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INTER- AND INTRASPECIES DIFFERENTIATION IN THE GENUS *Helianthus* BY RAPD ANALYSIS

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SUMMARY

PCR with random primers was used for inter- and intraspecies differentiation in the genus *Helianthus*. Species-specific primers as well as polymorphic ones between lines were observed. A phenogram, reflecting the genetic relation between wild and cultivated *Helianthus* accessions, was constructed. PCR analysis could effectively classify and identify species most related to *H. annuus* which will be used for the improvement of cultivated sunflower.

Key words: Sunflower, RAPD, polymorphism, phylogeny

INTRODUCTION

Molecular-genetic polymorphism investigations that analyze variability of separated DNA sites are becoming one of the most efficient approaches in plant genetics.

RAPD analysis is widely used for taxonomy of various genera (Devos, 1992), species (Igbal, 1995; Faroog, 1995) and for the differentiation of intraspecies units (Sweeny, 1995; Mackil, 1995; Stiles, 1993). PCR analysis is incorporated in genetic and breeding programs to classify the relation of various cultivars, lines or hybrids as well as wild ancestors and related species in many crops.

Sunflower is one of the most important agricultural crops in which the level of genetic investigation permits the use of molecular markers for breeding purposes. Germplasm of related species and hybrid development are used for the improvement of this crop and these technologies demand genotype control.

The origin and phylogeny of many representatives of the genus *Helianthus* remain insufficiently investigated. Therefore, the classification and distribution of some species within sections were repeatedly subjected to revision (Heiser, 1969; Анащенко, 1974; Schilling, 1981). The intensive breeding of sunflower and the necessity to introduce new germplasm sources, which carry genes for resistance and other valuable agronomic traits, require more detailed information about *H. annuus* relationship to the progenitors and related wild species.

Our previous paper (Сиволап, 1998) described the genetic similarity within a set of sunflower inbred lines in which polymorphisms were studied by random primed PCR. It also indicated a possibility of PCR analysis application in sunflower breeding.

The objectives of this work were:

- to compare the ability of the RAPD technique for the inter- and intraspecies differentiation in sunflower and
- to define more precisely the phylogenetic relationship between some representatives of the genus *Helianthus*.

MATERIALS AND METHODS

The materials under study included 35 species and subspecies of *Helianthus* genus, two closely related to the genus *Helianthus* coming from the genera *Simsia* and *Titonia*, and 30 inbred lines of *Helianthus annuus*. The material was kindly provided by Prof. V.V. Burlov, Dr. R.M. Serbay (Plant Breeding and Genetic Institute, Odessa, Ukraine) and Dr. A.Ph. Pershin, Dr. V.V. Tolmachev (Institute of Oilseed Crops, Zaporozhye, Ukraine).

DNA was extracted from 5-days-old seedlings and roots as described previously (Сиволап, 1998). PCR mix in 20 μl consisted of: 50 mM KCl, 20 mM Tris-HCl pH 8.4, 4 mM $MgCl_2$, 0.01% Tween-20, 20 mM of each dNTP, 0.2 μM primer, 20 ng DNA and 1 unit Tag-polymerase. A "Bioterm" thermal cycler was used, programmed for one initial denaturation step for 3 min at 94°C, then for four cycles of amplification: 1 min at 94°C, 2 min at 39°C, 2 min at 72°C; and finally for 30 subsequent cycles where annealing of the primer occurred for 1.6 min at 47°C, followed by a terminal extension step of 8 min at 72°C. Amplification products were analyzed after electrophoresis in 2% agarose gels and ethidium bromide staining.

Computer program TREE (Сиволап, 1998) was used to construct dendrograms reflecting the genetic relationship between the analyzed samples. At the first step, TREE defines genetic distances (D) in accordance with

$$D = - \ln F,$$

where $F=2N_{xy}/(N_x+N_y)$, and F – similarity coefficient, N_{xy} – common bands of amplification for both X- and Y-sample, N_x and N_y – bands of X- and Y-sample.

