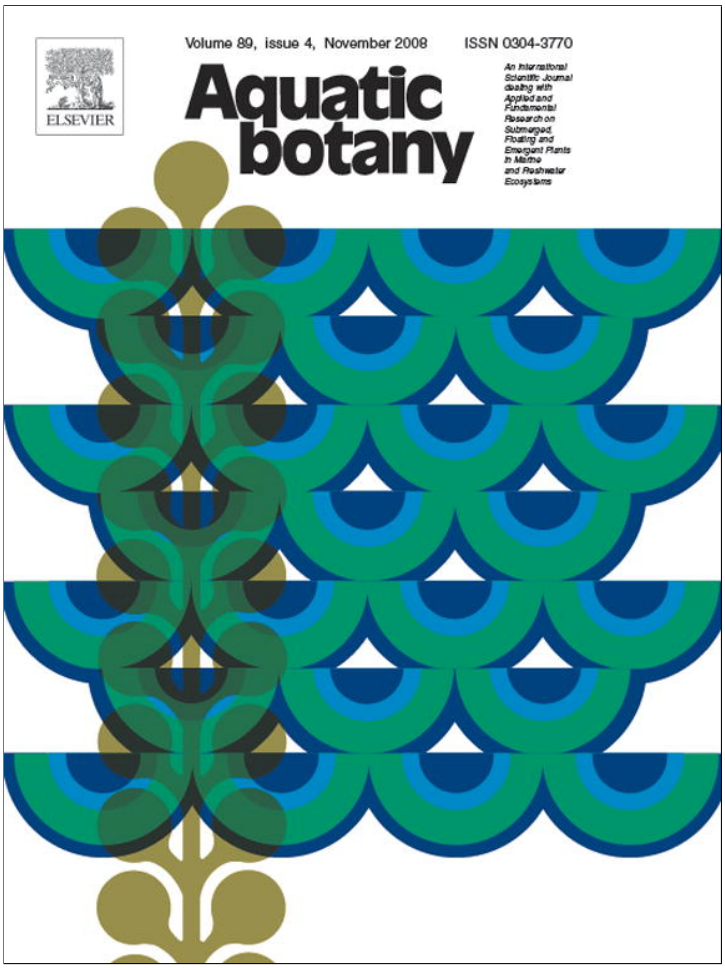

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Amounts of nuclear DNA in marine halophytes

Jasna Dolenc Koce [a,*], Siniša Škondrić [b], Tinka Bačič [a], Marina Dermastia [a,c]

[a] University of Ljubljana, Biotechnical Faculty, Department of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia
[b] University of Novi Sad, Faculty of Science, Department of Biology and Ecology, Trg Dositeja Obradovića 2, 21000 Novi Sad, Serbia
[c] National Institute of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia

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ABSTRACT

The amount of nuclear DNA, expressed as the *C*-value, was estimated for 13 marine halophytic plant species from six families. Plant material was collected in the nature reserve of the Strunjan saltpan in the Northern Adriatic and comprised all halophytic species inside the investigated area. Reproductive region of the shoot or root tips of halophytes were dissected, nuclei were Feulgen stained and 2*C*-values were measured by DNA image cytometry as follows: *Crithmum maritimum* (4.38 pg DNA), *Artemisia caerulescens* (6.43 pg), *Aster tripolium* (21.43 pg), *Inula crithmoides* (3.63 pg), *Atriplex portulacoides* (1.83 pg), *A. prostrata* (1.51 pg), *Salicornia europaea* (2.75 pg), *Salsola soda* (2.62 pg), *Sarcocornia fruticosa* (5.91 pg), *Suaeda maritima* (2.11 pg), *Limonium angustifolium* (5.06 pg), *Puccinellia palustris* (8.15 pg) and *Ruppia cirrhosa* (4.65 pg). With the exception of the *C*-value estimate for *A. caerulescens*, which has been listed in the Plant DNA *C*-values Database, the *C*-values represent the first estimates for all the examined species. In addition, the *C*-value for *R. cirrhosa* is also the first report for the family Ruppiaceae. The investigated halophytes had a smaller genome size compared to other known *C*-values for species within a particular family and also when compared to the mean values of dicots and monocots. The study also showed that halophylic annuals have a smaller genome size (2.49 pg) than perennial ones (7.45 pg DNA).

