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SSR Analysis of Nuclear DNA of Annual and Perennial Sunflower Species (Helianthus L.)

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Abstract: Genotyping of 29 species of genus Helianthus L., including 5 annual and 24 perennial species from the collection of the N. I. Vavilov All-Russian Institute of Plant Genetic Resources has occurred, for this purpose were selected 52 SSR markers, with localization on all the 17 linkage groups of the sunflower genome. All the studied sunflower samples had unique SSR loci banding patterns. The mean PIC value varied was 0,72, which indicates the high resolution of this SSR based system for sunflower nuclear genome investigations. The discriminatory power of the marker system allowed us to classify all the sunflower species and provide the molecular barcoding. The UPGMA dendrogram, reflecting the genetic differences between 29 species of the genus Helianthus L., was constructed. Allele distribution data of the studied sunflower samples is a database that can be used to determine the levels of genetic variability, provide molecular barcoding and control the genetic integrity of collection sunflower samples.

Keywords: SSR Markers, Polymorphism, UPGMA, Sunflower

Introduction

The investigations of plant genomes variability is an up today issue with both fundamental and applied interest. The application of genomic data enables the determination of the genetic diversity and relationships of various plant species, as well as the development of barcoding systems for agricultural and collection samples. To study the variability of the sunflower genome, a number of different criteria were used: morphological (Schilling and Heiser, 1981), chemical (Spring and Schilling, 1989; 1990), isoenzyme (Carrera and Poverene, 1995; Cronn *et al*., 1997), RFLP (Gentzbittel *et al*., 1994; Berry *et al*., 1994; Schilling, 1997), DNA sequence data (Vischi *et al*., 2006; Timme *et al*., 2007), transcriptomes (Baute *et al*., 2015; Smith *et al*., 2018), as well as variations in the number of retrotransposons copies (Mascagni *et al*., 2015; 2018). However, even nowadays, for the identification of annual and perennial wild-growing sunflowers species, the Heizer's classification (Heiser *et al*., 1969), based on visual assessment of the morphological characteristics, is still predominantly used. Nevertheless, such classification has some disadvantages and limitations, for instance, the morphological characters are not always clearly expressed at different stages. Thus the application of DNA markers revealed new possibilities for studying genetic diversity and relationships at the intraspecific and generic levels (Knapp *et al*., 2001). The most effective and simplest molecular methods for assessing genetic polymorphism are PCR-based techniques. Among such techniques, the Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR) markers are widely used, allowing rapid detection of the variability of a large number of genome loci (Sivolap and Solodenko, 1998; Sossey-Alaoui *et al*., 1998; 1999; Markin *et al*., 2016; Suresha *et al*., 2017; Yang *et al*., 2018; Uma *et al*., 2018). The spectra of DNA fragments, obtained as a result of their amplification, can be used as genetic markers for species identification and barcoding, as well as for determining taxonomic differences between species. The aim of the current investigation is genotyping of annual and perennial sunflower species from the collection of the N. I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) based on the polymorphism of microsatellite markers.

Materials and Methods

The objects of the study were samples of 5 annual and 24 perennial species of sunflower from the collection of the N. I. Vavilov All-Russian Institute of Plant Genetic Resources (Table 1).

Using sunflower map from NCBI database (https://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=4232&query=Helianthus), we selected 52 SSR markers, with localization on all the 17 linkage groups (chromosomes) of the sunflower genome (Table 2).

Table 1: The list of sunflower samples from the VIR collection according to the Schilling and Heiser (1981) species classification

