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STUDY OF THE GENETIC DIVERSITY OF SOME WILD SUNFLOWER SPECIES USING ISSR MARKERS

Elena Laura Conțescu[*] and Florin Gabriel Anton[*]

National Agricultural Research and Development Institute Fundulea, 915200 Fundulea, Călărași County, Romania
[*]Corresponding authors. E-mail: contescu_elena_laura@yahoo.com; gabriel.anton@incda-fundulea.ro

ABSTRACT

Wild sunflower species are adapted to diverse habitats and possess considerable variability for biotic and abiotic traits, therefore wild germplasm can be a real source for improving the narrow genetic base of the cultivated sunflower crop by introgression of new genes which were previously identified in wild genotypes. In order to effectively exploit these genes in cultivated genotypes, researchers need more information regarding the quantity and distribution of the genetic variability available within the wild sunflower species, which can offer a multitudine of valuable traits for traditional or molecular breeding.

This study was conducted to reveal the genetic diversity of wild and cultivated sunflower genotypes using ISSR markers. From a total of 19 ISSR markers, which were used to evaluate the genetic diversity, eight markers (UBC808, UBC823, UBC834, UBC836, UBC840, UBC845, UBC853, 17899A) clearly differentiated the wild sunflower genotypes from the wild sunflower and/or the cultivated sunflower.

The primers amplified a total of 120 alleles ranging from 10 to 22 alleles per marker. A wide range of fragment length was detected among the accessions, from 140 to over 1500 bp. The neighbor-joining dendrogram, based on Rogers genetic distance, of the genotypes studied consisted of two main clusters of different sizes: five entries were grouped into cluster A and the remaining six entries were grouped into cluster B. It is interesting that genotypes of *Helianthus anuus* L., *Helianthus maximiliani* and *Helianthus argophyllus* were clustered together. Another aspect observed, refers to interspecific variability for *Helianthus debilis* genotypes. Regarding the PCA, the first two principal axes accounted for 26.8% (CP1) and 16.1% (CP2) of the total variation, respectively, together explaining 42.9% of the total variability.

In the future, this molecular genetic information can be combined with morphological and biochemical data to improve the sunflower breeding program.

Keywords: sunflower, wild genotypes, ISSR, diversity.

INTRODUCTION

Helianthus annuus L. (sunflower) is a part of the *Asteraceae* family, one of the most diverse and extensive family of flowering plants. The *Helianthus* genus consists in 53 species and 19 subspecies, from which 14 are annual species and 39 are perennial (Schilling and Heiser, 1981).

Due to the high economic importance of the crop, breeders make continuous efforts to obtain new lines and hybrids, adapted to the constantly climatic changes, with high yield, high oil content, tolerance to diseases and pests, etc. Unfortunately, their already hard work, is hampered by the narrow genetic basis of the cultivated species (Rîşnoveanu et al., 2019), which can be significantly improved with an infusion of genes from wild species, real sources of genetic variability for the cultivated sunflower.

A way to transfer those useful traits in cultivated sunflower was the interspecific hybridization, technique developed back in 1916 by Sazyperow who produced the first interspecific hybrids between *Helianthus argophyllus* and cultivated sunflower, like a solution for improving resistance to rust.

Wild species of sunflower where used further for resistance genes to pathogens and parasites like: alternaria (*H. debilis*), powdery mildew (*H. debilis*, *H. argophyllus*), charcoal rot (*H. maximiliani*), phomopsis (*H. debilis*, *H. argophyllus*, *H. maximiliani*), rust (*H. maximiliani*, *H. argophyllus*), downy mildew (*H. argophyllus*), *Sclerotinia sclerotiorum* (*H. argophyllus*, *H. debilis*, *H. maximiliani*), parasite broomrape (*H. debilis*, *H. maximiliani)*,

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sunflower moth (*H. maximiliani*), stem weevil (*H. argophyllus)*, drought tolerance (*H. argophyllus*) and other useful traits (Petcu and Păcureanu, 2012; Seiler et al., 2017; Saucă et al., 2018).

The most adequate method for determining the degree of diversity within and between the sunflower populations is based on molecular marker use, respectively polymerase chain reaction (PCR) amplifications. Techniques like RAPD (Random Amplified Polymorphic DNA - small number of polymorphisms), AFLP (Amplified Fragment Length Polymorphism - large number of bands) or SSR (Simple Sequence Repeat - knowing the primer sequence and high cost) can have significant limitations in their use. Instead, markers like ISSR (Inter Simple Sequence Repeat) have the ability to overcome these limitations and can be used to detect the genetic variations in determining mutant varieties due to the high level of polymorphism provided by this type of markers.