1. Introduction

Interest in research on the amount of DNA in plant nuclei has increased since the discovery of the key role of DNA in biology in the middle of the last century. The amount of DNA in the unreplicated haploid nuclear chromosome complement was defined by Swift (1950) as the 1*C*-value and termed holoploid genome size (Greilhuber et al., 2005). It has been suggested that the genome size is an important biodiversity character with a predictive value in systematics, ecology and evolution (Bennett and Leitch, 2005b). Data about the nuclear DNA amount are collected in Plant DNA *C*-values Database (Bennett and Leitch, 2005a), which is the largest database of nuclear DNA amounts available for any group of organisms. At the moment, 5150 plant species are listed in the database: 4427 of them are angiosperms representing 1.8% of all angiosperm species, and approx. 50% of angiosperm families. While it is practically impossible to obtain *C*-values for all taxa, estimates for 10–20% of angiosperms would be adequate for all conceivable uses, provided they were carefully targeted to be representative of various taxonomic groups, geographical regions and life forms in the global flora (Bennett and Leitch, 2005b). Therefore, several gaps in our knowledge on genome size have been identified and among them was the severe under-representation in the DNA *C*-values database of plant life forms from halophylic environments (Bennett and Leitch, 2005b).

Halophytes are mostly herbaceous angiosperms (Nybakken, 2001), which are able to grow in habitats excessively rich in salts, such as salt marshes, sea coasts and saline or alkaline semi-deserts and steppes. Although halophytes represent only 2% of terrestrial plant species, they are present in about half the higher plant families and represent a wide diversity of plant forms. For their successful growth in a unique and unpredictable environment, special physiological adaptations evolved that enable them to absorb water from soils and from seawater which have solute concentrations that non-halophytes could not tolerate. Despite their polyphyletic origin, halophytes appear to have evolved the same basic mechanism of osmotic adjustment, which makes them a rich source of potential new salt tolerant crops (Glenn et al., 1999) and many genes associated with salt tolerance have been discovered (Khan et al., 2007). Halophytes significantly participate to the biocenoses of bays, estuaries and their surrounding salt marshes, which are among the most productive systems of the biosphere (Lefeuvre et al., 2000), but often impacted by intense

* Corresponding author. Tel.: +386 1 4233388; fax: +386 1 2573390.
E-mail address: jasna.dolenc.koce@bf.uni-lj.si (J.D. Koce).

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anthropogenic activities that cause pollution and damage from construction and development (Ungar, 1991).

The aim of the present work was to estimate the size of the holoploid genome for marine halophyte species from the Strunjan saltpan on the Slovenian coast of the Northern Adriatic. The saltpan is the northernmost saltpan in the Mediterranean region, and together with the adjacent freshwater lagoon, represents an area under the combined effects of salt- and freshwater. The saltpan is a nature and landscape reserve and is also included in Natura 2000—an EU-wide network of nature protection areas established under the 1992 Habitats Directive and the centrepiece of EU nature and biodiversity policy (http://ec.europa.eu/environment/nature/natura2000/index_en.htm). Most of the halophytes at the site are vulnerable (V) or endangered species (E) according to IUCN (Anon., 2002; Wraber and Skoberne, 1989) and their study is thus of great conservation importance. The results of this work will therefore directly contribute to knowledge of the biodiversity of the area and since many of the halophytic species are cosmopolite, the results will be also relevant for any halophylic environment.

2. Materials and methods

2.1. Plant material

For the purpose of the study, plants, seeds (where available), shoot tips and/or flower buds of 13 halophyte species were collected at the Strunjan saltpan (Slovenia; longitude: 13°60.4′N, latitude: 45°53.2′E). Species were determined using relevant local keys (Martinčič et al., 2007). Herbarium vouchers are deposited at LJU. The halophyte species belong to six families: Apiaceae, Asteraceae, Chenopodiaceae and Plumbaginaceae, which are eudicots, and two monocot families, Poaceae and Ruppiaceae.

