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Determination of Genome Size Differentiation and Ploidy Levels in Some Citrus Rootstock Populations

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ABSTRACT
Determining the ploidy level of plant material used in breeding is important, especially for biotechnological applications. The genetic diversity in citrus enables the development of rootstocks and cultivars adapted to various climates and soils. Various suitable rootstocks are used in commercial citrus production.This study was conducted to determine the genome size and ploidy levels of citrus rootstocks widely used worldwide using flow cytometry. The rootstocks used in the study included Gou-Tou, C-35, Troyer citrus, Taiwanica, Citremon, Yuzu, Sunki mandarin, Flying Dragon, Yuma citrus, Macrophylla and Chinese orange. Fresh leaf tissue from each rootstock was mixed with triploid Tahitian lemon leaf tissue, used as a standard species, and cell nuclei were isolated. The cells stained with propidium iodide were analysed by flow cytometry, and histograms and cytograms were obtained. According to the results, although all species had diploid genome sizes, differences were observed between species in terms of genome volume. Yuzu seedlings were found to have the largest genome size (0.808 pg/2C), while Flying Dragon trifoliate had the smallest genome size (0.700 pg/2C).

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Bazı Turunçgil Anaç Popülasyonlarında Genom Büyüklüğü Farklılıklarının ve Ploidi Seviyelerinin Belirlenmesi

ÖZET
Bitki ıslahında kullanılan materyalin ploidi seviyesinin belirlenmesi, özellikle biyoteknoloji uygulamaları açısından önemlidir. Turunçgillerdeki genetik çeşitlilik, farklı iklim ve topraklara uyumlu anaç ve çeşitlerin geliştirilmesini sağlamaktadır. Ticari üretimde çeşitli uygun anaçlar kullanılmaktadır. Bu çalışma, dünya genelinde yaygın olarak kullanılan turunçgil anaçlarının flow sitometri ile genom büyüklüklerini ve ploidi seviyelerinin belirlenmesi amacıyla yürütülmüştür. Çalışmada Gou-Tou, C-35, Troyer Citrange, Taiwanica, Citremon, Yuzu, Sunki mandarin, Flying Dragon, Yuma Citrange, Macrophylla ve Çin portakalı anaçları kullanılmıştır. İlgili anaçlardan elde edilen taze yaprak dokuları standart tür olarak kullanılan triploid Tahiti limon yaprak dokusu ile karıştırılmış ve hücre çekirdekleri izole edilmiştir. Propidium iodid ile boyanmış hücreler flow sitometri ile okunmuş ve histogram ve sitogramlar elde edilmiştir. Elde edilen sonuçlara göre, tüm türlerin diploid genom büyüklüğüne sahip olmalarına karşın genom hacmi açısından türler arasında farklılıkların olduğu belirlenmiştir. Yuzu fidanlarının en büyük (0.808 pg/2C), Flying Dragon trifoliatın ise en küçük genom hacmine sahip olduğu (0.700 pg/2C) tespit edilmiştir.

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INTRODUCTION

Citrus, originating in southeast Asia, are commonly cultivated in Türkiye as in the rest of the world. According to the FAO Statistical Database (FAO, 2022) 4.348.742 tons of citrus fruits from different species were produced in Türkiye in 2022. Citrus fruits have a very significant share of global fruit production. Citrus species have been cultivated in subtropical and tropical regions for years. Rootstocks play a vital role in citrus cultivation by mitigating challenges such as adverse climates, subpar soil conditions, and various biotic and abiotic stresses, thus ensuring optimal production (Narukulla at al., 2023). Rootstock selection is very important for citrus cultivation. Rootstock has very large effects on tolerance to cold and diseases, adaptation to different climate and soil conditions, fruit yield and quality. As a result, the use of rootstocks for citrus species has become mandatory. An ideal citrus rootstock has high ability to adapt to different soil conditions, is compatible with citrus species and varieties, resistant to biotic and abiotic stress conditions and has high polyembryony rates. Gou-Tou, C-35, Troyer Citrange, Taiwanica, Citremon, Yuzu, Sunki mandarin, Flying Dragon, Yuma Citrange, Macrophylla and Chinese citrus rootstocks are among the rootstocks commonly used in citrus cultivation (Davies & Albrigo, 1994). As a significant proportion of citrus rootstocks have a tendency toward nucellar embryony, seeds display the same characteristics as main plants and they display performance like clonal proliferation.

