MATERIAL AND METHODS

Plant material

Inbred restorer lines R 107, R 110, R 114 and R 120 in the F_9 generation derived from the interspecific hybrid *H. annuus* (hybrid Albena) × *H. salicifolius* by direct organogenesis method were used to analyze the genetic variation.

DNA extraction

RAPD analysis was carried out using genomic DNA extracted by a method according to Doyle and Doyle (1990) with some modifications. Sunflower leaf tissue was ground to a fine powder in liquid nitrogen. The frozen powder (2.5 g) was transferred to 15 ml hot hexadecyltrimethylammonium bromide (CTAB) extraction buffer [(2% CTAB, 100 mM Tris HCl (pH 8.0), 20 mM EDTA, 1% $Na_2S_2O_5$, 0.2% β-mercaptoethanol)] and incubated at 65°C for 30 min. with occasional shaking. An equal volume of chloroform : isoamylalcohol (24 : 1 v/v) was added and mixed by inversion, then centrifuged at 6000 rpm, 4°C for 10 min. The aqueous phase was transferred to a fresh tube and re-extracted with an equal volume of chloroform : isoamylalcohol (24 : 1 v/v) and centrifuging at 5000 rpm, 4°C for 10 min. The aqueous phase was removed and transferred to a fresh tube again and precipitated in 1.0 ml ammonium acetate (10 M), 1.0 ml sodium acetate (3 M, pH 5.5) and 2/3 VT 2-propanol (4°C). Lastly, the precipitated DNA was dried and resuspended in TE buffer (10 mM Tris HCl (pH 8.0) and 1 mM EDTA, pH 8.0). After treatment with RNase, the DNA concentration was measured by using a flourometer (Model TKO 100, Hoefer Scientific Instruments, Serva, Germany).

DNA amplification

Short 21 RAPD primers (10 bp) were utilized for the amplification of random DNA sequences (Table 1). Primer selection was based on the information content, clarity and reproducibility of banding patterns. Amplifications were carried out in a 20 μl volume containing: 1.5 units polymerase Stoffel fragment "Goldstar" (Eurogentec), 1 × reaction buffer, 6 mM $MgCl_2$, 0.4 mM each of dATP, dCTP, dGTP and dTTP, 25 ng of template DNA, 10 × Stoffel buffer, add 20 μl H_2O and 0.3 μM of primer. The amplifications were performed using a Thermal cycle 9600. The thermal cycle was programmed for a first denaturation step of 4 min. at 94°C followed by 45 cycles of 2 min. at 94°C, 1 min. at annealing temperature 36°C and 2 min. extension step at 72°C.

Amplification products were resolved by gel electrophoresis in 2% agarose in 0.5 TBE (89 mM Tris pH 8.0, 89 mM boric acid and 0.5 M EDTA), stained with etidiumbromide and visualized on a UV screen.

Molecular sizes of the amplification products were estimated by using a 100 bp DNA ladder (Gibco BRL, Life Technologies).

Analysis of amplification profiles

Amplification profiles of *Helianthus* genotypes were compared with each other and bands of DNA fragments were scored as present (1) or absent (0). The computer program "RFLPscan", (Scanalytics, Billerica, MA, USA) was used to estimate the data for all the 21 primers.

The similarity was then analyzed on the basis of the number of shared amplification products according to Nei and Li (1979). A dendrogram based on similarity coefficients was generated with the programme "NTSys" (Version 1.80, Exeter Software, Setauket, NY, USA) by using the unweighted pair group method of arithmetic means (UPGMA).

