

DNA content estimation of Fig and Black Mulberry using flow cytometry

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Abstract- In this study, fig and black mulberry DNA contents were estimated using DAPI fluorescence stain in flow cytometry. The 2C DNA contents of the fig and black mulberry were found as 0.82 pg and 8.34 pg, respectively. The calculated 1C value of genome size of fig is 401.8 Mbp and that of black mulberry is 4086.6 Mbp. The ratio of 2C DNA content and 1C genome of the black mulberry was 10.17 times that of the fig although fig is diploid and black mulberry is decosaploid.

Index terms: *Ficus carica*, *Morus nigra*, DAPI, DNA content, flow cytometry, genome size.

Conteúdo de ADN estimado de Figueira e Amoreira-Preta utilizando citometria de fluxo

Resumo- Neste estudo, os teores de ADN de figueira e amoreira-preta foram estimados utilizando a coloração fluorescente DAPI em citometria de fluxo. Os conteúdos de ADN 2C da figueira e da amoreira-preta foram encontrados na faixa de 0,82 pg e 8,34 pg, respectivamente. O valor calculado de 1C para os tamanhos do genoma da figueira e da amoreira-preta foi alterado de 401,8 Mpb e 4.086,6 Mpb, respectivamente. A proporção do conteúdo de ADN 2C e do genoma 1C da amoreira-preta foi de 10,17 vezes a da figueira, embora figueira seja diploide e amoreira-preta decosaploide.

Termos para indexação: *Ficus carica*, *Morus nigra*, DAPI, conteúdo de ADN, citometria de fluxo, tamanho do genoma.

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Introduction

The fig (*Ficus carica* L., $2n=2x=26$, diploid, CONDIT, 1928) and black mulberry (*Morus nigra* L., $2n=22x=308$, decosaploid, AGAEV; FEDOROVA, 1970; BASAVAIAH et al., 1990) belong to the Eusyce section and Moreae tribe, respectively, of the Urticales order Moraceae family which comprises 37 genera and approximately 1,100 species. Fig is the most produced in Turkey and black mulberry is one of the relatives of fig in the Moraceae. These two fruit species have of economic and social importance in agriculture, and health benefits. Both genera are perennial and evergreen in tropics and deciduous in subtropics and temperate climates (WEIBLEN, 2000; FLAISHMAN et al., 2008; HE et al., 2013; VIJAYAN et al., 2014). The genus *Ficus* includes approximately 700 dioecious and gynodioecious species. The fertilization of fig is carried out by the symbiotic fig wasp, *Blastophaga psenes* L. The fig is the oldest cultivated plant according to the fossil records of dried fruit (syconia) and seeds (drupelets) found in the ancient ruins in the Gilgal Village of the lower Jordan Valley dated back to 11,400 years before present (KISLEV et al., 2006). Anatolia is in the center of origin of the cultivated figs. Turkey (274,535 t, 25.1%) is the leading country for fig production in the world (1,093,189 t) (FAOSTAT, 2012). The genus *Morus* has about 10-13 recognized species (HE et al., 2013). There are at least 24 species (KAFKAS et al., 2008) or 68 species having different ploidy levels (ANIL KUMAR et al., 2012) species. The fertilization of mulberry trees is carried out by wind. The mulberry originated from the Himalayan foothills and has distributed between 50°N and 10°S latitudes in Asia, Middle Asia, Europe, North, Central, and South America, and Africa. Its leaves have been used for feeding of silkworms, *Bombyx mori* L. (VIJAYAN et al., 2014). Turkey produces 76,400 t mulberries (TÜİK, 2013). According to the recently published draft genome sequence of *Morus notabilis* (HE et al., 2013), its estimated genome size is 357 Mbp and contains 330 Mb genome assembly, 128 Mb repetitive sequences, and 29,338 genes, 60.8% of which are supported by transcriptome sequencing. The 1C DNA value corresponds to the DNA amount in the unreplicated haploid nucleus chromosome complement where the letter C stands for a “constant” (TUNA et al., 2001; BENNETT; LEITCH, 2011). The 2C DNA values represent the DNA content of a diploid somatic nucleus. The conversion factor is as follows: 1 pg = 980 Mbp (TUNA et al., 2001). There are 6,287 angiosperm species listed at the Plant DNA C-values database (BENNETT; LEITCH, 2010). Feulgen densitometry (FD) and flow cytometry (FC) are the two mostly used methods in DNA content estimation. The latter has been used to estimate the nuclear DNA content and genome size of an unknown sample comparing with a reference standard (GALBRAITH et al., 1983;