Variations in genome size constitute a significant aspect in evolutionary studies and the characterization of species. In addition genome sizes are very important for identification of significant citrus genotypes (Šimoníková et al., 2022). With flow cytometry analyses, genome volumes and different ploidy structures may be determined in citrus species and varieties. For calculation of the nuclear DNA content of a species, it is necessary to determine ploidy level, chromosome counts and how big chromosomes are. Before beginning breeding studies, differences in ploidy level among individuals to be used as genotype and identification of these differences is very important in terms of breeding studies being successful. Flow cytometry is currently the most sensitive, rapid and reliable method used for determination of nuclear DNA content, and use with this aim has become more common in recent years (Tuna, 2014). Determination of ploidy level in plants with traditional methods includes counting chromosomes during mitosis division in preparations made from root tip tissues with light microscopy; however, this method is very demanding, slow and performing ploidy analysis of many plant species takes time (Nix et al., 2024). Additionally, it is not useful for determination of ploidy levels in plant species with small chromosomes and high ploidy levels, and may cause misclassification of species. As the plant sample for ploidy level determination increases, the light microscopy method may not be sufficient. As studies may not be sufficient for determination of ploidy level using root tip tissues, the flow cytometry method has become a method chosen for ploidy analyses in recent years due to being convenient, rapid, sensitive and reliable (Johnson et al., 1998; Brummer et al., 1999; Tuna, 2014). As chromosomes are found in the nuclei of cells in plants, there is a close correlation between the nuclear DNA amount and ploidy level. As a result, due to the convenience, speed and reliability of the flow cytometry method, in recent times ploidy analysis has been performed by determining the nuclear DNA content in plant cells with the flow cytometry method. Flow cytometry analysis has many advantages compared to the chromosome count method in traditional methods such as preparation of samples being easy, it is rapid and there is no need for a root tip cell in mitosis to perform analyses, just a small leaf tissue is sufficient (Nakandala et al., 2023). Flow cytometry was first developed as a method to identify the nuclear DNA content of organisms and it is a very important and easily applied method in these studies (Salameh, 2014). The flow cytometry method is a method based on analysis of light after being absorbed by cells as they pass singly through fluorescence detectors generally (Soni et al., 2024). However, in order to ensure light absorption by cells, the cell nuclei from enzymatically degraded leaf samples are freed and cell nuclei stained with fluorescent stains like DAPI or PI are used to determine nuclear DNA amounts. With the flow cytometry method, one person can analyze over 100 plants per day, it performs chromosome counts very rapidly, provides accurate and reliable results, and increases significance and this situation has led to flow cytometry currently being the most chosen method for chromosome counts. However, the disadvantage of the method is that it must be set to the plant species to be analyzed (Ellialtioglu et al., 2000). Applications where flow cytometry is commonly used in plants include determination of ploidy level, nuclear DNA content and estimation of genome volume (Palomino et al., 2003). Determining the genome size in citrus species through flow cytometry offers several advantages:

a) Utilization in plant breeding: The knowledge of citrus genome size can be employed in plant breeding. Genome size aids in understanding inter-species relationships and degrees of relatedness. Additionally, it is crucial for comprehending plant traits such as adaptability, stress tolerance, and productivity.

b) Diversity and hylogenetic analyses: Variations in genome size among different citrus species and cultivars aid in understanding species relationships and phylogenetic structures.

c) Plant biotechnology applications: Genome size information is crucial in transgenic studies and plant biotechnology applications. It can be used to evaluate the effectiveness and efficiency of genetic manipulations in target plants.

d) Conservation and conservation biology: Genome size information is essential for the conservation and preservation of rare or threatened citrus species. It can assist in managing and conserving populations of endangered species by informing conservation strategies.

e) Agricultural productivity and quality improvement: Genome size helps in identifying genetic traits that may impact agricultural productivity and quality. This information can contribute to the development of agricultural practices and processes aimed at improving productivity and quality.

In conclusion, determining genome size in citrus species through flow cytometry provides valuable information and opportunities for various fields including plant breeding, molecular studies, conservation biology and horticulture.

In this study, the nuclear DNA amounts in seedling populations of citrus genotypes were examined with the flow cytometry method and ploidy levels and genome volumes were determined. Thus, the ploidy levels among genotypes were investigated for presence of significance from a statistical viewpoint.

MATERIAL and METHOD

Plant materials

In this study citrus rootstocks's (Gou-Tou, C-35, Troyer Citrange, Taiwanica, Citremon, Yuzu, Sunki mandarin, Flying Dragon, Yuma Citrange, Macrophylla and Chinese orange rootstocks) seeds and seedlings were used during the experiment. The characteristics of citrus rootstocks used as materials in the scope of the study are specified below. Tahitian lime (*Citrus latifolia* Tan.) was used as a control in the study.

Gou Tou Sour Orange (*Citrus aurantium* L. var. Gou Tou), A rootstock of Chinese origin, apart from being resistant to tristeza disease, there is no information about soil wants or resistance to other diseases. In Florida plants grafted onto Gou Tou rootstock have larger crown structure compared to those grafted on other bitter oranges (Saunt, 2000). Gou Tou sour orange is reported to reduce yield in grapefruits (Louzada et al., 2008). Gou Tou sour orange is tolerant of *Phytophthora citrophthora* and *Phytophthora* parasitical diseases (Matheron et al., 1988).

