UDC 633.16: 631.543
Original scientific paper

CHARACTERIZATION OF F_1 INTERSPECIFIC HYBRIDS BETWEEN WILD *HELIANTHUS ANNUUS* L. POPULATIONS AND CULTIVATED SUNFLOWER

Sreten TERZIĆ, Jovanka ATLAGIĆ and Dejana PANKOVIĆ

Institute for Field and Vegetable Crops, Novi Sad, Serbia

Terzić Sreten, Jovanka Altagić and Dejana Panković (2006): *Characterization of F_1 interspecific hybrids between wild Helianthus annuus l. populations and cultivated sunflower.* - Genetika, Vol. 38, No. 2, 159-168.

Phenotype, chromosomes pairing and pollen vitality were compared between parental populations and F_1 hybrids of interspecific cross between *Helianthus annuus* L. and cultivated sunflower. The investigation of the simple sequence repeats (SSR) polymorphism was also used to test the hybrid nature of F_1 populations.

The phenotypic traits of F_1 hybrid plants were either closer to the wild species or intermediate. Irregular chromosome pairing was found in only 0 to 10% of meiocytes in the meiosis of F_1 hybrid plants. Interspecific crosses were confirmed with SSR markers in all hybrid combinations. Alleles that were not present in parental DNA were frequently observed in F_1 hybrids. That is additional evidence that those hybrid combinations were not produced by self-fertilization.

The results suggest that SSR markers can be efficiently used for the F_1 hybrid characterization in crosses between closely related species, in which, the changes of phenotype, meiosis and pollen vitality are not always significant.

Corresponding author: Terzić Sreten, Institute for field and vegetable crops, Maksima Gorkog 30, Novi Sad, Serbia, Tel. ++381 21 4898429, Fax: ++381 21 413833, e-mail: terzic@ifvcns.ns.ac.yu

Key words: Sunflower, wild species, interspecific hybridization, phenotype, analysis of meiosis, pollen viability, SSR

INTRODUCTION

There are 49 species of sunflower that make the genus *Helianthus* (flower of the sun in Greek), of which 13 are annual and 36 perennial (SCHILING and HEISER, 1981). They are all from North America. The haploid number of chromosomes in diploid species is 17. There are diploid (2n=2x=34), tetraploid (2n=4x=68) and hexaploid (2n=6x=102) species (GEORGIEVA-TODOROVA, 1976). All annual species are diploid.

All annual species except for *H. agrestis* have been crossed and backcrossed with cultivated sunflower using the classical method of hybridization (JAN, 1997).

Irregular meiosis in F_1 hybrids between cultivated sunflower and annual wild species has been found by HEISER (1961). By analyzing the meiosis of annual wild species, CHANDLER *et al.* (1986) found that they differ in 0-6 translocations and 0-8 paracentric inversions.

Phenotypic traits of F_1 plants have for long been one of the basic markers of their hybrid nature. The advantage of molecular markers in comparison to morphological and biochemical, is that they do not change under the influence of environment and that they can be detected in early phases of plant development. The development of microsatellite markers for sunflower made the analysis of genetically close populations possible (TANG *et al.*, 2002). Now it is possible to differentiate between close taxons and individuals in those taxons (TANG and KNAPP, 2003). Microsatellites (SIMPLE SEQUENCE REPEATS) are repeated short sequences of DNA (2-10 bp, 5-50 copies) that can be found in genomes of fungi, plants and animals. They are very variable and can be found on every 6-7 kb in plant genomes (CARDLE *et al.*, 2000). The goal of this research is to characterize F_1 interspecific hybrids between wild *Helianthus annuus* L. populations and cultivated sunflower, by comparing the results of phenotype analysis, meiosis, pollen vitality and SSR markers.