2.2. Measurement of nuclear DNA amount

Shoot tips and flower buds were fixed at the collection site for 90 min in 4% buffered formaldehyde, post-fixed in 3:1 methanol:acetic acid for 24 h at 4 °C and stored in 96% ethanol at −20 °C (Dolenc Koce et al., 2003). Different tissues were used for the measurement of the amount of nuclear DNA, depending on the phenological state of the species (Table 1). In most cases, the reproductive region of the shoot was used and further dissected to obtain ovulary, anther, embryo or fruit tissue, where mitotic divisions are frequent. Only in *Suaeda maritima*, *Limonium angustifolium* and *Puccinellia palustris*, seeds were germinated on a Petri dish to harvest young root tips, which were fixed as described above. Regardless the origin of the selected tissue, meristematic regions were dissected, hydrolyzed in 5N HCl for 90 min at 20 °C, stained with Feulgen reagent (pararosaniline chloride, BDH, UK) overnight in a refrigerator, washed in several changes of SO_2–water for a total of 45 min and in distilled water (Greilhuber and Temsch, 2001). Squash preparations of dissected and stained tissues were prepared in a drop of 45% acetic acid. The cover slips were removed by the dry-ice method; the slides were washed in 96% ethanol for 5 min and air dried. Root tips of the calibration standard species *Pisum sativum* cv. 'Kleine Rheinlaenderin' were processed simultaneously with the halophyte specimen during all experimental procedures.

Nuclear DNA content was measured densitometrically by DNA image cytometry, using the interphase-peak method (Vilhar et al., 2001) and expressed as the 2*C*-value. The image analysis instrumentation was as described in Bačič et al. (2007) and was calibrated according to Vilhar and Dermastia (2002). Integrated optical density (IOD) was measured for approx. 200 interphase nuclei per slide and at least 10 slides were measured per species. Arbitrary units of IOD were converted to picograms of DNA using the calibration standard *P. sativum* with a 2*C*-value of 8.84 pg DNA (Greilhuber and Ebert, 1994).

2.3. Statistical analysis

Statistical analysis was made with Excel (Microsoft) and Prism 4 (GraphPad) software. The mean 2*C*-values ± standard error (S.E.) of the families and higher taxonomic groups (monocots, eudicots, angiosperms) were calculated using data from the Plant DNA *C*-values Database (Bennett and Leitch, 2005a). The genome size data of halophytes were compared to different groups of angiosperms using the *t*-test.

3. Results

In the present study were included all halophytes that grow inside the boundaries of the saltpan Strunjan. The halophytes in the investigated area were *Crithmum maritimum*, *Artemisia caerulescens*, *Aster tripolium*, *Inula crithmoides*, *Atriplex portulacoides*, *A. prostrata*, *Salicornia europaea*, *Salsola soda*, *Sarcocornia fruticosa*, *S. maritima*, *L. angustifolium*, *P. palustris* and *Ruppia cirrhosa*. They are herbaceous perennials and annuals with a late flowering period, since most of them flower in summer and autumn (Table 1).

First genome size estimates were determined for 12 out of 13 examined halophyte species (Table 2). Among them is the first holoploid genome size estimate for a plant from the family Ruppiaceae. The measured *C*-values ranged from 1.51 pg DNA in *Atriplex prostrata* to 21.43 pg DNA in *Aster tripolium*. The smallest amounts of nuclear DNA were measured in halophytes from the family Chenopodiaceae. In *A. prostrata* and *A. portulacoides* different *C*-values were measured within the same tissue, ranging from 0.37 to 2.62 pg DNA in *A. prostrata*, and from 0.66 to 3.41 pg DNA in *A. portulacoides*. The 2*C*-values for both species given in Table 2 are the mean 2*C*-values and the high variation of the measured data is indicated by the large standard error. With the exception of *Aster tripolium* all other halophytes had 2*C*-values smaller than 10 pg DNA (Table 2). Since no metaphase chromosomes were clearly seen in our sample preparations, we were not able to determine the chromosome numbers and ploidy levels in particular nuclei. Therefore, data from Flora Europaea (Tutin et al.,

Table 1
List of halophytes collected in the Strunjan saltpan, including the origin of the tissue dissected for genome size measurement

