**Genome size in aquatic and wetland plants: fitting with the large genome
constraint hypothesis with a few relevant exceptions**

**Oriane HIDALGO, Sònia GARCIA, Teresa GARNATJE, Màrius MUMBRÚ,
Aysun PATTERSON, Josep VIGO, Joan VALLÈS**

Oriane Hidalgo · Sònia Garcia · Màrius Mumbrú · Joan Vallès

Laboratori de Botànica - Unitat Associada CSIC, Facultat de Farmàcia, Universitat de
Barcelona, Av. Joan XXIII s.n., 08028 Barcelona, Catalonia, Spain

Oriane Hidalgo

Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3AB, United
Kingdom

Teresa Garnatje

Institut Botànic de Barcelona (IBB-CSIC-ICUB), Passeig del Migdia s.n., Parc de
Montjuïc, 08038 Barcelona, Catalonia, Spain

Aysun Patterson

Division of Botany, Department of Biology, Faculty of Science, Istanbul University,
34134 Istanbul, Turkey

Josep Vigo

Secció de Ciències Biològiques, Institut d'Estudis Catalans, C. Carme 47, 08001
Barcelona, Catalonia, Spain

Unitat de Botànica, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 643,
08028 Barcelona, Catalonia, Spain

Correspondence: joanvalles@ub.edu

Running title Genome size in aquatic and wetland plants

Abstract Genome size was assessed by flow cytometry in 55 populations of 53 taxa with different kinds of relationship with freshwater environments (25 populations of aquatic plants, 24 of wetland plants and six occurring both in aquatic and wetland habitats). Nuclear DNA content is provided for the first time for one family, five genera, 28 species, four subspecies and one variety. Around three quarters of the results obtained belong to the lowest levels of nuclear DNA content (very small and small values), which agrees with the large genome constraint hypothesis. Nevertheless, some remarkably high values were also found. Further studies would still be required to increase the set of water-dependent plants with known nuclear DNA amount in view of clearly establishing any relationship between genome size and aquatic habitat. A synthesis of the current knowledge on genome size in water-dependent angiosperms shows a distribution of C-values biased toward very small genomes, a tendency especially strong in wetland plants, where species of $< 1\text{pg}/2C$ are four times more frequent than in angiosperms as a whole.

Keywords Aquatic plants, flow cytometry, genome size, large genome constraint hypothesis, nuclear DNA content, wetland plants

Introduction

Genome size is a basic biological trait of living organisms, usually expressed as the so-called C-value, where C originally accounts for constant, meaning constant within a species, although the existence of some genuine intraspecific variation is accepted (Bennett and Leitch 2005, and references therein). Swift (1950) coined the term C-value to indicate the DNA content of the nucleus of an unreplicated gamete, i.e. of the haploid complement of an organism. Greilhuber et al. (2005) detailed genome size terminology and established, among others, the terms holoploid (2C, DNA amount of the nucleus of a somatic cell) and monoploid genome size (1Cx, DNA content in a basic chromosome set -x- of an organism). Genome size shows a very large diversity, neither proportional to organism complexity nor according to phylogenetic position and relationships. This has been called the C-value paradox or the C-value enigma (Gregory 2005, and references therein). In plants, nuclear DNA amounts show an almost 2400-fold variation, 2C-values ranging from 0.13 to 304.4 pg (Greilhuber et al., 2006; Pellicer et al., 2010a).

Genome size is intimately related to other karyological and cytogenetic characters, especially chromosome number and ploidy level (e.g. Pellicer et al. 2010b, and references therein). Bennett (1972) developed the nucleotypic theory to highlight the non-coding face of nuclear DNA amount, i.e. its physical effects reflected in cell cycle duration, life cycle type and their adaptive implications in plants. Among the latter features, ecological parameters have often been reported as correlated with genome size, temperature, altitude and water availability being some of the aspects considered (Grime and Mowforth 1982; Bennett 1987; Rayburn and Auger 1990; Knight et al. 2005; Suda et al. 2007; García-Fernández et al. 2012, and references therein).

Aquatic (including helophytes and hydrophytes) and wetland (very variable in life type, encompassing therophytes, geophytes, hemicryptophytes and even

phanerophytes) plants occupy particular ecological niches and are adapted to special development and life cycle conditions. Strictly aquatic plants are relatively scarce in terms of taxonomic diversity: around 370 genera comprising about 1,200 species (Margalef 1983). Wetland plants constitute a more numerous group with fuzzy borders, but in any case, they are taxa with a particular dependence on, tendency to or use of water.