MATERIAL AND METHOD

The crosses were made with 28 populations of the annual wild species *H. annuus* (ANN1963, 1977, 1983, 2110, 2116, 2125, 2129, 2134, 2137, 2138, 2141, 2155, 2157, 2159, 2162, 2165, 2168, 2170, 2171, 2174, 2175, 2177, 2180, 2186, 2188, 2196, 2197, 2210). Cytoplasmatic male sterile line HA26A and lines in the breeding process (PHBC1 and PHBC2) were used as the mother lines. Low seed set is frequent in interspecific hybridization and because of that, the seed germination method was applied (CHANDLER and JAN, 1985).

Phenotypic traits were evaluated on ten plants per population using the sunflower descriptor (IBPGR, 1985). Branching was described so that the zero

marked non-branched plants, one - basal branching, two - apical branching, three - complete branching with central inflorescence and four - complete branching without the central inflorescence. Number of ray flowers, plant height, inflorescence diameter, and leaf shape, were also evaluated. Leaf shape was calculated by dividing the leaf length and width. The type of inheritance was determined by calculating the significance of difference between mean values of parental and F1 populations using t-test (BOROJEVIĆ, 1965).

Anthers of 3-5 plants have been taken from all F1 and parental populations for the analysis of meiosis. Acetocarmin method was used to check the regularity of chromosome pairing, their number and regularity of diakinesis, metaphase I, anaphase I and telophase II (GEORGIEVA-TODOROVA, 1976). Pollen vitality was determined by differential staining of vital and abortive pollen grains (Alexander, 1969). Samples of pollen were taken in the field as a mixture from several plants of the same population or two separate samples from two plants of the same population. Three preparations of each sample were made and complete male sterility was also registered.

The isolation of DNA was performed from frozen leaves using a modified CTAB method (HUGO *et al.*, 1998). After the PCR amplification, fragments were separated by horizontal electrophoresis on 2% high-resolution agarose gel (TANG *et al.*, 2002). The obtained bands were stained with ethidium bromide and analyzed with a computer program "Image master 1D Elite" (Pharmacia Biotech). The differences in fragment length greater than 20 bp were accepted as significant. Parental populations were tested in the first part of research, to establish which primers reveal alleles specific for the wild species. Primers ORS5, ORS7, ORS16, ORS78, ORS166, ORS509, ORS533 and ORS595 were used for that test (TANG *et al.*, 2002). The appearance of alleles in the sample of F1 population that were found in the sample of father and not in the sample of mother, was taken as an evidence of a successful interspecific cross (PANKOVIĆ i *sar.*, 2004; ATLAGIĆ *et al.*, 2003).

RESULTS AND DISCUSSION

Cross compatibility and analysis of phenotype

Hybridization was successful with 25 populations of 28 used and F1 seed was found on 54 of 106 pollinated inflorescences. The total number of F1 seed was 2503 and 169 F1 plants were obtained. Several types of inheritance were scored for every analyzed phenotypic trait. Partial domination (pd) or domination (d) of wild parent was scored in most of the hybrid combinations, while domination of cultivated sunflower (d^c) was found in only three hybrid combinations. Intermediate type of inheritance was scored for leaf shape (i) and heterosis (h) was found only for inheritance of plant height. In majority of hybrid combinations, plants were completely branched with central inflorescence (68 of 108 plants) (Table 1.).

Table 1. - Type of inheritance of phenotype traits and type of branching in F1 generation of sunflower

Hybrid combination	Height	Inflorescence diameter	Number of ray flowers	Leaf shape	Type of branching
F_1 *H. annuus*	$4h^{\alpha}$, 3d, $2d^{c}$, 1pd	5pd, 3d, 3i	6i, 4d, 1pd	8i, $1d^{c}$, 1d, 1pd	$0^{5\beta}$, 1^{22}, 2^{1}, 3^{68}, 4^{12}

$^{\alpha}$ 4h - Heterosis was scored in four hybrid combinations, 3d Domination of wild sunflower in three hybrid combinations, $2d^{c}$ - Domination of cultivated sunflower was scored in two hybrid combinations
$^{\beta}$ 0^{5}, 1^{22} - Five plants were not branched, twenty two basaly branched, etc.