Family	Species	Life cycle[a]	Tissue
Apiaceae	*Crithmum maritimum* L.	HP	Ovulary
Asteraceae	*Artemisia caerulescens* L.	HP	Ovulary
	Aster tripolium L.	HP	Ovulary
	Inula crithmoides L.	HP	Ovulary
Chenopodiaceae	*Atriplex portulacoides* L.	A	Ovulary
	Atriplex prostrata Bouch. ex DC.	A	Ovulary
	Salicornia europaea L.	A	Anther
	Salsola soda L.	A	Embrio
	Sarcocornia fruticosa (L.) A.J. Scott	HP	Ovulary
	Suaeda maritima (L.) Dum.	A	Root tip
Plumbaginaceae	*Limonium angustifolium* (Tauch) Degen	HP	Root tip
Poaceae	*Puccinellia palustris* (Seen.) Podp.	HP	Root tip
Ruppiaceae	*Ruppia cirrhosa* (Petagna) Grande	HP	Fruit

Life cycle is indicated as: A: annual; HP: herbaceous perennial.
[a] Data from Martinčič et al. (2007).

Table 2
Genome size of halophytes

Species	2C-value ± S.E. (pg DNA)	CV (%)	Chromosome number (2n)[a]	Ploidy level[a]
Crithmum maritimum	4.38 ± 0.08	7.21	20	2×
Artemisia caerulescens	6.43 ± 0.08	4.65	18	2×
Aster tripolium	21.43 ± 0.27	6.95	18	2×
Inula crithmoides	3.63 ± 0.08	9.81	18	2×
Atriplex portulacoides	1.83 ± 0.18	48.82	36	4×
Atriplex prostrata	1.51 ± 0.22	52.30	18	2×
Salicornia europaea	2.75 ± 0.03	6.49	18	2×
Salsola soda	2.62 ± 0.04	5.81	18	2×
Sarcocornia fruticosa	5.91 ± 0.25	16.61	54	6×
Suaeda maritima	2.11 ± 0.04	3.56	36	4×
Limonium angustifolium	5.06 ± 0.12	12.06	36	4×
Puccinellia palustris	8.15 ± 0.26	9.5	42	6×
Ruppia cirrhosa	4.65 ± 0.21	17.74	40	4×

2C-values are presented as mean value ± standard error (S.E.) of at least 10 slides, from each of which the nuclear DNA amount of 200–300 interphase nuclei was measured. CV: coefficient of variation.
[a] Data from Tutin et al. (1968–1980).

1968–1980) are listed in Table 2. The examined halophytes are diploids, tetraploids and hexaploids.

The genome size of halophytes was compared to other species within the same family with known genome size data, listed in the Plant DNA *C*-values Database (Bennett and Leitch, 2005a) (Fig. 1). The estimated 2*C*-values of halophytes ranked low in comparison with other taxa in the family. The 2*C*-value of 5.06 pg DNA of *L. angustifolium* is even the lowest estimate in the family Plumbaginaceae with mean 2*C*-value 9.95 pg DNA. A 2*C*-value lower than the mean value of the family was also estimated for *C. maritimum*. It has 18% less DNA than the average 2*C*-value of 5.16 pg DNA for the family Apiaceae. In the Asteraceae, *A. caerulescens* and *I. crithmoides* have smaller genome than the mean 2*C*-value of the family (e.g. 9.70 pg DNA), but the 2*C*-value of *Aster tripolium* is 1.2-fold higher. The lowest *C*-values of the halophytes were estimated for species from the family Chenopodiaceae. The *C*-values of *A. prostrata* and *A. portulacoides* were lower than the mean 2*C*-value of 1.98 pg DNA calculated for this family. Other species from the family had up to 40% larger amount of nuclear DNA and *S. fruticosa* threefold larger. Among the investigated monocot halophytes, *Puccinelia palustris* has a 28% smaller genome than the mean 2*C*-value of 11.40 pg DNA estimated for the Poaceae. The nuclear DNA amount of the monocot *R. cirrhosa* was 4.65 pg and this is the first estimate not only for the genus, but for the whole family Ruppiaceae as well.

