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Aquatic Botany 77 (2003) 17–25

Aquatic botany

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Genome size of Adriatic seagrasses

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Received 20 February 2002; received in revised form 30 January 2003; accepted 7 April 2003

Abstract

Genome size (C-value) was measured in four species of Adriatic seagrasses with interphase-peak DNA image cytometry. The estimated 2C-value was 1.5 pg DNA for *Zostera noltii* ($2n = 12$), 1.2 pg for *Zostera marina* ($2n = 12$), 1.1 pg for *Cymodocea nodosa* ($2n = 28$) and 6.2 pg for *Posidonia oceanica*, using *Pisum sativum* (2C-value = 8.84 pg) as the calibration standard. Seagrass leaves were fixed in 4% buffered formaldehyde to mitigate stoichiometric error due to tannins and post-fixed in 3:1 methanol:acetic acid (MAA). DNA was stained with the Feulgen reaction after hydrolysis in 5 M HCl for 90 min at 20 °C. Comparison of genome size of seagrasses with the data for other species of Alismatidae indicated that the ancestral genome of Alismatidae was relatively small.

Keywords: Seagrasses; Alismatidae; Genome size; C-value; DNA image cytometry

1. Introduction

Seagrasses are a polyphyletic group comprising about 60 marine angiosperm species growing in the littoral zone of tropical and temperate seas (Kuo and McComb, 1989). Seagrass meadows are a major source of primary production, responsible for 15% of the total excess carbon produced in the global ocean (Duarte and Chiscano, 1999). They provide habitat and food for associated organisms and modulate sedimentary and biogeochemical processes. Seagrasses are thus an important component of marine ecosystems; however, their distribution is in decline due to anthropogenic disturbances (Nybakken, 2001).

Four of five Mediterranean seagrass species (Den Hartog et al., 1987) occur in the northern Adriatic Sea, along the coast of Slovenia and the western coast of the Istria peninsula

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doi:10.1016/S0304-3770(03)00072-X

in Croatia (Jogan, 1994). *Cymodocea nodosa* and *Zostera noltii* are common, but *Zostera marina* and *Posidonia oceanica* are rare. *P. oceanica* is listed in the Red Data List of Threatened Vascular Plants in Slovenia. Its only natural habitat in Slovenia is about 50 m wide and 1 km long and is protected as a natural monument (http://www.dragonja.mbss.org/Zusterna/indexS.html).

The ecology of seagrasses has been extensively investigated (Duarte, 1999), whereas studies on their phylogeny are limited (Les, 1988; Les et al., 1997), and little is known about their population genetics (Reusch, 2001). The morphology of chromosomes has been analysed in several seagrass species (Den Hartog et al., 1987; Uchiyama, 1989; Kuo et al., 1990), but prior to our study nuclear genome size has been measured for only one species, *P. oceanica* (Cavallini et al., 1995). Very recently genome size estimate was published also for *Z. marina* (Hanson et al., 2003).

Data on nuclear genome size, which are usually expressed as DNA C-value (Swift, 1950), are used in diverse scientific disciplines, including systematics, ecology, conservation, cell and molecular biology, and physiology (Bennett et al., 2000). Genome size data are required in detailed analyses of genome structure and evolution. The choice of species for future genome sequencing projects will depend on knowledge of their genome sizes (Hardie et al., 2002). However, presently available plant C-value data are fragmentary (Bennett et al., 2000), and a first genome size estimate is still not available for about half of the angiosperm families (Hanson et al., 2003).

In the present study, we measured genome size in all species of an ecologically defined group—in seagrasses of the northern Adriatic Sea. For two of the four investigated species, we provide the first genome size data. Genome size was estimated with DNA image cytometry, which is a modern version of Feulgen densitometry (Hardie et al., 2002), and has been recently added to the methods available for genome size measuring in plants (Vilhar et al., 2001). We optimised the method with a special regard to specific problems expected to be associated with seagrass tissues, such as interference of tannins with the staining reaction (Greilhuber, 1986).

2. Material and methods

2.1. Plant material

Samples of seagrasses were collected in up to 2 m deep coastal waters. *Z. marina* L. was collected near Fiesa (Slovenia; 45°31.4′N, 13°35.2′E), *P. oceanica* (L.) Del. near Koper (Slovenia; 45°32.7′N, 13°43′E), *C. nodosa* (Ucria) Asch. and *Z. noltii* Hornem near Novigrad (Croatia; 45°19′N, 13°33.5′E). Species were determined using the determination key of Jogan (1994). Herbarium vouchers were deposited at LJU (numbers 131 863; 132 425; 131 769; 131 770).

