Journal of Balkan Science and Technology
journal homepage: www.jbst.nku.edu.tr/

Flow Cytometric Characterization of White Clover Populations (*Trifolium repens* L.) Growing in Natural Grasslands of Tekirdag, Turkey

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Research Article

Keywords:
White clover
Flow cytometer
Nuclear DNA content
Ploidy

Received: 20.04.2022
Accepted: 02.05.2022
Published:31.05.2022

DOI: 10.55848/jbst.2022.7

ABSTRACT

White clover (*Trifolium repens* L.) is a stoloniferous and perennial species that is the major forage legume of livestock systems in temperate regions of the world. The objectives of the study were to determine nuclear DNA content and ploidy level of the native white clover populations growing in natural pastures of the Tekirdag region, Turkey. Nuclear DNA content analysis was carried out by flow cytometer using propidium iodide (PI) as a fluorochrome. The mean 2C nuclear DNA content of clover populations analysed in the study varied between 2.307 ± 0.063 and 2.345 ± 0.048 pg. Differences among the clover populations were not significant statistically. Based on the results of the study, all white clover genotypes investigated in the study were determined as tetraploid with $2n = 4x = 32$ chromosomes.

1. Introduction

The flow cytometer was originally invented to count and analyse blood cells automatically for medical purposes and was introduced to the medical community by Wallace H. Coulter in 1956 [1]. It is a unique technique to analyze optical characteristics (fluorescent intensity and scattered light) of single cells, their organelles, or other microscopic particles such as nuclei and chromosomes in liquid suspension when they cross a beam of light. The technology used in flow cytometers is similar to inkjet printers and, therefore, has the ability to analyze up to 1000 particles per second. In addition to this, some flow cytometers can sort simultaneously up to four subgroups for further use or investigation [2]. These superior characteristics of flow cytometry made it an invaluable tool for biomedical research and clinical applications, and therefore, its usage has rapidly become widespread. It took more than two decades before FCM began to be employed in analysis of plant cells with the advancements in science and technology. Friedrich Otto Heller used the flow cytometer to analyse plant cells for the first time in 1973 [3]. However, it didn't draw the attention of the plant community mainly due to inconvenience of the protocol used for preparing samples from solid tissues. For approximately 10 years, no one again tried to analyse plant cells by using the flow cytometer. The easy and rapid sample preparation procedure developed by Galbraith et al. [4] was a major breakthrough for plant FCM, which triggered its usage in plant science [5]. It is the original protocol that today plant scientists use in their laboratories. Since then, the number of publications on plant cytometry have been increasing gradually. Nuclear DNA content analysis is by far the most common application of flow cytometry in plants.

The nuclear DNA content is the total amount of DNA present within each cell nucleus of a eukaryotic organism. It is expressed in picograms (10^{-12} gram) as "C" values to avoid confusion with ploidy level [6,7]. In publications, the nuclear DNA content value is presented as either a 1C or 2C value. The amount of DNA in a haploid nucleus is known as the 1C value while the 2C value represents the amount of DNA included in a diploid somatic nucleus. Sometimes, 1Cvalue and genome size are also used synonymously since both terms represent the DNA amount of one copy of chromosomes (haploid nucleus). The number of nucleotide pairs can also be calculated roughly by using the following equation: 1 pg = 985 million base pairs [7].

The nuclear DNA content is generally constant among cells of an individual, and relatively constant among individuals of a given species with same ploidy level (6). However, it differs more than 2000-fold between plant species, ranging from 0.0648 pg/1C in *Genlisea margaretae* HUTCH [8] to 132.45 pg/1C for *Trillium camschatcense* KER GAWLER [9]. These characteristics of the nuclear DNA content make it genome (species) specific, and therefore, it is a basic information required in many areas of biology such as taxonomy, evolution, and genetics [10 ,11, 2, 12]. The variation observed among different plant species is directly correlated with some organismic traits such as minimum generation time

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[13], growth in different latitudes and ecological conditions [7,14,15,16], water relations in conifers [17,18,19], seed size [16, 20], physiology [21], and development [22]. Polyploidy and repetitive DNA sequences, especially retrotransposons, are the two major contributing factors to great interspecific variation [23,24,25].