Further statistical analyses were conducted to test the differences among monocot and dicot halophytes and among annual and perrenial halophytes. The mean 2*C*-value of dicot halophytes was 6.04 ± 1.99 pg DNA and was not significantly smaller than the mean 2*C*-value of monocot halophytes of 6.40 ± 1.75 pg DNA ($p = 0.936$). The mean 2*C*-value of dicot halophytes was 8% smaller than the currently calculated mean 2*C*-value of 2458 eudicots, which is 6.56 pg DNA ($p = 0.861$) (Bennett and Leitch, 2005a). The mean 2*C*-value of monocot halophytes of 6.40 pg DNA was 70% smaller than the calculated mean 2*C*-value of 1885 monocot species of 21.83 pg DNA ($p = 0.076$).

The mean *C*-values of all annual halophytes, namely *A. portulacoides*, *A. prostrata*, *S. europaea*, *S. soda* and *S. maritima* (Table 1) rank low among the examined halophyte species (Table 2). The mean 2*C*-value of the annual halophytes was 2.49 ± 0.19 pg DNA and was 66% lower than the mean 2*C*-value of the perennial halophytes, which was 7.45 ± 2.06 pg DNA ($p = 0.012$).

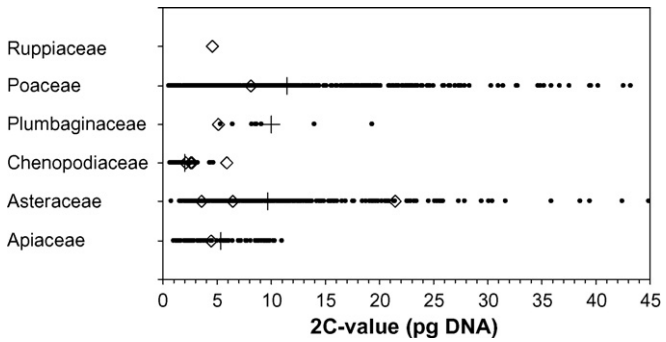

Fig. 1. Genome size of angiosperm families with halophytes from the Strunjan saltpan. Genome size data from this study (◇), plant DNA *C*-values database (Bennett and Leitch, 2005a; ●) and the mean 2*C*-value of the family (+) are presented to demonstrate the size of the halophyte genome in comparison to other species from the same family.

4. Discussion

In this study, first genome size data are presented for 12 out of 13 examined halophyte species from the Strunjan saltpan. In the current version of the Plant DNA *C*-values database (Bennett and Leitch, 2005a) halophytic species are very under-represented and only the *C*-value estimate for *A. caerulescens* is included. This is the report of Torrell and Valles (2001) and their 2*C*-value estimate of 6.66 pg DNA agrees well with our value of 6.43 pg DNA (Table 2).

From the available data in the Plant DNA *C*-values database several classes of genome size have been defined (Leitch et al., 1998). According to this classification, very small genomes are considered to be those with 2*C*-values lower than 2.8 pg DNA; small ones with 2*C*-values between 2.8 and 7.0 pg DNA, medium ones with 2*C*-values between 7.0 and 28.0 pg DNA; large ones with 2*C*-values between 28.0 and 70.0 pg DNA; and very large with 2*C*-values larger than 70.0 pg DNA. The mean holoploid genome size of the investigated halophytes ranged from 1.51 to 21.43 pg DNA and was generally small (Table 2). The smallest amounts of nuclear DNA were measured in halophytes from the family Chenopodiaceae. *A. portulacoides*, *A. prostrata*, *S. europaea*, *S. soda* and *S. maritima* have *C*-values under 2.8 pg DNA and thus belong to the group with very small genome size. *I. crithmoides*, *C. maritimum*, *R. cirrhosa*, *L. angustifolium*, *S. fruticosa* and *A. caerulescens* have small genomes. The only investigated halophytic species with medium genome size were *P. palustris* and *Aster tripolium*. The first one with its 2*C*-value of 8.15 pg DNA ranked low in the medium genome size class and the second with a 2*C*-value of 21.43 pg DNA was positioned at the beginning of the last third in that class. Notably,

no species were found in the classes of large or very large genome sizes.

