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Original article

Molecular Phylogenetic Analysis and Characterization of Wild Sunflower Species

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Abstract

As it is known, sunflower is a very important oil crop that is generally used in Turkey and the world. To compensate of Turkish vegetable oil need, with increasing population day by day, it needs to increase the sunflower yield. To increase sunflower seed yield and production as well as the quality, the planted cultivars should have the resistant genes for better adaptation capability to bad environmental conditions as well as new better specifications for higher yielding. Sunflower wild species growing in very hard environments are the main resources for these purposes. There are many conducted studies to give proper information to sunflower breeders until now for classifying wild species. In addition to classical phylogenetic, it is so important that searching of relationships and specifications of sunflower wild species utilizing from molecular phylogenetic studies given more appropriate and reasonable information. In this study, molecular phylogenetic analysis was performed by using 14 SSR markers that are high polymorphic in sunflower to define of the phylogenetic relationship among wild sunflower species with using 52 different species in Helianthus genus. PCR products obtained as a result of SSR analysis were measured with a capillary electrophoresis. The frequencies of the obtained alleles were analyzed using GenAlex 6.5 and PIC values were analyzed using CERVUS programs. A total of 134 different alleles were obtained for 8 SSR loci. Remained 4 SSRs were monomorphic and 2 of them did not produce scorable products. The most polymorphic SSR was ORS662 marker with 19 alleles. The least allele (10) was seen in ORS331 marker. The average number of alleles per locus was calculated as 16. Then, the UPGMA dendrogram was created based on similarity matrices. According to the dendrogram, it was observed that the closest species to cultivated sunflower (H. annuus) was H. eggertii. The similarity index between the two species was calculated as 0.589. After H. eggerti, the most similar species were H. pauciflorus, H. praecox and H. decapetalus, respectively. With this study, 8 markers that could distinguish the similarities among sunflower species were determined and the results were obtained about the proximity of the species to each other.

Keywords: Wild Sunflower, Helianthus genus, Molecular Phylogenetic, SSR, Dendrogram

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INTRODUCTION

The sunflower (*Helianthus*) genus belonging to the *Asteraceae* family, is one of the largest and most diverse families, with a chromosome number of n=17. The *Helianthus* genus consists of 51 species and 19 subspecies. Among these, 14 are annual species and 37 are perennial species. Each species has its own unique advantageous or disadvantageous features. This broad genetic variation is the result of numerous processes that occurred during their evolutionary history. The genetic diversity of sunflower is very important in the development of cultivated sunflowers (Seiler *et al*., 2017). To gain a comprehensive understanding of these processes, it is necessary to reconstruct the wild species from which they were derived in a broader context. These types of evaluations can provide insights into the ancestors' lineages that led to early domestication and modern varieties and explain the factors that influence the distribution of genetic diversity across gene pools (Park and Burke, 2020).

Sunflower, with many different wild species, exhibits a wide range of variability in terms of its characteristics and adaptation to extreme climatic conditions. Understanding the relationships between these species is extremely important because each species has the potential to provide advantageous traits in the development of cultivated sunflowers (Jocic *et al.,* 2015; Kantar *et al*., 2015; Kaya *et al*., 2016). Phylogenetic studies are conducted to gain comprehensive information about the history of sunflower and to understand the relationships between its species. Molecular phylogeny is a branch of phylogenetic that analyzes the differences in DNA sequences to gather information about the evolutionary relationships between organisms or genes. The development of molecular techniques has facilitated many studies (Sujatha *et al*., 2008).

The identification, examination, and classification of organisms have been greatly simplified through phylogenetic and molecular studies. Molecular markers, in particular, are powerful tools used across all organisms. As a result, plant molecular systematics has also greatly advanced. Molecular markers are preferred over other markers (such as morphological and biochemical markers) because they can be applied to any tissue or developmental stage regardless of environmental conditions, require a small amount of material, and represent the entire genome (Fu *et al*., 2016).