Intermediate result of the program is a matrix of distances between the samples under consideration. Then the matrix is subjected to the unweighted pair grouping with arithmetic means (UPGMA).

Table 1: Investigated species and subspecies of the genus *Helianthus*

Species		Section	Series
H.annuus (lines 2B, OD20, 1036, 3369)	2n	*Helianthus*	*Helianthus*
H.debilis ssp. *tardiflorus*	2n	*Helianthus*	*Helianthus*
H.debilis ssp. *silvestris*	2n	*Helianthus*	*Helianthus*
H.petiolaris	2n	*Helianthus*	*Helianthus*
H.petiolaris ssp. *petiolaris*	2n	*Helianthus*	*Helianthus*
H.argophyllus	2n	*Helianthus*	*Helianthus*
H.praecox	2n	*Helianthus*	*Helianthus*
H.praecox ssp. *praecox*	2n	*Helianthus*	*Helianthus*
H.praecox ssp. *hirtus*	2n	*Helianthus*	*Helianthus*
H.praecox ssp. *rungonii*	2n	*Helianthus*	*Helianthus*
H.bolanderi	6n	*Helianthus*	*Helianthus*
H.mollis	2n	*Divaricati*	*Corona-Solis*
H.maximiliani	2n	*Divaricati*	*Corona-Solis*
H.divaricatus	2n	*Divaricati*	*Corona-Solis*
H.giganteus	2n	*Divaricati*	*Corona-Solis*
H.grossesseratus	2n	*Divaricati*	*Corona-Solis*
H.nuttallii	2n	*Divaricati*	*Corona-Solis*
H.salicifolius	2n	*Divaricati*	*Corona-Solis*
H.strumosus	2n	*Divaricati*	*Corona-Solis*
H.hirsutus	4n	*Divaricati*	*Corona-Solis*
H.decapetalus	4n	*Divaricati*	*Corona-Solis*
H.californicus	6n	*Divaricati*	*Corona-Solis*
H.tuberosus	6n	*Divaricati*	*Corona-Solis*
H.eggertii	6n	*Divaricati*	*Corona-Solis*
H.resinosus	6n	*Divaricati*	*Corona-Solis*
H.microcephalus	2n	*Divaricati*	*Microcephali*
H.laevigatus	4n	*Divaricati*	*Microcephali*
H.rigidus	6n	*Divaricati*	*Atrorubentes*
H.macrophyllus	6n	?	?
H.multiflorus	6n	?	?
H.laetiflorus	6n	?	?
H.pauciflorus ssp. *rigidus*	6n	?	?
H.pauciflorus ssp. *subrhomboideus*	6n	?	?
H.scaberimus	2n	?	?
H.trachelifolius	2n	?	?
Titonia speciosa			
Simsia foetida			

Level of polymorphism (P) was defined as

$$P = \frac{\text{number of polymorphic RAPD-fragments}}{\text{total RAPD-fragments}} \times 100$$

Appearance frequency (AF) of RAPD-fragments was calculated as

$$AF = \frac{\text{number of lines possessing any fragment}}{\text{total number of lines}}$$

Representation of RAPD-fragment with given appearance frequency (R) was estimated as

$$R = \frac{\text{number of RAPD-fragments with given AF}}{\text{total number of RAPD-fragments}} \times 100\%$$

RESULTS AND DISCUSSION

Selection of corresponding primers has an important influence on the significance of the PCR analysis. For detection of variability on interspecies level, it is desirable to use primers that are monomorphic at the intraspecies level.

A high level of polymorphisms was detected in sunflower with random primed PCR. For evaluation of interspecies variability, primers revealing about 25 – 37% of polymorphism on the intraspecies level were used. Variability of inbred lines was studied using the most polymorphic primers, which showed 50–74% polymorphism between the lines.