DOLEŽEL et al., 1998; DOLEŽEL; GREILHUBER, 2010; TİRYAKİ; TUNA, 2012). Different fluorochromes (fluorescent dyes) are used in FC. DAPI, DIPI, Hoechst 33258, and Hoechst 33342 bind to AT-rich regions whereas chromomycin A₃, mithramycin, and olivomycin bind to GC-rich regions. Ethidium bromide (EB) and PI intercalate to DNA molecules independent of base sequences (DOLEŽEL et al., 1998). DAPI has been chosen for staining the nuclear DNA in FC since it is cheaper, safer, and more environment friendly than the others (OCHATT, 2008). The published nuclear DNA contents of some fruit trees change as follows: diploid banana, *Musa* spp. $2n=2x=22$, 2C=1.108 pg, 1C=534 Mbp (LYSÁK et al., 1999; ASIF et al., 2001); diploid olive, *Olea* spp. $2n=2x=46$, 2C=2.90-3.07 pg, 1C=1,453-1,502 Mbp (LOUREIRO et al., 2007a; BRITO et al., 2008); diploid coffee, *Coffea* spp. $2n=2x=22$, 2C=0.95 pg (CROS et al., 1995; OHRI, 1998). Diploid barley (*Hordeum vulgare* L., $2n=2x=14$, 2C=10.65-10.68 pg, 1C=5,022-5,218 Mbp, TİRYAKİ; TUNA, 2012; TUNA et al., 2001; BENNETT; LEITCH, 2011) and diploid common vetch (*Vicia sativa* L., $2n=2x=12$, 2C=3.69-3.79 pg, 1C=1,808-1,857 Mbp, KAHLAOUI et al., 2009) are among the internal reference standards used in FC. The 2C DNA contents per nucleus for diploid fig were estimated as 1.41±0.01 pg using FD (OHRI; KHOSHOO, 1987) and as 0.73±0.03 pg using propidium iodide (PI) (LOUREIRO et al., 2007b). The estimated genome size of *F. carica* is 1C=356-357 Mbp (LOUREIRO et al., 2007b; BENNETT; LEITCH, 2011). The 2C DNA content of *F. elastica* was estimated to 1.52 pg using DAPI and PI (KOLÁŘ et al., 2012). The 2C DNA content of *F. bengalensis* was 1.45±0.01 pg using FD (OHRI; KUMAR, 1986). These were the four records for *Ficus* spp. DNA content determination. There were only three published articles on *Morus* spp. The 2C nuclear DNA content of diploid *Morus alba*, *M. bombycis*, *M. latifolia*, and *M. rotundiloba* were 2C=0.79 pg using EB (HORJALES et al., 2003). The 2C=0.704-0.746 pg was found using DAPI (4',6-diamidino-2-phenylindole) and PI (YAMANOUCHI et al., 2010). The 2C DNA content was 1.70±0.02 pg using FD (OHRI; KUMAR, 1986). The estimated genome size of *M. alba* is 1C=345-366 Mbp (YAMANOUCHI et al., 2010) or 1C=386 Mbp (HORJALES et al., 2003). During the literature search, no report was observed on *M. nigra* 2C DNA content. To acquire some knowledge on the DNA content on cultivated fig and black mulberry will help to facilitate genomics studies. The objective of this study was to determine 2C DNA content of fig and black mulberry using DAPI in flow cytometry. This is the first report on determining of 2C DNA content of fig and black mulberry using DAPI.