Table 1: List of the primers used to characterize interspecific progenies in *Helianthus*

Primer	Primer sequence (5`-3`)	Ta*
A-12	5` TCG GCG ATA G 3`	36
A-16	5` AGC CAG CGA A 3`	36
AE-01	5` TGA GGG CCG T 3`	36
AE-03	5` CAT AGA GCG G 3`	36
AH-15	5` CTA CAG CGA G 3`	36
AJ -19	5` ACA GTG GCC T 3`	36
AJ-20	5` ACA CGT GGT C 3`	36
AK-05	5` GAT GGC AGT C 3`	36
AK-08	5` CCG AAG GGT G 3`	36
AO-14	5` CTA CTG GGG T 3`	36
AO-18	5` GGG AGC GCT T 3`	36
AO-20	5` GGC TTGCCT G 3`	36
AP-04	5` CTC TTG GGC T 3`	36
AP-05	5` GAC TTC AGG G 3`	36
AS -12	5` TGA CCA GGC A 3`	36
AT-12	5` CTG CCT AGC C 3`	36
AV -10	5` ACC CCT GGC A 3`	36
AW -17	5` TGC TGC TGC C 3`	36
AW -18	5` GGC GCA ACT G 3`	36
AW -19	5` GGA CAC AGA G 3`	36
W-04	5` CAG AAG CGG A 3`	36

Ta*: annealing temperature actually used

RESULTS AND DISCUSSION

Inbred restorer lines R 107, R 110, R 114 and R 120 derived from the interspecific hybrid *H. annuus* (cv. Albena) × *H. salicifolius* by direct organogenesis method were used to analyze the genetic variation.

The RAPD primers generated a higher degree of polymorphism in sunflower and resulted in distinct and clear patterns. Analyses were carried out on those fragments that were well visible. The comparison of the amplification profiles of F_9 plants and parents was based on the presence or absence of fragments.

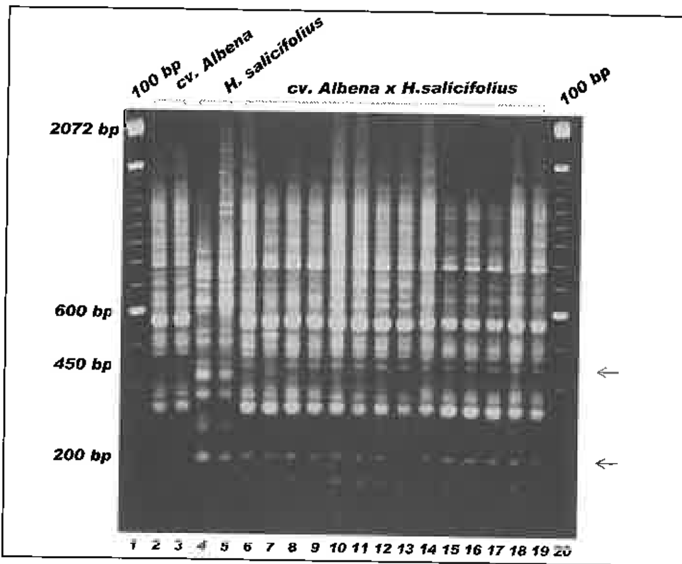

Figure 1: *Genetic profile of interspecific sunflower hybrids progenies (Helianthus annuus × H. salicifolius) based on primer OPAE-01. Lines 2 and 3: maternal parent (cv. Albena). Lines 4-5: paternal parent (H. salicifolius); Lines 6-19 interspecific hybrid progenies: 6-9-line R 110; 10-13-line R 120; 14-15-line R 107 and 16-19-line R 114; Lines 1 and 20: 100 bp ladder. (Gibco BRL, Life Sciense Technology)*