Characteristics of the primers revealing polymorphism are presented in Table 2.

Table 2: Characteristics of primers revealing polymorphism

Primer	Sequence of primer 5′ - 3′	G – C Content (%)	No. of analyzed representatives		No. of analyzed RAPD-loci		P (%)	
			S	L	S	L	S	L
P 28	CAAACGTCGG	60	37	30	19	20	100	65
P 36	CCGAATTCGC	60	37	30	15	13	100	65
P 37	CTGACCAGCC	70	37	30	12	18	100	67
P 38	GATACGTTGTC	46	37	30	16	19	100	74
P 39	CCAGTTCGCC	70		30		19		53
P 43	AGTCAGCTGC	60		30		20		65
P 44	GGACCCCGCC	90		30		12		50
P 46	GGTTGGGGAG	70		30		14		71
P 48	GCGGTGCTCG	80		30		19		53
P 49	GACAGCCTAC	60		30		21		52
P 53	GTCTAAGTCG	50		30		19		74
P 55	GTTTTCCTCG	50	37		20		100	
Total					82	194	100	64

S-species, L-inbred line

Fragments amplified by PCR ranged in their sizes from 400 to 1400 bp. Depending on the primer the number of fragments varied between 12 and 20 (for species) and from 12 to 21 (for lines). In total, 82 RAPD loci for wild representatives and 194 (124 of these were polymorphic) for cultivated sunflower were detected. The level of polymorphism reached 100% between species and 64% between lines. Gentzbittel *et al.* (1992) investigated RFLP in *Helianthus* genus and reconstructed a phylogeny of this taxon. According to their data, the degree of interspecies differences reached 0.73 and for inbreds – 0.59. Thus, a greater potential of the PCR analysis as a source of probable markers could be demonstrated.

RAPD loci were divided according to the appearance frequency into groups (Table 3). The percentage of RAPD loci with certain appearance frequency in the set of species and lines under study, designated as "representation", reflects the contribution of "rare" (AF=0.1), "usual" (AF=0.3 – 0.8) and "non-polymorphic" (AF=1) PCR loci to the total pattern of amplified fragments.

Table 3: Classification of amplified RAPD loci by the appearance frequency in the investigated set of species and lines

Appearance frequency of RAPD loci (AF)	No. of RAPD loci with given AF		Representation of RAPD loci (%)	
	S	L	S	L
0.1	4	7	5	3.6
0.2	11	12	13	6.2
0.3	11	13	13	6.7
0.4	17	13	21	6.7
0.5	17	12	21	6.2
0.6	11	12	13	6.2
0.7	3	19	4	9.8
0.8	4	13	5	6.7
0.9	4	24	5	12.4
1.0	0	69	0	35.5
	82	194	100	100

S-species, L-line

Most of the loci present in the species have an AF=0.4 or 0.5 (representing 21% of the observed fragments), whereas for the self pollinated lines the most distributed loci were non-polymorphic (35% of the total number).

No fragment was revealed as unique for only one genotype. Amplification patterns were reasonably specific, especially for the representatives of the wild flora.

By characterizing the primer specificity it is possible to report the availability of species-specific primers, e.g., P28, P55 revealed considerably more "rare" PCR loci in the wild species, as well as primers, e.g., P37 was more suitable for differentiation of inbreds (Table 4).

The comparison of amplification patterns of the various genome representatives allowed us to obtain quantitative integrative evaluation of their divergence. On

the basis of the RAPD analysis, by comparing PCR products of species and lines, genetic distances (D) were estimated. On the average, interlines genetic distances were to a considerable extent shorter then interspecies ones.