Molecular methods have been used in genetic characterization to investigate the phylogeny of wild sunflower species and their hybrids (Schilling, 1997; Suresha *et al*., 2017; Mwangi *et al*., 2019; Burke *et al.,* 2004). By examining DNA sequence differences among sunflower varieties, inter-species relationships can be studied and phylogenetic trees can be constructed. Molecular methods are used to detect DNA sequence variations. Various molecular methods are employed to detect these differences in sunflowers (Sahranavard *et al*., 2015). In addition to RFLP, AFLP, RAPD, and SSR markers, SNPs, cpDNA regions, and rRNA regions enable the investigation of genetic diversity and the phylogenetic tree in sunflowers (Liu and Burke, 2006). The first high-resolution phylogenetic tree for sunflowers was created using nuclear 16S and 26S rDNA regions (Timme *et al*., 2007). In 2014, researchers used

chloroplast DNA from 80 different wild and cultivated forms obtained from the Vavilov Research Institute in Russia to study the polymorphism of the sunflower chloroplast genome. Fu *et al*., (2016) studied in China with using three cpDNA regions (ndhF, matK, rbcL) to generate a phylogenetic tree for this family. A study based on the variation of nuclear genome size was conducted, and a comprehensive database was created for 49 *Helianthus* species, with variability in the genus being investigated and a comparative phylogenetic analysis presented (Qiu *et al*., 2019).

SSR markers are a powerful technique for evaluating genetic diversity and obtaining comprehensive information about species, thanks to their high polymorphism, repeatability, ease of use, and frequent occurrence in the genome. In a study by Zia *et al*. (2014), SSR markers were used to estimate the genetic diversity of 44 different sunflowers. A total of 22 markers were used, and the overall polymorphism was observed to be 45.19%, with variability ranging from 25% to 100%. In another study, Zeinalzadeh-Tabrizi *et al*. (2018) used 21 SSRs to estimate the genetic diversity of 68 *Helianthus* genotypes, observing 49 polymorphic bands.

In another study, Markin *et al*. (2020) genotyped 29 *Helianthus* species using 52 SSR markers, finding an average PIC value of 0.72, demonstrating the high resolution of the SSR-based system. The discriminatory power of SSR markers allowed for the classification of sunflower species. In another study, 110 SSR markers were used in a DNA-based study to check the genetic purity of 23 sunflower parents and their 60 hybrids, showing polymorphism in 92 markers at a rate of 83.63% (Ahmed *et al*., 2022).

Markers are important tools for molecular phylogenetic. Sunflowers have many different wild species, mostly originating from North America, each with distinct advantageous and disadvantageous traits. It is important to know the wild species to develop cultivated sunflower varieties, as each serves as a valuable genetic resource (Seiler and Marek, 2011). Therefore, it is crucial to use the genetic resources of both wild and cultivated *Helianthus* species. Wild species, in particular, hold a wealth of genetic material for resistance to many diseases and abiotic stress conditions (Jocic *et al*., 2015; Kantar *et al*., 2015; Kaya *et al*., 2016). To obtain successful new varieties, knowledge of the genetic distances between genetic resources, as well as sufficient biological, taxonomic, genetic, and agronomic information, is essential. The identification of molecular markers linked to the locus carrying the desired trait in wild species has accelerated this process. The use of these markers in breeding programs to select individuals carrying the desired trait, in other words, MAS (Marker-Assisted Selection), offers significant advantages in developing new varieties (Sahranavard *et al*., 2015). In sunflower cultivation, the use of beneficial genes from interspecies hybrids obtained through hybridization, including genetic material volume, heterosis, resistance, and improved hybrid quality and yield, is essential. Knowing the genetic origin of wild species and their evolutionary changes is important for the development of cultivated sunflowers. Phylogenetic studies are conducted to gain

comprehensive knowledge of this process and uncover the relationships between ancestors. SSR markers can be used for the phylogenetic analysis of wild species (Qiu *et al*., 2019).

In this study, SSR (Simple Sequence Repeat) markers have been selected due to their advantages, such as frequent repetition in the genome, high polymorphism, repeatability, speed, and ease of use. SSR markers can be used for various purposes, such as constructing genetic maps, population analyses, and selection assisted by markers. These markers can also be used in cases of intraspecific confusion (Nimmakayala *et al*., 2010). Microsatellites are the smallest repeating units in DNA, with repeat motifs ranging from 1-6 base pairs. When the sequences surrounding these regions (flanking regions) are known, suitable primers can be designed, approximately 20-25 base pairs long, and amplified using the PCR technique. The difference in the number of repeating nucleotide sequences in the amplified region creates polymorphism in SSR markers.

In the study, the relationships between the species of the *Helianthus* genus, as well as their evolutionary closeness, have been examined using SSR markers. The aim of this study was to determine the relationships between wild sunflower species and to investigate the closeness of the species to each other in the evolutionary process. The markers used in the study were the ones determined as a result of the literature researches, which can be used between species and are known to show polymorphism in sunflower.