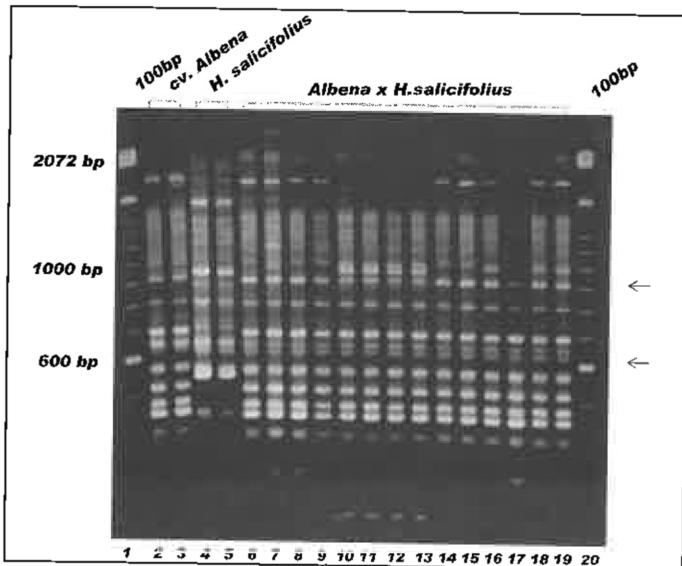

Figure 2: *Genetic profile of interspecific sunflower hybrids progenies (Helianthus annuus × H. salicifolius) based on primer OPAS-12. Lines 2 and 3: maternal parent (cv. Albena). Lines 4-5: paternal parent (H. salicifolius); Lines 6-19 interspecific hybrid progenies: 6-9-line R 110; 10-13-line R 120; 14-15-line R 107 and 16-19-line R 114; Lines 1 and 20: 100 bp ladder. (Gibco BRL, Life Sciense Technology)*

In total twenty one (10 base) primers with random sequences were applied to characterize DNA sequences of the lines R 107, R 110, R 114 and R 120 obtained from the interspecific cross. Two of the total of 21 primers used (OPAE-01 and OPAS-12) gave polymorphic products for the interspecific hybrid progeny, which were missing in the cultivated sunflower. The two primers amplified polymorphic fragments for all of the studied hybrid progenies. The primer OPAS-12 gave polymorphic products for line R 120 only.

In our study RAPD fingerprint obtained through primer OPAE-01 revealed considerable variation in the hybrid progenies. Figure 1 demonstrates some fragments in the interspecific hybrid progenies with a size of 200 bp and 450 bp which were specific for the genotype of the wild species *H. salicifolius*, only. There were also bands specific for the genotypes of cultivated sunflower.

Genetic profile based on primer OPAS-12 (Figure 2) showed bands with sizes 630 bp and 950 bp at hybrid progenies which were typical for wild *H. salicifolius*. Bands with size 630 bp were common for all four hybrid progenies, while band 950 bp was specific for line R 120 only.

These DNA fragments obtained with primers OPAE-01 and OPAS-12 indicate a probable introgression from *H. salicifolius* DNA into the obtained inbred lines. In spite of the fact that the lines investigated were selfed for nine generations, proportions of the wild alien genome(s) are still detectable and are highly specific for the *H. salicifolius* used for the interspecific hybridization.

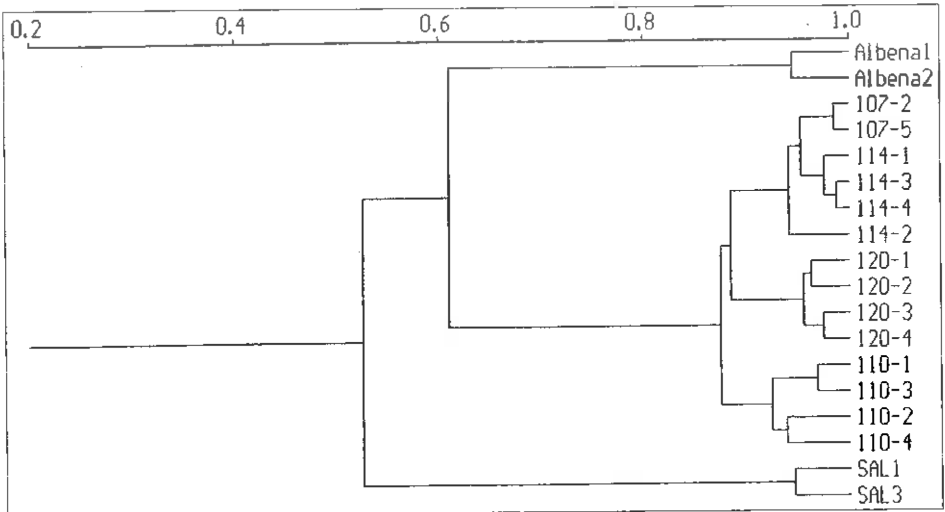

Figure 3: Dendrogram constructed from a matrix of RAPD-marker based genetic similarities between H. annuus (cv. Albena), H. salicifolius and interspecific hybrids