Table 4: Characteristics of primer specificity

Primer	No. of PCR loci		No. of PCR loci with appearance frequency																			
			0.1		0.2		0.3		0.4		0.5		0.6		0.7		0.8		0.9		1.0	
	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L
P 28	19	20	2	1	1	1	1	2	4	2	5	1	2	2	3	2	1	2	-	-	-	7
P 36	15	13	-	-	3	1	3	3	5	-	2	2	2	1	-	1	-	3	1	-	-	2
P 37	12	18	-	2	-	1	-	-	3	4	2	-	4	1	-	2	1	-	2	2	-	6
P 38	16	19	1	-	1	2	2	2	3	-	5	2	2	-	-	2	2	1	-	5	-	5
P 55	20	-	2	-	6	-	5	-	2	-	3	-	1	-	-	-	-	-	1	-	-	-
P 49	-	21		1		-		-		2		1		2		2		1		2		9
Total	82	83	4	4	11	5	11	7	17	8	17	6	11	6	3	9	4	7	4	9	-	29
%			5	5	13	7	13	9	21	9	21	7	13	7	4	11	5	9	5	11	-	35

S-species, L-line

A phenogram reflecting the genetic relationships between wild *Helianthus* species and cultured representatives of *H. annuus* was obtained. On the constructed scheme (Figure 1) it is possible to recognize several clusters. Morphological classification of *Helianthus* (Schilling, 1981) divided the genus into 4 sections and 8 series. In the phenogram obtained after RAPD analysis, the general tendency of the grouping of the certain taxonomic units has been saved.

The clusters with the species of the series *Corona-Solis* and *Helianthus* are clearly distinguished. The representatives of *H. annuus*, breeding lines 2B, OD20, 1036, 3369, are united in a separated group being part of a large subcluster which included all species from *Helianthus* section.

The genetic distances between subspecies *H. praecox rungonii* and *H. praecox hirtus* do not exceed those between inbred lines of the cultivated sunflower. This indicates that their attribution to different subspecies cannot be explained by the RAPD analysis. Taking into consideration that there are no sharp criteria for subspecies differentiation, the range of genetic distances should be considered as a possible argument for such justification. The short genetic distances between *H. annuus, H. laetiflorus, H. salicifolius, H. bolandery, H. petiolaris* and *H. tuberosus* permit to recommend these species as donors of valuable agronomic traits for improving cultivated sunflower. The species *H. multiflorus, Simsia foetida, H. rigidus* are genetically most distant from the cultivated sunflower. According to Schilling & Heiser (1981), the group of species from the series *Microcephali* is closest to the representatives of the series *Corona-Solis*.

Representatives of *H. laevigatus* and *H. microcephalus* also show genetic relatedness with such species of *Corona-Solis* as *H. californicus* and *H. giganteus*. *H. rigidus* was classified by Schilling & Heiser (1981) as a member of the section

