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SHORT COMMUNICATION

# Nuclear DNA content estimations in wild olive (*Olea europaea* L. ssp. *europaea* var. *sylvestris* Brot.) and Portuguese cultivars of *O. europaea* using flow cytometry

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**Abstract** Olive tree (*Olea europaea* L.) is an economically important woody fruit crop widely distributed in the Mediterranean regions. In this work the genome size of six Portuguese cultivars of olive (*O. europaea* ssp. *europaea* var. *europaea*) and wild olive (*O. europaea* spp. *europaea* var. s*ylvestris*) was estimated for the first time. The nuclear DNA content of *O. europaea* cultivars ranged between 2.90 ± 0.020 pg/2C and 3.07 ± 0.018 pg/2C and the genome size of wild olive was estimated as 3.19 ± 0.047 pg/2C DNA. These results suggest a low intraspecific variation at least among the studied cultivars and between them and wild olive. This is not in accordance with previous results in some Italian cultivars where high genome size heterogeneity was found. The methodology presented here seems appropriate for genome size estimations within this genus and opens good perspectives for a large screening of estimation of nuclear DNA content among *O. europaea* cultivars and *Olea* species that could clarify this issue.

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## Introduction

The genus *Olea* L. (Oleaceae) consists of around 30 species distributed in Europe, Asia, and Africa. Olive tree (*Olea europaea* L.) is a woody fruit crop widely distributed in the Mediterranean regions and comprises several economically important cultivars and wild olive genotypes. The Mediterranean basin provides ideal conditions for olive growing, and supplies more than 97% of the world production of olive (Rugini 1995). Despite of great economical importance, little attention was given to the cytogenetics of this species ($2n = 46$ chromosomes) with most of the studies concerning the genetic diversity of this species using molecular markers (e.g. Gemas et al. 2004).

Despite being a useful descriptor for characterization of plant genetic resources (e.g. Jarret et al. 1994), nuclear DNA content of olive cultivars was only determined for the first time by Rugini et al. (1996), who used Feulgen cytophotometry to estimate the 2C nuclear DNA content of cvs. 'Frantoio' and 'Leccino'. More recently and using the same technique, Bitonti et al. (1999) estimated the genome size of cvs. 'Dolce Agogia' and 'Pendolino'. The results of these studies indicated a

high genome size intraspecific variation among the studied Italian cultivars. Bitonti et al. (1999) also analysed the genome size of other *Olea* species and verified that it was considerably lower than the genome size of *O. europaea* cultivars.

In recent years, flow cytometry (FCM) became the dominant technique for genome size and DNA ploidy level analyses in plant sciences (for a review of its applications see Doležel 1997), mainly because of its rapidity, ease and accuracy, which is unsurpassed by other classical methodologies as Feulgen microspectrophotometry (Doležel and Bartos 2005).

Until now, this technique was never applied in the study of nuclear DNA content of *O. europaea* and its cultivars. In this study, FCM was applied to estimate for the first time the genome size of six economically important Portuguese cultivars of *O. europaea* ssp. *europaea* var. *europaea* and wild olive (*O. europaea* ssp. *europaea* var. *sylvestris*). Our goal was to verify the occurrence of intraspecific variation within this species and compare the obtained values with previous ones, already available for other cultivars of this species.

## Material and methods

Plant material of six *O. europaea* cultivars was collected from healthy mature field trees (Table 1) of the Germplasm collection of the Direcção Regional de Agricultura de Trás-os-Montes, Mirandela Portugal. The wild olive plant material was obtained from a field tree in Porto de Mós, Portugal. Young leaves were collected and kept in moistened paper for a maximum period of 2 days until FCM analysis.

Nuclear suspensions were obtained from leaves following the protocol developed by Galbraith et al. (1983). In brief, nuclei were released from cells by chopping half a young leaf (1–2 $cm^2$) of both *O. europaea* and *Pisum sativum* cv. 'Ctirad' (internal reference standard with 2C = 9.09 pg of DNA, Doležel et al. 1998; kindly provided by Jaroslav Doležel, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Olomouc, Czech Republic), in a glass Petri dish containing 1 mL of LB01 nuclear isolation buffer (Doležel et al. 1989) [15 mM TRIS, 2 mM $Na_2EDTA$, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl and 0.1% (v/v) Triton X-100, pH 7.5]. The buffer had to be supplemented with 15 mM of ß-mercaptoethanol to reduce the interaction between secondary compounds and propidium iodide (PI) staining. Also, the chopping of *O. europaea* leaves was quick (30 s) and not very intense. The nuclear suspension was then filtered through a 50 µm nylon filter and nuclei were stained with 50 µg $mL^{-1}$ PI (Fluka, Buchs, Switzerland). Also,