MATERIAL AND METHOD

Material

In this study, the collection of currently available annual and perennial wild Helianthus species, obtained from the U.S. Department of Agriculture, Iowa, has been used as genetic materials (Table 1). The genetic material from wild Helianthus species were planted in Wild Sunflower Garden in Trakya University, Edirne, Turkey (https://sunflower.trakya.edu.tr/). Since there were 4-5 accessions from each *Helianthus* species in the project material list, there were sufficient alternative samples for each species in the study.

Table 1. Wild sunflower species used in the study and their origins.

#	Species	Accession #	Type	Country of Origin
1	*Helianthus agretis*	3	Annual	US Florida
2	*Helianthus annuus*	9	Annual	US South Dakota
3	*Helianthus argophyllus*	14	Annual	US Florida
4	*Helianthus atrorubens*	23	Perennial	US N. Carolina
5	*Helianthus bolanderi*	27-30	Annual	US California
6	*Helianthus californicus*	32-33	Perennial	US California
7	*Helianthus carnosus*	35-36	Perennial	US Florida
8	*Helianthus cusickii*	40	Perennial	US Washington
9	*Helianthus debilis subsp. cucumerifolius*	46-47	Annual	US Georgia
10	*Helianthus debilis subsp. silvestris*	49	Annual	US Texas
11	*Helianthus decapetalus*	52-55	Perennial	US Ohio
12	*Helianthus divaricatus*	63	Perennial	US Virginia
13	*Helianthus eggertii*	65-67	Perennial	US S. Carolina
14	*Helianthus exilis*	68	Annual	US California
15	*Helianthus giganteus*	74-77	Perennial	US N. Carolina
16	*Helianthus glaucophyllus*	81	Perennial	US Tennessee
17	*Helianthus gracilentus*	83	Perennial	US California
18	*Helianthus grosseserratus*	87-88-89-91	Perennial	US North Dakota
19	*Helianthus heterophyllus*	95	Perennial	US Alabama
20	*Helianthus hirsutus*	97-99	Perennial	US Oklahoma
21	*Helianthus laciniatus*	102-105	Perennial	US Texas
22	*Helianthus laevigatus*	107-109	Perennial	US Virginia
23	*Helianthus longifolius*	111-113	Perennial	US Georgia
24	*Helianthus maximiliani*	114-116	Perennial	US Kentucky
25	*Helianthus mollis*	120-124	Perennial	US Missouri
26	*Helianthus neglectus*	127-130	Annual	US New Mexico
27	*Helianthus niveus subsp. canescens*	131	Annual	US Arizona
28	*Helianthus nuttallii*	136	Perennial	Canada Alberta
29	*Helianthus nuttallii subsp. nuttallii*	138-141	Perennial	US Oregon
30	*Helianthus nuttallii subsp. rydbergii*	144	Perennial	US North Dakota
31	*Helianthus occidentalis subsp. plantagineus*	148-149	Perennial	US Texas
32	*Helianthus paradoxus*	150-151	Annual	US Texas
33	*Helianthus pauciflorus*	152-154	Perennial	Canada Manitoba
34	*Helianthus pauciflorus subsp. subrhomboideus*	160-162	Perennial	US Iowa
35	*Helianthus petiolaris*	164	Annual	US Missouri
36	*Helianthus petiolaris subsp. petiolaris*	168-169	Annual	US New Jersey
37	*Helianthus porteri*	182	Annual	US Georgia
38	*Helianthus praecox*	184	Annual	US Texas
39	*Helianthus praecox subsp.hirtus*	185	Annual	US Texas
40	*Helianthus praecox subsp. praecox*	186	Annual	US Texas

41	*Helianthus praecox subsp. runyonii*	188	Annual	US Texas
42	*Helianthus pumilus*	191	Perennial	US Colorado
43	*Helianthus radula*	197	Perennial	US Florida
44	*Helianthus resinosus*	198	Perennial	US Mississippi
45	*Helianthus salicifolius*	203	Perennial	US Kansas
46	*Helianthus silphioides*	207-208	Perennial	US Arkansas
47	*Helianthus simulans*	212	Perennial	US Georgia
48	*Helianthus smithii*	214-216	Perennial	US N. Carolina
49	*Helianthus strumosus*	223	Perennial	US Indiana
50	*Helianthus tuberosus*	231	Perennial	US South Dakota
51	*Helianthus winteri*	237	Perennial	US California
52	*Helianthus laetiflorus*	239	Perennial	US Virginia

Method

The study was conducted in Trakya University Genetics and Bioengineering Molecular Genetics Lab in 2021-2022 with collected samples from Wild Sunflower Garden, Trakya University, Edirne, Turkey.

