Turkish Journal of Field Crops
field-crops.org

DOI: *10.17557/tjfc.1650798*
ISSN: 1301-1111
e-ISSN: 3023-6657
2025, 30(1), 110-120

A Flow Cytometry-Based Assessment of the Nuclear DNA Content and Ploidy Level of Wild Sunflower Species

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ARTICLE INFO

Research Article
Received: 4 March 2025
Accepted: 15 May 2025
Published: 23 June 2025

Keywords:
Flow cytometry
Helianthus
Nuclear DNA content
Ploidy
Wild sunflowers

Citation: Kaya, Y., Savas Tuna, G., Beser, N., & Tuna M. (2025). A Flow Cytometry-Based Assessment of the Nuclear DNA Content and Ploidy Level of Wild Sunflower Species. *Turkish Journal of Field Crops*, 30(1), 110-120. https://doi.org/10.17557/tjfc.1650798

ABSTRACT

Sunflower genus (*Helianthus* spp.), native to North America, includes 37 perennials and 14 annual species, many of which possess valuable genes related to biotic and abiotic stress tolerance that can enhance yield and yield-related traits in cultivated sunflower. However, it is crucial to characterize wild species to incorporate these beneficial traits into breeding programs. The study aim was to determine the nuclear DNA content of 133 sunflower accessions representing 52 species of the genus by flow cytometer and use the obtained information to determine their ploidy levels and verify their taxonomic identities. Based on flow cytometrical analysis, nuclear DNA content of the individual plants and the average values of accessions of the same species generally showed a high degree of similarity, except in some cases. However, the average 2C nuclear DNA content of *Helianthus* species was estimated to range from 5.61 pg (*H. porteri*) to 27.05 pg (*H. tuberosus*). The differences among the species were statistically significant. Based on the study results, it was determined that approximately 80% (41 species) of the wild species (15 annuals, 26 perennials) are diploid. The ploidy level of the remaining species exhibited variability, and 12 species were tetraploid (4x) while 2 species were hexaploid (6x). The study results indicated that some accessions of some species were either identified incorrectly or they are progenies of natural interspecific hybrids. The study's results confirm that flow cytometer can be used to characterize the sunflower genetic resources before including them in breeding programs.

1. INTRODUCTION

Sunflower (*Helianthus annuus* L.) (2n=34), a member of the genus *Helianthus* included in the Compositae family, is one of the most important crops grown mainly for its highly quality edible oil (Gül and Coban, 2020). The genus consists of 52 species (14 annual and 37 perennial) that contributed many desirable characteristics to cultivated sunflower (Kaya, 2014; 2016).

Due to their importance in sunflower breeding, wild sunflower species have been collected from the geographic areas where they are growing naturally through expedition trips for over 60 years and preserved in gene banks. Approximately 2500 accessions of wild sunflower species are currently stored in the USDA-ARS collection, the most complete collection in the world (Seiler and Gulya, 2004). However, it is well known that collection of genetic resources of the crop species are quite challenging due to difficulties in identification of the species in the nature when they are growing together with their relatives (Vižintin and Bohanec, 2008). Same is true for wild sunflower species since their identification is problematic especially in the field, due mainly to natural variation and interspecific hybridization. Ploidy is another important factor contributing to the complexity of the species classification as several ploidy series exists in the *Helianthus* genus (Seiler and Gulya, 2004; Natali et al., 2008; Makarenko et al., 2020). Therefore, taxonomic identity and ploidy of the sunflower gene bank accessions should be confirmed before they were included in the breeding programs. However, at the species level, reliable identification may require years of training and specialization on one plant taxon. On the other hand, the classical ploidy analysis method of counting chromosomes using a light microscope is time-consuming, requires skill and experience, and highly depends on rapidly dividing cells (Brummer et al., 1999; Tuna et al., 2001, 2004). Getting freshly growing root tips with rapidly dividing cells suitable for cytological preparations were also big problem in the current study. Additionally, the method is inadequate when many samples needed to be analyzed, as in the case of this study (Tuna et al., 2001).

Since chromosomes are primarily composed of nucleic acids and are in the cell nucleus, there is a strong positive correlation between ploidy level and nuclear DNA content. Due to this correlation, nuclear DNA content values can be used to estimate ploidy level. Nuclear DNA content in plants was previously determined by Feulgen microspectrophotometry of root tip or shoot tip mitotic cells (Bennett and Smith, 1976). In recent years, flow cytometry has become the preferred technique for estimating the nuclear DNA content because of its ease, quickness, and accuracy (Rayburn et al., 1989; Heslop-Harrison, 1995; Mabuza et al., 2023).