I/n	Species	Chromosome number, 2n	Introduction number in the VIR collection	Section	Subsectio
	Annual				
1.	*H. annuus* L.	34	598276	Helianthi	-
2.	*H. annuus* L.	34	545522		
3.	*H. annuus* L.	34	441098		
4.	*H. annuus* L.	34	549513		
5.	*H. annuus* L.	34	506067		
6.	*H. annuus* L.	34	545563		
7.	*H. annuus* L.	34	545616		
8.	*H. annuus* L.	34	545736		
9.	*H. annuus* L.	34	436863		
10.	*H. annuus* L.	34	545500		
11.	*H. annuus* L.	34	545598		
12.	*H. annuus* breeding form VIR 119	34	-		
13.	*H. debilis* Nutt.	34	560395		
14.	*H. praecox* Engelm. and A. Gray	34	560400		
15.	*H. agrophylus* Torr. and A. Gray	34	1000		
16.	*H. petiolaris* Nutt.	34	503232		
	Perennial				
17.	*H. ciliaris* DC.	68, 102	-	Ciliares	Ciliares
18.	*H. californicus* DC.	102	530447		Corona-solis
19.	*H. decapetalus* L.	34, 68	440439		
20.	*H. trachelifolius* Mill.	34, 68	-		
21.	*H. divaricatus* L.	34	2099		
22.	*H. eggertii* Small	102	-		
23.	*H. giganteus* L.	34	489235		
24.	*H. grosseserratus* M. Martens	34	545698		
25.	*H. hirsutus* Raf.	68	560389		
26.	*H. maximiliani* Schrad.	34	2099		
27.	*H. mollis* Lam.	34	2102		
28.	*H. nuttallii* Torr. and A. Gray	34	-		
29.	*H. salicifolius* A. Dietr.	34	440074	Divaricati	
30.	*H. strumosus* L.	68, 102	440679		
31.	*H. tomentosus* Michx.	102	2107		
32.	*H. tuberosus* L.	102	2111		
33.	*H. laevigatus* Torr. and A. Gray	68	-		Microcephali
34.	*H. microcephalus* Torr. and A. Gray	34	-		
35.	*H. smithii* Heiser	68	-		
36.	*H. occidentalis* Riddel subsp. plantagineus (Torr. and A.Gray) Heiser	34	441062		Atrorubente
37.	*H. rigidus* Desf.	102	545658		
38.	*H. angustifolius* L.	34	1889		
39.	*H. floridanus* A. Gray ex Chapm.	34	-		Angustifolii
40.	*H. simulans* E. Watson	34	545659		

Table 2: SSR markers used for genotyping of sunflower

№	SSR marker	Linkage group	Amplicon size, bp	PIC
1	ORS 610	1	144	0,59
2	ORS 509		198	0,56
3	ORS 552		200, 246, 500	0,68
4	ORS 1194	2	180, 217, 280, 300, 380	0,82
5	ORS 1045		155	0,83
6	ORS 653		312, 500	0,97
7	ORS 545	3	100, 180	0,61
8	ORS 1021		280, 309	0,58
9	ORS 488		179	0,53
10	ORS 963	4	100, 300, 340, 600	0,93
11	ORS 785		100, 161, 200	0,75
12	ORS 1217		300, 431	0,68
13	ORS 1024	5	224, 250	0,97
14	ORS 1159		200, 255, 400	0,99
15	ORS 1120		250, 300, 321, 400, 600	0,95
16	ORS 650	6	100, 412	0,99
17	ORS 381		100, 216, 550	0,96
18	ORS 1256		150, 180, 210	0,68
19	ORS 426	7	334	0,91
20	ORS 966		372	0,31
21	ORS 901		407	0,32
22	ORS 1043	8	204	0,86
23	ORS 243		170	0,94
24	ORS 894		150, 252, 350	0,90
25	ORS 1265	9	222, 250	0,63
26	ORS 887		252	0,88
27	ORS 1220		257	0,68
28	ORS 878	10	203, 320	0,58
29	ORS 437		342	0,59
30	ORS 691		200, 447	0,66
31	ORS 625	11	204, 300	0,61
32	ORS 1214		369	0,35
33	ORS 697		238, 450	0,66
34	ORS 502	12	120	0,59
35	ORS 946		191	0,37
36	ORS 810		398	0,97
37	ORS 707	13	100, 160	0,52
38	ORS 1179		315	0,99
39	ORS 799		143	0,76
40	ORS 578	14	238, 300, 600, 800	0,98
41	ORS 398		298	0,89
42	ORS 1086		140	0,68
43	ORS 151	15	180, 220, 454	0,73
44	ORS 687		168	0,71
45	ORS 857		212	0,68
46	ORS 899	16	323	0,98
47	ORS 656		196	0,80
48	ORS 788		263	0.94
49	ORS 996		150, 292, 700	0,79
50	ORS 297	17	225	0,73
51	ORS 727		192, 210, 250, 280, 300	0,66
52	ORS 1097		130, 161	0,68