The nuclear DNA content measured by flow cytometry has also been commonly used as a feasible alternative to classical chromosome counting by light microscopy in ploidy analysis due to its tight positive correlation with the number of chromosomes [26, 27]. The greatest advantage of the method is its speed, ease, and accuracy in ploidy screening without requiring dividing cells as with the classical method. FCM is especially useful if there are many samples to screen and the organism has a high number of chromosomes.

White clover (*Trifolium repens* L.) is a stoloniferous and perennial allotetraploid species that is one of the most important forage legumes of livestock systems in temperate regions of the world. It is generally grown in mixtures with grass species, such as perennial ryegrass (*Lolium perenne* L.), fescues (*Festuca* spp.) and cocksfoot (*Dactylis glomerata*) [28]. It is found commonly in the natural flora of Turkey as well, although it is not cultivated [29]. Eastern Mediterranean, East Africa, and South America are the diversity centers for the clovers. The results of recent studies support the Mediterranean origin of the genus [30]. Turkey has high genetic diversity and is rich in clover species as it is located in the center of the geographical region where the genus originated. Therefore, clover populations growing naturally in the flora of Turkey are important genetic resources to use in breeding programmes.

The objectives of this study are to determine nuclear DNA content of natural clover populations growing in the Tekirdag region of Turkey by flow cytometer and to use the information to determine ploidy levels of the populations.

2. Materials and Methods

2.1. Plant Materials

The clover plants were dug up in early spring from two natural pastures (Koseilyas and Yagcı) around Tekirdag and campus of Tekirdag Namik Kemal University, and transferred to pots (Fig 1). The plants were kept outdoors and they were grown in natural conditions during the study.

2.2. Nuclear DNA Content Analysis

The nuclear DNA content of the white clover populations was determined by flow cytometer (Partec, CyFlow® Space Münster, Germany) equipped with a green solid-state laser (Cobolt Samba, 532 nm, 100 mW). Suspensions of intact nuclei were prepared using commercial kits (CyStain PI absolute P) manufactured by Sysmex Partec GmbH (Münster, Germany). The fresh leaf tissues of clover plants grown in pots were used in analysis, with common vetch (*Vicia sativa*) as internal standard. Approximately 20 mg leaf tissue from both clover and the standard were simultaneously chopped in a petri dish with 0.5 ml of extraction buffer. The homogenized solution was transferred into a glass tube through a 30 µm filter, and 2 ml of staining buffer was added to each tube. Before FCM analysis, samples were incubated at room temperature in the dark for at least 1 hour. A total of 10 individual plants for each population were analysed in the study. The results of each sample were analyzed by a software called Flomax, specifically dedicated to the partec flow cytometers. The 2C nuclear DNA content of each clover genotype was calculated by using mean fluorescence intensities of the G1 peaks of the sample and standard with the following formula.

$$\text{DNA cont. of sample} = \frac{\text{Fluorescence intensity of sample (mean of G1 peak)}}{\text{Fluorescence intensity of standard (mean of G1 peak)}} \text{ X DNA cont. of } \textit{Standard}$$

Fig. 1 Map of location sites.

The mean 2C value for each population was calculated as the average of 10 genotypes. Only the results of samples that had a coefficient of variation (CV) less than 3% were used in the calculation of the mean.

2.3. Ploidy Analysis

Ploidy levels of the clover plants were determined by correlating nuclear DNA content of plants determined by flow cytometry with their chromosome number determined by counting chromosomes of at least one individual for each group. The rest of the plants included in the same group were assumed to have the same chromosome number.