2.2. Experimental design

Genome size of seagrasses was measured with DNA image cytometry. Several experiments were conducted to optimise the method. The potential interference of tannins with

staining intensity was tested in *Z. noltii* using two fixatives, formaldehyde and methanol–acetic acid (MAA). Hydrolysable gallotanins were detected in hand-cut cross sections of fixed leaves with 3% $FeCl_3$ (Greilhuber, 1986). In addition, genome size was measured with flow cytometry.

2.3. Fixation of samples for image cytometry

In all investigated seagrass species, the basal part of the shoot was dissected from the rhizome, the outer most leaves were removed and the remaining leaves were trimmed to retain the lower 1 cm. Samples were fixed in 4% phosphate-buffered neutral formaldehyde for 1.5 h at room temperature, followed by post-fixation in 3:1 methanol:acetic acid for 24 h at 4 °C (Greilhuber and Temsch, 2001). Fixed samples were stored in 96% ethanol at −20 °C. To test the influence of the fixative on the accuracy of genome size measurement, some *Z. noltii* samples were fixed directly in MAA for 24 h at 4 °C (Greilhuber and Temsch, 2001).

Root tips of the calibration standard species *Pisum sativum* L. cv. 'Kleine Rheinlaenderin' were fixed and further processed in the same vials as the tissues of seagrasses, as recommended by Greilhuber and Temsch (2001).

2.4. Measurement of nuclear DNA amount with image cytometry

Prior to staining with the Feulgen reagent, samples were hydrolysed in 5 M HCl at 20 ± 0.1 °C. The influence of hydrolysis time on the intensity of Feulgen staining was investigated in formaldehyde-fixed tissues of *P. oceanica* and *P. sativum*. The obtained hydrolysis curves were similar to the results of Greilhuber and Temsch (2001), and the optimal hydrolysis time of 90 min was subsequently used for all formaldehyde-fixed material. Tissues fixed in MAA were hydrolysed for 60 min (Greilhuber and Baranyi, 1999).

After hydrolysis, samples were washed in ice-cold distilled water for 5 min, stained with the Feulgen reagent (pararosaniline chloride, BDH, UK) for 120 min at 20 °C, washed in several changes of SO_2–water for a total of 45 min and washed in distilled water (Greilhuber and Temsch, 2001). Stained tissue was dissected and squashed in a drop of 45% acetic acid. The cover slips were removed with the dry-ice method, the slides were briefly washed in ethanol, air dried for 1 h, mounted in DPX (Fisons, UK) and dried in the dark for at least 3 days.

Nuclear DNA amount was measured with DNA image cytometry, using the interphase-peak method (Vilhar et al., 2001). The image analysis instrumentation was as described in Vilhar et al. (2001), and was calibrated according to Vilhar and Dermastia (2002). Integrated optical density (IOD) and nuclear area were measured for 200 to 300 interphase nuclei per slide. *P. sativum* was used as the calibration standard (2C-value = 8.84 pg DNA, Greilhuber and Ebert, 1994) for conversion of nuclear DNA amount from arbitrary units to picograms of DNA. The Feulgen-stained slides were also used to determine the number of chromosomes.

2.5. Measurement of nuclear DNA amount with flow cytometry

Plants were uprooted and kept in an aerated container with seawater until measurement. Fresh young leaves of examined seagrass species and leaves of the standard species *Trifolium*

repens (2C-value = 2.07 pg DNA, Arumuganathan and Earle, 1991) were chopped in isolation buffer LB01 (Doležel et al., 1989) with a sharp razor blade to isolate nuclei. The samples were stained with 50 μg/ml propidium iodide (Sigma, D) and treated with 50 μg/ml RNase (Sigma, D). At least 10 000 nuclei were analysed per sample with a Partec PAS flow cytometer, equipped with 488 nm argon laser. The ratio between the 2C peak mean of the seagrass and the standard species was used for conversion of nuclear DNA amount from arbitrary units to picograms of DNA.

3. Results and discussion

3.1. Optimisation of DNA image cytometry

The first step in DNA image cytometry is the choice of a suitable tissue, which should ideally contain rapidly dividing cells without substances that interfere with the Feulgen staining. Root tips are commonly used for this purpose. The investigated seagrass species have firm roots, so root tips were mostly torn when we pulled them out of the sediment. In the northern Adriatic Sea, these species rarely produce flowers and seeds (Jogan, 1994), hence root tips could not be obtained from seedlings either. Therefore, we investigated the possibility to use leaf tissue for genome size measurement.