Material and Methods

The nuclear DNA content was determined from leaflets of barley, common vetch, fig, and black mulberry plants. The methods were used from TUNA et al. (2001) and TAVARES et al. (2014) with some modifications. For intact nuclei isolation, leaf tissues of sample and standard species were used alone and then simultaneously in flow cytometry. Each of 1.0 cm² (20-50 mg) of leaf tissues of *H. vulgare*, *V. sativa*, *F. carica*, and *M. nigra* analyzed one by one to observe corresponding G1 and G2 peaks. Then, leaf tissues of fig or black mulberry were mixed together with 1.0 cm² (20-50 mg) of leaf tissues of diploid common vetch and barley as internal reference standards. While fig leaf sample was used with common vetch leaf standard (1:1), black mulberry leaf sample was used with barley leaf standard (1:1) since their peak sizes were comparable. Leaf samples from both plants of each species pair were simultaneously chopped with a sharp razor blade for approximately 30 s in a plexiglass Petri dish containing 400 µl of ice-cold extraction buffer (stock: 24 ml MgSO₄, 25 mg dithiothreitol, 625 µl Triton X-100 stock (1.0 g Triton X-100 in 10 ml ddH₂O)). The nuclear suspension was filtered through a 30 µm nylon filter (Partec CellTrics®, Görlitz, Germany) to remove cell fragments and large leaf debris. Then, nuclei were stained using the kit CyStain® UV Precise P (Partec GmbH., Münster, Germany) with 1600 µl of DAPI (1×) solution. After a 2-3 min incubation period in darkness, the relative fluorescence intensity (FI) of nuclei per G1 peak was analyzed in a CyFlow® Space flow cytometer (Partec GmbH., Münster, Germany) equipped with a green solid state laser for monitoring DAPI fluorescent dye excitation (>435 nm, YAMANOUCHI et al., 2008, or 365 nm YAMANOUCHI et al., 2010). It is recommended to 10-15 particles per second during the excitation recording. DNA content was calculated as follows:

$$2C \text{ or Nuclear DNA content (pg)} = (\text{mean position of unknown sample peak}) / (\text{mean position of known standard peak}) \times \text{DNA content of known standard. } 1C = (\text{nuclear DNA content (pg)} \times 980 \text{ Mbp}) / 2.$$

Results and discussion

In the beginning of the experiment, barley, common vetch, fig, and black mulberry leaf samples were analyzed alone (data not presented). Their corresponding G1 and G2 peaks were observed and compared. When fig and common vetch leaf samples were analyzed, it was seen that G1 peak of fig (115.94) was 4.5 folds smaller than common vetch (517.96) (Fig 1, Table 1). G1 peak of black mulberry (163.53) was 1.2 fold smaller than that of barley (208.77) (Fig 2, Table 1). The estimated