C-35 citrange (*Poncirus trifoliata* (L.) Raf. X *Citrus sinensis*. Osb. 'Ruby'), A rootstock obtained by hybridization of Ruby Blood orange and trifoliate orange. It is tolerant of gummosis (*Pytophthora citrophthora* (Smith & Smith) Leon.) and Tristeza diseases and resistant to nematodes. Resistance to cold is equivalent or slightly better than Carrizo citrange. Trees have moderate size and those grafted on Troyer have 25 % smaller crown. Good compatibility with sandy, sandy-clayey and clayey soils; however, is more susceptible to limey soils than Carrizo citrange (Saunt, 2000).

Troyer citrange (*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* (L.) Osb.), A sweet orange and trifoliate orange hybrid. Generally, it has trifoliate properties, with more compliant characteristics in terms of environmental conditions and compatibility with varieties so it is used more often in recent years and in most cases is chosen as alternative to sour orange rootstock. Proliferation with seed and grafting is easy, growth is moderate, yield is high, maturation and fruit setting are early, effects on fruit quality are high and economic lifespan is at moderate levels (Ozcan & Ulubelde, 1984).

Macrophylla (Alemow) (*Citrus macrophylla* Wester), Important features are resistance to salinity and boron. Generally, it has good compatibility with all varieties. However, it is used as rootstock for lemon and lime mainly due to susceptibility to Tristeza and *Xyloporosis* diseases. It appears to be tolerant of Exocortis and Psorosis diseases. Varieties grafted on it grow rapidly and set early fruit. However, quality of fruit is negatively affected. It is susceptible to cold.

Flying Dragon (*Poncirus trifoliata* var. Monstrosa), The trifoliate clone Flying Dragon was found in America in 1915. This rootstock ensures tight crown formation for lime, grapefruit and tangelo, and has the effect of dwarfing mandarin and orange. It is very sensitive to calcium and chlorosis. It develops excessively slowly on mild sandy soils. It is resistant to tristeza (CTV) virus and *Phytophthora* spp. (root neck rot). It is sensitive to Exocortis. The Eureka group showed incompatibility with lemons. Due to showing dwarfing effect on all citrus types and varieties, it is appropriate for dense planting, ensuring convenient harvesting of grafted varieties. It has positive effects on fruit quality, like the trifoliate rootstock. The body having zigzag form and many thorns makes grafting difficult (Aubert & Vullin, 1998). The Flying Dragon rootstock which is resistant to cold and humid conditions and sensitive to high-lime soils, does not have a tendency to form nucellar plants at high rates (Ashkenazi et al., 1992; Ferguson & Chaparro, 2004).

Sunki mandarin (*Citrus sunki* (Hayata) hort ex. Tanaka), It is very widely used as rootstock in China. It is tolerant of Tristeza and Xyloporosis, but sensitive to Exocortis. Studies have reported Sunki is susceptible to *Phytophthora*

brown rot. This rootstock is tolerant of salt, has moderate resistance to low temperatures, and can withstand chlorosis in limey soils. It is a polyembryonic rootstock Louzada et al. (2008) reported adaptation to limey soils was good, and that it was tolerant to iron chlorosis. Fruit yield, fruit juice amounts and sugar content in fruit juice was equivalent or superior to fruit obtained from trees grafted on bitter orange (Saunt, 2000).

Yuzu (*Citrus junos* Sieb. ex Ten.), It is a common rootstock in the southern regions of China. From China, production spread to Japan and it forms an important commercial rootstock in Japan. Proliferation from seeds is easy, with slow growing features. It is a rootstock with high fruit quality and yield. It is resistant to *Phytophthora*, fungus and nematodes. It is tolerant of tristeza, dwarfing and spalling diseases. It has moderate levels of resistance to limey and salty soils. It has high resistance to low temperatures and polyembryony tendency.

Taiwanica (*Citrus taiwanica* Tan. and Shim.), It is a rootstock that is easily proliferated from seeds, and has moderate levels of tree growth, fruit quality and yield. It is resistant to *Phytophthora* disease, very susceptible to fungal disease and susceptible to nematode damage. It is tolerant of tristeza, dwarfing and spalling diseases. It has moderate levels of resistance to limey soils, with weak resistance of saline soil conditions. It has moderate resistance to low temperatures and is a rootstock with very high tendency for polyembryony.

Yuma Citrange (*P. trifoliata* × *C. sinensis*), A trifoliate orange hybrid. It matures in the months of October-November. It is a rootstock susceptible to iron deficiency. It has smaller fruits than Citrumelo. It forms trees of moderate size, with trifoliate leaves, and crown volume in global structure. It has low tendency toward polyembryony, and forms zygotic plants at high rates (Jaskani et al., 2006). It is a very suitable rootstock for grapefruit. In terms of features like susceptibility to disease and nematodes, fruit quality and adaptation to soil types, Yuma citrange is similar to Carrizo and Troyer citranges.

Chinese bitter orange (*C. myrtifolia* Rafinesque), Chinese bitter orange, susceptible to CTV, matures from January-March. Leaves are small and don't have pointed tips. Fruit are small and rounded, with variability in seed numbers from low to high, and it forms small tress. The peel of the fruit has moderate roughness, with color ranging from orange to dark orange. It originated in China.