Carefully determined nuclear DNA content by flow cytometry remains generally constant among all individuals of a species and across all cells of an individual, while it can vary up to 10,000-fold among the species. Therefore, nuclear DNA content is specific to species information. This characteristic of the nuclear DNA content makes it an essential information to use in the characterization of species' genomes, investigating their relations and evolutions (Vogel et al., 1999; Tuna et al., 2001; Ozkan et al., 2003; Leitch and Bennett, 2004; Tuna et al., 2005; 2006; Ozkan et al., 2006). Hence, it is widely utilized for the taxonomic identification, classification, and determination of ploidy levels of species (Dolezel, 1997; Ohri, 1998; Lu et al., 1998. Baack et al., 2005; Tuna et al., 2004; Savas Tuna et al., 2016; 2017).

Few studies have previously employed flow cytometry to analyze *Helianthus* species. Michaelson et al., (1991) reported that the average DNA content in root and shoot tip nuclei of wild *Helianthus* species ranged from 6.01 to 7.95 pg in aneuploids (2n = 17 to 33) and diploids (2n = 34). Sujatha and Prabakaran (2006) utilized flow cytometry to evaluate interspecific hybrids derived from diploid cultivated sunflower and two hexaploid species (*H. tuberosus* and *H. resinosus*), aiming to identify plants with the desired diploid status. Kantar et al., (2014) investigated genomic diversity in sunflower accessions by assessing average nuclear DNA content using flow cytometry. Kallamadi and Sujatha (2016) measured the nuclear DNA content of 43 sunflower species out of 52 investigated in this study via flow cytometry using DAPI as fluorochrome and confirmed that the ploidy levels of the species were consistent with previous reports. However, preferential binding to AT-rich regions can cause DAPI to introduce up to a 15% deviation in nuclear DNA content analysis. This characteristic of DAPI make it unsuitable to use in determination of absolute nuclear DNA content. In addition, that study did not use an internal standard that eliminates errors due to random instrument drift and variation in the sample preparation and staining (Dolezel and Bartos, 2005). Due to these two drawbacks, the validity of the results of this recent study is low.

The objective of the current study is to determine the nuclear DNA content of 133 wild *Helianthus* accessions from 52 different species by flow cytometry analysis using propidium iodide as flourochrome, to confirm their taxonomic identity and ploidy level before incorporating them into sunflower research and breeding programs in Turkey.

2. MATERIALS AND METHODS

In the present study, 133 wild sunflower (*Helianthus spp*.) accessions, representing 52 species from the USDA gene bank, were planted in the Wild Sunflower Garden at Trakya University, Edirne, Turkey, and were investigated from 2020

to 2022. The nuclear DNA content analysis was performed in the Plant Genetics and Breeding Laboratory of the Department of Field Crops, Tekirdag Namik Kemal University, Tekirdag, Turkey by using a Partec CyFlow Space flow cytometer. Samples of intact nuclei were isolated from fresh leaf tissues of young (5-6 weeks old) and healthy plants using Partec commercial kit (CyStain® PI Absolute P). Nuclei isolations were done according to the manufacturer's protocol (Figure 1). Common vetch (*Vicia sativa*, cv. kara elci) has been used as internal standard in the study because it has the most suitable genome size for all sunflower species investigated. Briefly, the procedure is as follows:

1. Approximately 20 mg of fresh leaf tissues was collected from both young and healthy sunflower plants and standard and placed in a petri dish.

2. 500 µl of isolation buffer was added into the Petri dish.

3. both sample and standard leaf tissues were chopped simultaneously with a sharp razor blade into small pieces (30-60 seconds).

4. The cut leaf sample was further shaken in the petri dish for 15-20 seconds.

5. The leaf suspension was transferred to a glass tube, through a 30-33 µm mesh filter, to separate the nuclei from the residual plant tissue material.

6. 2 ml of flow cytometry staining solution was added to the isolated nuclei sample.

7. Samples were incubated in a dark environment at 37°C for approximately 1 hour.

8. After the incubation period is completed, the samples were loaded into the flow cytometry device and subjected to analysis. Minimum 3000 nuclei analyzed for each sample.

The absolute nuclear DNA content of a sample was calculated in picograms (1pg = 10-12 g) using the values of the fluorescence intensities of the G1 peaks of the sample and the standard, using the formula given below.

Nuclear DNA content (pg) = (Fluorescence intensity of G1 peak of sample) / (Fluorescence intensity of G1 peak of the standard) × DNA content of the standard.

The leaves tissues of three single plants for each accession were analyzed separately and their average was used as mean of the accession. The confidence intervals which are a simple statistical procedure was used to analyze nuclear DNA content results. Nuclear DNA content of each accession was correlated with their ploidy by counting mitotic chromosomes of at least one plant from each group created based on their nuclear DNA content. It was assumed that the remaining plants within the same group, characterized by similar DNA content, possess the same chromosome number (ploidy level).

The average nuclear DNA content of all accessions was compared among themselves (1-3 samples for each accession) using confidence intervals, which is a simple statistical method. The confidence interval (0,95) for each mean was calculated using the equation below.