Analysis of meiosis and pollen vitality

In the diakinesis of F_1 plants, beside of bivalent, a small percentage of uni- and multivalent chromosome configurations were found. Similar percent of fast and lagging chromosomes and chromosome bridges were found in other phases of meiosis (Table 2.).

Table 2. - Characteristics of meiosis in the species H. annuus and it's hybrids with cultivated sunflower

Phase	Characteristics	*H. ann.*	F_1 *H. ann.*
Diakinesis	Number of bivalents per cell	17.00(91)	17.02(366)
	% of meiocytes with		
	Univalents	0	1.16
	Bivalents	100	97.38
	Multivalents	0	1.45
	Trivalents	0	0.0
	Quadrivalents	0	1.16
	Hexavalents	0	0.29
Metaphase I	Fast chromosomes	0	7.26
Anaphase I	Lagging chromosomes	0.21	1.95
	Chromosome bridges	0	3.57
Telophase II	Lagging chromosomes	0	0.79
	Pollen vitality (%)	79-100 (95)	10-100 (91)

Chromosome pairing in diakinesis of F1ANN populations was irregular in less than 3% of analyzed meiocytes. Quadrivalents were found in 1.16% of meiocytes with multivalent configurations and hexavalents in 0.29%. Mean number of bivalents per meiocyte varied from 16.81 to 17.42 and the percent of meiocytes with regular diakinesis (17^{II}) was very high (97%). In other phases of meiosis there were 0.8-7.26% of meiocytes with irregularities. The largest percent of meiocytes with irregularities was found in metaphase I.

Restoration of male fertility occurred in most of the hybrid combinations. Percent of male sterile plants in F_1 hybrid generation was 6.04%. Pollen vitality in

the populations of the wild species was in average 95% and in F1 generation 91% (Table 2.).

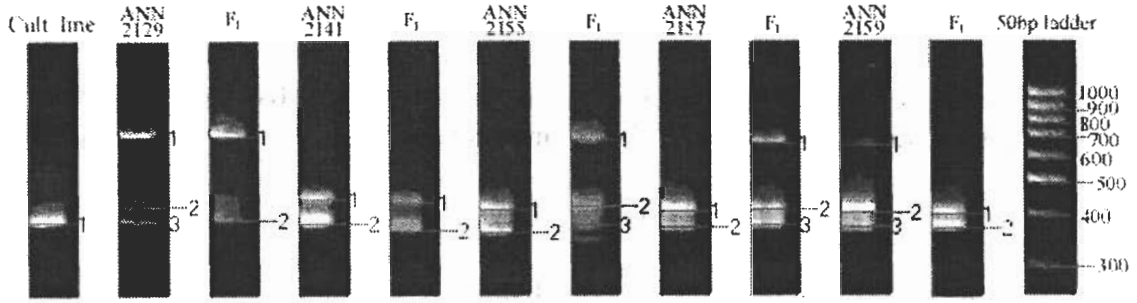
Characterization of F1 plants with SSR markers

Only primers ORS5 and ORS595 amplified SSR fragments on one locus on genomic DNA and showed existence of three alleles for every locus. Other primers amplified SSR fragments on two or three locuses (Table 3.).

Table 3. - Primers that were used to analyze the variability of genomic DNA in parental populations and number of locuses in which the SSR fragments were amplified

Primer	ORS5	ORS7	ORS16	ORS78	ORS166	ORS509	ORS533	ORS595
Locus number	1	2	2	2	3	2	2	1

One of the obtained markers per primer combination was usually codominant and the other(s) was (were) dominant. Following SSR primers were used for the identification of all F1 plants: ORS166, ORS533 and ORS595 (Picture 1. and Table 4.).