Germination

Seeds belonging to wild sunflower species procured for the study were sown in petri dishes, 10-15 seeds per dish. A 25 ppm solution of ethephon (a synthetic plant growth regulator) was added to the seeds. The seeds were kept at 26°C and rinsed with distilled water before being transferred into ozonized water. The petri dishes were labeled with the species name and a sort number and were left under appropriate conditions to germinate (Figures 1 and 2). Germinated seeds were transferred to seed trays containing peat-based soil (Figure 2). Once plants had grown sufficiently in the soil, they were moved to the wild sunflower collection garden. Leaves from the species grown in this garden were used as material for the study.

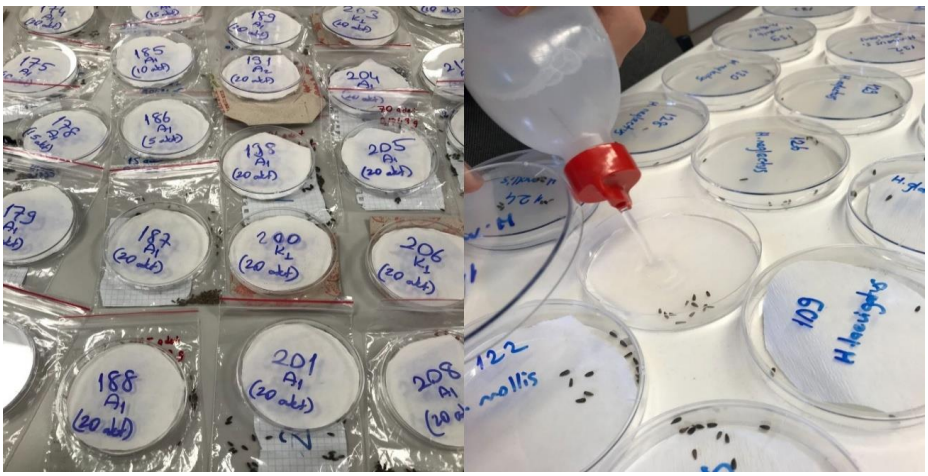

Figure 1. Seeds placed in Petri dishes – Adding ethephon to the seeds

Figure 2. Seed germination and transferring to seed trays after germination

DNA Isolation

Leaf tissues were placed in tubes with 3 mm metal beads and immersed in liquid nitrogen. The tissues were ground into powder using beads and a tissue grinder (RETSCH MM400). The powdered tissues were subjected to genomic DNA (gDNA) isolation using the NORGEN Plant/Fungi DNA Isolation Kit (Product #26200). The DNA concentration was measured using an OPTIZEN NanoQ spectrophotometer.

Amplification of SSR Markers

Fourteen SSR markers were chosen for the study due to their high polymorphism features. Information regarding these selected SSR markers is presented in Table 2. gDNAs purified from leaf tissues were subjected to PCR with 14 markers. Amplification reactions were prepared containing 50 ng DNA, 1X PCR buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.5 µM SSR primers and 1 U Taq polymerase (Invitrogen). Amplification was performed in a T100 Bio-Rad thermal cycler (CA, USA) by following the cycle of 3 min at 94°C for initial denaturation and 35 times, 45 sec at 94°C for denaturation, 45 sec at 53-56°C for primer annealing, 45 sec at 72°C for extension and 5 min at 72°C for final extension. PCR products were analyzed and separated by capillary electrophoresis.

Table 2. Information about selected SSR markers in the study and their specifications