By using the similarity coefficient of Nei and Li, 1979 a dendrogram was generated through UPGMA analysis with the aim to determine the genetic distance of the studied genotypes. The cluster analysis (Figure 3) distinguished three main groups

(the interspecific progenies, cv. Albena and *H. salicifolius*). The constructed scheme revealed that the species *H. salicifolius* was distinctly separated from the other genotypes in the study (cv. Albena and the interspecific lines), forming their own genetic pool.

Based on the RAPD data the cluster analysis distinguished a main cluster including all interspecific progenies (R 107, R 110, R 114, and R 120) divided into several smaller groups. Lines R 107 and R 114 demonstrated the highest degree of similarity and were located at considerable distances from genotypes R 120 and R 110. The newly released line R 107 possessed a higher degree of homogeneity than R 114, R 120 and R 110. All hybrid progenies were placed at considerable distance from the cultivated parent (cv. Albena), but the degree of similarity between them was higher than with the wild parent *H. salicifolius*.

There are just a few publications on the use of molecular markers for identification of F_1 interspecific and intergeneric sunflower hybrids and their progenies. While Kräuter *et al.* (1991) and Faure *et al.* (1998) have used RFLP markers, Köhler *et al.* (1999) have applied AP-PCR technique.

In our study we also used short 10 base RAPD primers. The interspecific cross (*H. annuus* (cv. Albena) × *H. salicifolius*) was obtained according to the method of direct organogenesis (Encheva *et al.*, 1992). The available literature does not provide data on the obtaining of these hybrid combinations with that method. The cluster and coordinates analysis confirmed the hybrid nature of the interspecific progenies. Cultivated sunflower (cv. Albena), the *H. salicifolius* and their hybrid lines were clearly distinguishable. From the constructed scheme (Figure 3) it is evident that *H. salicifolius* and cultivated sunflower did not form clusters with any of the hybrid progenies. The discriminating power of molecular markers, in comparison to morphological and biochemical markers is higher, therefore they are generally superior for the assessment of genetic diversity due to better sensitivity and efficiency (Lamkey and Lee, 1993).

The wild *Helianthus* species are a potential source of genes for resistance to diseases and pests and of considerable variability for most agronomic traits and qualities of seed (Thomson *et al.*, 1981) and could be included in interspecific and intergeneric crosses for increasing genetic variability in cultivated sunflower (Seiler, 1992, 1997; Škorić and Rajčan, 1992; Škorić *et al.*, 1995; Köhler *et al.*, 1997; Thomson *et al.*, 1981).

In our study RAPD analysis indicated increased genetic variation in hybrid progenies. This genetic variation is a valuable source of resistance to diseases, parasites and improved agronomic indices. The phytopathological evaluation of parental forms and the obtained hybrid progenies (Encheva *et al.*, 2006) showed complete resistance to *Phomopsis* (line R 114) and to *Alternaria* (line R 120). This resistance probably comes from the wild species *H. salicifolius*, which, according to the investigation of Christov, (1990, 1996) and Christov *et al.* (1996) has shown

complete resistance to *Phomopsis*. Among the sources of resistance to *Phomopsis* and *Alternaria*, Škorić, 1987 pointed out *H. salicifolius*.

As a result of interspecific hybridization, line R 107 was developed, which showed 100% resistance to parasite *Orobanche cumana* Wallr. against artificial infection background. The resistance of the line R 107 comes from the wild *H. salicifolius*, which according to Christov *et al.* (1996) has complete resistance to the parasite under both field and laboratory conditions. The female form (cv. Albena) in the interspecific cross is, on its part, susceptible to the diseases and parasite mentioned above.