P (×1-t 0.05 S x< µ< x1+ t 0.05Sx) = 0.95

In the formula, t0.05 is the “t” statistic and $S_{\bar{X}} = \sqrt{\frac{S^2}{n}} = \frac{S}{\sqrt{n}}$

n is the number of plants analyzed in each population and s is their standard deviation. It was assumed that the means with overlapping confidence intervals were not different from each other. In this respect, the analysis is the same as the t test performed to compare means (Steel and Torrie, 1960).

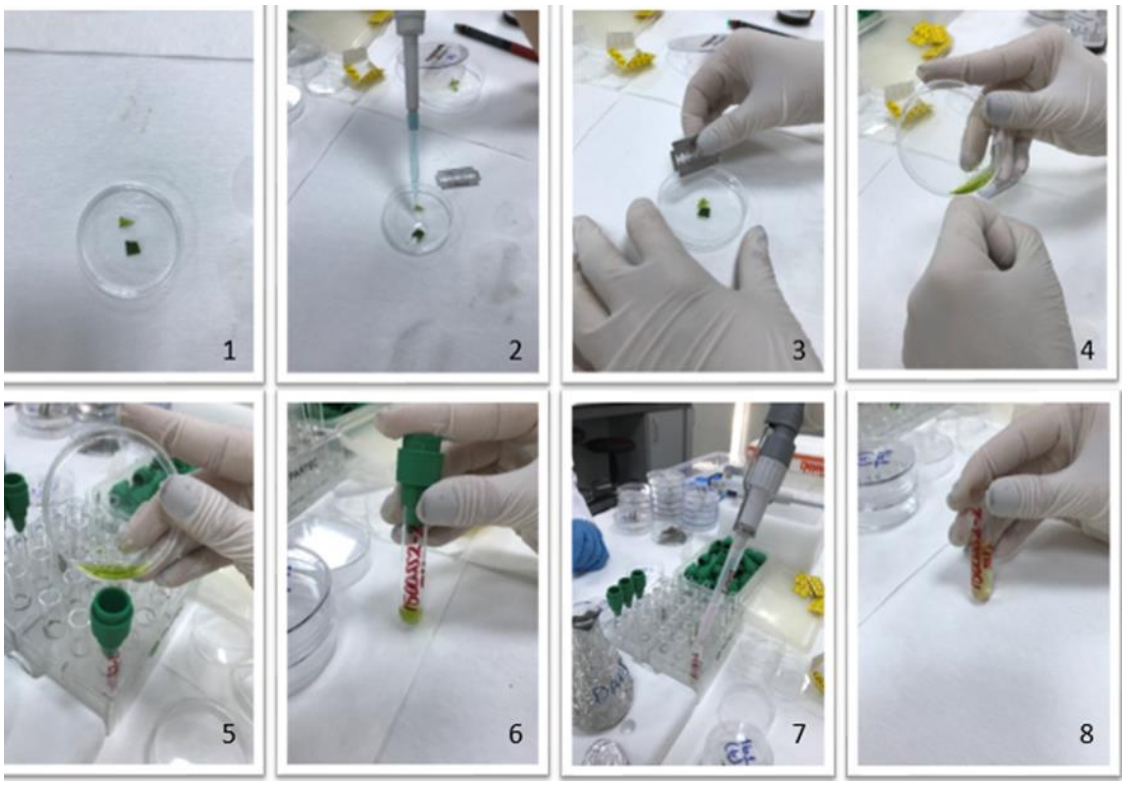

Figure 1. Isolation of nuclei from plant leaves for nuclear DNA content analysis

Cytological samples were prepared by using root tip meristem tissues. Root tips were harvested from adult plants growing in the pots, in early morning, treated with cold water for about 20 hours, fixed in farmer solution and stored at 4.0 ºC until further use. Prior to squashing, the root tips were washed a few times, for 5 minutes, in 0.01 M citric acid – sodium citrate buffer (pH 4.8) and enzymatically digested at 37°C in an enzyme mixture comprising of 1% (w/v) cellulose (Calbiochem), 1% (w/v) cellulase, Onozuka R-10; (Serva) and 20% (v/ v) pectinase (Sigma) for approximately 2 h.

The root tips were washed again in diluted citrate buffer (1X) to stop the enzyme reaction and remove the excess enzyme. Then, the root tips were carefully transferred to a container containing 45% acetic acid, and the meristem part of the root tips was taken out using special needles under a stereo microscope and transferred on a glass slide. A drop of 45% acetic acid was applied on the meristem, causing the tissue to disintegrate and further a coverslip was placed on it. The preparation was examined immediately under a phase-contrast microscope, and samples containing dividing cells were preserved for subsequent staining procedure.

Cytological preparations were stained using DAPI dye. For this purpose, the preparations containing dividing cells were kept at -80 ˚C for approximately 2 hours and the coverslip on the preparation was carefully removed with the help of a razor blade. The preparations were dried at room conditions for 24 hours, and then a small drop of DAPI dye was added to the preparation, to the area where the cells were located, and the coverslip was covered again. After this stage, the cytological preparation was stored in a cold and dark environment until chromosome counting was performed.