2.4. Cytological Preparations

Somatic chromosome preparations were made using root tips. Root tips were harvested from plants growing in pots in early morning and treated with cold water (4°C) for 20 hours, followed by fixation in ethanol:acetic acid (3:1, v/v). Preparations were performed as described by Jenkins and Hasterok [31]. First, the roots were taken out of the fixative and washed in 0.01M citric acid-sodium citrate buffer (pH 4.8, 5 min, 4 times), and then digested enzymatically at 37 °C in a mixture comprising 20% (v/v) pectinase (Sigma-Aldrich Corp., St. Louis, MO, USA), 1% (w/v) cellulase (Calbiochem, San Diego, CA, USA), and 1% (w/v) cellulase Onozuka R-10 (Serva) for 3 hours. Digested meristem was transferred onto a slide in a drop of 45% acetic acid and squashed. The coverslips were removed from the slides by placing them in the freezer at –80°C for a minimum of 1 hour. The slides were air dried overnight and stained with DAPI (4′,6-diamidino-2-phenylindole).

2.5. Image Capturing

Images of the cells with well distributed chromosomes were captured by CCD digital camera (SPOT RT SLIDER) attached to the epifluorescence microscope (Olympus BX 51).

2.6. Statistical analysis

Confidence intervals, a simple statistical procedure, was used to compare the mean 2C nuclear DNA content of the clover populations [32]. Population means with overlapping confidence intervals were assumed to be similar. This is equivalent to conducting a simple t test to compare specific means.

3. Results and Discussion

This is the first study reporting 2C nuclear DNA content of native white clover populations collected from natural pastures of Tekirdag by using flow cytometry. Technically, measurement of nuclear DNA content was efficient in all white clover genotypes, and flow cytometric analysis of each of the individual plants generated high resolution histograms (Fig 2) with CV of the G1 peaks lower than 3%, indicating the precision of the data obtained in the study. A typical flow histogram obtained for each genotype is presented in Fig 2, where the PI-fluorescence intensities (G1 peaks) of both clover genotype and standard are shown over the 1024 channels. The wide peak at the lowest channels (left of the histogram) corresponds with debris. *Vicia sativa* (cultivar nilüfer) was excellent as an internal standard for white clover since its G1 peak was unambiguously distinguishable from the G1 peak of white clover genotypes. The mean 2C nuclear DNA content of the clover populations varied between 2.307 ± 0.063 and 2.345 ± 0.048 pg while the 2C nuclear DNA content of the white clover genotypes used in the study ranged from 2.22 to 2.41 pg. The differences among the clover populations were not significant statistically. The 2C nuclear DNA content values obtained in the study are presented in Table 1. Nuclear DNA content analysis has been carried out on white clover in various studies prior to this study. The reported 2C nuclear DNA content values of white clover varied between 2.07 and 2.67 pg in the previous studies (33, 34, 35, 38). The 2C DNA content of white clover determined in the current study is in the range of the 2C values reported for white clover. Vizithin et al. and Rizza et al. reported 2C nuclear DNA content of white clover as 2.23 pg and 2.22 pg, respectively [33, 34]. Those 2C values are closer to the 2C values of white clover obtained in the current study than in other studies. Arumuganathan and Earle reported the 2C nuclear DNA content of white clover as 2.07 pg [35]. This value was the lowest 2C value reported for white clover although it used FCM, as did the other studies mentioned above. The reason for these small differences among different studies can be attributed to the different standards and genotypes used. For example, Arumuganathan and Earle used chicken red blood cells (CRBC) as internal standard while different plant species with known nuclear DNA content were used as internal standard in the others. Based on the results of those previous studies carried out using flow cytometry, white clover has a limited intraspecific nuclear DNA content variability (≤ 5%), which is consistent with the generally accepted view of constancy of genome size at the species level. This may be related with the absence or rarity of interspecific hybridization events in the genus Trifolium [36]. Evans suggested that the rarity of wide hybridization in the forage legumes may well be associated with their predominant adaptation to insect pollination [37]. However, Campbell et al. reported more than 20% nuclear DNA content variation (2.22 - 2.67 pg) among white clover varieties and breeding lines of various geographic origin, which contradicts with the results of the other previous studies [38]. The reason for the high intraspecific variation reported in the later study can be due to the method, since less accurate feulgen densitometry was used in the study carried out by Campbell et al. [38].