**Table 1** Nuclear DNA content of *Olea europaea* ssp. *europaea* var. *europaea* cultivars and wild olive (*O. europaea* ssp. *europaea* var. *sylvestris*). The values are given as mean and standard deviation of the mean (SD) of the nuclear DNA content (pg/2C) and as mean of the 1C genome size in Mbp. The range of values (min.–minimum, max.–maximum) obtained for each cultivar and wild olive is also presented. The mean coefficient of variation (CV, %) of the 2C peak, the number of genotypes tested (*n*) and the number of replicates made per genotype (R) are also given

| Subspecies and variety | Cultivar | Nuclear DNA content (pg/2C) | | | | 1C genome size (Mbp)** | CV (%) | *n* (R) |
|---|---|---|---|---|---|---|---|---|
| | | Mean* | SD | Min. | Max. | | | |
| *europaea* var. *europaea* | 'Redondal' | 3.04a | 0.042 | 2.96 | 3.09 | 1488 | 3.83 | 3 (2) |
| *europaea* var. *europaea* | 'Santulhana' | 3.07a | 0.018 | 3.06 | 3.11 | 1500 | 3.18 | 3 (2) |
| *europaea* var. *europaea* | 'Negrinha' | 3.07a | 0.013 | 3.06 | 3.09 | 1502 | 2.76 | 3 (2) |
| *europaea* var. *europaea* | 'Madural' | 3.05a | 0.012 | 3.03 | 3.07 | 1492 | 3.16 | 3 (2) |
| *europaea* var. *europaea* | 'Verdeal' | 2.97b | 0.020 | 2.95 | 3.00 | 1453 | 2.66 | 3 (2) |
| *europaea* var. *europaea* | 'Cobrançosa' | 3.03ab | 0.035 | 2.96 | 3.07 | 1480 | 2.93 | 3 (2) |
| *europaea* var. *sylvestris* | – | 3.19c | 0.047 | 3.12 | 3.24 | 1558 | 3.32 | 1 (5) |

* Means followed by the same letter are not statistically different according to the multiple comparison Tukey-Kramer test at $P \leq 0.05$

** 1 pg DNA = 978 Mbp according to Dolezel et al. (2003)

50 μg $mL^{-1}$ RNase (Sigma, St Louis, USA) was added to nuclear suspension to prevent staining of double stranded RNA. Samples were kept on ice and analysed within a 10 min period in a Coulter EPICS XL (Coulter Electronics, Hialeah, Florida, USA) flow cytometer. The instrument was equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm. PI fluorescence was collected through a 645-dichroic long-pass filter and a 620-band-pass filter. Doublets, partial nuclei, nuclei with associated cytoplasm and other debris were removed from analysis using a specific gating region defined in a linear-fluorescence light scatter (FL) pulse integral versus FL pulse height cytogram. For each sample at least 5,000–10,000 nuclei were analysed.

The nuclear genome size of *O. europaea* was estimated according to the following formula:

$$O.\ europaea\ \text{2C nuclear DNA content (pg)} = \frac{O.\ europaea\ \text{2C peak mean}}{P.\ sativum\ \text{2C peak mean}} \times 9.09 \quad (1)$$

Conversion into base-pair numbers was performed using the following factor: 1 pg = 978 Mbp (Doležel et al. 2003).

Three field trees were analysed for each *O. europaea* cultivar and two replicates for each tree were performed in two different days to avoid possible instrumental drift. One field tree of wild olive was analysed five times in two different days.

Genome size data was analysed using a one-way ANOVA (SigmaStat for Windows Version 3.1, SPSS Inc., USA) procedure. A Tukey-Kramer multiple comparison test was used for pair-wise comparison.

## Results and discussion

The determination of the nuclear DNA content of *O. europaea* Portuguese cultivars ranged between 2.90 ± 0.020 pg/2C (cv. 'Verdeal') and 3.07 ± 0.018 pg/2C (cv. 'Santulhana'). The genome size of wild olive was estimated as 3.19 ± 0.047 pg/2C DNA (Table 1). Statistical analyses revealed significant differences between wild olive and all tested cultivars and, with the exception of cv. 'Cobrançosa', between cv. 'Verdeal' and the remaining cultivars. Nevertheless, the difference between extreme values of genome size is only of 3.3% for the Portuguese cultivars, and only of 6.9% between wild olive and cv. 'Verdeal'. It is therefore difficult to attest with certainty that these differences are real and not derived from technique related variations. In spite of that, previous estimations of genome size within this species using the Feulgen cytophotometry technique, revealed high nuclear DNA content heterogeneity among cultivars (Rugini et al. 1996; Bitonti et al. 1999). In these works, the genome size of several Italian cultivars of olive was estimated and the obtained values ranged from 3.90 pg/2C (cv. 'Dolce Agogia') to 4.66 pg/2C (cv. 'Pendolino'), which accounts for a 16.3 % difference. Bitonti et al. (1999) found that these DNA content determinations were positively correlated with the copy number of DNA repeats in the genomes studied, indicating that these genomes could differ in their amount of repetitive DNA.