Marker Code	Primer Forward	Primer Reverse	Repeat Motifs	Alleles (bç)
ORS309	CATTTGGATGGAGCCACTTT	GATGAAGATGGGGAATTTGTG	$(A)_{19}$	107-148
ORS331	TGAAGAAGGGTTGTTGATTACAAG	GCATTGGGTTCACCATTTCT	$(CT)_{12}(AC)_{13}$	185-198
ORS381	CCAACGGTGATGTAACTAGGAA	GTTCTCCTGGATAGCTCGACA	$(AC)_{11}$	100,216,550
ORS398	CACGTCCTAAATTAAGTAGGAACGA	CCAAGACCTCCGTTGAGCTAT	$(AT)_9(GT)_{10}$ $(AT)_2(GT)_6$	298
ORS423	TCATATGGAGGGATCTGTTGG	AAGCAACCATAATGCATCAGAA	$(GT)_{18}$	375-393
ORS613	GTAAACCCTAGGTCAATTTGCAG	ATCTCCGGAAAACATTCTCG	$(AG)_{16}$	204-247
ORS788	CTGGATAAAGATGGGATAAAGAGAG	GGACCCACCAAGATTTGTTTT	$(AG)_{13}$	252-296
ORS887	TCGAAAACGACTAATCCAACTTTC	GAGCATGAACAAGAATTGACACA	$(AC)_{11}$	224-249
ORS899	GCCACGTATAACTGACTATGACCA	CGAATACAGACTCGATAAACGACA	$(AC)_{19}$	323-341
ORS1120	TAACGTGTGCAGGTCTGTCTAAA	TGCAAATAATAAGATAATGACCGATT	$(GT)_{13}(GA)_{16}$	250-300
ORS963	CCTCCTAGGGTGTGAGGATGAG	TCGAACTCTGGCTCTTGTAGTTG	$(GT)_{10}$	340
ORS1043	CCAAACCGTCATGTTCTATGTTC	AGTGTGATTGCGAATTGTAGTGC	$(CT)_{16}$	204
ORS662	CGGGTTGGATATGGAGTCAA	CCTTTACAAACGAAGCACAATTC	$(AG)_{16}$	314
ORS243	GGGATGACGTGCGTTTGG	ACCACCATTTCTACCGTTTCTC	$(GGT)_7$	234

Data Processing and Dendrogram Construction

Allele sizes for each marker locus obtained from capillary electrophoresis were organized into tables in the appropriate format. The allele frequencies for 12 different SSR markers across all sunflower materials analyzed were individually calculated using the **GenAlex 6.5** software. The resulting allele data were statistically evaluated to construct genetic distance and similarity matrices (Figures 4.11 and 4.13). Based on the similarity matrices, a UPGMA dendrogram was generated using the **DarWin** software (Figure 4).

RESULTS

SSR Loci and Diversity in Wild Sunflower Species

The analyses of the 8 markers were performed with GenAlex (Peakall and Smouse, 2006) (Table 3). A total of 134 alleles were obtained for the 8 analyzed SSR markers. The highest number of alleles (24) was observed in ORS788 marker. The number of alleles per locus was calculated as 16. Alleles belonging to each marker, allele frequencies and the distribution of alleles belonging to loci among 50 cultivars screened and PIC value are given in Table 3. Genetic similarity and distance between the

cultivars were calculated by DARwin 6.0 program according to Nei, 1987) and a dendrogram showing the phylogenetic relationships of the cultivars was created by using the neighbor joining method (Figure 5).

Table 3. Allele information of polymorphic SSR markers used in the study

Marker	Number of Observed Alleles	Minimum Allele Size (bç)	Maximum Allele Size (bç)	PIC (Polymorphic Information Content)
ORS309	13	106	270	0.846
ORS423	14	214	246	0.890
ORS331	10	174	216	0.799
ORS381	15	126	398	0.909
ORS788	17	230	289	0.899
ORS887	15	219	259	0.881
ORS1043	12	190	212	0.887
ORS662	19	140	332	0.918

To determine the genetic relationships among wild sunflower species,0 14 different markers were screened. Subsequently, the marker loci for each marker were measured using a capillary electrophoresis device. During the analyses, 4 SSRs were found to lack polymorphism and 2 SSRs did not yield reliable results despite repeated attempts; thus, they (ORS243, ORS398, ORS899, ORS1120, ORS963, ORS613**)** were excluded from the evaluation. The other 8 SSRs (ORS309, ORS331, ORS381, ORS423, ORS1043, ORS788, ORS887, ORS662) showed high polymorphism and analyzed in detail using the AATI Fragment Analyzer. The different alleles of the ORS887 marker on 10 species, representing the results obtained by capillary electrophoresis, are presented in Figure 3. This marker produced 21 different alleles in the screened samples, with a maximum of 259 bp and a minimum of 219 bp. In another analysis with with ORS788 marker, 24 alleles were observed with a maximum size of 289bp and a minimum size of 230bp in Figure 4.