Chromosome counts were made using an epifluorescent microscope. Images of the cells with well distributed chromosomes were captured by an epifluorescence microscope (Olympus BX 51) with Rt Slider CCD digital camera attachment.

3. RESULTS AND DISCUSSION

Nuclear DNA content of 133 accessions from 52 different sunflower species investigated in the study was determined successfully. The nuclear DNA content of the following 25 wild sunflower species were determined for the first time in this study: *H. glaucophyllus, H. gracilentus, H. laciniatus, H. laetiflorus, H. longifolius, H. silphioides, H. nuttalii s. nuttalii, H. nuttalii, H. nuttalii s. rydbergi, H. occidentalis s. plantagenius H. occidentalis s. occidentalis, H. pauciflorus s. subrhomboideus, H. pauciflorus s. pauciflorus, H. pumilus, H. simulans, H. smithii, H. floridanus, H. californicus, H. ciliaris, H. cusickii, H. atrorubens, H. eggerti, H. strumosus, H. praecox s. hirtus (Annual), H. deblis s. silvestris (Annual), H. praecox s. runyoni.*

The mean 2C nuclear DNA content of the sunflower accessions ranged from 4,22 pg (PI 597908, *H.debilis s. cucumerifolius*) to 27,06 pg (PI 664699, *H. smithii*) as the mean 2C nuclear DNA content of the sunflower species ranged from 5.77 pg (*H.debilis s. cucumerifolius*) to 25.70 pg (*H. resinosus*) (Table 1 and 2). Unfortunately, seeds of some accessions had very low germination and therefore it was not possible to get 3 seedlings for each accession investigated in the study. The average value of the two or the only one seedling was used for those accessions based on the number of available seedling (Table 1). Due to the same reason, the average for some species was calculated based on three accessions, while for others it was based on two. Some species were represented by only one accession in the study; therefore, the value of that single accession was considered as the average for the species (Table 2). The difference between minimum and maximum mean nuclear DNA content of the species was approximately 5-fold. The differences observed among both accessions and species were found to be statistically significant. The flow histograms of two individuals from different species presented in Fig 2.

A careful examination of Table 1 and 2 reveals that the nuclear DNA content of individual plants within the same accession and the mean nuclear DNA content of accessions of the same species are notably similar. As a result of this similarity, SD values were generally low indicating the sensitivity of the nuclear DNA content analysis carried out in the study. However, it has been observed that some SD values were higher than the acceptable upper limit of 0.5 (Tuna et al., 2001). When accessions or species with such high SD values are reexamined, it has been observed that at least one sample of these accessions or species has a different nuclear DNA content value compared to the others. The reason for this difference may be mechanically mixed seeds during harvest, seed processing, packaging or misidentification of the species during the expedition trips. Some of those individuals with different nuclear DNA content value may even be interspecific hybrids (Kaya and Vasilevska-Ivanova, 2021). Those accessions or species have been accepted as mixed. In other words, they include interspecific hybrids or individuals from different species (Tuna et al., 2001).

The nuclear DNA content values of the species analyzed in the study were generally in agreement with the values reported in the literature (Sims and Price, 1985; Michaelson et al., 1991; Kantar et al., 2014; Kallamadi and Mulpuri, 2016; Sahin et al., 2022). The small differences between the results of the current and previous studies could be due to accessions, methods, fluorescent dye and internal standards used.

Table 1. The mean 2C nuclear DNA content (pg) of wild sunflower accessions

Helianthus species	Mean	SD	$T*S_x$	Confidence interval	
				Lower	upper
H. smithii-664699	27,06	0,023	0,019	27,044	27,082
H. resinosus-664678	25,70	1,081	0,884	24,816	26,584
H. tuberosus-664619	24,78	2,030	1,660	23,117	26,436
H. laevigatus-503226	24,52	0,023	0,019	24,504	24,542
H. agrestis-468415	23,83	0,404	0,330	23,503	24,164
H. smithii-664698	23,62	0,012	0,009	23,607	23,626
H. agrestis-673205	23,43	0,153	0,125	23,308	23,558
H. decapetalus-468697	23,20	0,755	0,617	22,579	23,814
H. eggertii-649978	22,73	0,032	0,026	22,707	22,760
H. laevigatus-503229	22,65	0,571	0,467	22,179	23,114
H. atrorubens-503206	21,41	0,193	0,158	21,252	21,568
H. pauciflorus s. pauciflorus-494613	21,37	0,035	0,028	21,342	21,398
H. laetiflorus-503236	20,98	0,012	0,009	20,974	20,993
H. californicus- 649941	20,48	0,189	0,155	20,329	20,638
H. tuberosus-650105	20,33	1,181	0,965	19,361	21,292
H. hirsutus-468735	18,71	0,609	0,498	18,212	19,208
H. hirsutus-435703	17,87	0,006	0,005	17,862	17,871
H. decapetalus-503245	17,38	0,040	0,033	17,350	17,416
H. ciliaris-653552	16,23	0,074	0,060	16,166	16,287
H. strumosus-547223	15,12	0,000	0,000	15,120	15,120
H. floridanus-664733	13,23	0,006	0,005	13,222	13,231
H. floridanus-468715	12,89	0,085	0,070	12,820	12,960
H. simulans-664806	12,40	0,035	0,029	12,368	12,425
H. anomalus-649861	11,60	0,361	0,295	11,305	11,895
H. radula-468876	11,36	0,295	0,241	11,119	11,601
H. occidentalis s. plantagenius -664789	11,29	0,318	0,260	11,027	11,546
H. pumilus-650048	11,01	0,069	0,057	10,953	11,067
H. deserticola-468702	10,90	0,458	0,375	10,525	11,275