In the leaves of seagrasses, specialized cells accumulate different phenolic compounds, in particular tannins (Kuo and McComb, 1989), which may interfere with the Feulgen staining (Greilhuber, 1986, 1988). In the investigated species, we detected small amounts of tannins in the leaf base, in the intercalary meristem and in young leaves. The number and the size of the tannin cells in the mesophyll increased from the proximal to the distal part of the leaf lamina and in older leaves.

To investigate the possible influence of tannins on the accuracy of genome size measurements in seagrasses, we compared tissues of *Z. noltii* fixed in two different fixatives. Namely, tannin diffusion causes reduction of DNA staining in tissues fixed in commonly used acetic alcohol fixatives, e.g. MAA (Greilhuber, 1986, 1988). This stoichiometric error can be mitigated with formaldehyde fixative, which polymerises tannins in the vacuoles (Greilhuber, 1986). The intensity of Feulgen staining was almost 50% lower in MAA-fixed leaves than in formaldehyde-fixed leaves. A 20–70% reduction in the intensity of the Feulgen staining has been reported before for different plant tissues containing tannins (Greilhuber, 1986, 1988).

We used few collected intact root tips of *Z. noltii* to investigate whether the source tissue affected the accuracy of the measurement. We detected no significant difference between the intensity of Feulgen staining measured for root tips (2C-value = 57.0 ± 4.0 arbitrary units; $N = 3$) and for young leaves (2C-value = 59.2 ± 4.3; $N = 5$; *t*-test $P = 0.74$).

3.2. Genome size of Adriatic seagrasses

On the basis of the above observations, the youngest leaves were dissected from shoots fixed in formaldehyde and used for genome size measurement with image cytometry in the investigated seagrasses. The 2C-value estimates were 1.1 pg for *C. nodosa*, 1.2 pg for *Z. marina*, 1.5 pg for *Z. noltii*, and 6.2 pg for *P. oceanica* (Table 1).

Table 1
Nuclear DNA amount of northern Adriatic seagrasses

Species	Number of chromosomes	Image cytometry	Flow cytometry	
		2C (pg)	2C (pg)	*t*-test
Zostera noltii	12	1.54 ± 0.04 (7)	0.94 ± 0.03 (4)	***
Zostera marina	12	1.22 ± 0.06 (11)	–	–
Cymodocea nodosa	28	1.08 ± 0.06 (7)	0.64 ± 0.01 (5)	***
Posidonia oceanica	(20)[a]	6.25 ± 0.13 (11)	7.27 ± 0.10 (6)	***

Image cytometry: each microscope slide of the seagrass species was measured against one slide of the standard species *Pisum sativum* (2C-value = 8.84 pg DNA; Greilhuber and Ebert, 1994). Flow cytometry: leaves of the seagrasses and of the standard species *Trifolium repens* (2C-value = 2.07 pg DNA; Arumuganathan and Earle, 1991) were processed together and stained with propidium iodide. Each sample was measured four times. The mean 2C-value ± S.E. is shown, with the number of independent samples per slides in parenthesis. The difference between the two methods for each species was analysed with *t*-test.

[a] Data from Den Hartog et al. (1987).

*** $P < 0.001$.

Genome size in *Z. marina* was significantly smaller than in *Z. noltii* (*t*-test, $P < 0.01$). This difference in genome size between the two species is in agreement with previous observations that *Z. marina* chromosomes are smaller and dot-like, compared to longer and thread-like chromosomes of *Z. noltii* (Den Hartog et al., 1987), and that the total chromosome length is lower in *Z. marina* than in *Z. noltii* (Uchiyama, 1989).

The previously published 2C-values of seagrasses were 5.6 pg DNA for *P. oceanica* (Cavallini et al., 1995) and 0.6 pg for *Z. marina* (Hanson et al., 2003). Both values are lower than our genome size estimate for the respective species (Table 1). Notably, acetic alcohol fixative was used in both studies (Cavallini et al., 1995, L. Hanson, pers. commun.). With respect to the results of our image cytometry optimisation experiments, these discrepancies in genome size estimates among different laboratories are more likely due to stoichiometric errors caused by tannins than to true biological variation in genome size within species.