Instead of resistance, the new lines R 107 and R 114 were characterized by positive transgression in comparison to parental forms for traits such as number of branches, length of branches and diameter of branch head (Encheva and Christov, 2006). The negative transgression was establish for lines R 107, R 114 and R 120 for the indexes plant height, number of leaves and vegetation period.

CONCLUSION

We have successfully applied molecular markers to investigate diversity of sunflower cultivar, wild sunflower species, as well as to identify interspecies hybrids.

The results confirm that polymorphism occurred in the amplification PCR profiles of *H. annuus*, *H. salicifolius* and *H. annuus* × *H. salicifolius*, *i.e.*, the RAPD analysis confirmed the hybrid nature of the F_9 material obtained from interspecific cross. RAPD markers indicated also a possible introgression of wild genome portions into the obtained interspecific hybrid progenies

Our data confirmed the conclusion drawn by Köhler *et al.* (1999) that the lines which originate from the same interspecific cross form their specific cluster, *i.e.*, the related lines could be grouped into a separate gene pool.

We were able to demonstrate that RAPD could be used for characterization of interspecific progenies in sunflower at a late stage of selection (F_9) in which an increased genetic variation was discovered. This genetic variation is a valuable source of resistance to diseases, parasites and improved agronomic indices.

The interspecific hybrids provide valuable initial material to enlarge genetic variation in sunflower and genetic markers could help the breeders to characterize this new material.

REFERENCES

Anisimova, I.N., Tumanova, L.G., Gavrilova, V.A., Diagileva, A.V., Pasha, L.I., Mitin, V.A. and Timofeeva, G.I., 2009. Genomic instability in sunflower interspecific hybrids. Genetika 45(8): 1067-1077.

Atlagić, J., Panković, D. and Pekanović, A., 2003. Backcrosses in interspecific hybridization in sunflower. Genetika 35(3): 187-197.

Breton, C., Serieys, H. and Berville, A., 2010. Gene transfer from wild *Helianthus* to sunflower: topicalities and limits. Oleagineux. Corps Gras. Lipides 17(2): 104-114.

Christov, M., 1990. Study on the wild species from genus *Helianthus* with a view of their use in sunflower breeding. Ph.D Thesis, Sofia (Bg): p. 208.

Christov, M., 1996. Characterization of wild *Helianthus* species as sources of new features for sunflower breeding. *In:* P.D.S. Caligari & D.J.N. Hind (eds). Compositae. Biology and Utilization. Proceedings of the International Compositae Conference, Kew, 1994. (D.J.N. Hind, Editor-in-Chief), Vol. 2: 547-570. Royal Botanic Gardens, Kew, 2: 603-615.

Christov, M., Shindrova, P. and Encheva, V., 1996. Transfer of new characters from wild *Helianths* species to cultivated sunflower. Gent. a Selecht. 32(4): 275-286.

Christova-Cherbadzi, M., Atanasova, R., Batchvarova, R., Christov, M. and Ivanova, I., 2007. Characterization of hybrids between *H. annuus* L. and the subspecies *subrhomboideus* (Rydberg) Heiser of perennial hexaploid *H. pauciflorus*. Helia 30(47): 37-50.

Doyle, J.F. and Doyle, J.L., 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Encheva, J., Christov, M. and Ivanov, P., 1992. Use of direct organogenesis *in vitro* from immature embryos of interspecific and intergeneric hybrids of *Helianthus annuus* L. Proceedings of the 13th International Sunflower Conference, Pisa (Italy), 7-11 September, Vol. II: 1455-1460.

Encheva, J., Christov, M., Kohler, H., Shindrova, P., Encheva, V. and Friedt, W., 2004. Interspecific hybrids between *Helianthus annuus* and *H. tuberosus*: RAPD analysis, disease resistance, combining ability. B.J.A.S. 10(2): 169-175.