Table 1. (continued)

H. silphioides-673159	10,79	0,020	0,016	10,774	10,806
H. deserticola-649883	10,67	0,231	0,189	10,478	10,856
H. anomalus-468638	10,40	0,173	0,142	10,258	10,542
H. exilis-649891	10,03	0,379	0,310	9,724	10,343
H. longifolius-650000	9,990	0,000	0,000	9,990	9,990
H. paradoxus-673239	9,927	0,046	0,038	9,889	9,964
H. paradoxus-673253	9,797	0,244	0,199	9,597	9,996
H. longifolius-664680	9,727	0,116	0,095	9,632	9,821
H. exilis-664633	9,533	0,252	0,206	9,328	9,739
H. exilis-649901	9,533	0,306	0,250	9,284	9,783
H. bolanderi-673280	9,500	0,361	0,295	9,205	9,795
H. bolanderi-673294	9,433	0,208	0,170	9,263	9,604
H. mollis-478309	9,287	0,100	0,082	9,205	9,369
H. maximilliani-613794	9,273	0,692	0,566	8,707	9,840
H. bolanderi-435641	9,200	0,346	0,283	8,917	9,483
H. occidentalis s. occidentalis-435788	9,037	0,055	0,045	8,992	9,082
H. glaucophyllus-468721	8,970	0,459	0,376	8,594	9,346
H. giganteus-468720	8,930	0,087	0,071	8,859	9,001
H. argophyllus-468651	8,833	0,404	0,330	8,503	9,164
H. mollis-441066	8,763	0,422	0,345	8,419	9,108
H. niveus-650020	8,700	0,361	0,295	8,405	8,995
H. porteri-649917	8,667	0,208	0,170	8,496	8,837
H. cusickii- 649962	8,590	0,036	0,029	8,561	8,619
H. cusickii- 649966	8,507	0,067	0,054	8,452	8,561
H. anomalus-664638	8,500	0,100	0,082	8,418	8,582
H. argophyllus-664803	8,500	0,608	0,497	8,003	8,997
H. occidentalis s. plantagenius -494592	8,493	0,248	0,203	8,290	8,696
H. nuttalii-592341	8,460	0,036	0,029	8,431	8,489
H. grossesseratus-586889	8,460	0,300	0,246	8,214	8,706
H. niveus-613758	8,433	0,379	0,310	8,124	8,743
H. occidentalis-664648	8,433	0,006	0,005	8,429	8,438
H. gracilentus-673286	8,427	0,012	0,009	8,417	8,436
H. grossesseratus-468727	8,427	0,214	0,175	8,252	8,602
H. grossesseratus-649994	8,407	0,031	0,025	8,382	8,432
H. nuttallii s. rydbergii-597918	8,390	0,104	0,085	8,305	8,475
H. maximilliani-613757	8,347	0,326	0,266	8,080	8,613
H. salicifolius-664759	8,340	0,216	0,177	8,163	8,517
H. maximilliani-468746	8,323	0,006	0,005	8,319	8,328
H. nuttalii s. nuttallii-586905	8,317	0,268	0,219	8,098	8,536
H. pauciflorus s. subrhomboideus-435869	8,310	0,269	0,220	8,090	8,530
H. nuttalii-420182	8,237	0,029	0,024	8,213	8,260
H. argophyllus-490291	8,233	0,208	0,170	8,063	8,404
H. niveus-435774	8,233	0,153	0,125	8,108	8,358
H. giganteus-649984	8,233	0,681	0,557	7,676	8,790
H. argophyllus-649865	8,200	0,100	0,082	8,118	8,282
H. cusickii- 649958	8,167	0,115	0,094	8,072	8,261
H. nuttalii s. nuttallii-531044	8,167	0,058	0,047	8,119	8,214
H. divaricatus-435675	8,163	0,144	0,117	8,046	8,281
H. salicifolius-664783	7,983	0,045	0,037	7,946	8,020
H. porteri-673214	7,900	0,400	0,327	7,573	8,227
H. porteri-649911	7,833	0,321	0,263	7,570	8,096
H. grossesseratus-468725	7,747	0,159	0,130	7,616	7,877
H. argophyllus-649863	7,733	0,153	0,125	7,608	7,858
H. laciniatus-653545	7,627	0,006	0,005	7,622	7,631
H. annuus- 597890	7,533	0,074	0,060	7,473	7,594
H. debilis-653611	7,433	0,321	0,263	7,170	7,696
H. maximilliani-586892	7,430	1,360	1,112	6,318	8,542
H. praecox s. hirtus – 435855	7,410	0,000	0,000	7,410	7,410
H. winteri-673290	7,333	0,058	0,047	7,286	7,381