Citremon (*Poncirus trifoliata* (L.) Raf. X *Citrus lemon* (L.) Burm), Most of these hybrids show abnormal small leaf features. They die in the germination stage or a short while after. Large leaved plants survive, fruit has many seeds and rough structure, trees show rapid development like lemon.

Sampling Method:

Two hundred seeds from the following citrus rootstocks were sown and germination rates were precisely determined during four weeks. Seedling populations comprising 100 plants from each rootstock were obtained.

Laboratory Analysis:

Seed Selection:

Seeds that exhibited no abortive properties, were plump, and did not display any fungal pathogen symptoms were selected within the scope of the study. The seeds demonstrated a high germination rate in the pre-germination test.

Planting the Selected Seeds and Determination of Germination Rates:

Seeds were provided from open pollinated mature fruits of Gou-Tou sour orange, C-35 citrange, Troyer citrange, Taiwanica, Citremon, Yuzu, Sunki mandarin, Flying Dragon trifoliate orange, Yuma Citrange, Macrophylla and Chinese orange rootstocks trees in Citrus orchards in Adana – Türkiye. The seeds were then dried in a shaded area and treated with CAPTAN'H® fungicide. 200 seeds from each genotype were sown in growing media containing of vermiculite No:3 in greenhouse. Seedlings were counted 15 days later after seed germination then seedlings with three developed leaves were transferred into the plastic pots containing peat moss. Subsequently, the plants were subjected to a series of periodic maintenance operations in order to ensure optimal growth and development. The seedlings grown well without any blemishes were used for cytometry analysis.

Isolation and Staining of Nuclei:

To release cell nuclei, approximately 50 mg of fresh tissue form each seedling leaf was mixed with Tahiti lime (*Citrus latifolia* Tan.) leaf pieces, which were used as a control, and chopped into small pieces with a sharp razor in a sterile Petri dish containing 300 µl of nuclei buffer (pH 7.4) of the following composition: 0.14 M NaCl, 0.003 M KCl, 0.012 M NaH_2PO_4, 0.002 M KH_2PO_4, 0.1 % Triton 100, 50 µg of RNase and 100 µl of dithiothreitol. For measurements of absolute DNA values, Tahiti lime leaf tissues were included as an internal standard, as previously described by (Ollitrault & Michaux-Ferriere, 1994; Ollitrault et al., 1994). Tahiti lime was described as

triploid and nuclear DNA content was found to be 1.17 pg / 2C by (Ollitrault & Michaux-Ferriere, 1994; Ollitrault et al., 1994). The suspension was filtered through a 50 µm pore nylon filter into microcentrifuge tubes. After filtration, 100 µl of propidium iodide was added for staining of the DNA. Then the suspensions were incubated for approximately 5 min at room temperature. After incubation, each sample was run on a flow cytometer (Seker et al., 2003). For estimation of DNA content of nuclei, the relative fluorescence of nuclei was measured by using a CA-III Flow Cytometer (Partec GmbH, Münster, Germany) with an Argon laser light source operating at a wavelength of 488 nm. Histograms and cytograms were evaluated on DPAC Software (Partec GmbH, Münster, Germany). From 2000 to 5000 nuclei were counted per flow cytometry measurement. The nuclear DNA contents of different seedlings were calculated by comparison of relative positions for G_{0-1} peaks corresponding to the sample nuclei and the nuclei isolated from Tahiti lime or mungbean, respectively. This permits accurate determination of the unknown DNA content (Seker et al., 2003).

Data analysis and estimation of nuclear genome size: The nuclear DNA contents of the different rootstock seedlings were calculated by comparison of the relative positions for the G_{0-1} peaks corresponding to the sample nuclei and the nuclei isolated from Tahiti lime, respectively. This permits accurate determination of the unknown DNA content. Calculation was made according to the formula:

$Q = R \times (E / S)$

where Q = unknown DNA content (pg / 2C), R = standard 2C DNA content (1.17 pg), E = sample G_{0-1} peak mean, and S = standard G_{0-1} peak mean.

Statistical Analysis

Statistical analyses were carried out with the genome results obtained from each seedling. Analysis of variance was used to determine statistical signifcant diference of genome size variation data by using SAS software. The mean separation was done using Fisher's least signifcant diference (LSD) test if the F test was signifcant at $P<0.05$.