There are no large and comprehensive genome size studies about aquatic and humid-habitat plants. Some attention has been paid to seagrasses (Cavallini et al. 1995; Dolenc Koce et al. 2003) and halophytes (Dolenc Koce 2008). Concerning freshwater-related plants, some specific taxonomic groups partly, mostly or entirely formed by these kind of organisms have been investigated. Genera *Callitriche*, *Veronica*, *Juncus*, *Potamogeton*, *Nasturtium* and *Schoenus* (Pijnacker and Schotsman 1988; Albach and Greilhuber 2004; Schönswetter et al. 2007; Ceccarelli et al. 2008; Morozowska et al. 2010; Kaur et al. 2012), the families Podostemaceae and Lemnaceae (Oropeza et al. 2002; Wang et al. 2011), and the Nymphaeales lineage (Pellicer et al. 2013) are included among these.

Wetland and aquatic taxa have been chosen for the present study as they have in common a stronger relationship to or dependence on fresh water than most plants, which could be reflected in genome size variation, as other ecological parameters are known to do so. Goin et al. (1968) associated high and low nuclear DNA amounts in frogs to life cycles either strictly associated with the vicinity of water or not so dependent on the wetness, respectively. Bennett (1987) supposed that analogous examples should exist in plants. With the idea that a certain relationship could be detected between genome size and water-linked life and given the relative scarcity of data on nuclear DNA content in plants with this particular ecological appetency, the aim

of this paper is to perform genome size assessments in a rather large set of aquatic and wetland plants, with the objectives of 1) increasing the knowledge of this parameter in this type of organism, and 2) elucidating possible relationships between these data and ecological factors, basically addressing the question whether easy water availability and/or water dependence are or not reflected in terms of genome size variation regarding plants not so strongly linked to fresh water; specifically, we aim to test the large genome constraint hypothesis (Knight et al., 2005), according to which plants with large genomes are rare in extreme environments.

Materials and methods

Plant material

Plant material for the present study is constituted by 55 populations (25 of aquatic plants, 24 of wetland plants and six occurring both in aquatic and wetland habitats) of 53 taxa. Plants were collected mostly in wild populations in Catalonia (Iberian Peninsula) and Eastern Thrace and Central Anatolia (Turkey). Taxonomic data and sources as well as plant origins are presented in Table 1, and detailed localities are provided in Online Resource 1. Leaf material of five (exceptionally four or three) individuals of each population was placed separately in slightly humid paper tissues and kept at 4-6°C for 1-5 days (depending on the closeness of the collection places to the laboratory facilities), until the moment of its flow cytometric analysis. Herbarium vouchers were prepared and are deposited either in the herbarium ISTF, of the Istanbul University (Turkish plants), or in the herbarium BCN, of the *Centre de Documentació*

de Biodiversitat Vegetal, Universitat de Barcelona (the remaining plants) (Online Resource 1).

Flow cytometric analyses

Nuclear DNA content of each population was assessed by flow cytometry at the *Centres Científics i Tecnològics (Universitat de Barcelona)*. Leaf material of each studied individual was chopped with a razor blade together with leaf material of an internal standard in 1,200 µl of LB01 buffer (Doležal et al. 1989), supplemented with 100 µg/ml of ribonuclease A (RNase A, Boehringer). The resulting liquid with chopped plant material was filtered and the nuclei suspension was stained with 36 µl of propidium iodide (1 mg/ml, Sigma-Aldrich Química, Madrid, Spain) to a final concentration of 60 µg/ml and kept on ice for 5-20 min. Measurements were carried out with an Epics XL (Coulter Corporation, Hialeah, Florida, USA), with the following configuration of the instrument: excitation of the sample was carried out using a standard 488 nm air-cooled argon-ion laser at 15mW power; forward scatter, side scatter and red (620 nm) fluorescence for propidium iodide were acquired. One run was done per preparation, in which 8000 particles were measured. Two independent replicates of each individual and five individuals per population (three in a very few cases) were analysed. All the analyses for a given population were performed on the same day (see more methodological details in Garnatje et al. (2004). *Lycopersicon esculentum* Mill. ‘Montfavet 63-5’ (2C = 1.99 pg; Lepers-Andrzejewski et al. 2011), *Petunia hybrida* Vilm. ‘PxPc6’ and *Pisum sativum* L. ‘Express long’ (2C = 2.85 pg and 8.37 pg, respectively; Marie and Brown 1993) were used as internal standards, covering a large range of genome sizes. Seeds of the standards were provided by the *Institut des Sciences*