Genomic DNA was isolated from sunflower leaf tissue, with our modifications (Markin *et al*., 2016). PCR The PCR was carried out in 25 μL reaction mixture of the following composition: 67 mm Tris-HCl buffer, pH 8.8, 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgSO_4$, 0.1 mM mercaptoethanol, 0.25 mM of each dNTP (dATP, dCTP, dTTP and dGTP), 400 nM primers, 2.5 units of Taq polymerase and 15 ng of DNA template. Amplification was performed in the thermocycler Palm Cycler (Corbett Research, Australia). Thermal regime of the reaction was chosen individually for each pair of primers on the basis of their sequences. For majority of

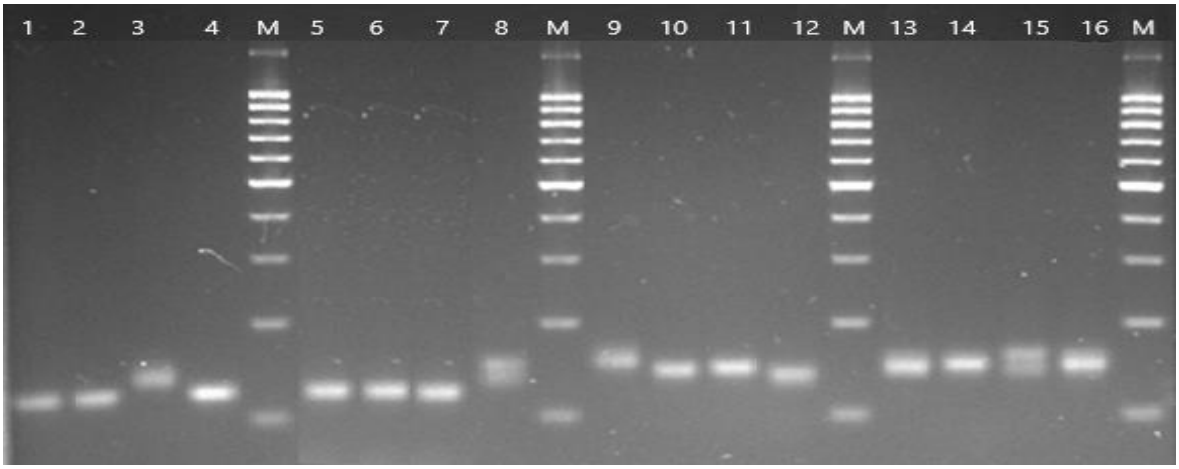
reactions the optimal thermal regime was as follows: (1) denaturation at 94°C for 4 min, (2) 35 cycles at the following thermal and time regime: denaturation 94°C - 20 sec, annealing 58°C - 20 sec, elongation 72°C - 60 sec (3) final elongation at 72°C for 10 min. The primer sequences of the SSR markers are taken from the GenBank NCBI.

The amplicons were separated by electrophoresis in 3% agarose gel supplemented with ethidium bromide in Tris-Borate buffer. The obtained gels were analyzed with the Gel-Documenting System (GelDoc 2000, BioRad, United States). 100+ bp DNA Ladder (Evrogen, Russia) was used as a molecular weight marker. All the procedures were performed in 3-5 replicates.

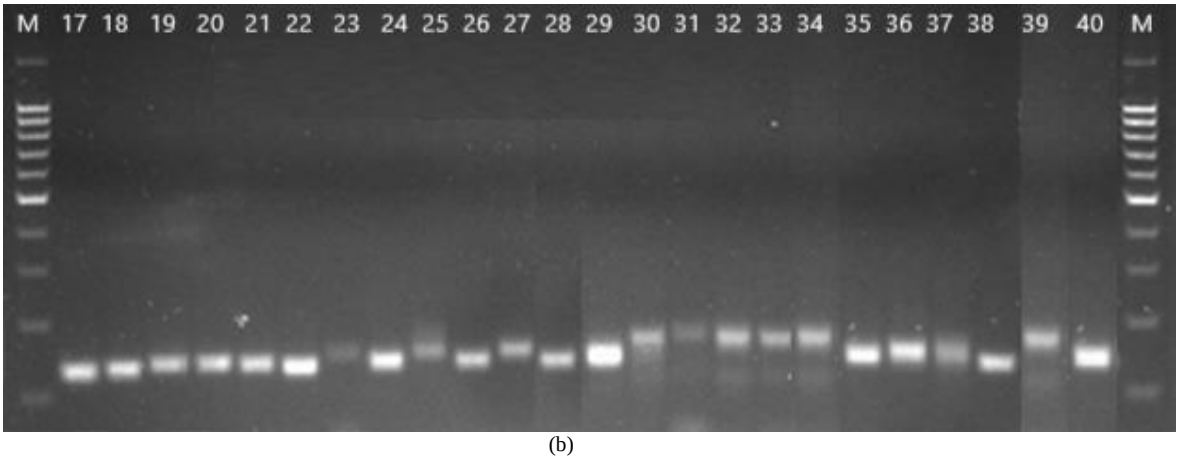
For the estimation of SSR loci polymorphism, the Polymorphism Information Content (PIC) value was used. PIC = $1-\sum pi^2$, where Pi is the frequency of the i-th allele among the total number of alleles (Nei, 1973). For determination of the genetic differences in sunflower samples as well as for dendrogram construction, the TREECON program (Van de Peer and De Wachter, 1993) was applied.