We evaluated the results obtained with image cytometry according to the proposed quality control standards (Vilhar et al., 2001). The coefficient of variation of the 2C peak (CVp) for each measured slide was just below the recommended upper limit of 6% (Vilhar et al., 2001) in *Z. noltii* (5.6 ± 0.1%, mean ± S.E., *N* indicated in Table 1), but higher in *P. oceanica* (7.4 ± 1.1%), *C. nodosa* (8.4 ± 1.5%) and in *Z. marina* (9.7 ± 0.8%). This variation of nuclear DNA amount measurements was the lowest we could achieve with any of the tested experimental procedures. Hence, the proposed 6% limit for the coefficient of variation of the 2C peak may be too stringent for measurements of plant material containing different interfering compounds.

Additionally, we measured 2C-values of the seagrasses with flow cytometry, after quantitative staining of DNA with propidium iodide. Compared to image cytometry, the flow cytometry data were significantly lower in *Z. noltii* and *C. nodosa*, and higher in *P. oceanica* (Table 1). The upper limit for the coefficient of variation of the 2C peak obtained with flow cytometry has been recommended only for medical applications, and is 3% (Ormerod et al., 1998). In our study, the coefficient of variation of the 2C peak was in this range in *P. oceanica* (2.6 ± 0.1%), but was higher in *Z. noltii* (11.1 ± 0.3%) and in *C. nodosa*

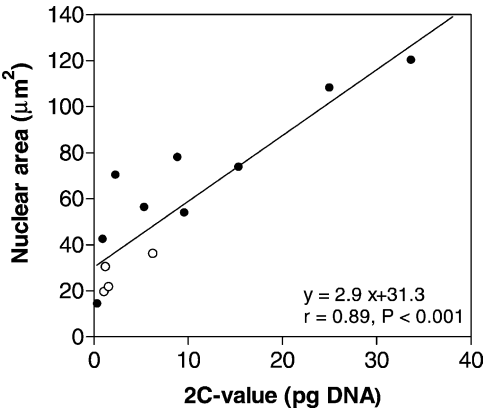

Fig. 1. Relationship between 2C-value and nuclear area. (○) Seagrasses (data from Table 1); (●) species from small to large 2C-value: *Arabidopsis thaliana*, *Raphanus sativus*, *Glycine max*, *Zea mays*, *Pisum sativum*, *Hordeum vulgare*, *Secale cereale*, *Vicia faba*, *Allium cepa* (2C data from Vilhar et al., 2001). The correlation between 2C-value and nuclear area was analysed with linear regression.

(15.2 ± 1.7%). It is therefore possible that some compounds from seagrass leaves interfered with measurements, as has been reported by Price et al. (2000) for sunflower.

To summarise, image cytometry proved to be more practical than flow cytometry for several reasons. Flow cytometry requires fresh material, therefore special transportation of living seagrass specimens to the laboratory and immediate measurement of nuclear DNA amount was needed. On the other hand, DNA image cytometry allowed for fixation of plant material at the collecting site and long-term storage until measurements. Furthermore, few dividing cells were present even in the youngest leaves, so only one prominent peak was observed with flow cytometry and it was therefore not clear whether it represented the 2C or the 4C nuclei. With interphase-peak DNA image cytometry, it was possible to corroborate the position of the 2C and the 4C peak on the basis of the few cells determined to be in late telophase (2C) or in early prophase (4C).

DNA image cytometry also allowed for measurement of nuclear size. We compared the area of the 2C nuclei in the investigated seagrasses and in nine plant species with genome sizes ranging from 0.3 to 33 pg DNA. Linear regression analysis showed a positive correlation between genome size and the nuclear area (Fig. 1), which is in agreement with previous observations by Van't Hof and Sparrow (1963). The relatively small nuclear size in all four seagrasses is consistent with a relatively small genome size measured in these species (Fig. 1).

3.3. Genome size and phylogeny of Alismatidae

We compared genome size of all four investigated seagrass species with known genome sizes of other monocots. The 2C-value of seagrass species was smaller than the median value of 11.5 pg for 1494 monocot species (data from Bennett and Leitch, 2001; and this study). The 2C-values of *Z noltii*, *Z. marina* and *C. nodosa* were all in the first 10 percentiles

