genotype identification. The alleles typical for male inbred lines as well as for females were obtained in some loci (ORS 78, ORS 815, ORS 595, Ha 1209). Identification based of genotype formula consisted of information about allele size (in bp) corresponding to polymorphic loci (in coded mode) may be used as a DNA-passport of some lines or hybrids. For example, the genotype formula of the inbred line Od 1036 is:

$$A_{131}B_{162}C_{202}D_{181}E_{163}F_{141}G_{126}H_{155}I_{164}$$

A set of 9 SSR loci was used for testing genetic purity of 6 inbred lines of cultivated sunflower that represented a part of the Ukrainian sunflower germplasm. Twenty individual plants per inbred were analyzed. No heterogeneity was detected in any line. Amplification patterns of the hybrids contained the alleles of both parent lines (Figure 3).

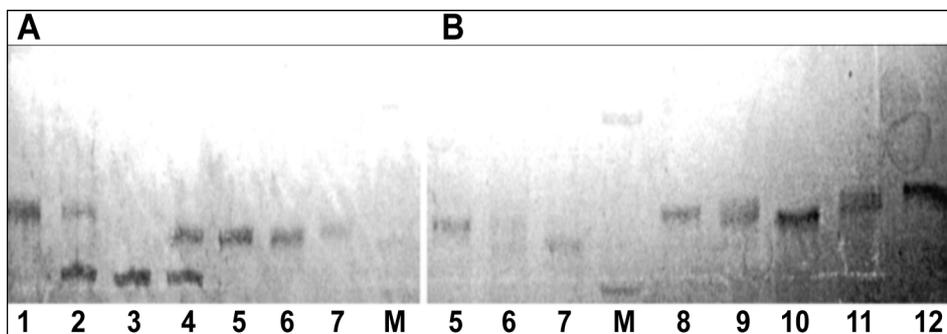


Figure 3: Amplification patterns of SSR loci ORS 509 (A) and ORS 78 (B) of: 1 - Od 1036, 2 - Od 122, 3 - 4B, 4 - Od 123, 5 - Od 3369, 6 - Zgoda, 7 - RHA CD, 8 - BO, 9 - BOO, 10 - Orange, 11 - Odor, 12 - Od 1036, M - molecular weight marker (pUC 19/ MspI).

A testing of individual plants of the hybrid Od 123 showed that some plants were true hybrids and some were not (Figure 4). This situation was confirmed by the analysis of different informative loci.

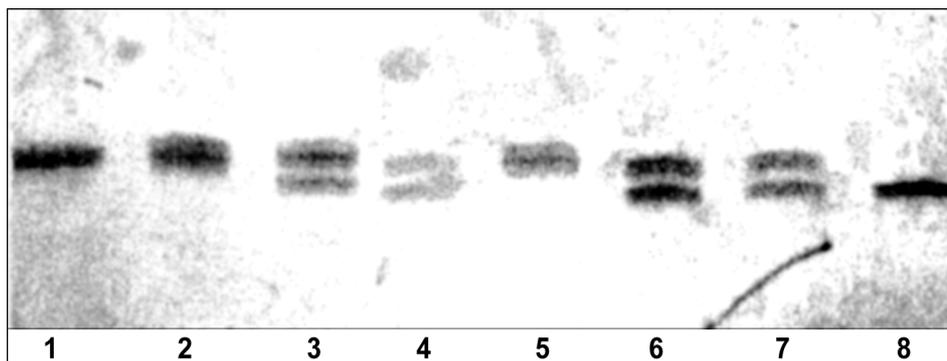


Figure 4: Amplification patterns of SSR locus ORS 78 of individual plants of hybrid Od 123 (2-7) and their parent lines Od 3369 (1) and 4B (8).

Analysis of allelic composition for 4 polymorphic loci was sufficient to differentiate the investigated genotypes. It needs at least 2 informative SSR loci (*i.e.*, polymorphic for parents lines) to identify some hybrids while genotyping of sufficient excerpts of F_1 plants permits a "hybridity range" estimation. It is possible that an increased set of inbreds and hybrids would require an investigation of a larger number of SSR loci. Genetic diversity range of commercial germplasm will be a determining factor for the development of the best system of SSR markers that allows differentiation and precise identification of genotypes.

CONCLUSIONS

SSR markers provide a powerful system for differentiating sunflower genotypes and identifying commercial inbred lines. Analysis of microsatellite loci of breeding material may be used to define genetic purity, "hybridity range" and DNA registration of lines.