RESULTS and DISCUSSION

Germination rates the seeds of different citrus rootstocks were given in (Table 1) and compared in (Fig. 2). According to the obtained results germination rates were differed statistically important among the rootstocks. The highest germination rate was found on Troyer citrange (16.3 %) at the end of first week. The lowest rates were found in Flying Dragon (5.0 %), Taiwanica (5.3 %) and Citremon (6.2 %). Troyer citrange seeds can be considered that having earliest tendency for germination. Seed germination continued to increase rapidly in all surveyed rootstocks. The highest germination percentage was determined on C-35 Citrange (40.5 %) whereas the lowest in Yuzu (19.0 %) 14 days after seed sowing. While the highest germination rate was found in C-35 Citrange (95.2 %), Troyer Citrange (94.5 %), Macrophylla (92.6 %) and Yuma citrange (88.9 %) rootstocks whereas the lowest rate was observed in Yuzu rootstock (68.3 %) after three weeks of sowing. All viable seeds were germinated at the end of four weeks of sowing. The highest germination percentages were obtained from C-35 citrange (98.0 %) and Troyer citrange (96.5 %) rootstocks whereas the lowest germination percentage was determined in Yuzu (74.0 %). To summarize, 1921 seedlings were obtained at the end of seed germination studies from 2200 seeds. The number of seedlings per genotype was recorded maximum in C35 citrange (196 seedlings) whereas the minimum in Yuzu (148 seedlings) followed by Flying Dragon trifoliate orange (163 seedlings). The difference for germination rates could be due to genotypic difference. A study conducted by Cimen (2020), C35 citrange had the lowest germination rate under in vitro germination conditions. Contrarily, C35 citrange had the highest germination rate in our research. The reason for the high difference between two researches could be origin of seed source and seed conservation conditions. Some of the citrus genotypes like C-35 citrange and Troyer citrange produced two or more seedlings from one single seed due to nucellar embryony (Navarro & Juarez, 2007) stated that Troyer citrange has the highest graft success if two weeks old seedlings used for shoot tip grafting. The seedlings obtained from each rootstock were uniform for further evaluation.

Flow cytometry analyses are rapid, preparation of nuclear suspensions is easy and statistical distribution of DNA content of large populations is completed rapidly. Cutting fresh leaf tissues from young seedlings with a sharp razor ensures mixing of large numbers of cell nuclei with the nuclear buffer solution. Leaves from young citrus seedlings and Tahiti lime were mixed and prepared for analysis and flow cytometry formed two large peaks showing G_{0-1} peaks for both species. Calculation of total genome in G_{0-1} peak ratio was successfully completed as published by many researchers. Results obtained from flow cytometry analyses show seedlings of the species used in this study had diploid genome. The G_{0-1} peak ratios were smaller than the Tahiti lime genome used as control species. The mean genome volumes for seedling species analyzed in this study are given in (Table 2) and genome size variations among species are given in (Fig. 1). According to the obtained results, Yuzu (0.808 pg) has the largest genome while Flying Dragon trifoliate had the smallest genome (0.700 pg). There were statistical

differences determined between the seedling populations of the eleven different rootstocks. The Yuzu rootstock is easily proliferated from seeds, has slow growth characteristic, and is a rootstock with high fruit quality and yield. Yuzu has high resistance to low temperatures and polyembryony tendencies. Though it is resistant to *Phytophthora*, fungus and nematodes and tolerant of tristeza, having dwarfing effect, it has moderate resistance to lime and salty soils so commercial use in the world has not become widespread.

Çizelge 1. Narenciye anaç tohumlarının çimlenme oranları*
*Table 1. Germination rates of citrus rootstock seeds**

Rootstock species and hybrids	Germination rates (%)				
	7 days	14 days	21 days	28 days	Total number of seedlings
Yuzu CRC	6.8 cd	19.0 e	68.3 d	74.0 d	148
Chinese sour orange	8.1 bcd	22.7 de	78.0 bcd	88.0 abc	176
Yuma Citrange SRA	14.3 abc	36.5 abc	88.9 a	91.0 abc	182
C-35 Citrange	15.0 ab	40.5 a	95.2 a	98.0 a	196
Macrophylla	12.1 abcd	30.2 abcd	92.6 a	94.5 ab	189
Troyer Citrange	16.7 a	38.0 ab	94.5 a	96.5 a	193
Gou-tou	7.5 bcd	28.8 bcde	81.0 bc	82.5 bcd	165
Citremon	6.2 d	30.1 abcd	80.7 bc	89.0 abc	178
Taiwanica	5.3 d	24.6 de	78.5 bcd	82.0 cd	164
Sunki Mandarin	9.7 abcd	25.5 cde	75.2 cd	83.5 bcd	167
Flying Dragon	5.0 d	22.1 de	74.8 cd	81.5 cd	163
Significance	**	**	**	**	

*P value= 0,0132 (P<0.05).
**The differences among the values are statistically significant.