Genuine intraspecific variation involving differences in heterochromatic sequences are known but rather rare. An example of this was described in the subspecies pair *Scilla bithynica* Boiss. spp. *bithynica*, which presents many large C-bands and a 1C genome size of 29.2 pg and *S. bithynica* ssp. *radkae*, with few small C-bands and a 1C genome size of 22.9 pg of DNA (Greilhuber 1998). On the other hand, several reports concerning intraspecific variation that suggested a rather plastic genome were further rebutted after reinvestigation of the original material (for a review see Suda 2004). In most of these cases, the supposed intraspecific variation was a result of technical artefacts and/or taxonomic errors (Greilhuber et al. 1998). Methodological errors that cause non-stoichiometric binding of the fluorochrome are often responsible for such variations. As an example, hot hydrolysis (1 M HCl, 60°C), which is not recommended because the staining optimum lasts for a very short time, is often used by many authors (e.g. Cavallini et al. 1996; Bitonti et al. 1999).

An interesting case of a supposed plastic genome, which was further disproved is sunflower, *Helianthus annuus* L. Cavallini et al. (1996) using

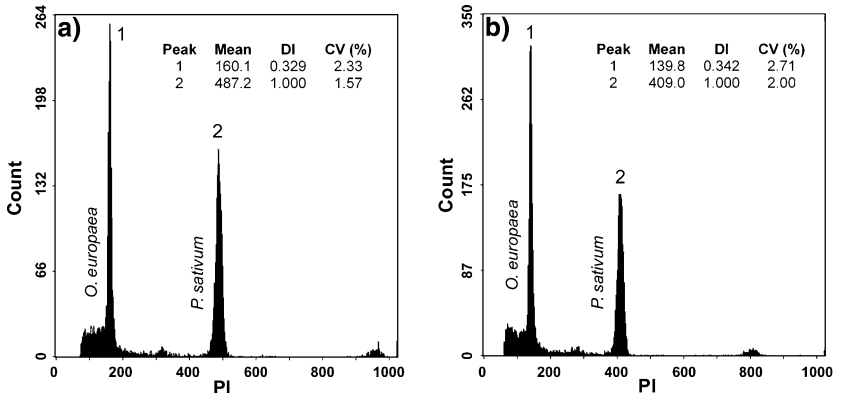


**Fig. 1** Histograms of relative fluorescence intensity obtained after simultaneous analysis of nuclei isolated from *Pisum sativum* cv. Ctirad (2C = 9.09 pg DNA, as an internal reference standard) and *Olea europaea*: (**a**) ssp. *europaea* var. *europaea* cv. 'Verdeal' and (**b**) ssp. *europaea* var. *sylvestris*. The mean channel number, DNA index (DI = mean channel number of sample/mean channel number of reference standard) and coefficient of variation (CV, %) value of each peak is also given

Feulgen cytophotometry, found a 1.58 fold genome size variation between lines of *H. annuus* and suggested a correlation between these nuclear DNA changes and the degree of methylation and amount of repetitive DNA. Also in this species, Johnston et al. (1996) and Price and Johnston (1996) using FCM, found a 287% intraspecific difference and claimed that the genome size of sunflower varied with light quality and quantity. Nevertheless, subsequent re-evaluation by Price et al. (2000) revealed that these differences were caused by a technical artefact caused by the release of cytosolic compounds during the nuclei isolation procedure. These compounds interfered with the analysis and their levels were influenced by the quantity and quality of the light. As cytosolic compounds, like phenols, are common, at least in woody plant species, intraspecific genome size variation must be interpreted with caution, until an independent confirmation is made.

Olive trees, as other woody plants, appeared to be difficult species to isolate nuclei in proper conditions for FCM estimation of nuclear DNA content. In initial experiments this was the case, as nuclei isolated in LB01 buffer without protectants such as ß-mercaptoethanol, metabissulfite or polyvinyl pirrolydone (PVP-10) were very instable and histograms of relative fluorescence intensity presented peaks with poor resolution (coefficient of variation, CV > 5.0%), suggesting the possible interference of cytosolic compounds (data not shown). The addition of ß-mercaptoethanol (an anti-oxidant used to suppress the interference of secondary products, Galbraith et al. 2002), and the reduction of tissue chopping intensity (to decrease the release of cytosolic compounds), were fundamental to obtain nuclear fluorescence stability and peaks with good CV values (Fig. 1, Table 1). Also, very low background noise was obtained in histograms, leading to the conclusion that the followed methodology provided unbiased estimations of nuclear DNA content for this species. This procedure has therefore the potential to be used in the genome size estimation of other *O. europaea* cultivars and olive species.

Taking in consideration the low heterogeneity of the nuclear DNA content estimations found in this work, and the contrary data obtained previously for Italian cultivars of olive, which point in the direction of intraspecific variation, it would be interesting to estimate the genome size of a vast number of olive cultivars (there are up to 2,600 identified cultivars, Rugini and Lavee 1992) and related species to ascertain which case is correct. If the intraspecific variation is confirmed, FCM and other genome characters can be used to easily identify some *Olea* cultivars and help understanding the relationships between species within *Olea* genus and the phylogenesis of cultivated olive.

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