du Végétal, CNRS, Gif-sur-Yvette (France). Nuclear DNA contents (2C) were calculated multiplying the known DNA content of the standard by the quotient between the peak positions (mode) of the target species and the standard in the histogram of fluorescence intensities, assuming a linear correlation between the fluorescent signals from the stained nuclei of the unknown specimen, the known internal standard, and the DNA amount (Doležal 1991).

Databases consulted

Kew plant DNA C-values database (<http://data.kew.org/cvalues>) and FLOWer, a plant DNA flow cytometry database (<http://botany.natur.cuni.cz/flower/index.php>) were accessed on August 2014 in order to establish the novelties in genome size assessments, and to compare, with the necessary analyses, those obtained in the present paper with those previously reported in the same or in related taxa. In addition, the Index to Plant Chromosome numbers (IPCN, www.tropicos.org/Project/IPCN) was also consulted in August 2014 to check the chromosome numbers reported for the studied taxa.

Statistical analyses

One-way ANOVA analyses were carried out to test if there are statistically significant differences of means of genome size among habitat types (aquatic, wetland, aquatic/wetland), life cycles (annual, perennial) and life forms (chamaephyte, geophyte, hemicryptophyte, hydrophyte, phanerophyte, therophyte).

Results and discussion

Genome size data (2C-values in pg and 1C-values in Mbp) of the taxa studied are provided in Table 1, together with previous nuclear DNA amount data, and chromosome numbers reported for each taxon whenever available. Fluorescence histograms of six taxa, representing different genome sizes and internal standards used, are provided in Fig. 1. Half-peak coefficients of variation (HPCV) of 2C peaks were mostly low (mean HPCVs were 4.12% for target plants and 2.57% for internal standards), indicating reliable results of flow cytometric analyses.

Genome size information is provided for one pteridophyte, 25 monocot and 27 eudicot plant taxa. Nuclear DNA amounts are provided for the first time for one family (Trapaceae), five genera, 28 species (including three subspecies and one variety) and one subspecies (of a species with previously assessed genome size in another subspecies). These novelties are indicated with an asterisk (*) in Table 1. Holoploid genome size values (2C) range from 0.44 to 43.06 pg (mean 4.66 pg), with a 98-fold variation. For the two taxa for which two populations were studied, the results are very similar in one case and quite different in the other one (discussed in the subheading devoted to ploidy level).

Comparison with previously known values

Genome size was previously assessed in 24 of the taxa here reported (Table 1). Previous nuclear DNA amounts were assessed by Feulgen microdensitometry in 17 taxa, by flow cytometry in two taxa and by both methods in five taxa.

Small and large genomes

Most 2C-values obtained are relatively small. According to the genome size categories in plants established by Leitch et al. (2005), 33 out of the 55 populations with nuclear DNA amount estimated in the present paper (60%) have very small genome sizes ($2C < 2.8$ pg), and out of this, 33% are lower than 1 pg. Eleven populations (20%) have small ones ($2.8 \leq 2C < 7$ pg), whereas 10 (18%) have intermediate ($7 \leq 2C < 28$ pg), only one (2%) large ($28 \leq 2C \leq 75$ pg) and there is no very large genome size in our sample ($2C > 75$ pg). So around three quarters of the values obtained belong to the lowest levels of nuclear DNA content. However, since data available in the above-mentioned plant DNA C-values database (Fig. 2) show that the vast majority of angiosperms possess small or very small genomes, this cannot be considered as a specific characteristic of aquatic or wetland plants. Nevertheless, as it will be detailed in the next subheading, most aquatic and, even more, wetland plants follow the large genome constraint hypothesis (Knight et al., 2005), although with some remarkable exceptions.

The large genome constraint hypothesis addressed: Genome size and its relationship with life cycle, growth form and water dependence

The differences in nuclear DNA content in annual and perennial plants and their life-cycle implications have been largely addressed, mostly from Bennett (1972). Annual plants often exhibit lower genome sizes than perennials, although some clear exceptions have been reported (e.g. Vallès et al. 2011, 2013). No significant differences have been found in genome size averages between annual and perennial habits ($p=0.454$). Genome size values for the annual taxa of our dataset are all small or very small (cf. the

precedent subheading), but the lowest values in the studied populations correspond to perennials. In any case, it has to be remarked that annual taxa are very scarce in the present dataset (only four out of 55 populations, plus one taxon that may behave both as annual and perennial).