The observed more than sevenfold range of the obtained *C*-values in *A. prostrata* and *A. portulacoides* was probably due to the presence of some nuclei with endoreduplicated DNA. Endoreduplication is a variant of the cell cycle in which chromosomal DNA is replicated without intervening mitotic division, leading to endopolyploid nuclei with polytene chromosomes (Joubès and Chevalier, 2000). It may occur in specific cells or tissues inside the organism. Endopolyploidy is common in the family Chenopodiaceae and has been found, among others, in *Atriplex rosea* (Barow and Meister, 2003).

To highlight the significance of genome size of halophytic plant species, their *C*-values were compared to the data from the Plant DNA *C*-values database (Bennett and Leitch, 2005a).

Within the plant family to which particular investigated halophytic species belong (Fig. 1), the estimated 2*C*-values ranked low in comparison with other taxa in this family. Moreover, the 2*C*-value of 5.06 pg DNA of *L. angustifolium* is even lower than currently the lowest estimate in the family Plumbaginaceae for *L. vulgare* (e.g. 5.3 pg DNA) (Bennett and Leitch, 2005a). Lower amount of nuclear DNA than the mean of the family had also the halophytes from families Apiaceae and Poaceae. Although the lowest *C*-values of the halophytes were estimated for species from the family Chenopodiaceae, these *C*-values were mostly higher than the mean 2*C*-value of this family. It is noteworthy that the majority of the available genome size data for members of the family Chenopodiaceae are known only for diploid species (Bennett and Leitch, 2005a), while most of the halophytic species in our study were polyploids (Table 2) and thus higher values might be expected. The genome size estimate of *R. cirrhosa* is the first data for the family Ruppiaceae as well and no comparison with related species or genera was possible. The family Ruppiaceae is related to seagrass families Posidoniaceae and Zosteraceae. The holoploid genome size of *R. cirrhosa* is 25% smaller than the genome size of *Posidonia oceanica*, having a 2*C*-value of 6.25 pg DNA, but higher than in species from the genus *Zostera*, which have very small genome sizes—approx. 1.4 pg DNA (Dolenc Koce et al., 2003).

Although monocots have generally larger genomes than dicots (Leitch et al., 1998; Vinogradov, 2001; Bennett and Leitch, 2005a), this trend was not observed in the present study when differences among halophytes were tested. Dicot halophytes had on average 5% smaller genome size than monocot halophytes, but the difference was not significant. When we compared halophyte genome size data to the mean genome size of eudicots and monocots, halophytes had on average 8 and 70% smaller genome than other species, respectively. The difference was especially in case of monocots substantial but not significant due to the almost 1000-fold range of nuclear DNA amount of monocots (2*C*-values from 0.30 to 254.80 pg DNA; Bennett and Leitch, 2005a) and possibly due to the small number of halophytes compared.

The mean *C*-values of all annual hylophytes, namely *A. portulacoides*, *A. prostrata*, *S. europaea*, *S. soda* and *S. maritima* (Table 1) rank low among the examined species (Table 2). This observation is in accordance with the suggestion of Bennett (1972) and Leitch et al. (1998) that annuals tend to have lower genome sizes than perennials. The obtained data also agree with a statistical analysis of the present *C*-values from the Plant DNA *C*-values database (Bennett and Leitch, 2005a) which shows that the mean 2*C*-value of annual angiosperms is 7.83 ± 0.28 pg DNA and is significantly lower than the mean 2*C*-value of perennials of 14.89 ± 0.38 pg DNA ($p < 0.0001$). The mean 2*C*-value of the annual halophytes was 66% lower than the mean 2*C*-value of the perennial halophytes.

To summarize, the present genome size analysis shows that in general halophytes have smaller genome size in comparison with known genome sizes within a particular plant family or in comparison with higher taxon levels. Several studies on genome sizes have shown that plants with large genomes are more sensitive to extreme environments, and are thus excluded from them (Bennett, 1987; Knight and Ackerly, 2002; Knight et al., 2005; Vidic, personal communication). The results of the present study are in accordance with these predictions. The Strunjan saltpan, with an extreme salt concentration and high insolation that causes high temperatures, is a clear example of an extreme habitat in which plant species with large genomes are not able to survive. Therefore, the exclusion of species with large genome sizes from the investigated area may be related to the extreme living conditions in saltpans.

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