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REFERENCES

- Akagi, H., Yokzeki, Y., Inagaki, A., and Fujimura, T., 1998. Origin and evolution of twin microsatellites in the genus *Oryza*. *Heredity* 81: 187-197.
- Cronn, R., Brothers, M., Klier, K., Bretting, P., and Wendel, J., 1997. Allozyme variation in domesticated annual sunflower and its wild relatives. *Theor. Appl. Genet.* 95: 532-545.
- Hongtracul, V., Huestis, G., and Knapp, S., 1997. Amplified fragment length polymorphisms as a tool for DNA fingerprinting sunflower germoplasm: genetic diversity among oilseed inbred lines. *Theor. Appl. Genet.* 95: 400-407.
- Maughan, P., Saghai Maroof, M., and Buss, R., 1995. Microsatellite and amplified sequence length polymorphisms in cultivated and wild soybean. *Genome* 38: 715-723.
- McCouch, S., Chen, X., Panaud, O., Temnykh, S., Xu, Y., Cho, Y., Huang, N., Ishii, T., and Blair, M., 1997. Microsatellite marker development, mapping and applications in rice genetics and breeding. *Plant. Mol. Biol.* 35: 89-99.
- Paniego, N., Eschaide, M., Munoz, M., Fernandez, L., Torales, S., Faccoi, P., Fuxan, I., Carrera, M., Zandomeni, R., Suarez, E., and Hopp, H., 2002. Microsatellite isolation and characterization in sunflower (*Helianthus annuus* L.). *Genome* 45: 34-43.
- Roder, M., Plaschke, J., Konig, S., Borner, A., Sorrells, M., Tanksley, S., and Ganai, M., 1995. Abundance, variability and chromosomal location of microsatellites in wheat. *Mol. Gen. Genet.* 246: 327-333.
- Senior, M., and Heun, N., 1993. Mapping maize microsatellites and polymerase chain reaction confirmation of the target repeats using a CT primer. *Genome* 36: 884-889.
- Sivolap, Yu., Solodenko, A., 1998. Inter- and intraspecies differentiation in the genus *Helianthus* by RAPD analysis. *Helia* 21: 9-18.
- Tang, S., Yu, J.-K., Slabaugh, M., Shintani, D., and Knapp, S., 2002. Simple Sequence Repeat Map of the Sunflower Genome. *Theor. Appl. Genet.* 105: 1124-1136.
- Yu, K., Park, S., and Poysa, V., 1999. Abundance and variation of microsatellite DNA sequences in beans (*Phaseolus* and *Vigna*). *Genome* 42: 27-34.
- Yu, J.-K., Mangor, J., Edwards, E., Thompson, L., and Knapp, S., 2000. Simple sequence repeats in sunflower: length polymorphisms among elite inbred lines for several motifs. In: Abstracts of Plant and Animal Genome Conference VII. 9-12 January, 2000, San Diego, Calif. Available at <http://www.intl-pag.org/pag/8/abstracts/pag8735.html>.

DETERMINACIÓN DE LA PERTENENCIA GENOTÍPICA DEL GÉNERO *Helianthus* SOBRE LA BASE DE LA SECUENCIA MICROSATÉLITE

RESUMEN

El análisis de las secuencias microsatélites fue utilizada para la evaluación de la genética del girasol cultivado y el salvaje, y su utilidad para la identificación de genotipos, determinación de la pureza genética y definición del éxito de hibridación. En total fueron investigados 13 SSRs. Los alelos únicos fueron encontrados en algunas especies salvajes. Ocho de 13 loci SSR eran polimorfos para las líneas consanguíneas investigadas. Cuatro de los microsatélites investigados son oportunos y suficientes para la identificación del genoma y la determinación de los progenitores híbridos. Fue propuesta la versión de registro de genotipos de girasol, mediante el método de DNA.

ÉTABLIR LES GÉNOTYPES *Helianthus* D'APRÈS LES SÉQUENCES MICROSATELLITES

RÉSUMÉ

L'analyse des séquences microsatellites a été utilisée pour évaluer la diversité génétique du tournesol cultivé et du tournesol sauvage et son utilité dans l'identification des génotypes, la détermination de la pureté génétique et la définition de "l'échelle d'hybridité". Treize SSR ont été testés en tout. Des allèles uniques ont été trouvés dans certaines espèces de tournesol sauvage. Huit des loci 13 SSR étaient polymorphiques pour les souches pures examinées. Quatre des microsatellites examinés conviennent et suffisent à l'identification des génomes et à la détermination des parents hybrides. On propose une version d'enregistrement des génotypes du tournesol au moyen de l'ADN.