ISSRs are DNA fragments of about 100-3000 bp, located between adjacent oppositely oriented microsatellite regions. The ISSRs advantage is that no sequence data for primer construction are needed; only low quantities of template DNA are required (Amiteye, 2021).

The ISSR technique has successfully been used to evaluate genetic diversity for diverse crops: wheat (Polido et al., 2020), corn (do Amaral Júnior et al., 2011), barley (Olgun et al., 2015), strawberry (Hussein et al., 2008), pinus (Parasharami and Thengane, 2012), leucadendron (Pharmawat et al., 2005) and also for sunflower (Mahmoud and Abdel-Fatah, 2012; Altindal, 2019; Darbani et al., 2020; Duca et al., 2020; Morsi et al., 2020; Dhutmal et al., 2021) and can be applied also to study genetic identity, parentage, gene mapping, etc. (Spooner et al., 2005).

Concidering all above, the objective of the present study was to detect genetic differences between the wild and the cultivated sunflower species using ISSR markers.

MATERIAL AND METHODS

Genomic DNA belonging to eight wild genotypes, two lines and one commercial hybrid (Table 1) was extracted from sunflower seeds. 2% CTAB (Cetyl Trimethyl Ammonium Bromide) extraction buffer was used for DNA isolation [Ciucă et al. (2020), with slight alterations]. RN-ase treatment was used for eliminate the RNA contamination. DNA quantification was verified by electrophoretic method (0.8% agarose gel; 0,5X TBE solution) and spectrophotometric method (spectrophotometer Beckman-Coulter Du Series 700).

Table 1. Sunflower genotypes used in this study

Accesions	Species	Type	Country of origin
P3MAX	*Helianthus maximiliani*	perennial wild	Romania
A1NE	*Helianthus neglectus*	annual wild	Bulgaria
A13ARG	*Helianthus argophyllus*	annual wild	Romania
A2DE	*Helianthus debilis*	annual wild	Bulgaria
A3DE			
LC 1085 B	*Helianthus anuus* L.	maintainer line	Romania
LC 1095 C	*Helianthus anuus* L.	restorer line	Romania
Performer	*Helianthus anuus* L.	hybrid	Romania

PCR amplification

Molecular analyses were performed with 19 high reproducible polymorphic ISSR molecular markers: UBC808, UBC810, UBC814.1, UBC823, UBC824, UBC834, UBC836, UBC840, UBC840.1, UBC841, UBC845, UBC853, UBC853.1, HB14, HB15, 17899A, 17899B, 17898A and 17898B. Markers sequences were identified in previous scientific papers (Khalil et al., 2019; Darbani et al., 2020; Dhutmal et al., 2021).

PCR amplifications were performed in a 20µl reaction volume which contained 20-25 ng template DNA, 1X buffer DreamTaq Green PCR Master Mix (Thermo

Scientific), 1.25 µM from each primer in a ProFlex (Applied Biosystem) thermocycler system.

After an initial denaturation cycle of 3 min at 95°C, the PCR program consisted of 40 cycles at 95°C for 30 sec, 45 sec at variable annealing temperature (47-52°C), 1 min 30 sec at 72°C. The samples were held at 20°C after a final extension for 10 min at 72°C.

After PCR amplification, the samples were loaded in 1.5% agarose gel (routine use), stained with ethidium bromide. A 50 DNA Ladder RTU (GeneDirex-1500 bp) was used as a molecular size standard, for estimating the appropriate band size of amplified products. Gels were visualized in a gel documentation system UVITEC.

RESULTS AND DISCUSSION

As a result of PCR analysis with the 19 ISSR primers used to identify polymorphism between the wild and cultivated genotypes, only 8 ISSR markers revealed positive results (clear and distinct bands) for all genotypes (Table 2).