Chromosome numbers of the clover genotypes were correlated with their nuclear DNA content by counting mitotic chromosomes of only one white clover genotype with a. microscope. Based on cytological investigations, chromosome number of the white clover genotype was determined as 2n = 4x = 32, indicating that the genotype was tetraploid (Fig 3). The rest of the white clover genotypes were assumed to be tetraploid with 2n = 4x = 32 chromosomes since they had similar 2C nuclear DNA content values. The results of cytological investigations obtained in the current study are in agreement with the known chromosome number of white clover [30,39].

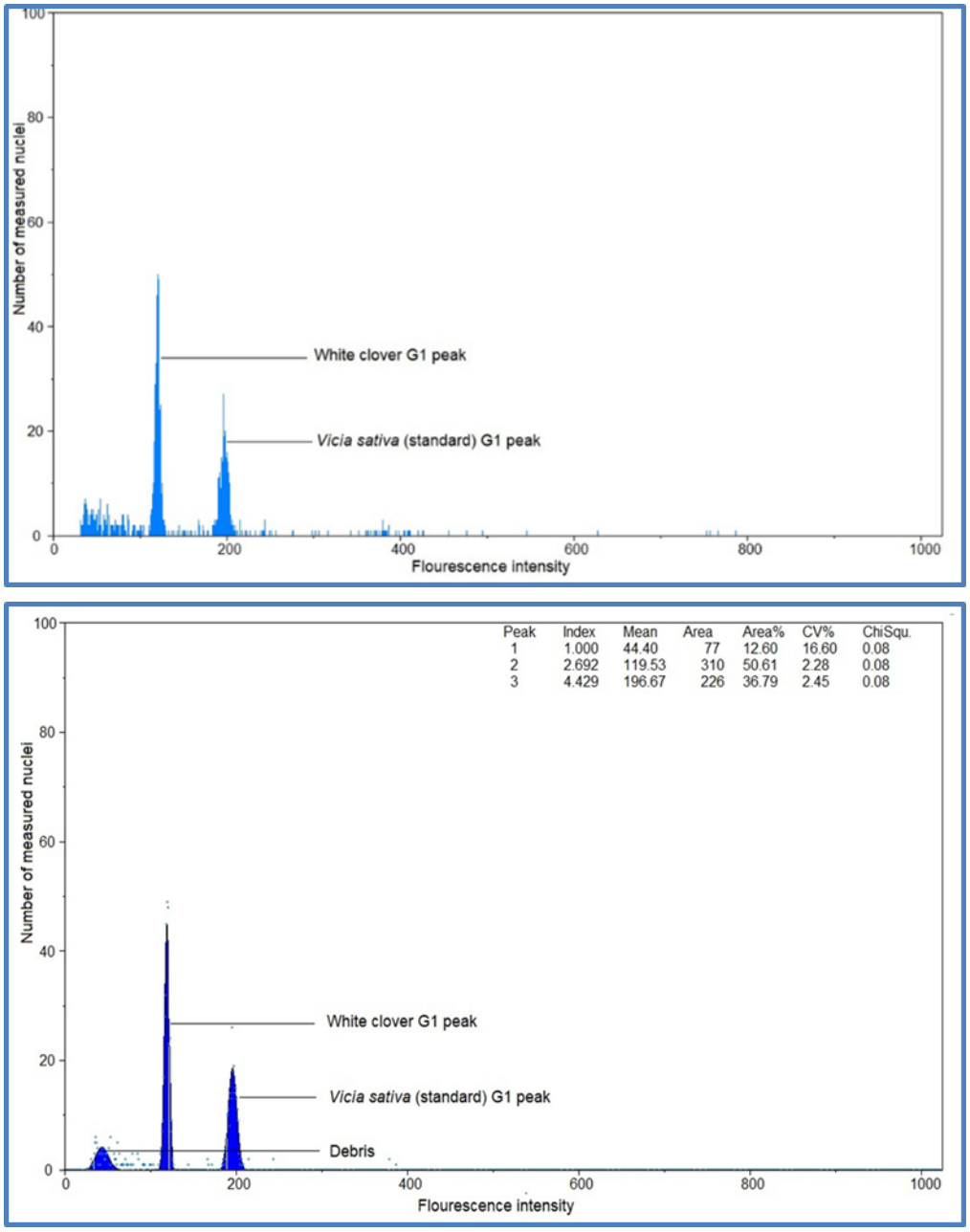

Fig. 2 Relative positions of G1 peaks of white clover and standard.

Table 1. Locations and 2C nuclear DNA content of the white clover populations.

Locations of Pop.	Nuclear DNA Content of Individual Plants (2C/pg)										Mean 2C/pg	SD	T*S_x	Confidence intervals	
	1	2	3	4	5	6	7	8	9	10				lower	upper
TNKU C.	2,37	2,22	2,27	2,40	2,33	2,37	2,34	2,26	2,28	2,27	2,307	0,063	0,052	2,255	2,359
Koseilyas	2,30	2,26	2,31	2,36	2,33	2,33	2,35	2,36	2,34	2,37	2,331	0,033	0,027	2,304	2,358