Table 1. (continued)

H. debilis s. Silvestris-613754	7,307	0,025	0,021	7,286	7,327
H. praecox-435855	7,300	0,458	0,375	6,925	7,675
H. debilis-613753	7,300	0,000	0,000	7,300	7,300
H. debilis-597908	7,267	0,321	0,263	7,004	7,530
H. debilis-649870	7,233	0,058	0,047	7,186	7,281
H. debilis-613754	7,233	0,058	0,047	7,186	7,281
H. praecox-435853	7,100	0,436	0,356	6,744	7,456
H. petiolaris-586911	7,100	0,100	0,082	7,018	7,182
H. bolanderi-673141	7,067	0,252	0,206	6,861	7,272
H. debilis-435654	7,067	0,252	0,206	6,861	7,272
H. debilis-468672	7,067	0,208	0,170	6,896	7,237
H. petiolaris-586922	6,967	0,503	0,412	6,555	7,378
H. petiolaris-597922	6,967	0,208	0,170	6,796	7,137
H. gracilentus-664644	6,937	0,025	0,021	6,916	6,957
H. praecox-468846	6,900	0,100	0,082	6,818	6,982
H. petiolaris-597924	6,800	0,265	0,216	6,584	7,016
H. petiolaris-592355	6,800	0,100	0,082	6,718	6,882
H. petiolaris-586919	6,767	0,231	0,189	6,578	6,956
H. petiolaris-503232	6,767	0,115	0,094	6,672	6,861
H. praecox-435847	6,733	0,058	0,047	6,686	6,781
H. petiolaris-649910	6,700	0,100	0,082	6,618	6,782
H. petiolaris-468818	6,667	0,208	0,170	6,496	6,837
H. petiolaris-586928	6,633	0,416	0,340	6,293	6,974
H. debilis s. cucumerifolius-435654	6,627	0,029	0,024	6,603	6,650
H. petiolaris-597923	6,600	0,200	0,164	6,436	6,764
H. petiolaris-613769	6,600	0,300	0,245	6,355	6,845
H. petiolaris-592359	6,567	0,153	0,125	6,442	6,692
H. petiolaris-547210	6,533	0,029	0,024	6,510	6,557
H. petiolaris-613765	6,500	0,000	0,000	6,500	6,500
H. petiolaris-586931	6,500	0,173	0,142	6,358	6,642
H. neglectus-435769	6,467	0,289	0,236	6,231	6,703
H. debilis s. cucumerifolius-613753	6,460	0,329	0,269	6,191	6,729
H. petiolaris fallax-468817	6,400	0,100	0,082	6,318	6,482
H. petiolaris-468842	6,400	0,100	0,082	6,318	6,482
H. petiolaris s. petiolaris-586922	6,310	0,565	0,462	5,848	6,772
H. neglectus-468773	6,267	0,058	0,047	6,219	6,314
H. praecox-468846	6,250	0,221	0,181	6,069	6,431
H. petiolaris fallax-435817	6,233	0,115	0,094	6,139	6,328
H. petiolaris s. petiolaris-468842	6,197	0,237	0,194	6,003	6,390
H. praecox s. praecox- 435847	6,160	0,000	0,000	6,160	6,160
H. petiolaris s. petiolaris-503232	6,153	0,551	0,450	5,703	6,604
H. praecox s. runyonii-494606	6,123	0,080	0,066	6,058	6,189
H. neglectus-435763	6,000	0,200	0,164	5,836	6,164
H. neglectus-673249	5,933	0,153	0,125	5,808	6,058
H. praecox s. runyonii-435853	5,497	1,097	0,897	4,600	6,394
H. debilis s. cucumerifolius-597908	4,223	0,193	0,158	4,065	4,382