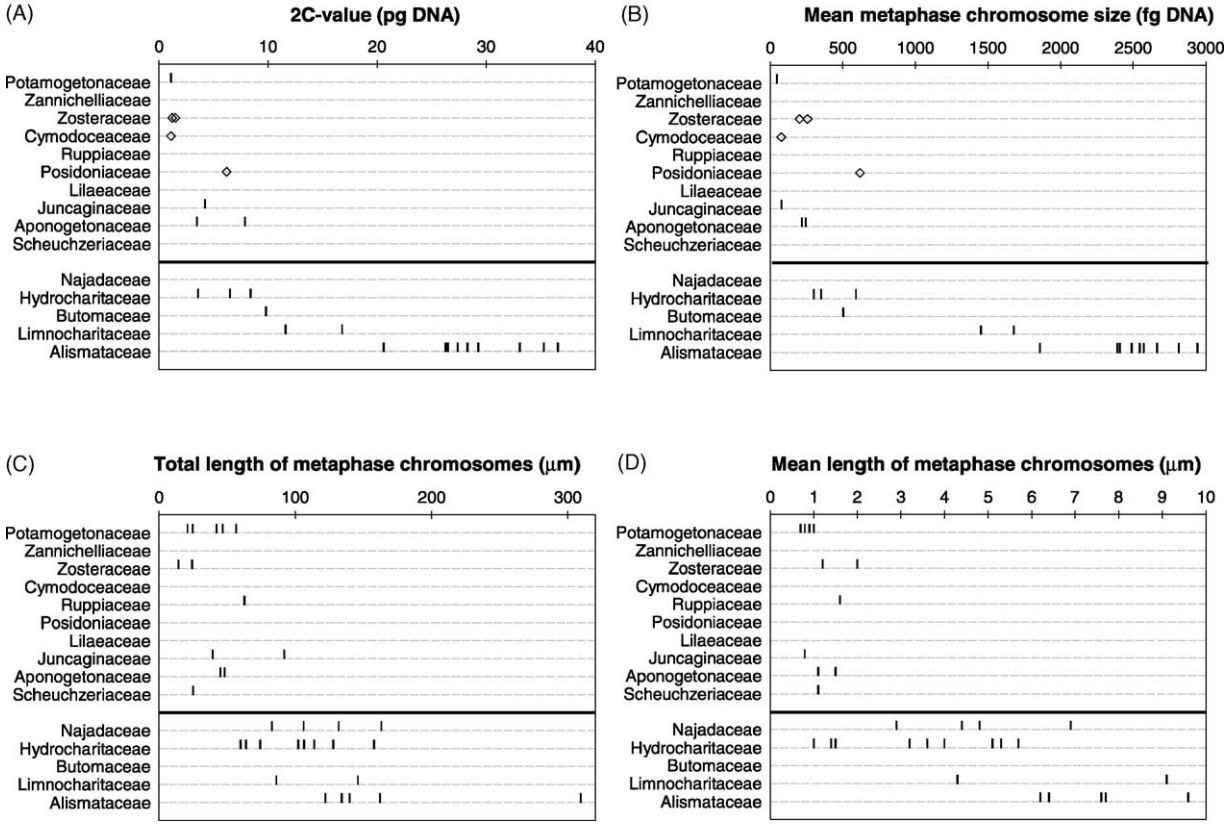

Fig. 2. 2C-value and chromosome size in Alismatidae. (A and B) Data from Bennett and Leitch (2001, ▮) and this study (◇); (C and D) data from Uchiyama (1989). Two major phylogenetic lineages of Alismatidae, according to Les et al. (1997), are separated with a bold horizontal line.

of the monocot values, and that of *P. oceanica* in the 31st percentile. Hence, the first three seagrass species had very small genomes compared to other monocots, while the genome of *P. oceanica* was in the middle range.

All seagrasses belong to the subclass Alismatidae. Phylogenetic studies based on analysis of chloroplast *rbcL* gene sequence data indicated two major lineages in this subclass (Les et al., 1997). The first major lineage, which includes seagrasses, is characterised by a small genome size, low total chromosome length and small chromosomes compared to the second lineage (Fig. 2, data from Uchiyama, 1989; Bennett and Leitch, 2001). Analysis of the evolutionary aspect of genome size variation in phylogenetic lineages of monocots revealed that early diverging branches display smaller genome sizes with a more narrow range of C-values, while large genomes are restricted to more derived families (Leitch et al., 1998). In this respect, it is likely that the ancestral genome of Alismatidae was small with small chromosomes, and a marked increase in genome size accompanied by an increase in chromosome size occurred only in the second major lineage (Fig. 2). Slow evolutionary rate and low species diversity, observed also in other hydrophytes (Les, 1988), may be one explanation for the preservation of the relatively small ancestral genome size in seagrasses.

Acknowledgements

We thank Prof. Tom Turk and Dr. Aleš Koce for help with collecting of the experimental material. J. Dolenc Koce was a recipient of a grant no. 38/12 from the Ministry of Education and Sport of the Republic of Slovenia.

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