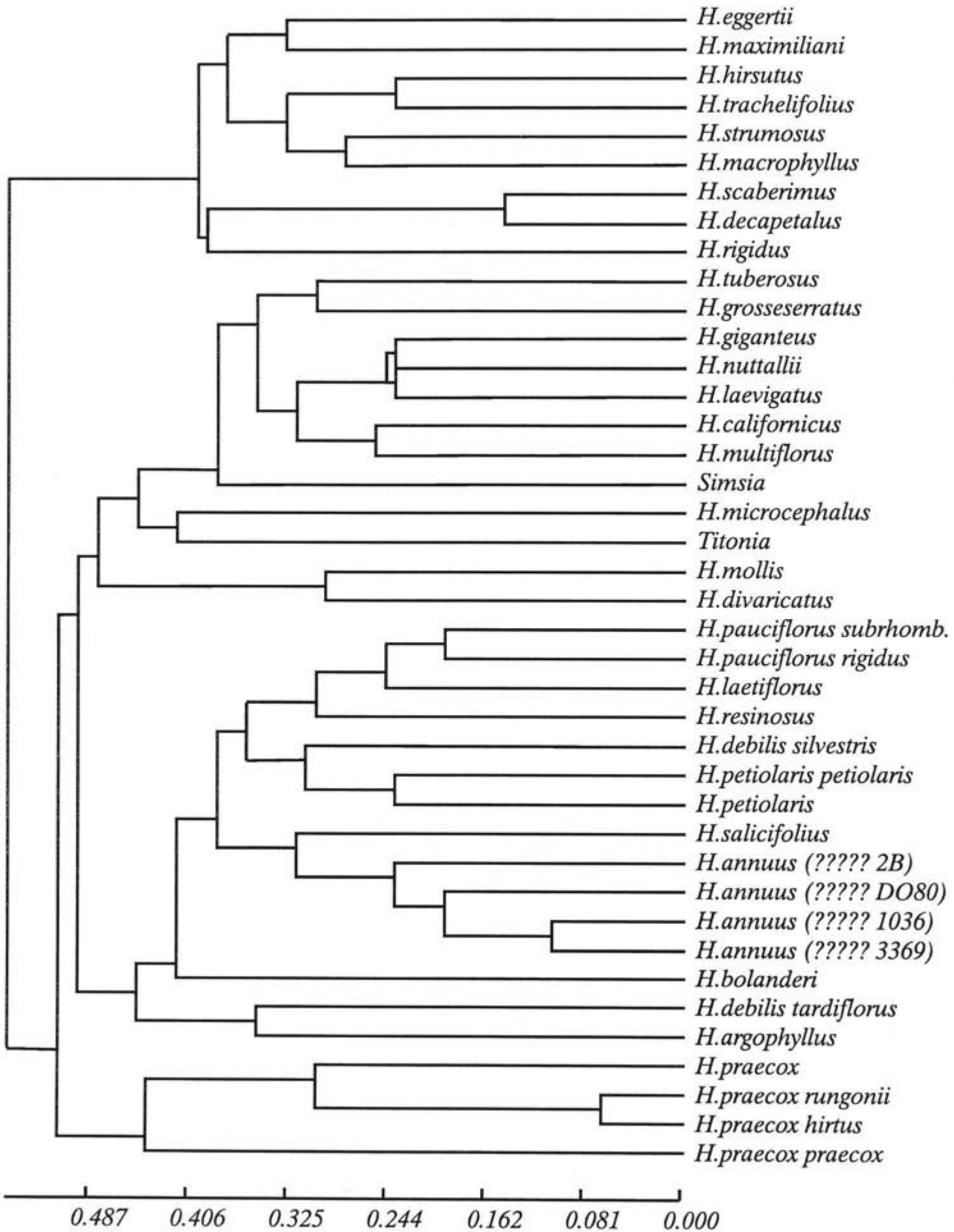

Figure 1: Phenogram reflecting the genetic relationship between wild Helianthus species and representatives of H. annuus

Divaricati (series *Atrorubentes*). Gentzbittel *et al.* (1992) included those species into the group of species of the series *Corona-Solis* : *H. hirsutus, H. resinosus, H. tuberosus* and *H. strumosus*. According to the data of the PCR analysis, *H. rigidus* is closest to *Corona-Solis* species *H. decapetalus, H. strumosus* and *H. hirsutus.*

One of the tasks of our investigation was a more accurate definition of the phylogenetic relationship between the species under study, which have not been presented earlier in Schillings's and Gentzbittel's classifications. Based on the DNA polymorphism analysis, *H. macrophyllus, H. scaberimus*, *H. multiflorus* and *H. trachelifolius* show significant genetic similarity to common representatives of the series *Corona-Solis*. Minimal genetic distances are observed between *H. scaberimus* and *H. decapetalus* (d=0.158), *H. trachelifolius* and *H. hirsutus* (d=0.241). According to our data, *H. strumosus* and *H. californicus* are most remote from *H. macrophyllus* and *H. multiflorus* species (d=0.286 and 0.259, respectively). This allows to group these species into the series *Corona-Solis* (section *Divaricati*).