2C DNA contents of fig and black mulberry were 0.82 pg and 8.34 pg, respectively (Table 1). The ratio of 2C DNA content of the black mulberry was 10.17 times of the fig. The calculated 1C values (1C=980 Mbp) of fig and black mulberry genomes were 401.8 Mbp and 4086.6 Mbp, respectively. The ratio of 1C genome of the black mulberry was 10.17 times of the fig. The estimated 2C DNA content and calculated 1C genome values of fig were more than those of LOUREIRO et al. (2007b). The reason for this discrepancy can be attributed to a fluorochrome difference used in this study. While DAPI was used in the current study, PI was used in LOUREIRO et al. (2007b). The CV values of the current study were deviated from 2.53 to 4.67. PI-stained *F. elastica* samples had higher CVs than DAPI-stained samples (KOLÁŘ et al. 2012). The 2C DNA content of black mulberry was much higher than in the work done by HORJALES et al. (2003). In their work, HORJALES et al. (2003) used intercalating EB stain. They used diploid *Morus* spp. whose 2C DNA content is very similar to that of diploid *F. carica* in the current study. In other words, the 2C DNA contents of diploid *Morus* spp. and diploid *F. carica* are similar in number. On the other hand, YAMANOUCHI et al. (2010) found 2C DNA content (0.704-0.746 pg) close to that obtained by HORJALES et al. (2003) (0.79 pg). It can be said that the results obtained from decosaploid *M. nigra* in the current study is in agreement with both YAMANOUCHI et al. (2010) and HORJALES et al. (2003) since they used diploid *Morus* spp. Nevertheless, OHRI; KUMAR (1986) used probably a tetraploid *Morus* sp. (2C=1.70 pg) in their work which can be in accordance with a decosaploid *M. nigra* (2C=8.34 pg) in the current study. The results obtained from the current study after nuclei staining with DAPI (binding preferentially to AT-rich regions) did not agree with those obtained using FD (OHRI; KHOSHOO 1987, DOLEŽEL et al. 1998). Higher FI was obtained using laser FC compared to FD (DOLEŽEL et al. 1998).

The present work showed that samples of unknown DNA content and the standards of known DNA content should be first prepared one by one and then, simultaneously with each other in order to see the peak positions. DAPI can give a better resolution of DNA content histograms using with lamp-based FC (BUITENDIJK et al. 1997). Due to preferential binding of DAPI to AT-rich regions of DNA, the estimation of DNA content observed after DAPI staining might be expected to differ from that of PI staining. It was stated that DAPI may be preferred as the fluorochrome for genome size estimation and PI was found to be less reliable (RAYBURN et al. 1992). It would be interesting to compare DAPI with PI in fig and black mulberry. PI dye could be used to make DNA content estimation for fig and black mulberry in the future. This is a preliminary study to estimate the DNA content and genome size of black mulberry. The DNA content and genome size of diploid

M. alba and *M. rubra*, and decosaploid *M. nigra* (AGAEV; FEDOROVA 1970) need to be determined in the same study. Previous knowledge from the published results can help to choose reliable internal standards before start to analyze the sample plant. Both common vetch and barley can be used to estimate DNA contents of fig and black mulberry, respectively. In the following experiments, other standards could be used for *F. carica* such as *Arabidopsis thaliana* (L.) Heynh. accession Stoc (0.9 pg, 435 Mbp),

Raphanus sativus L. (1.11 pg, 544 Mbp), *Lycopersicon esculentum* (syn. *Solanum lycopersicum* L.) (1.96 pg, 1C=960 Mbp), and that for *M. nigra*, such as *Zea mays* L. (5.43 pg, 1C=2,261 Mbp), *Pisum sativum* L. (9.09 pg, 1C=4,454 Mbp), *Secale cereale* L. (16.19 pg, 1C=7,933 Mbp) (DOLEŽEL; GREILHUBER 2010). The peaks for these suggested standards appear less than three orders of the DNA content of the sample need of fig and mulberry.