We tried to detect differences between genome sizes in strictly aquatic taxa (26 populations) and in those occupying wetland habitats (28 populations; one population of one taxon with capacity to live in both situations has not been taken into account for this analysis). There are statistically significant differences in mean genome sizes among the habitat types ($p < 0.0001$). Species with preferences for both aquatic and wetland habitats ($2C = 13.96$ pg) show significant differences in genome size in respect to the species growing only in wetlands ($2C = 2.24$ pg) and aquatic habitats ($2C = 4.38$ pg). No significant differences have been found in genome size averages among life forms ($p = 0.829$).

Distribution of genome size across a wider dataset of wetland and aquatic angiosperms (Online Resource 2, where the only fern studied in the present work has not been taken into account) shows a C-value bias toward very small genomes, a tendency more accentuated in wetland plants where species with $2C < 1$ pg are four times more frequent than in angiosperms as a whole (Fig. 2A). Difference in genome size classes in aquatic and wetland plants might be explained by difference in preferential life growth forms. Indeed, hydrophytes are the largely most represented category of aquatic plants (all of them, therophytes excepted) and display a similar distribution of genome size class frequencies to aquatics, and similarly do the hemicryptophytes in the wetland category (Fig. 2A-B; Online Resource 2). Our results suggest that growth forms in water-dependent taxa do not contribute equally to the genome size classes, with the hemicryptophytes over-represented in the lowest genome

size category and the hydrophytes the main contributors in all remaining categories (Fig. 2C). Similar relationships between growth forms and genome size classes have been previously reported, with e.g. the geophytes over-represented in larger classes (in Lebanon Flora, Bou Dagher-Kharrat et al. 2013; in Balkan flora, Pustahija et al. 2013).

Knight et al. (2005) postulated the large genome constraint hypothesis, suggesting that plants with large genomes are under-represented in extreme environments, because high DNA amounts are a problem for species abundance and differentiation there. This agrees with the large majority of small and very small genomes in the plants studied, considering water-dominated ecosystems as extreme, in the sense that plants have developed particular and dramatic morphophysiological and reproductive adaptations to water in such environments (Les and Philbrick 1993). However, our results suggest that wetland plants are even more constrained toward small genome sizes than aquatic plants (Fig. 2A). This may be due to the fact that the challenge that wetland plants face is double: as the aquatics, they have to deal with an aquatic environment, but they also have to undergo dryer periods. Hydrophytes evolved to lose terrestrial features to fully adapt to aquatic environments while most wetland plants have retained characteristics typical of terrestrial plants and developed a high degree of plasticity in response to their life in a variable environment (Cronk and Siobhan Fennessy 2001; Hamann and Puijalon 2013). Our results include three outstandingly large 2C-values obtained in two aquatic (*Alisma plantago-aquatica*, *Ranunculus lingua*, the latter the taxon with the biggest genome size in its genus, containing aquatic and non-aquatic representatives) and one wetland (*Caltha palustris*) taxa, which suggests that such large genome sizes are exceptions in water-dependent

environments rather than the more likely ones as was hypothesised by Goin et al. (1968).

Genome size and ploidy level

As it has been widely reported, genome size is a very good indicator of ploidy level (Suda et al. 2007; Garcia et al. 2008; Pellicer et al. 2010b; Hidalgo and Vallès 2012). We will therefore make some comments on this parameter in the studied taxa in the light of the present data and the previous ones, when available. Unfortunately we could not perform chromosome counts in the studied populations (mostly because of sampling periods and at least partly due to the intrinsic difficulty of collecting seeds in these kind of plants, which do not flower every year), but nuclear DNA content information can be brought together with some chromosome numbers collected from the literature for discussion.

Our C-value results and those previously published suggest the existence of different ploidy levels in some species considered: *Alisma plantago-aquatica*, *Cardamine amara*, *Elodea canadensis*, *Eriophorum angustifolium* and *Nelumbo nucifera*, this being mostly confirmed by chromosome numbers previously reported for these taxa (Table 1). *Ranunculus lingua*, the species with the largest genome size among those studied in this paper, is likely a high polyploid, as its commonest chromosome number, $2n = 128$, suggests. The result here obtained of $2C = 42.06$ pg is close to one of the previously reported for this taxon (42.35 pg), while the others available (50.20, 50.30, 50.40 pg) could represent either one more chromosome complement, $x = 8$ being one of the basic chromosome numbers in the genus *Ranunculus*, or simply reflect intraspecific variation or even methodological differences.