Table 2. Markers sequences, annealing temperature, percentage polymorphism

ISSR markers	Sequence	Annealing T°C	No. of bands	No of polymorphic bands	Percentage polymorphism
UBC 808	AGAGAGAGAGAGAGAGC	47°C	20	20	100.00
UBC 823	TCTCTCTCTCTCTCTCC	52°C	13	12	92.30
UBC 834	AGAGAGAGAGAGAGACYT	50°C	13	12	92.30
UBC 836	AGAGAGAGAGAGAGACYA	52°C	15	15	100.00
UBC 840	GAGAGAGAGAGAGAGAYT	30°C	17	16	94.11
UBC 845	CTCTCTCTCTCTCTCTRG	50°C	12	11	91.66
UBC 853	TCTCTCTCTCTCTCTCRT	52°C	10	10	100.00
17899A	CACACACACACAAG	47°C	22	22	100.00
	Total		**122**	**118**	
	Average/primer		15		**96.29**

A total of 122 polymorphic bands were obtained with ISSR markers UBC808 (Figure 1), UBC823 (Figure 1), UBC834 (Figure 2), UBC836 (Figure 2), UBC840 (Figure 3), UBC845 (Figure 3), UBC853 (Figure 4) and 17899A (Figure 4).

ISSR markers 17999A (22 bands) and UBC808 (20 bands) produced highest number of polymorphic bands, whereas UBC845 (12 bands) and UBC853 (10 bands) produced lowest number of bands. Average percentage polymorphism was 96.29% (Table 2), the size of PCR amplified products ranged from 160 bp to more than 1500 bp.

PCR analysis with marker UBC808 revealed similarities between all *Helianthus debilis* ascensions but also some specific bands for *Helianthus debilis*-D4 (550 bp, 690 bp). Unique products were detected on wild sunflower *Helianthus argophyllus (*450 bp) and *Helianthus neglectus* (570 bp). The highest number of polymorphic bands were detected on wild sunflower *Helianthus debilis*-D4 (9 bands) and the lowest for cultivated hybrid Performer (2 bands).

The results obtained by marker UBC823 revealed a common PCR product of 850 bp between wild and cultivated sunflower and also common products of 700 and 1150 bp between all *Helianthus debilis* ascensions, *Helianthus neglectus* and *Helianthus argophyllus*. Unique PCR product of 470 bp was detected for *Helianthus neglectus*. The number of bands obtained with this marker varied between eight (*H. debilis*) and three (*H. maximiliani*).

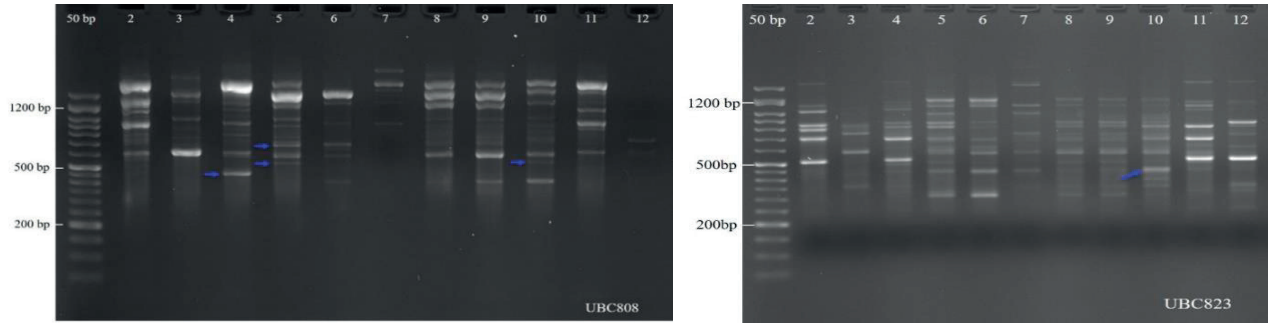

Figure 1. Electrophoretic pattern obtained with markers UBC808 (left) and 823 (right): 1- 50 bp Ladder; 2- maintainer 1085, 3- *H. maximiliani*, 4- *H. argophyllus*, 5-9- *H. debilis,* 10- *H. neglectus*, 11- restorer 1095, 12- Performer

PCR analyses with marker UBC834 revealed unique products of 290 and 370 bp for *Helianthus maximiliani* and a 420 bp product for *Helianthus argophyllus*. The number of bands resulted after PCR analysis with this marker was between seven (maintainer fertility line 1085) and three (*H. neglectus, H. debilis*).

PCR with marker UBC836 revealed unique amplification products of 820 bp for wild *Helianthus maximiliani* and a 680 bp *product for Helianthus neglectus*. Maximum number of bands was six (*H. debilis*-A3), the minimum was three *(H. maximiliani)*.