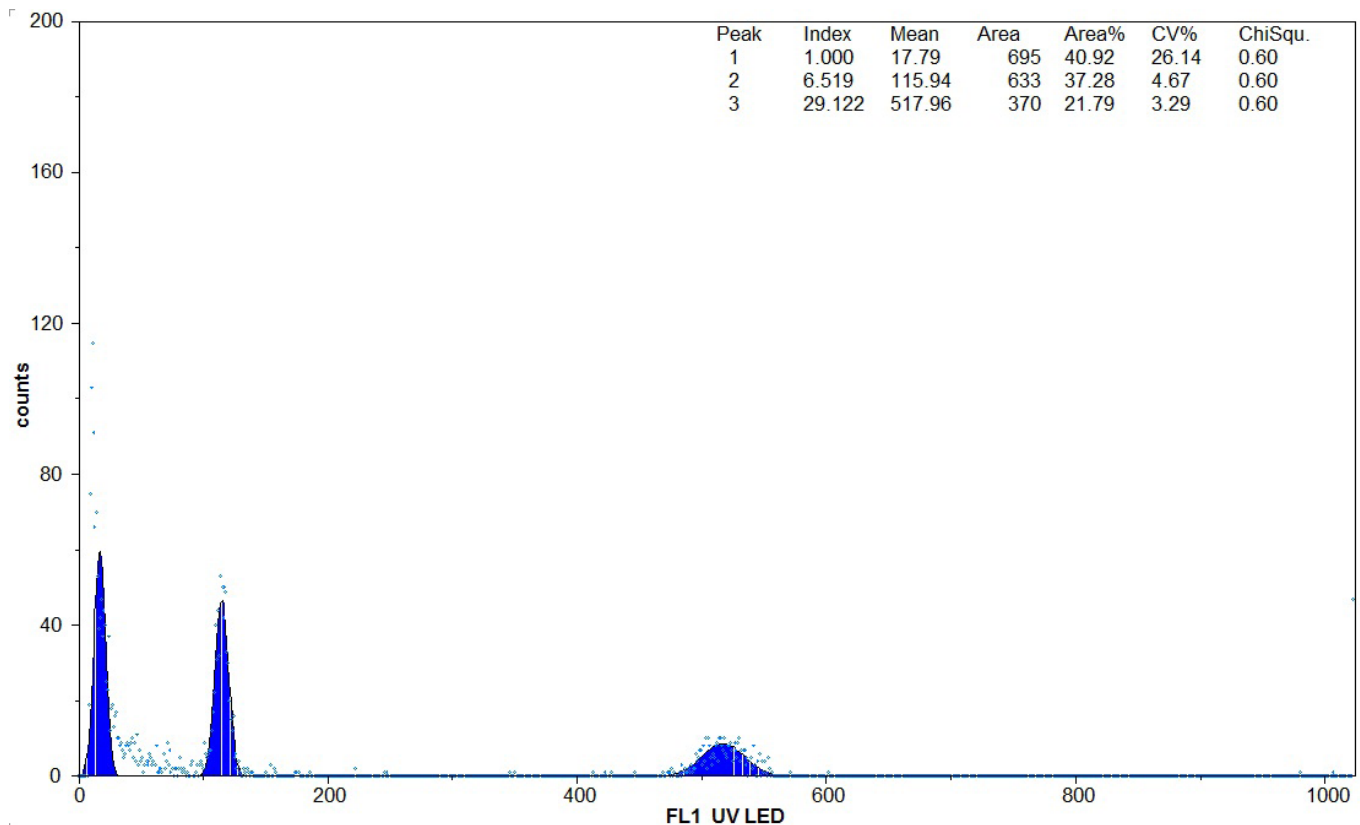


Figure 1. Fluorescence intensity (relative DNA content) of *Ficus carica* (peak 2) and *Vicia sativa* (peak 3) in flow cytometry. The first peak was a default for the DNA or cell noise signal.

Table 1. Expected DNA amount of fig (*Ficus carica*) and black mulberry (*Morus nigra*) compared to that of standards, barley (*Hordeum vulgare*) and common vetch (*Vicia sativa*)

Sample species	Standard species	Sample FI value	CV	Index	Standard FI value	CV	Index	DNA amount of standard (pg)	DNA amount of sample (pg)
Fig	Common vetch	115.94	4.67	6.519	517.96	3.29	29.122	3.65 Common vetch	0.82 Fig
Black mulberry	Barley	163.53	2.53	9.276	208.77	2.70	11.842	10.65 Barley	8.34 Black mulberry