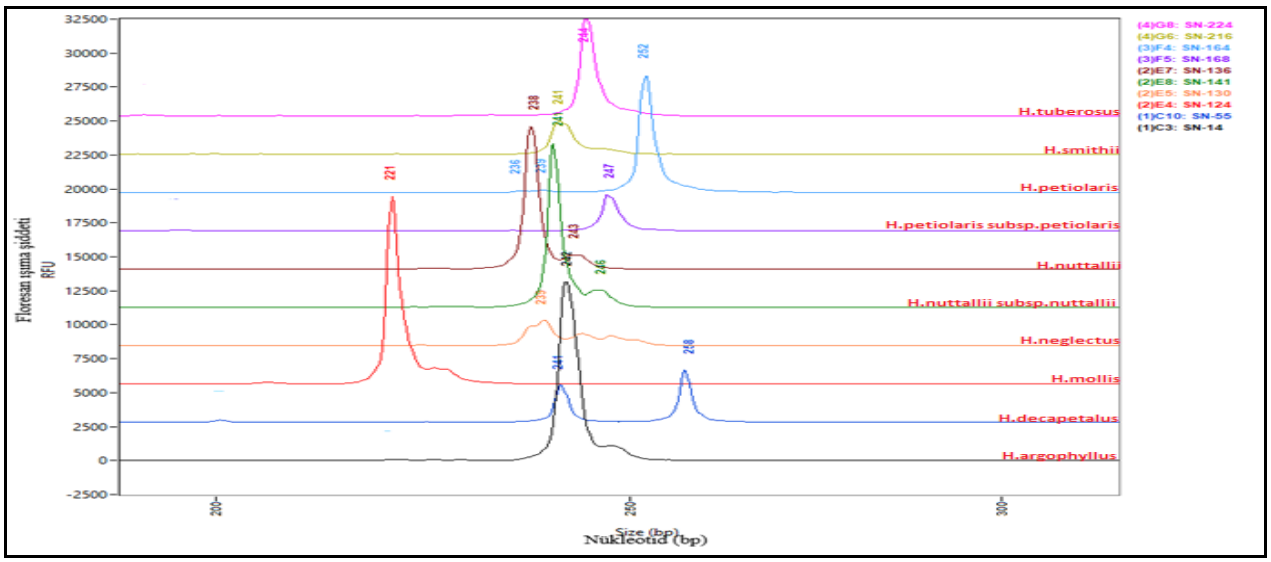

Figure 3. DNA fragments of different alleles obtained with ORS887 marker

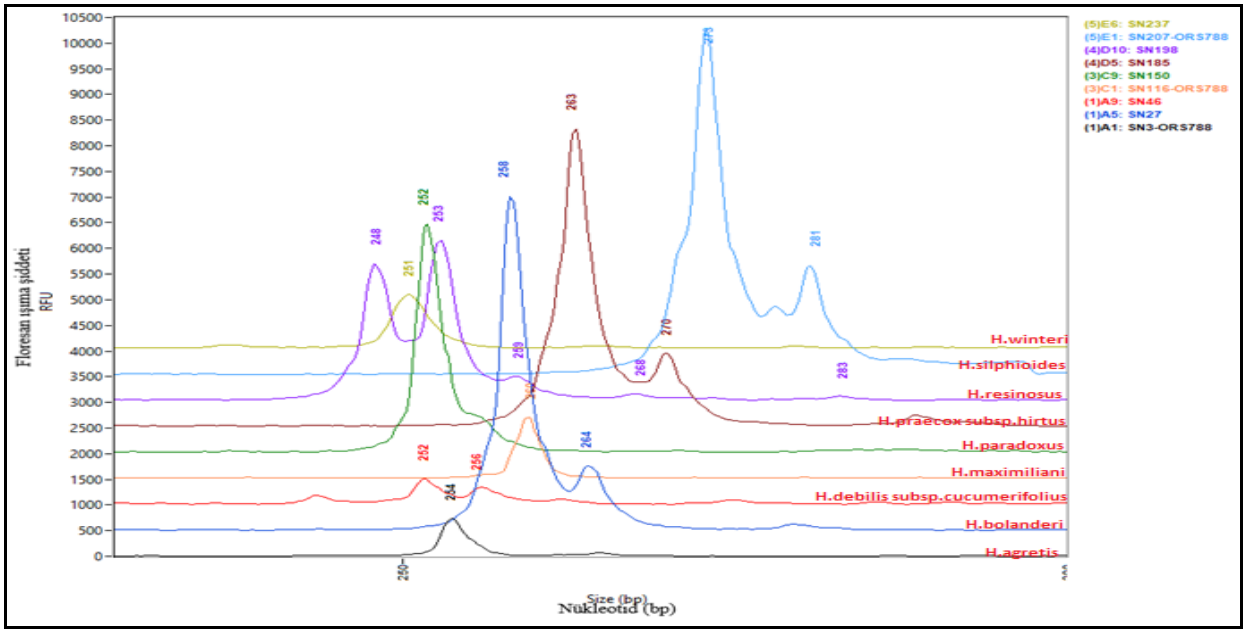