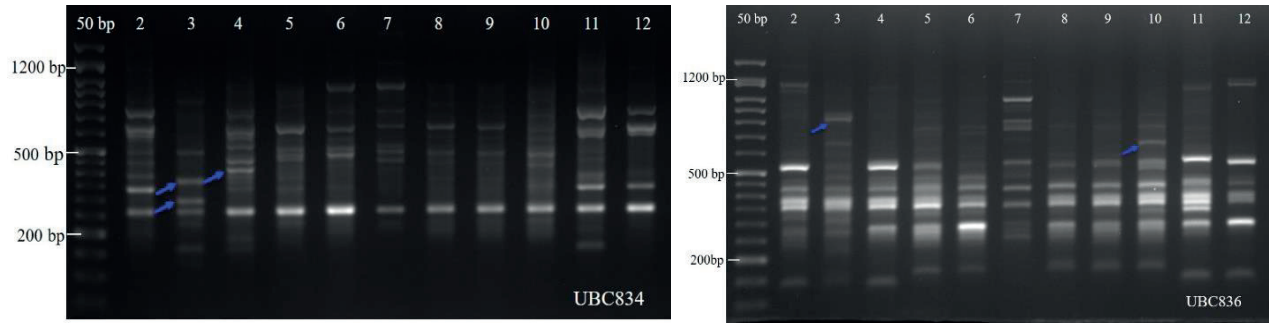

Figure 2. Electrophoretic pattern obtained with markers UBC834 (left) and 836 (right): 1- 50 bp Ladder; 2- maintainer 1085, 3- *H. maximiliani*, 4- *H. argophyllus*, 5-9- *H. debilis,* 10- *H. neglectus*, 11- restorer 1095, 12- Performer

As a result of the PCR reaction with marker UBC840 unique profiles of 850 bp (*H. neglectus)* and 500 bp (*H. argophyllus)* were obtained. The number of bands obtained with this marker varied between eight (*H. neglectus*) and three (*H. maximiliani*).

PCR reaction with ISSR marker UBC845 revealed unique products of 300 and 900 bp for *Helianthus debilis*-D4 and a product of 475 bp for *Helianthus neglectus*. The maximum number of bans was six (*H. debilis*-D4) and the minimum was one (*H. maximiliani*).

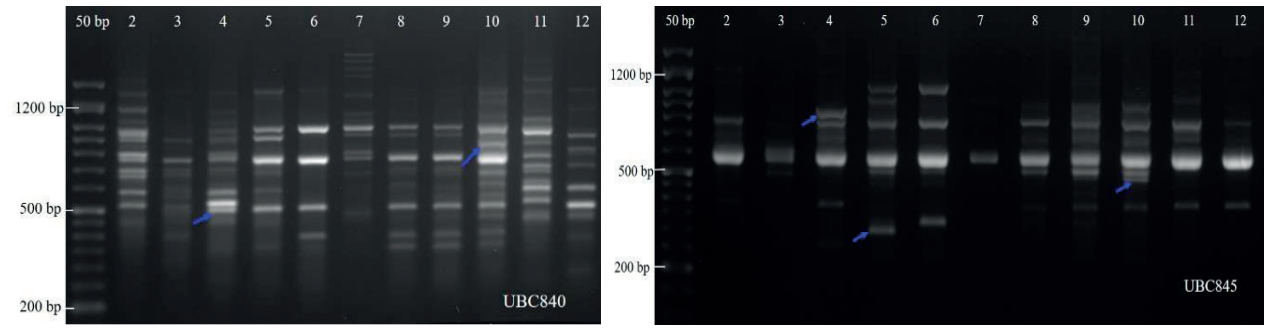

Figure 3. Electrophoretic pattern obtained with markers UBC840 (left) and 845 (right): 1- 50 bp Ladder; 2- maintainer 1085, 3- *H. maximiliani*, 4- *H. argophyllus*, 5-9- *H. debilis,*10- *H. neglectus*, 11- restorer 1095, 12- Performer

As a result of PCR analysis with marker UBC853 only ten polymorphic bands were obtained, from which three where unique products (690 and 750 bp for *H. maximiliani* and one product of 650 bp for *H. argophyllus*). The maximum number of bans was four (*H. argophyllus*) and the minimum was one (Performer).

