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Flow cytometric analysis of DNA content for four commercially important crabs in China

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Abstract

The genome size (C-value) of an organism is referring to the DNA content of its non-replicated haploid chromosome complement, generally deduced from measuring somatic diploid nuclei. We presented genome size (C-value) data obtained by flow cytometry for four commercially important crabs (*Portunus trituberculatus*, *Charybdis japonica*, *Scylla paramamosain*, and *Eriocheir sinensis*) common in the coast of China. *Gallus domesticus* (2C=2.5 pg) was used as the internal standard. The results showed that the C-value for *P. trituberculatus*, *C. japonica*, *S. paramamosain*, and *E. sinensis* were (2.31±0.01) pg, (2.33±0.03) pg, (1.64±0.02) pg, and (2.29±0.03) pg, respectively. The C-value of *P. trituberculatus*, *C. japonica* and *S. paramamosain* were reported for the first time. The data represented by the four species indicated that they had lower DNA contents than average DNA values in crustaceans ((4.99±0.48) pg), and three of the four values were very similar if not identical. The results provide useful data for future studies in the fields of biodiversity, species conservation, and phylogeny of these commercial crabs. They will also be helpful in instructing the hybridization breeding program and estimating the cost of the whole genome sequencing project.

Key words: genome size, flow cytometry, crabs, Portunidae, Grapsidae

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1 Introduction

An organism's C-value is an important feature that defines the genetic characteristic of a species by determining its capacity to undergo evolutionary changes (Petrov, 2001; Vinogradov, 2004; Fafanđel et al., 2008). Scientific disciplines, which profit from the knowledge of C-values are numerous, and include molecular biology, systematics and ecology (Bennett et al., 2000; Griffith et al., 2003). Moreover, knowledge of genome size is a useful and necessary prerequisite for the research of whole genome sequencing. Normally, two approaches are applied for the determination of C-value of a given organism: analysis of DNA extracted from a large number of cells, and measurement of individual nuclei. The second approach offers much higher precision, but is technically more demanding. Flow cytometry is one of the most sensitive techniques extensively used for quantifying cellular DNA by measuring individual nuclei (Crissman et al., 1979; Shapiro et al., 1988; Petrov, 2001; Fafanđel et al., 2008). It has been used to determine the nuclear DNA content of many animal species, including vertebrates from fishes to mammals (Tiersch et al., 1989; Birstein et al., 1993; Gregory, 2002; Yi and Streelman, 2005; Smith and Gregory, 2009), mollusks (Allen Jr, 1983; Dillon Jr, 1989; Fafanđel et al., 2008) and crustaceans (Bachmann and Rheinsmith, 1973; Doležel and Bartoš, 2005; Fafanđel et al., 2008; Jeffery, 2012).

Genome size research is a field that is currently expanding rapidly and becoming more important as whole genome sequencing becomes more frequent. To our knowledge, there are more than 60 000 species of crustaceans. But, only 330 crustaceans have genome size data in the Animal Genome Size Database (Gregory, 2016). The current number of genome size estimations is partly in comparison with the level of diversity of crustaceans. Statistics reveal that crustaceans, as a whole, have larger genomes (1C=(4.99±0.48) pg, mean±standard error, 284 species) compared to mollusks ((2.14±0.09) pg, 221 species) or polychaete annelids in invertebrates. Furthermore, the range in C-values (460-fold variation from 0.14 pg to 64.62 pg; Gregory, 2016) makes crustaceans a worthy focus for studying of genome size.

Portunus trituberculatus, *Charybdis japonica*, *Scylla paramamosain* and *Eriocheir sinensis* have become most important economic crabs in the last 30 years in China. The production of two mariculture crabs *P. trituberculatus* and *S. paramamosain* have now reached 240 000 t (Fishery Bureau, Ministry of Agriculture, China, 2014) while *E. sinensis* with more than 700 000 t. As farming of crabs is growing as an aquaculture enterprise, the need to identify the genomic mechanisms is becoming more important. Many molecular genetic studies for aquatic animals have being performed to assist the breeding program, including whole genome sequencing (Chen et al., 2014; Zhang et al., 2012).

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Here we provided genome size data estimated by flow cytometry for these four commercially important crabs. Knowledge of genome size is a prerequisite step for the development of genomic resources. The presented results should provide useful data and will be a guide to help the genome sequencing project for crabs.

2 Materials and methods

E. sinensis was collected in Binzhou, Shandong Province and *S. paramamosain* in Ningbo, Zhejiang Province. *P. trituberculatus* and *C. japonica* were sampled in Qingdao, Shandong Province (Fig. 1). Samples were immediately brought to laboratory in aerated seawater and acclimated in aquaria with running seawater at field temperature at least 24 h before analyses. Ten individuals for each species were collected and kept alive before extracting cell suspensions. The average weight for *E. sinensis*, *S. paramamosain*, *P. trituberculatus* and *C. japonica* were 153, 108, 173 and 76 g, respectively.