According to the obtained data, *H. laetiflorus* and both subspecies of *H. pauciflorus* are close relatives. They show almost identical genetic distances to *H. rigidus*, d=0.469 between *H. laetiflorus* and *H. rigidus*, d=0.443 between *H. pauciflorus subrhomboideus* and *H. rigidus*. Earlier these species were considered as synonymous (Анащенко, 1974). In view of the RAPD analysis it is possible to put them into the series *Atrorubentes* (section *Divaricati*).

CONCLUSIONS

Genetic distances between species of the genera *Helianthus, Simsia, Titonia* and representatives of *H. annuus* are shown. A possibility of using the PCR analysis for the purposes of systematics and selection of the species closest to *H. annuus* is demonstrated.

REFERENCES

Devos, K.M., Gale, M.D., 1992. The use of RAPD markers in wheat. Theor. Appl. Genet. 84: 567-572.

Igbal, M.J., Paden, D.W., Ryburn A.L., 1995. Assessment of genetic relationships among rhododendron species, varieties and hybrids by RAPD analysis. Scientia Horticulture 63: 215-223.

Faroog, S., Shah, T.M., Arif M., 1995. Utilization of RAPD markers for the identification of cultivated and wild rice species. Pakistan Journal of Botany 27: 127-138.

Sweeny, P.M., Danneberger, T.K., 1995. RAPD characterization of *Poa annua* L. populations in golf course greens and fairways. Crop Science 35: 889-894.

Mackil, D.J., 1995. Classifying *japonica* rice cultivars with RAPD markers. Crop. Science 35: 889-894.

Stiles, J.I., Lemme, C., Sondur, S., Morshidi, M., Manshardt, R., 1993. Using RAPD for evaluating genetic relationships among papaya cultivars. Theor. Appl. Genet. 85: 697-701.

Heiser, C.B., 1969. The North American sunflowers (*Helianthus*). Mem. Torrey Bot. Club 22: 1-218.

Анащенко, А.В., 1974. К систематике рода *Helianthus* L. Ботанический журнал 59: 1472-1481.

Schilling, E., Heiser, C., 1981. Infrageneric classification of *Helianthus* (*Compositae*). Taxon 30: 393-403.
Сиволап, Ю.М., Солденко, А.Е., Бурлов, В.В., 1998. РАПД-анализ молекулярно-генетического полиморфизма подсолнечника (*Helianthus annuus*). Генетика 34: 266-271.
Gentzbittel, L., Perrault, A., Nicolas, P., 1992. Molecular phylogeny of the *Helianthus* genus, based on nuclear restriction fragment length polymorphism (RFLP). Mol. Biol. Evol. 9: 872-892.

DIFERENCIACION INTER E INTRAESPECÍFICA DEL GENERO *Helianthus* POR ANÁLISIS RAPD

RESUMEN

El análisis de PCR con "primers" al azar fue utilizado con el objetivo de diferenciar entre y dentro, de especies de girasol. Primers específicos asi como polimórficos entre especies y entre líneas fueron revelados. Se construyeron cladogramas, reflejando la relación genética entre accesiones de *Helianthus* cultivado y silvestre. El análisis PCR pudo clasificar y seleccionar las especies más relacionadas a *H. annuus* para ser usadas en la mejora del girasol cultivado.

DIFFÉRENCIATION INTER ET INTRASPÉCIFIQUE DU GENRE *Helianthus*, PAR ANALYSE RAPD

RÉSUMÉ

La PCR avec des amorces choisies au hasard a été utilisée pour la différenciation inter et intraspécifique chez le tournesol. Des amorces spécifiques d'espèces ou polymorphes entre lignées ont été mises en évidence. Un cladogramme, reflétant les relations génétiques entre accessions cultivées et sauvages d'*Helianthus* a été construit. L'analyse PCR pourrait permettre de classer et repérer efficacement les espèces les plus proches d'*H. annuus*, qui doivent être utilisées pour l'amélioration du tournesol cultivé.