The two taxa for which two populations were studied also could likely present different ploidy levels. For *Caltha palustris*, 2C-values (19.54 and 19.62 pg) indicate constancy in ploidy level, although several chromosome numbers suggesting a long polyploid series have been reported for this taxon (Table 1), which is in agreement with the genome size data previously available (2C = 22.40, 31.10, 33.00 pg; Table 1). There is one extreme outlier result of 2C = 1.30 pg (Table 1; Bennett and Leitch 1995, from Baum A., Kenton A.Y., Bennett M.D., pers. comm., 1989), which should be carefully revised. Conversely, for *Eleocharis palustris*, 2C-values (3.83 and 6.83 pg) suggest different ploidy levels (possibly related to subspecific taxa within this complex), lower in the Catalan population and higher in the Moroccan one, which would have suffered a slight genome downsizing (the double of the lower value being 7.66 pg), typical in many cases of polyploidy (Leitch and Bennett 2004). One of the previous records from this taxon (2C = 4.20, 11.05 pg; Table 1) would suggest still one more ploidy level. The only chromosome number reported for this species being $2n = 16$ (Table 1), this parameter should be further studied in this species to confirm this hypothesis. For *Parnassia palustris*, our assessment (2C = 3.66 pg) also significantly differs from the previously published one (2C = 2.35 pg, Siljak-Yakovlev et al. 2010) in a way that it could suggest two ploidy levels; according to chromosome counts published for this species, diploid to hexaploid -including triploid- populations have been detected, what would agree with the two genome size results found to date. Undoubtedly, chromosome counts are necessary in all cases to ensure ploidy levels, but some big 2C value changes as those commented above clearly point to the existence of several levels in some taxa.

Concluding remarks

This paper contributes a significant amount of genome size data for plants living in water or in places where water plays a relevant role. These plants are considered to have a range of variation in chromosome numbers and ploidy levels (Les and Philbrick 1993), which is confirmed with interspecific and intraspecific variation in genome size values found. The rather low nuclear DNA amounts found are basically in agreement with the large genome constraint hypothesis (Knight et al. 2005), although there are a few remarkable exceptions. Although the current dataset fills an important gap in genome size knowledge at specific, generic and familial levels, many water-linked taxa still lack such information. So, further research is needed to be definitely conclusive on the relationships between genome size and the important ecological and adaptive specificities of the aquatic habitat.

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Figure captions

Fig. 1 Fluorescence histograms of the genome size assessments by flow cytometry using propidium iodide of: a *Apium nodiflorum* 2C=1.59 pg (a) with *Petunia* 2C=2.85 pg (b) as internal standard (i.s.); b *Potamogeton lucens* 2C=1.64 pg (c) with *Petunia* (d) as i.s.; c *Polygonum amphibium* 2C=3.91 pg (f) with *Petunia* (e) as i.s.; d *Stratiotes aloides* 2C=9.80 pg (h) with *Pisum* 2C=8.37 pg (g) as i.s.; e *Alisma plantago-aquatica* 2C=23.33 pg (j) with *Pisum* (i) as i.s.; f *Ranunculus lingua* 2C=43.06 pg (m) with *Pisum* (k and l, 2C and 4C peaks, respectively) as i.s.

Fig. 2 Distribution of genome sizes in water-dependent angiosperm species. a Distribution of genome size classes in aquatic (n = 137) and wetland (n = 65) angiosperm plants, and the angiosperms as a whole (n = 7541); b Distribution of genome size classes in hydrophyte (n = 98) and hemicryptophyte (n = 59) life growth forms; c Life growth form according to the genome size classes. Genome size data are gathered from the present study, the plant DNA C-values database (<http://data.kew.org/cvalues>; using prime estimate option) and Pellicer et al. (2013). VS: very small, $2C \leq 2.8$ pg; S: small, $2.8 < 2C \leq 7$ pg; I: intermediate, $7 < 2C < 28$ pg; L: large, $28 \leq 2C < 70$ pg; VL: very large, $2C \geq 70$ pg.