Results and Discussion

According to molecular genetic analysis of 40 sunflower samples, it was determined that all 52 selected SSR markers provided well reproducible and informative data. The electrophoresis analysis of amplicons revealed 1-5 bands for each SSR marker. The variability in amplicon size was from 100 bp to 800 bp. In total, 99 allelic variations across all studied SSR loci were defined (Table 2). While analyzing electrophoregrams the differences between samples were observed according to amplicon size, presence/absence of amplicon and multiply banding (multiple loci). As an example, Fig. 1 show the SSR profiles of annual and perennial species of sunflower using primers for one loci ORS 610.



(a)



(b)

Fig. 1: SSR profiles of annual (A) and perennial (B) species of sunflower using primers for one loci ORS 610. The numbers indicated index number of species (Table 1). M – molecular weight standard (100+ bp DNA Ladder (Evrogen, Russia))

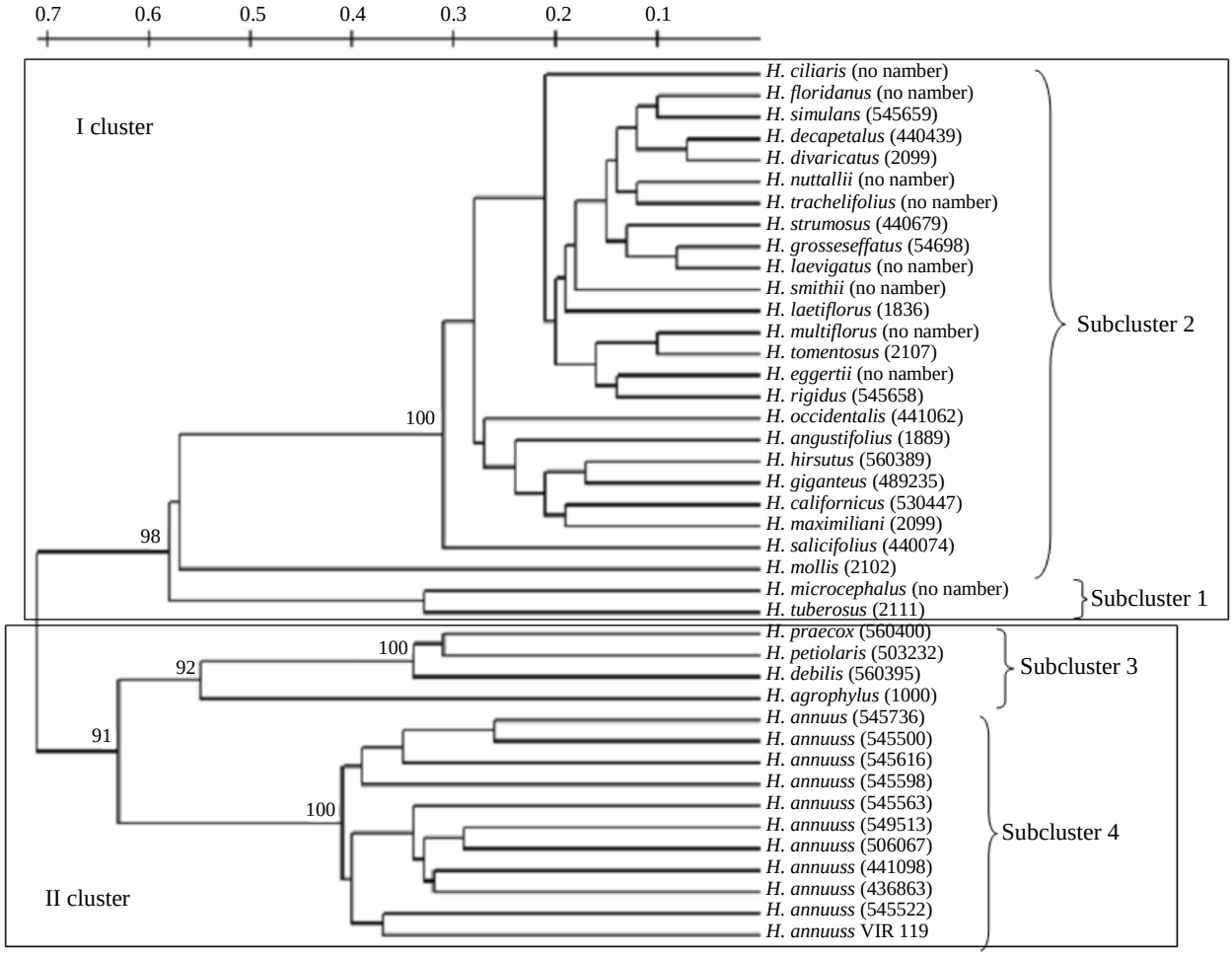


Fig. 2: UPGMA dendrogram of 42 sunflower samples based on 52 SSR loci. The introduction number of the studied samples is indicated in parentheses. The numbers indicate bootstrap values (only values greater than 90% are included

All the studied sunflower samples had unique SSR loci banding patterns. Thus the current approach indicated the effectiveness of SSR genotyping and made it possible to evaluate the genetic polymorphism of the samples. PIC values varied from 0,31 to 0,99 with the mean 0,72, which indicates the high resolution of this SSR based system for sunflower nuclear genome investigations. Moreover, the discriminatory power of the marker system allowed us to classify all the sunflower species and provide the molecular barcoding.

Based on the data obtained, the UPGMA dendrogram, reflecting the genetic differences between 29 species of the genus Helianthus L., was constructed. In the presented dendrogram (Fig. 2), the sunflower species are combined into two main clusters with high statistical reliability - 98% and 91% bootstrap. The first cluster includes all plants of perennial species of Helianthus L. and the second - all samples of annual sunflower. In turn, the perennial species cluster is divided into two subclusters: The first one includes only two species (*H. tuberosus* and *H. microcephalus*), while the second one - all other perennial species, in which the most divergent are *H. mollis*, *H. salicifolius* (Fig. 2). Annual species form two subclusters (subclusters 3 and 4), one is presented by *H. annuus* species, including the VIR 119 line and another one combines all other samples of annual species: *H. praecox*, *H. petiolaris*, *H. debilis* and *H. agrophylus* (Fig. 2). The obtained topology of the dendrogram of perennial species differs from the taxonomy data proposed by Schilling and Heiser (1981), which was constructed according to the analysis of morphological characters. There is no relationship between clusters and subsections identified by morphological characteristics. The reticulate speciation in the Helianthus L. genus and the high level of genetic variation can explain topological incongruence between current data and the classical ideas about the systematics of perennial sunflower species (Timme *et al*., 2007; Mascagni *et al*., 2017; 2018).

The high potential of SSR markers for investigations of plant genetic diversity was established in many studies (Ahmad *et al*., 2017; Wang *et al*., 2018; Parthiban *et al*., 2018). Current research of sunflower nuclear polymorphism based on analysis of52 SSR loci, allowed genotyping 29 species of the genus Helianthus L. Allele distribution data of the studied sunflower samples is a database that can be used to determine the levels of genetic variability, provide molecular barcoding, and control the genetic integrity of collection sunflower samples. Also, the dendrogram displaying the genetic relationships between the studied sunflower species was provided. Thus, the studied SSR markers are informative for assessing the level of genetic diversity of the genus Helianthus L.

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Author's Contributions

All the five authors equally participated in the laboratory study, data analysis and the entire process of the article preparation.

Ethics

This article is original and contains unpublished material. The authors declare that there is no conflict of interest regarding publication of this paper. The authors declare that no ethical issues are going to arise after the work has been published.

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