Picture 1. SSR fragments obtained with the primer ORS166. Positions on the gel (1-12) represent reactions with DNA and standard 50bp DNA ladder (Fermentas), with labeled bands from 300 to 1000 bp. Numbers with each sample represent the number of amplified fragments[1]

Table 4. - The length of amplified fragments of DNA (bp) in reaction with the primer ORS166[1]

Cult. line	ANN 2129	F1ANN 2129	ANN 2141	F1ANN 2141	ANN 2155	F1ANN 2155	ANN 2157	F1ANN 2157	ANN 2159	F1ANN 2159
	711					681		674	666	
	428	711	450	440	418	428	414	418	408	405
389	388	383	385	370	367	381	378	381	378	374

[1] The first sample is the cultivated sunflower and father and F1 populations are given in pairs after the first one. Fragments that were found in father and F1 populations and not in cultivated sunflower are written in bold fonts.

In the hybrid combinations in which the cross could not be confirmed with primers ORS166, ORS533 and ORS595 three more primers were used (ORS5, ORS78 and ORS509). One to three alleles were amplified per sample with the new set of primers. Obtained results confirmed that the cross was successful in the two remaining hybrid combinations. The number of alleles specific for the father, found in F_1 generation, varied from 1 to 3 (mean 1.8).

The success of crosses was also analyzed on the basis of fragments that were present in F_1 generation, but not in parental DNA's. The number of alleles specific for F_1 generation varied from 0 to 8 (mean 4.1). This is a clear proof that F_1 plants were not produced by self-fertilization i.e. the additional evidence of successful interspecific cross.

DISCUSSION

Cross compatibility and analysis of phenotype

The use of larger number of populations and pollinated inflorescences per population, led to increased number of successful crosses. The analyzed phenotype traits of F_1 plants were closer to the wild species (ATLAGIĆ, 1986). Several types of inheritance were found because of large variability in the wild species, poligenic determination of the analyzed traits and the influence of environmental factors on inheritance determination. Inflorescence diameter and type of branching were most informative for characterization of F_1 interspecific hybrids among all analyzed phenotype traits. The problem with this kind of characterization is that it has to be done at the end of the vegetative cycle and that it is partially under the influence of the environment.

Analysis of meiosis and pollen vitality

The pairing of chromosomes in diakinesis was regular in majority of analyzed wild species populations. Fast and lagging chromosomes were found in a very small percentage of meiocytes in the wild species. GEORGIEVA-TODOROVA (1976) and CHANDLER (1982) found regular diakinesis in annual wild species and a very small percentage of irregularities in the phases after the diakinesis (ATLAGIĆ, 1991).

F_1 hybrid populations from the cross between wild *H. annuus* L. and cultivated sunflower (*H. annuus* L. var. *macrocarpus* DC) most often had regular diakinesis in accordance with their genetic distance (5 of 9 analyzed hybrid combinations). Only one of nine analyzed hybrid combinations had close to 50% meiocytes with irregularities that included increased number of bivalents from 17 to 18 and 22. It is possible that such result is caused by hybrid nature of the wild population, which is indicated by lower pollen vitality (79%).

Pollen vitality in the F_1 hybrid combinations was under the influence of several factors. Sterile cytoplasm was inherited from the mother, male

fertility restoration genes from the father and irregular chromosome pairing was found in meiosis.

Regularity of meiosis is directly connected to pollen vitality and male sterility according to some authors (CHANDLER, 1986; BINSFELD *et al.*, 2001). They suggested that partial or complete sterility of F1 interspecific hybrids is caused by irregularities in meiosis. Lowered pollen vitality thus could be used as an indicator of interspecific hybridization (CHANDLER, 1986). On the other hand, several authors found that irregularities in meiosis do not have to influence the pollen vitality (HEISER, 1961; CHANDLER *et al.*, 1986) and that it is genetically controlled (GEORGIEVA-TODOROVA, 1984). ATLAGIĆ (1991) also considered that irregularities in meiosis (frequency and type) are the basic cause of sterility, but do not have to influence pollen vitality directly. Therefore, lowered pollen vitality is not necessarily the indication of an interspecific hybrid.