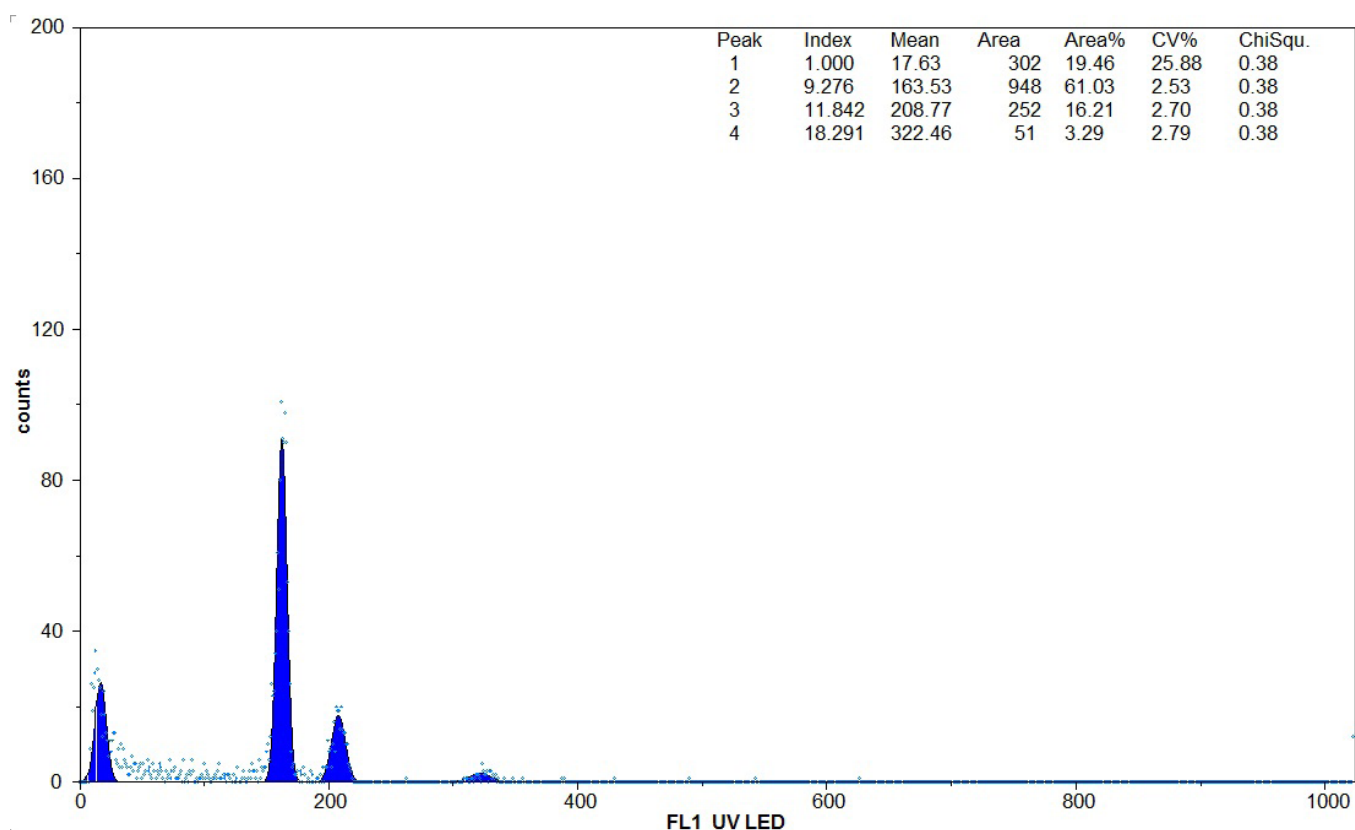


Figure 2. Fluorescence intensity (relative DNA content) of *Morus nigra* (peak 2) and *Hordeum vulgare* (peak 3) in flow cytometry. The first peak was a default for the DNA or cell noise signal.

Conclusion

The 2C DNA contents of the fig and black mulberry were found as 0.82 pg and 8.34 pg, respectively. The calculated 1C value of fig and black mulberry genomes were 401.8 Mbp and 4086.6 Mbp, respectively. The ratio of 2C DNA content and 1C genome value of the black mulberry was 10.17 times that of the fig although *F. carica* is diploid and *M. nigra* decaploid.

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