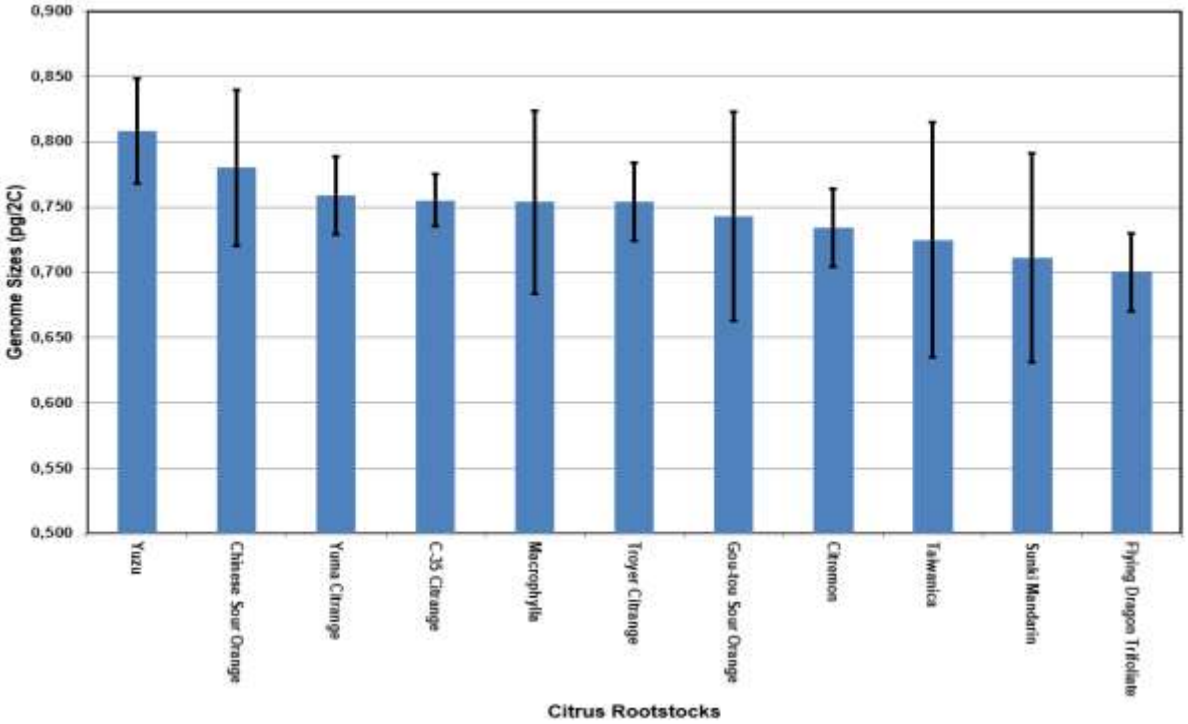
Çizelge 2. Turunçgil anaçlarının 2C DNA içeriklerinin karşılaştırılması*
*Table 2. Comparison of the 2C DNA content of the citrus rootstock species**

Rootstock species and hybrids	Ploidy level	Genome size (pg/2C) means
Yuzu CRC	diploid	0.808±0.04 a
Chinese sour orange	diploid	0.780±0.06 a
Yuma SRA	diploid	0.759±0.03 a
C-35 Citranj	diploid	0.755±0.02 a
Macrophylla	diploid	0.754±0.07 ab
Troyer Citranj	diploid	0.754±0.03 ab
Gou-tou	diploid	0.743±0.08 ab
Citremon	diploid	0.734±0.03 b
Taiwanica	diploid	0.725±0.09 b
Sunki Mandarin	diploid	0.711±0.08 b
Flying Dragon	diploid	0.700±0.03 b
Significance	-	**

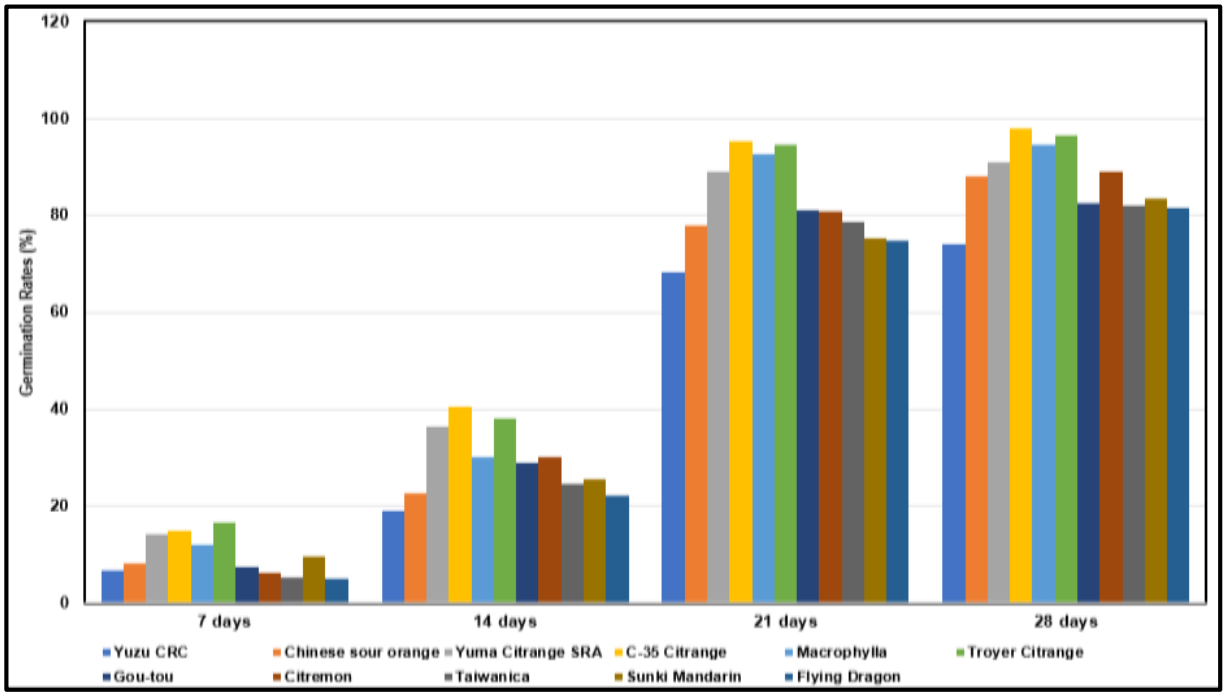
*P= 0,0421 (P<0.05).
**The differences among the values are statistically significant.