Figure 4. DNA fragments of different alleles obtained with ORS788 marker

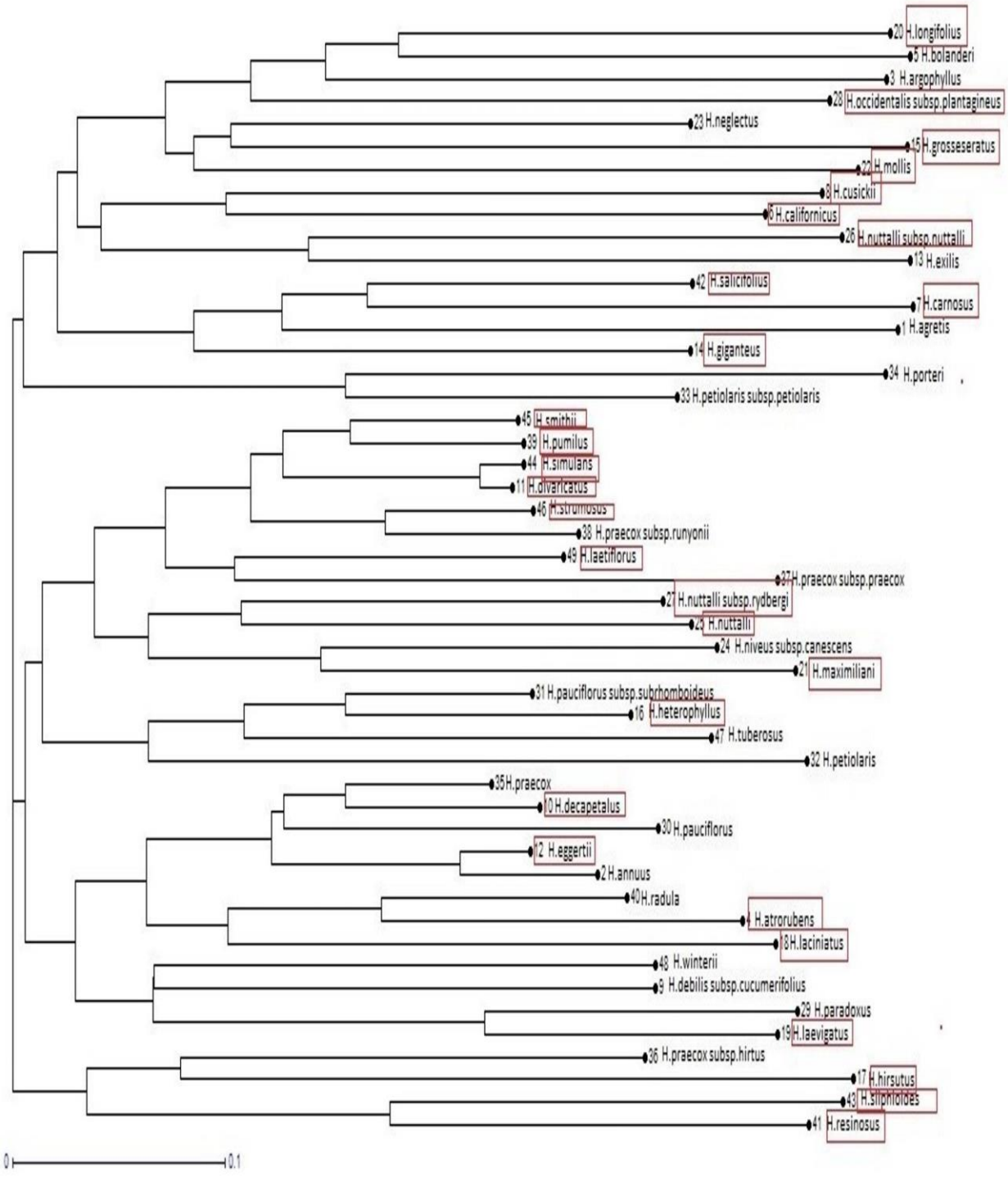

Figure 5. Dendrogram of 49 wild sunflower species (marked ones are perennial)