For all species, cell suspensions were prepared using hemolymphs, which were drawn directly from the heart using sterile syringes (Hameed et al., 2001). We used the mixture of sterile crab saline and anticoagulants (Bachère et al., 1988) when we extracted the blood from the crab. The formula of sterile crab saline was from Grau and Cooke (1992). Extracts were then filtered on a 48 μm mesh and centrifuged at 2 500 r/min for 20 min at 4°C. Then, the supernatant was replaced with 700 μL fresh PBS buffer (0.01 mol/L). Finally, each sample was stained on ice with 1 mL propidium iodide (PI). 100 mL final concentration consisted of 5 mg PI, RNase 2 mg, Triton-X-100 1 mL, 0.9% stroke-physiological saline solution 65 mL, and sodium citrate 100 mg, using NaOH to adjust pH to 7.2.

Fig. 1. The photograph of the four crabs in this study. a. *Portunus trituberculatus*, b. *Charybdis japonica*, c. *Scylla paramamosain*, and d. *Eriocheir sinensis*.

DNA analysis was carried out on a BD FACSAria Ⅱ flow cytometer with an anargon laser operated at a wavelength of 488 nm in a light-stabilized mode. The emitted light was collected through a 560 nm dichroic, and 600 nm long wave pass (LWP) filters. Finally, *Gallus domesticus* (1.25 pg/C) was chosen to act as the calibration standard and added to fresh PBS buffer (0.01 mol/L) before staining. The flow cytometer was regulated using *G. domesticus* cell samples which would be co-prepared and co-stained in the same tube with crab sample in the final test. The CV (coefficient of variance) was controlled below 3% (Marie and Brown, 1993). Percent of CV is a measure of peak distribution and refer to the standard deviation of the peak divided by the mean channel number, times 100.

The flow cytometer was regulated using standard Fluorescent Particles before we run the samples. The CV value was calculated by the software Diva Version 10 based on the formula: $CV = \frac{SE}{M} \times 100\%$ (*SE*: standard error; *M*: mean value). For cell cycle measurement 20 000 nuclei from all ten samples per species were analyzed. Genome size values of other crab species from Portunidae and Grapsidae were taken from the Animal Genome Size Database (Gregory, 2016). The haploid nuclear DNA content was expressed in picograms or million base pairs, where 1 pg=978 Mbp (Doležel et al., 2003). Data analysis and figure construction were carried out with the NovoExpress (with 30-day trial license).

3 Results and discussion

Many aspects can affect the accuracy of DNA content detection, such as the experimental method, cell sample preparation, and dye chosen. Flow cytometry was used in this study. Although the principle of flow cytometry is relatively straightforward, estimation of absolute DNA amount requires special attention to possible errors in sample preparation and analysis (Doležel and Bartoš, 2005). Flow cytometry should have some advantages with the high efficiency of sample preparation (Shapiro, 2003), good ability to measure DNA quickly in large populations of cells and better accuracy. Eighty-six have been obtained by flow cytometry among the 330 C-value records for crustaceans (Gregory, 2016).

Haemocytes of invertebrates have been successfully used for flow cytometry analysis in the last 15 years (Fafanđel et al., 2008). To date, there have been 19 Crustaceans C-value studies accounting for 5.69% of all the crustacean species. It has been confirmed that different types of cells could influence and even change the results of measurements and turn them to a nonproportional correlation of the DNA content (Birstein et al., 1993). Except for the internal standard, other factors including cytosolic compounds, growth state or tissue type of cell can cause an ex-

perimental error and may affect DNA content estimation (Prosperi et al., 1991; Rayburn et al., 1992). In order to minimize these negative factors, haemocytes were extracted from the live crabs with the same growth state in this study.

Meanwhile, *G. domesticus* was used as internal standard. It is accuracy to use the blood cell of *G. domesticus* as internal standard which has been reported in many animals (mammals, 188/778; birds, 764/896; reptiles, 20/418; fishes: ray-finned, 1016/1819; amphibians, 63/927) (No. of the species estimated using *G. domesticus* as internal standard/No. of the species have genome size data) and plants. Among 330 estimated genome sizes for crustaceans, there are 100 species in which the blood cell of *G. domesticus* is used as internal standard. Finally, the median fluorescence intensity for the DNA peaks of *P. trituberculatus*, *C. japonica*, *S. paramamosain* and *E. sinensis* were 521, 419, 354 and 440, respectively. Consistently, the median fluorescence intensity for the DNA peaks of *G. domesticus* was 282, 225, 270 and 240, respectively (Fig. 2).

The total nuclei numbers were not in the same for all samples. The *G. domesticus* had the largest number with 2 000, followed by the four commercially important crabs (*P. trituberculatus*, *C. japonica*, *S. paramamosain* and *E. sinensis*) from 50 to 400, respectively. At the same time, the CV of the *G. domesticus* DNA peaks was 2.8%. The variations between individuals within each of the species were below 5% (Table 1, Fig. 2). The C-value of *E. sinensis* was (2.29±0.03) pg, which was closely to the value estimated by Nick Jeffery using Feulgen image analysis densitometry (not published data) but bigger than the previous study ((1.72±0.25) pg) based on the Real-time PCR method (Zhu et al., 2007). Meanwhile, this is the first record of the C-value of these three species belonging to the family Portunidae.