Diploidy is the most common ploidy level in Citrus and its related genera with the basic chromosome number $x = 9$. However, some polyploid genotypes were found in Citrus and related genera. Tetraploid Hong Kong wild kumquat (*Fortunella hindsii* Swing.), Triploid Tahiti lime, tetraploid strains of *Poncirus trifoliata*, allotetraploid *Clausena excavata* Burm. F., tetraploid *Clausena harmandiana* and hexaploid *Glycosmis pentaphylla* are some examples of natural polyploidy found in the germplasm of the *Aurantioideae* subfamily.

Polyembryony is commonly observed in citrus and related genus of Clausena, Fortunella and Poncirus Withnucellar embryony several embryos occur from one seed and these plants carry all the characteristics of the mother plant. As a result, seedlings obtained from species and varieties with excess polyembryony tendency have diminished genetic expansion (Polatoz, 1995). Seedlings occurring from nucellar embryos may be used as rootstock due to having similar features to the mother. Tetraploid plants can be found in zygotic citrus seedling populations and polyploidy level could be reach up 2.5 % in Rutaceae according to the previous articles (Cameron, 1968; Rom & Carlson, 1987; Seker et al., 2003).



Şekil 1. Turunçgil anaçları arasındaki genom büyüklüğü farklılıkları (çubuklar standart hatayı göstermektedir)
Figure1. Genome size variations among citrus rootstocks (bar indicates the standard error)



Şekil 2. Turunçgil anaçlarının birinci, ikinci, üçüncü ve dördüncü hafta sonundaki çimlenme oranlarının karşılaştırılması
Figure 2. Comparison of germination rates of citrus rootstocks at the end of first, second, third and fourth weeks

A study by Seker et al. (2003), determined that trifoliate seedlings had the smallest genome among citrus species. As a result, both common trifoliate orange and Flying Dragon trifoliate orange were revealed to have small genomes. The Poncirus genus was determined to have smaller genome volume than the citrus genus. Again, Sunki mandarin was found to have smaller genome volume. The study carried out by Seker et al. (2003) determined Cleopatra mandarin has smaller genome than other citrus species.

Mandarins having small genome is considered to be effective on both species having small canopy volume, small

leaves and small fruit. As a result, Sunki mandarin and Cleopatra mandarin are two valuable rootstocks especially for mandarin species and varieties. C-35, Carrizo and Troyer citrange species have larger genome compared to trifoliates. The statistical differences between them are significant. The reason for this is associated with citranges having larger genome volume than the other parent of orange Cimen (2020) demonstrated C-35 citrange seedlings were diploid with 0.794 pg / 2C relative genome sizes. This result confirmed our findings in C-35 seedlings.

CONCLUSIONS

Different ploidy levels can have a decisive impact on plant development and morphology. For instance, variations in growth rates and sizes can be observed among diploid, triploid, and tetraploid plants. This information enables plant breeders to select rootstocks with desired traits and control plant growth. Rootstocks with different ploidy levels can also affect fruit yield and quality. For example, triploid rootstocks often reduce fruit size while enhancing fruit quality. Therefore, ploidy levels can be consciously chosen to increase fruit yield and quality for specific purposes. Certain ploidy levels may confer greater resistance to diseases and pests. This trait is crucial for maintaining plant health and increasing harvest yields. For instance, some triploid rootstocks exhibit higher resistance to root rot or nematodes. Different ploidy levels can influence plant crossability and reproductive ability. Knowing the ploidy level of a particular plant species helps determine which other plant species it can cross with, thus preventing unwanted crossbreeding. When selecting plants to be cultivated in a specific area, it is important to choose plants that can adapt to the climate, soil, and other environmental factors of that region. Ploidy levels can affect a plant's adaptation ability, assisting in the selection of rootstocks suitable for a particular region.