Table 2. The mean 2C nuclear DNA content (pg) of wild sunflower species

Helianthus species	Acces. No 1	Acces. No 2	Acces. No 3	Mean (pg/2C)	SD	T*S_x	Confidence intervals		Ploidy
							Lower	Upper	
H. resinosus	25,70			25,70					H
H. smithii	27,06	23,62		25,34	2,43	2,80	22,54	28,14	H
H. tuberosus	24,78	20,33		22,56	3,15	3,63	18,93	26,18	T
H. agrestis	23,83	23,43		23,63	0,28	0,39	23,24	24,02	T
H. laevigatus	24,52	22,65		23,59	1,32	1,52	22,06	25,11	T
H. eggertii	**22,73**			22,73					T
H. atrorubens	**21,41**			21,41					T
H. pauciflorus s. pauciflorus	21,37			21,37					T
H. laetiflorus	**20,98**			20,98					T
H. californicus	**20,48**			20,48					T
H. decapetalus	23,20	17,38		20,29	4,12	4,74	15,55	25,03	T
H. hirsutus	18,71	17,87		18,29	0,59	0,68	17,61	18,97	T
H. ciliaris	16,23			16,23					T
H. strumosus	15,12			15,12					T
H. floridanus	13,23	12,89		13,06	0,24	0,28	12,78	13,34	D
H. simulans	12,40			12,40					D
H. radula	11,36			11,36					D
H. pumilus	11,01			11,01					D
H. silphioides	10,79			10,79					D
H. deserticola	10,90	10,67		10,79	0,16	0,19	10,60	10,97	D
H. anomalus	11,60	10,40	8,50	10,17	1,56	1,80	8,37	11,97	D
H. occidentalis s. plantagenius	11,29	8,49		9,89	1,98	2,28	7,61	12,17	D
H. paradoxus	9,93	9,80		9,86	0,09	0,11	9,76	9,97	D
H. longifolius	9,99	9,73		9,86	0,19	0,21	9,64	10,07	D
H. exilis	10,03	9,53	9,53	9,70	0,29	0,33	9,37	10,03	D
H. bolanderi	9,50	9,43	9,20	9,38	0,16	0,18	9,20	9,56	D
H. occidentalis s. occidentalis	9,04			9,04					D
H. mollis	9,29	8,76		9,03	0,37	0,43	8,60	9,45	D
H. glaucophyllus	8,97			8,97					D
H. maximilliani	9,27	8,35	8,32	8,65	0,54	0,62	8,02	9,27	D
H. giganteus	8,93	8,23		8,58	0,49	0,57	8,01	9,15	D
H. niveus	8,70	8,43	8,23	8,46	0,23	0,27	8,19	8,73	D
H. grossesseratus	**8,46**	**8,43**	**8,43**	8,44	0,02	0,02	8,42	8,46	D
H. occidentalis	8,43			8,43					D
H. cusickii	8,59	8,51	8,17	8,42	0,22	0,26	8,16	8,68	D
H. nuttallii s. rydbergii	8,39			8,39					D
H. nuttalii	8,46	8,24		8,35	0,16	0,18	8,17	8,53	D
H. pauciflorus s. subrhomboideus	8,31			8,31					D
H. argophyllus	8,83	8,23	7,73	8,27	0,55	0,63	7,63	8,90	D
H. nuttalii s. Nuttallii	8,32	8,17		8,24	0,11	0,12	8,12	8,36	D
H. salicifolius	8,34	7,98		8,16	0,25	0,29	7,87	8,45	D
H. divaricatus	8,16			8,16					D
H. porteri	8,67	7,90	7,83	8,13	0,46	0,53	7,60	8,67	D
H. gracilentus	8,43	6,94		7,68	1,05	1,21	6,47	8,90	D
H. laciniatus	7,63			7,63					D
H. annuus	7,53			7,53					D
H. winteri	7,33			7,33					D
H. praecox s. hirtus	7,41			7,41					D
H. debilis s. silvestris	7,31			7,31					D
H. debilis	7,43	7,23	7,07	7,24	0,18	0,21	7,03	7,46	D
H. praecox	7,30	6,90	6,25	6,82	0,53	0,61	6,21	7,43	D
H. petiolaris	7,10	6,70	6,20	6,67	0,45	0,52	6,14	7,19	D
H. praecox	7,41	6,73	5,50	6,55	0,97	1,12	5,43	7,66	D
H. petiolaris fallax	6,40	6,23		6,32	0,12	0,14	6,18	6,45	D
H. petiolaris s. petiolaris	6,31	6,20	6,15	6,22	0,08	0,09	6,13	6,31	D
H. neglectus	6,47	6,27	5,93	6,22	0,27	0,31	5,91	6,53	D
H. praecox s. praecox	6,16			6,16					D
H. praecox s runyonii	6,12	5,50		5,81	0,44	0,51	5,30	6,32	D
H. debilis s. cucumerifolius	6,63	6,46	4,22	5,77	1,34	1,55	4,22	7,32	D

H= hexaploid, T=Tetraploid, D=Diploid

At the onset of the study, obtaining root tips from plants, suitable to prepare cytological preparations (with plenty of dividing cells) was challenging due to high number of different sunflower species and their requirements. The problem was partly solved by transferring the potted plants to a climate chamber with lower temperatures. Eventually, chromosome counting was successfully conducted on a limited number of accessions from different species with varying nuclear DNA content except hexaploids (Fig 3). Based on those cytological investigations, all accessions with mean 2C DNA content ranging from 4.22 to 13.23 pg had 2n = 2x = 34 chromosomes indicating that they were diploid (Figure 3). The chromosome number of *H. agrestis* accession (PI 468415) with a nuclear DNA content of 21 pg/2C was determined as 2n = 4x = 68 indicating that it was tetraploid (Figure 3). Although it was not possible to count chromosomes for each species, based on the data obtained in this study it can be speculated that species with DNA content from 15.12 pg to 23.63 pg are tetraploid with 2n=68 chromosome. It is possible to say this safely because accessions and species used in the study can be separated into 3 main groups based on their nuclear DNA content, and there is a clear gap (min. 2 pg) between groups as the data within each group is continuous type. In this case, the accessions and species with higher nuclear DNA content than 23.63 pg are hexaploids. However, this should still be confirmed by counting chromosomes of at least one individual plants for each species. Unfortunately, this was not possible in the current study since root tips of the plants for many species had very low dividing cells.