Yagci	2,39	2,34	2,37	2,41	2,36	2,38	2,34	2,29	2,36	2,25	2,345	0,048	0,039	2,310	2,388

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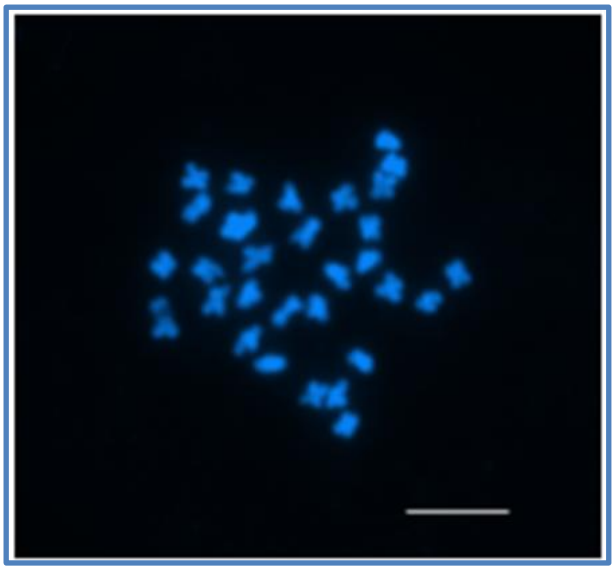

Fig. 3 Mitotic chromosomes of white clover plants (2n = 4x = 32, scale bar 10 μm).

4. Conclusion

In conclusion, the results of the current study prove one more time that flow cytometry is an excellent method to use for the analysis of nuclear DNA content and ploidy of *Trifolium* species due to its speed, ease, accuracy, and relatively low cost. The nuclear DNA content results obtained by flow cytometry can be used in taxonomic identification of *Trifolium* species and their classification, in determination of genome structure and ancestors of polyploids, in breeding programs involving interspecific and interploidy hybridization, and in the investigation of evolution within the genus *Trifolium*.

Declaration

Author Contribution: Conceive- M.T.; Design- M.T.; Experimental Performance, Data Collection, and/or Processing- G.S.T., G.Y., Y.I.S, B. C.; Literature review- G. S. T.; Writing- G.S.T.; Critical Review- M.T.

Conflict of interests: The authors have declared no conflicts of interest.

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