Compared with all ISSR markers used for PCR analyses, marker 17899A produced the highest number of bands (22), five of them were unique products belonging to wild sunflower *Helianthus maximiliani* (450, 520, 620, 950 and 1250 bp). Two unique products were also observed for wild *Helianthus argophyllus* (1100 and 1200 bp). Maximum number of bands was eight (maintainer line1085), the minimum was two (hybrid Performer).

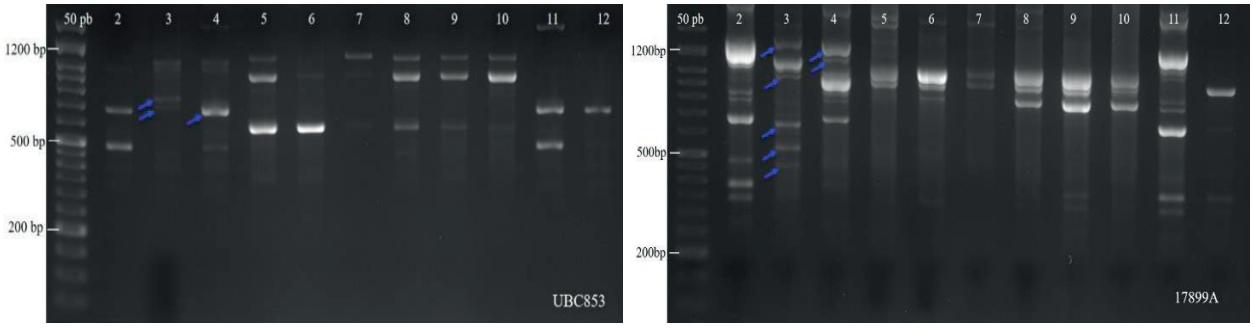

Figure 4. Electrophoretic pattern obtained with markers UBC853 (left) and 17899A (right): 1- 50 bp Ladder; 2- maintainer 1085, 3- *H. maximiliani*, 4- *H. argophyllus*, 5-9- *H. debilis,* 10- *H. neglectus*, 11- restorer 1095, 12- Performer.

Genetic distances and dendrogram

The bands which resulted after performing PCR analyses with ISSR markers UBC808, UBC823, UBC834, UBC836, UBC840, UBC845, UBC853 and 17899A (only markers with profiles for all genotypes were used) were listed as "1" for the existence of a band, missing the band was listed as "0".

Data was utilized for cluster analysis among and within wild and cultivated sunflower genotypes. Using Archaeopteryx software, part of BIO-R (a set of R programs) a dendrogram was constructed. Rogers genetic coefficient was used to estimate the genetic similarity matrix which allowed us to group all sunflower genotypes (wild and cultivated).

The ISSR's dendrogram gathered the eleven wild and cultivated sunflower accessions in two main clusters: A and B (Figure 5). Main cluster A is split into two subclusters: A1 - who included two genotypes: maintainer line 1085 and *Helianthus argophyllus,* and A2 - included three genotypes, from which *Helianthus maximiliani* formed a breakaway revealing less similarity with restorer line 1095 and hybrid Performer.

Cluster B which included only wild sunflower genotypes has split in two subclusters: B1 and B2. Subcluster B1 grouped tree *Helianthus debilis* genotypes, revealing the similarity between *Helianthus debilis*-D3 and *Helianthus debilis*-A3. Subcluster B2 gathered two *Helianthus debilis* genotypes and *Helianthus neglectus.*