Encheva, J. and Christov, M., 2006. Interspecific hybridization between cultural sunflower *Helianthus annuus* L. and *Helianthus salicifolius* L.-morphological and biochemical characterization. Helia 29(45): 107-116.

Encheva, J., Christov, M., Shindrova, P., Drumeva, M. and Encheva, V., 2006. New sunflower restorer lines, developed by direct organogenesis method from interspecific cross *Helianthus annuus* L. (cv. Albena) × *Helianthus salicifolius*-desease resistance, combining ability. Journal of Genetic and Breeding 60(2): 77-84.

Faure, N., Serieys, H., Griveau, Y., Kann, F., Tersac, M. and Berville, A., 1998. Characterization of interspecific and intergeneric hybrids between cultivated sunflower (*H. annuus* L.) and distant species from *Helianthus* genus and other related genera. Proceedings of the Fourth European Conference on Sunflower Biotechnology. October, 20-23, Montpellier, France.

Henn, H.J., Wiongender, R. and Schnabl, H., 1998. Regeneration of fertile interspecific hybrids from protoplast fusions between *Helianthus annuus* L. and wild *Helianthus* species. Plant Cell Reports 18: 220-224.

Kräuter, R., Steinmetz, A. and Friedt, W., 1991. Efficient interspecific hybridization in the genus *Helianthus via* "embryo-rescue" and characterization of the hybrids. Theor. Appl. Genet. 82: 521-525.

Köhler, H.,. Brahm, L., Rocher, T. and Friedt, W., 1997. Anwendung molekulargenetischer Techniken in der Resistenzzuchtung bei der Sonnenblume (*Helianthus annuus*) [Application of molecular methods in breeding for disease resistance in sunflower (*Helianthus annuus*)]. Vortr. Pflanzenzuchtung 36: 47-50.

Köhler, H. and Friedt, W., 1999. Genetic variability as identified by AP-PCR and reaction to mid-stem infection of *Sclerotinia sclerotiorum* among interspecific sunflower (*Helianthus annuus* L.) hybrid progenies. Crop. Science 39(5): 1456-1463.

Lamkey, K. R. and Lee, M., 1993. Quantitative genetics, molecular markers, and plant improvement. *In*: B.C. Imrie and J.B. Hacker [eds.] Focused plant improvement: Towards responsible and sustainable agriculture. Proc. 10th Australian Plant Breeding Australia pp. 104-115.

Mohan, G.S. and Seetharam, A., 2005. Studies on relationship among cultivated and wild sunflower through RAPD markers. Cytologia 70(3): 267-273.

Nei, M. and Li, W.H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76: 5269-5273.

Saftić-Panković, D., 2007. Application of molecular markers in sunflower breeding. Genetika 39(1): 1-11.

Seiler, G.J., Pomeroy, J.S. and Marinković, R., 1992. Valuable sunflower germplasm collected from the Northwestern United States. Helia 15(17): 1-6.

Schilling, E.E. and Heiser, C.B., 1981. Infrageneric classification of *Helianthus* (*Compositae*). Taxon 30: 393-403.

Škorić, D., 1987. *In* : Progress report 1984-1986. FAO Subnetwork. Genetic evaluation and use of *Helianthus* wild species and their use in breeding programs [ed. D. Škorić]. FAO, Rome, Italy, 1: 17.

Škorić, D. and Rajčan, I., 1992. Breeding of *Sclerotinia* tolerance in sunflower. *In*: Proceedings of the 13th International Sunflower Conference, Sept. 07-11, 1992, Pisa (Italy), Vol. I: 1257-1262.

Škorić, D., Atlagić, J., Marinković, R., Dozet, B. and Mihaljčević, M., 1995. *In:* FAO Working Group: Evaluation of wild *Helianthus* species, Progress Report 1994, July 25- 28, 1995, Bucharest (Romania) pp. 11-25.

Thompson, T.E., Zimmerman, D.C. and Rogers, C.E., 1981. Wild *Helianthus* as a genetic resource. Field Crops Res. 4: 333-343.