CONCLUSIONS

In the study, 12 different markers were used. SN49, SN81, SN83 (*H. debilis subsp silvestris, H. glaucophyllus, H. gracilentus*) species were not included in the study due to insufficient tissue or no results. Therefore, the remaining 49 species were genotyped using 8 polymorphic markers. As a result of the analyses performed with GenAlex, 49 species gave a total of 134 alleles. The most alleles (19) were obtained at ORS662 marker. The lowest allele number (10) was obtained from ORS331 marker. The average number of alleles per locus was calculated as 19 (Table 3). In this study, the maximum PIC value was 0.918 for the ORS662 marker and the minimum PIC value was 0.799 for the ORS331 marker. All of the markers used were found to be polymorphic.

According to the dendrogram given in Figure 5, the species were divided into 3 main groups based on obtained results of molecular analysis using SSR markers. The 3 main branches were divided into branches within themselves. In the 1st group, *H. resinosus* and *H. silphioides*; *H. hirsutus* and *H. praecox subps hirtus* are closely related.

Group 2 was divided into two subgroups. In the 2nd group, H. praecox was the species that left the ancestral lineage the earliest in the evolutionary process. The closest species to this species is *H. decapetalus. H. laevigatus* and *H. paradoxus, H. eggerti* and *H. annus, H. radula* and *H. atrorubens* were observed to be more similar. When the other branches of the 2nd group were analyzed, it was observed that *H. smithii - H. pumilus, H. simulans - H. divaricatus, H.strumosus - H.praecox subsp.runyonii* species were very close to each other. The 3rd group was again divided into more than one branch within itself. However, it was observed that *H. porteri* and *H. petiolaris subsp. petiolaris* species, which were divided into a single branch and formed taxa among themselves, were separate from the others and similar to each other. *H. longifolius - H. bolanderi, H. neglectus - H. grosseserratus, H. salicifolius - H. carnosus* are similar species. *H. eggerti* was genotypically the closest species to *H. annuus*, known as cultivated sunflower. Then *H. pauciflorus, H. decapetalus, H. praecox* are the most similar species respectively. Since they belong to the same subgroup, *H. radula, H. atrorubens* and *H. laciniatus* can also be said to be close.

In a previous study, 29 sunflower species were genotyped using 52 SSR markers. The average PIC value was 0.72. In their study, the PIC value of ORS381 SSR marker was calculated as 0.96, ORS788 marker as 0.94, ORS887 marker as 0.88 and ORS1043 marker as 0.86 (Markin *et al*., 2020). In our study, PIC values for these polymorphic markers were calculated as 0.90 for ORS381, 0.899 for ORS788, 0.881 for ORS887 and 0.887 for ORS1043. ORS marker showed polymorphism and PIC value was 0.96. DNA fragments belonging to this marker were 100,216,550 bc in size. In this study, the PIC value was found to be 0.90. When we compare the studies, it is seen that close results are obtained. Again, ORS398, ORS899 and ORS243 markers used in the study conducted by Markin *et al.* (2020) showed polymorphism. However, these markers were not found to be promising in our study.

Darvishzadeh *et al*. (2010), genetic distances between 28 sunflower genotypes were evaluated by using 38 SSR markers. The markers used showed low polymorphism. In the same study, it was observed that the PIC value for ORS613 marker was 0.32. However, quality results could not be obtained in the study. If we look at the other markers, it is seen that the PIC value of ORS423 is 0.49 and ORS331 marker is 0.34 and very low. However, in the study conducted, these values were found to be higher and calculated as 0.89 for ORS423 and 0.779 for ORS331. The reason for this difference is thought to be the difference in the material used.

Compared to the previous studies, this study was more comprehensive and all existing wild species were included in the study. Genotypically more distant species were identified. In this way, it was possible to identify and use genetically distant species in the studies carried out for the cultivation of sunflower of higher quality. At the same time, distinctive markers were determined for sunflower species. As a result, SSR markers have been found to be intra-species discriminative in genetic diversity studies in sunflower species. In conclusion, this study concluded that 8 markers could distinguish the similarities among sunflower species to determine the proximity of the species to each other.

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