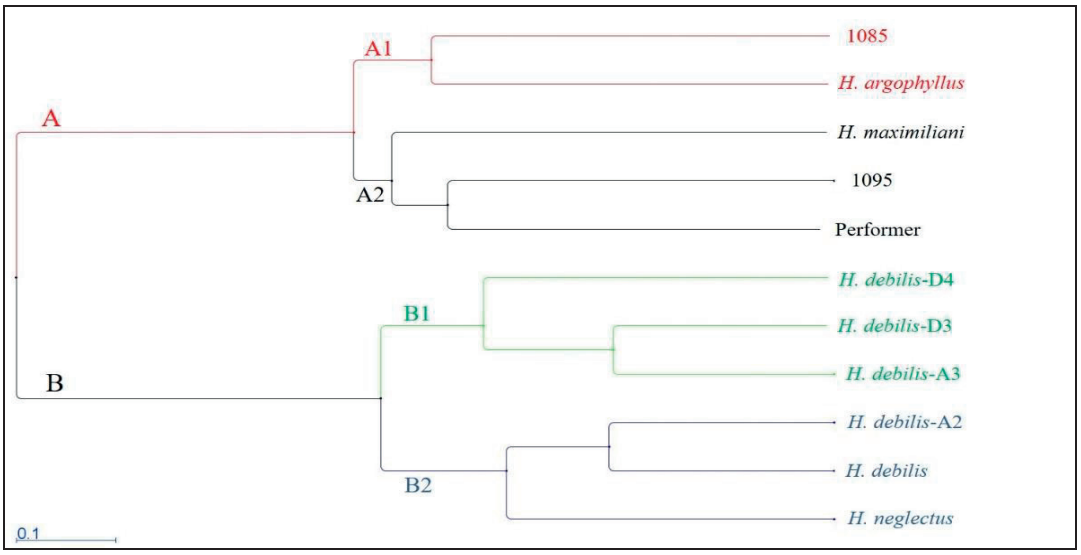

Figure 5. Dendrogram - differentiation between wild and cultivated sunflower genotypes based on ISSR analysis and Rogers's genetic distance

The PCA (Principal Component Analysis - Figure 6) confirmed the information provided by the dendrogram. The two principal axes accounted for 26.8% (CP1) and 16.1% (CP2) of the total variation, respectively, together explaining 42.9% of the total variability. These results suggest the genetic diversity of the studied sunflower genotypes, the ISSR marker analysis could ensure a better differentiation between genotypes.

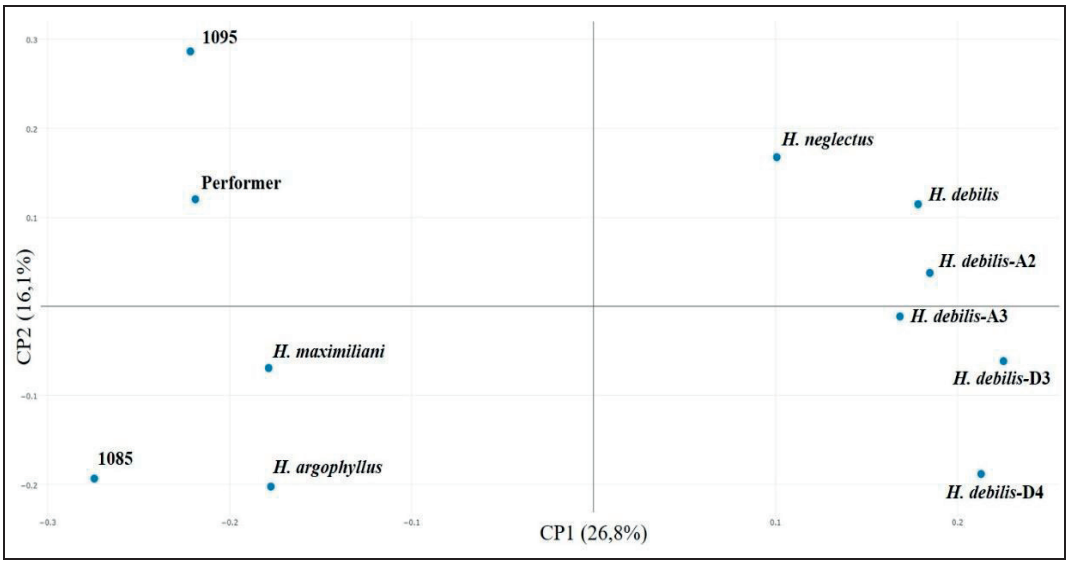

Figure 6. PCA - Distribution of sunflower wild and cultivated genotypes on the two first principal component analysis axes determined from ISSR genotyping

CONCLUSIONS

Results showed a high polymorphism between the genotypes, reflecting the genetic variability available in the wild and cultivated sunflower germplasm.

The efficiency of using ISSR markers was confirmed by the high level of polymorphism, respectively, 96.29%.

The study of genetic polymorphism in eight wild sunflower genotypes and three cultivated *Helianthus anuus* using 8 ISSR primers grouped them into two main clusters according their similarity.

The cultivated *Helianthus anuus* were grouped together in the same cluster with *Helianthus maximiliani* and *Helianthus argophyllus*, suggesting that these two wild

Helianthus species are more similar with cultivated *Helianthus*.

ISSR markers proved to be a successful tool for evaluating the genetic variation available in and between *Helianthus sp*. germplasm.

Supplementary analyses with a higher number of molecular markers and genotypes will allow to identify more genetic variance in *Helianthus* germplasm.

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