Characterization of F1 plants with SSR markers

In most of the hybrid combinations, fragments specific for father were revealed with first three primers in F1 generation. Some of the F1 populations had only one allele specific for the father even though analysis of phenotype, meiosis and pollen vitality indicated that they are interspecific hybrids. It is possible that such result is due to less intensive recombination because of the differences in genomes of the used species. BURKE *et al.*, (2004) compared genome maps of F1 hybrids and found more intensive recombination between lines of cultivated sunflower in comparison to interspecific cross with the species *H. annuus*. Both genome differences and heritable factors are responsible for a barrier to introgression between the species of the genus *Helianthus* (RIESEBERG *et al.*, 1995b).

RIESEBERG *et al.*, (1995a) used RAPD markers to analyze interspecific cross *H. annuus* X *H. petiolaris* and found that reorganization of hybrid genome occurred, in comparison to the parental one. Fragments of different sizes were obtained using the same primer. It is possible that alleles specific for father were not found in F1 generation because of genome reorganization by recombination. It can affect the position of an SSR marker, primer binding site and the length of markers (BURKE *et al.*, 2005). Recombination and errors in replication can change marker length, so that markers different than those in parents are found in F1 generation. If the primer binding site is affected, PCR amplification of the marker is disabled. Further analysis of the gels revealed that majority of F1 populations had alleles that were not present in either of parents. The number of such alleles varied from 0 to 8. These results confirm that F1 plants have been produced by hybridization and not self-fertilization.

Characterization of F1 interspecific hybrids with SSR markers is efficient and very useful when genetically close species are crossed, as the changes of phenotype traits in F1 generation are not always significant, pairing of chromosomes is regular and pollen vitality is high.

REFERENCES

ALEXANDER, M. P., (1969): Differential staining of aborted and non-aborted pollen. Stain Technology, USA, 11, 3: 117-123.

ATLAGIĆ, J., (1991): Karakteristike mejoze i fertilnosti biljaka F1 interspecific hibrida suncokreta. Doktorska disertacija, Poljoprivredni fakultet, Univerzitet u Novom Sadu.

ATLAGIĆ J., PANKOVIĆ D., PEKANOVIĆ A. (2003) Backcrosses in interspecific hybridization in sunflower. Genetika, Vol 35, No. 3, 187-197.

BINSFELD, P. C., WINGENDER, R., SCHNABL, H., (2001): Cytogenetic analysis of interspecific sunflower hybrids and molecular evaluation of their progeny. TAG, 102: 1280-1285.

BOROJEVIĆ, S., (1965): Način nasleđivanja i heritabilnost kvantitativnih svojstava u ukrštanjima raznih sorti pšenice. Savremena poljoprivreda, Novi Sad, 587-606.

BURKE, J. M., LAI, Z., SALMASO, M., NAKAZATO, T., TANG, S., HEESACKER, A., KNAPP, S. J., RIESEBERG, L. H., (2004): Comparative maping and rapid Karyotipic evolution in the genus *Helianthus*. Genetics, 167: 449-457.

BURKE, J. M., KNAPP, S. J., and RIESEBERG, L. H., (2005): Genetic consequences of selection during the evolution of cultivated sunflower. Genetics, 171: 1933-1940.

CARDLE, L., RAMSAY, L., MILBOURNE, D., MACAULAY, M., MARSHALL, D., and WAUGH, R., (2000): Computational and experimental characterisation of physically clustered simple sequence repeats in plants. Genetics, 156: 847-854.

CHANDLER, J. M., (1982): Chromosome Differentiation Among the Annual *Helianthus* Species. Disertation, University of California, Davis.

CHANDLER, J. M., and JAN, C. C., (1985): Comparations of germination techniques for wild *Helianthus* seeds. Crop sci. 25: 356-359.

CHANDLER, J. M., JAN, C. C., BEARD, H., (1986): Chromosomal differentiation among the annual *Helianthus* sp. Systematic Botany, 11(1): 354-371.

GEORGIEVA-TODOROVA, J., (1976): Interspecific relationships within the genus *Helianthus* (in Bulgarian). Bulgarian Academy of Sciences, Sofia, 1-169.