The results of chromosome count for the species used in the current study are generally consistent with previously reported results (Kallamadi and Mulpuri, 2016).

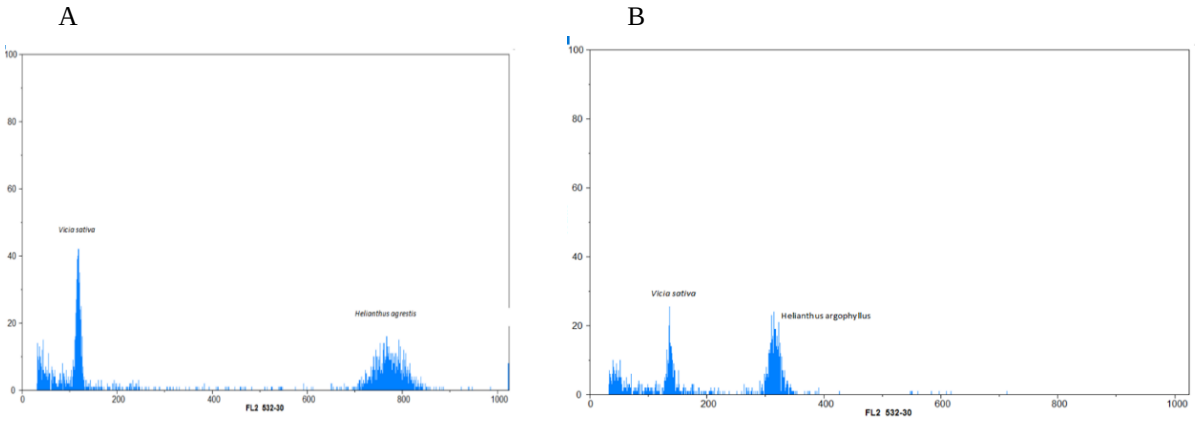

Figure 2. Histograms representing G1 peak position of samples and standards (A. PI 468415, *H. agrestis;* B. PI 468651, *H. argophyllus*)

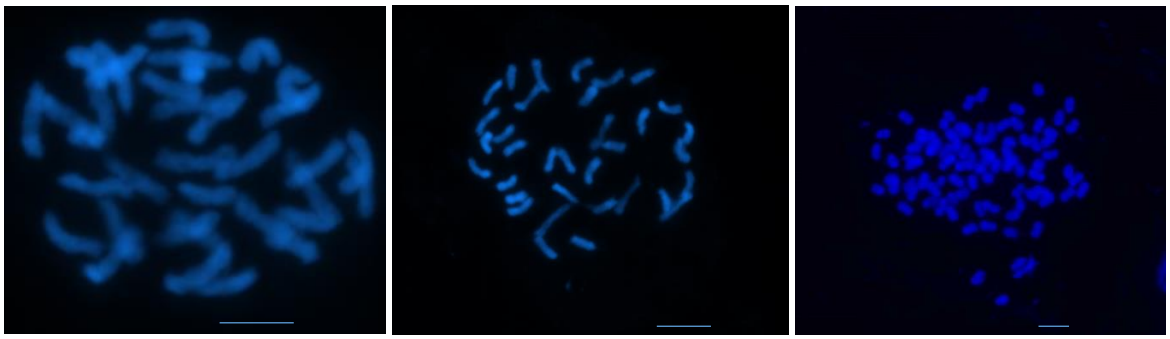

Figure 3. Mitotic chromosomes of PI 650001 (*H. longifolius*, 2n=2x=34), PI 673147 (*H. mollis*, 2n=2x=34) and PI 468415 (*H. agrestis*, 2n=4x=68), Bar is 10 µm.

4. CONCLUSION

In this study, nuclear DNA content and ploidy level of 52 wild sunflower species were determined using flow cytometry, the most accurate and reliable method. The performed analysis has shown that a significant variation (5-fold) in nuclear DNA content exists within the *Helianthus* genus. The study revealed that approximately 80% (41 species) of the 52 wild sunflower species (comprising 15 annuals and 26 perennials) were diploid (2x). The ploidy levels of the remaining species varied, with two species being hexaploid (6x) and twelve tetraploids (4x).

ACKNOWLEDGEMENT

The study results were funded by Trakya University with TUBAP 2020/62 project and by TUBITAK with 119O218 project. We would like to thank to Trakya University and TUBITAK for financial supports.

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