The determination of ploidy levels is typically accomplished successfully through studies utilizing flow cytometry technique (Kaya et al., 2020). Genome size refers to the amount of DNA in an organism's non-replicated haploid set of chromosomes (Swift, 1950). In diploid (2n = 2x) organisms, genome size refers to the amount of DNA contained in haploid (n number of chromosomes) chromosomes. Genome size is expressed as the C value, which is the amount of DNA content in the genome in picograms. The 2C value is the amount of DNA in the nucleus of a somatic cell, regardless of its ploidy level (Kaya & Sakiroglu, 2012; Tuna, 2014). Significant (about 1000-fold) differences are observed between species in terms of genome size (C value). On the other hand, the genome size remains constant among different individuals of a species and therefore becomes species specific. Therefore, the C values of species are extremely important for biology, genetics, taxonomy and evolution studies (Rees & Walters, 1965; Price & Bachmann, 1975; Ohri, 1998; Ozkan et al., 2003; Ollitrault et al., 2007). Flow cytometry is the newest, fast, sensitive and economical method used to determine genome size today. Since there is a very close relationship between the C values determined by flow cytometry and the chromosome numbers of the species, this parameter is also used to determine the ploidy levels of the species (Tuna et al., 2001; Mavioğlu Kaya, 2010). This study showed that nucellar seedling populations of Gou-Tou sour orange, C-35 citrange, Troyer citrange, Taiwanica, Citremon, Yuzu, Sunki mandarin, Flying Dragon trifoliate orange, Yuma Citrange, Macrophylla and Chinese orange rootstocks had only diploid genomes. In a study conducted by Guo et al. (2008), similar to our study results, they reported that the Gou-Tou sour orange rootstocks they used as study material was diploid. There were no polyploids in the surveyed seedling population due to high tendency to nucellar embriyony. Polyploidy may have great potential for citrus rootstock breeding. Autotetraploid and allotetrapoid rootstocks may have potential especially for dwarfing of citrus trees.

Determining ploidy levels in citrus is crucial due to its significance in plant cultivation, diversification, and breeding endeavors. Knowledge of ploidy levels plays a critical role in selecting parent plants for breeding programs. For instance, differential traits may exist between diploid (2n) and triploid (3n) variations in some citrus species, influencing the selection of desired traits in new cultivars. Ploidy levels can significantly impact fruit productivity, size, quality, and resilience. Thus, determining ploidy levels is essential for achieving higher yields and superior fruit quality. Certain ploidy levels can affect a plant's resistance to diseases and pests, hence informing the selection and cultivation of resistant varieties. Ploidy levels also influence a plant's reproductive capability; for example, triploid plants are often sterile and do not produce seeds, necessitating the selection of suitable plants for seed production. Additionally, the determination of ploidy levels serves as fundamental information in genetic research, guiding plant genetics and breeding efforts. Consequently, the identification of ploidy levels in citrus species is imperative for plant cultivation, breeding programs, and genetic research, contributing to the development of more productive, resilient, and high-quality cultivars and ensuring the sustainability of agriculture.

Polyploidy, the condition where an organism has more than two complete sets of chromosomes, plays a crucial role in the development of citrus fruits. It can result in larger fruit size, improved disease resistance, and enhanced stress tolerance, which can be beneficial for both the quality and yield of citrus crops. Polyploidy can also contribute to hybrid vigor and potentially improve the organoleptic qualities of citrus fruits, such as taste, color, and texture. However, as noted in the study, specific polyploid citrus species or varieties were not determined, which suggests

that further exploration into polyploid citrus cultivars could be valuable. To better understand and potentially develop polyploid citrus varieties, several methods can be employed to induce polyploidy:

Colchicine treatment: Colchicine is one of the most commonly used chemicals to induce polyploidy. It disrupts the process of chromosome segregation during cell division, leading to chromosome doubling. This method is often applied to meristematic tissues (such as root tips or shoot tips) of citrus plants to generate tetraploid individuals from diploid ones. Oryzalin treatment: Oryzalin, another chemical agent, can also be used to induce polyploidy. It works by inhibiting microtubule formation during cell division, preventing the separation of chromosomes, thus resulting in polyploid cells. In vitro culture: Tissue culture techniques can be used in conjunction with chemical treatments to generate polyploid plants. This method allows for the controlled environment required to induce polyploidy and regenerate whole plants from the treated cells. Somatic hybridization: This method involves fusing cells from different citrus species or varieties, followed by the induction of polyploidy in the hybrid cells. Somatic hybridization can lead to the creation of interspecific or intergeneric hybrids with desired traits, including polyploidy. Genome editing techniques: Newer genome editing technologies, such as CRISPR/Cas9, could also offer a precise approach to induce or modify polyploidy in citrus fruits, though this method is still in the experimental stages for polyploid induction.

By incorporating these methods into citrus breeding programs, researchers and growers can potentially create new, more resilient citrus varieties with improved agronomic and sensory qualities. Understanding and utilizing polyploidy could open up new avenues for the citrus industry, helping to meet the increasing global demand for high-quality fruits.

This study has laid the foundation for research aimed at determining ploidy levels in citrus rootstocks. The continuation and expansion of such studies are crucial for identifying new plant species and varieties, as well as elucidating which rootstocks exhibit resilience to various stress factors.

Author Contribution Statement

These authors contributed equally to this work.

Conflict of Interest

The authors declare that they have no conflict of interest.

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