GEORGIEVA-TODOROVA, J., (1984): Interspecific hybridization in the genus *Helianthus* L. Pflanzenzüchtung, 93: 265-279.

HEISER, CH. B., (1961): Morphological variation in *Helianthus petiolaris* with notes on related species. Evolution, 15: 247-258.

HUGO, R. P., MARIA, V. R., RUBEN H. V., (1998): A simple Method for Isolating High Yield and Quality DNA from Cotton (*Gossypium hirsutum* L.) Leaves. Plant Mol. Biol. Rep. 16: 1-6.

IBPGR, (1985): Deskriptor za suncokret (Descriptors for Cultivated and wild Sunflower, AGPG: International Board For Plant Genetic Resources, Rome, Italy, /85/54.

JAN, C. C., (1997): Cytology and Interspecific Hybridization. In: A.A. Schneiter *et al.*, (ed.) Sunflower Technology and Production. Agron. Monogr. 35. ASA, CSSA and SSSA, Medison, WI, 497-558.

PANKOVIĆ, D., SAKAĆ, Z., JOCIĆ, S., ŠKORIĆ, D., (2004): Molekularni markeri u oplemenjivanju suncokreta. Zbornik radova, Sveska 40: 301-311.

RIESEBERG, L. H., VAN FOSSEN C., and DESROCHERS, A., (1995a): Hybrid speciation accompanied by genomic reorganization in wild sunflowers. Nature, 375: 313-316.

RIESEBERG, L. H., RANDAL-LINDER C, SEILER, G. J., (1995b): Chromosomal and genic barriers to introgression in *Helianthus*. Genetics 141: 1163-1171.

SCHILING, E. E., HEISER, CH. B., (1981): Infrageneric classification of *Helianthus* (*Compositae*). Taxon, 30: (2) 393-403.

TANG, S., YU, J. K., SLABAUGH, M. B., SHINTANI, D. K., KNAPP, S. J., (2002): Simple sequence repeat map of the sunflower genome. TAG, 105 (8):1124-1136.

TANG, S., KNAPP, S. J., (2003): Microsatellites uncover extraordinary diversity in Native American land races and wild populations of cultivated sunflower. TAG, 106: 990-1003.

Received May 17, 2006
Accepted July 3, 2006

PROVERA USPEŠNOSTI MEĐUVRSNOG UKRŠTANJA POPULACIJA DIVLJE VRSTE *HELIANTHUS ANNUUS* L. SA GAJENIM SUNCOKRETOM

Sreten TERZIĆ, Jovanka ATLAGIĆ i Dejana PANKOVIĆ

Naučni institut za ratarstvo i povrtarstvo, Novi Sad, Srbija

I z v o d

Radi provere uspešnosti ukrštanja, posmatrane su promene u fenotipu, mejozi i vitalnosti polena F1 hibrida u odnosu na roditelje, kao i polimorfnost mikrosateliskih sekvenci DNK (SSR) u međuvrsnom ukrštanju divlje vrste *Helianthus annuus* L. sa gajenim suncokretom.

Osobine fenotipa F1 hibridnih biljaka su bile bliske divljoj vrsti ili intermedijarne. U mejozi F1 hibrida je bio prisutan mali broj mejocita (0 do 10%) sa nepravilnostima u parenju hromozoma. Priemnom SSR markera potvrđeno je da su hibridne kombinacije dobijene međuvrsnim ukrštanjem. U većini hibridnih kombinacija su nađeni aleli kojih nije bilo kod roditelja što je dodatni dokaz da one nisu nastale samooplodnjom.

Dobijeni rezultati ukazuju da se SSR markeri mogu efikasno koristiti za proveru uspešnosti ukrštanja filogenetski bliskih vrsta, kod kojih u F1 generaciji nisu prisutne značajne promene u fenotipu, mejozi i vitalnosti polena.

Primljeno 17. maja 2006.
Odobreno 3. jula 2006.