Haploid genome sizes for the other seven crab species belonging to the Portunidae and the nine crab species belonging to Grapsidae were also showed in Table 2. Great heterogeneity for C-value (1.07–15.17 pg) among the species of the family Portunidae was recorded. *Carcinus maenas* was the lowest with 1.07 pg, while *Necora puber* was the highest with 15.17 pg. The big divergence among different species in the same family might reflect a relationship between genome size and cell size (Gregory, 2001a, b), nucleus size (Beaulieu et al., 2007), metabolic rate (Xia, 1995) and/or other mechanisms (Yi and Streelman, 2005; Smith and Gregory, 2009) that govern these properties.

Known patterns in genome size indicate that complex and multiple mechanisms should operate at several levels of biological organization, and no single theory can account for genome size diversity (Bonnivard et al., 2009). In the present study, *P. trituberculatus* (2.31 pg) and *C. japonica* (2.31 pg) from family Portunidae almost have the same C-value (Table 1), close to that of *E. sinensis* (2.29 pg) but 40% larger than that of *S. paramamosain* (1.64 pg). The genome size diversity within or between family may contribute to the geographical distribution of these four species. The *S. paramamosain* is only naturally distributed in the

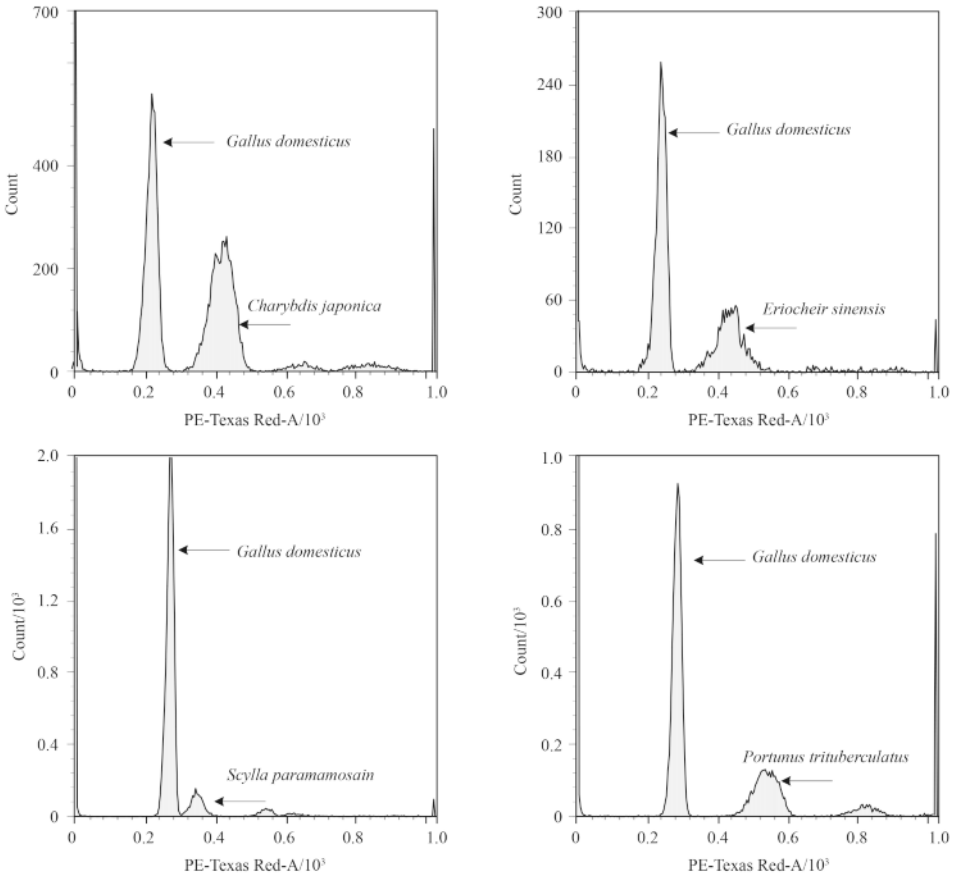

Fig. 2. Histograms of mixtures of four commercially important crabs and *G. domesticus* blood samples. The blood of *G. domesticus* was used as an internal standard. The PE-Texas Red-A represented the fluorescence value corresponded to PI-DNA complex fluorescence signal, which was detected by the flow cytometer using the special passway. The voltages of the PI channel were same for all samples analyzed.

Table 1. Comparison of mean C-value of family Portunidae and Grapsidae

Family	Species	Number of samples	1C DNA content		
			Mean/pg	SD/pg	Mean/G
Portunidae	*Portunus trituberculatus*	10	2.31	0.01	2.21
Portunidae	*Charybdis japonica*	10	2.33	0.03	2.22
Portunidae	*Scylla paramamosain*	10	1.64	0.02	1.57
Grapsidae	*Eriocheir sinensis*	10	2.29	0.03	2.19

Table 2. Comparison of mean C-value of family Portunidae and Grapsidae

Order	Family	Species	Common name	C-value/pg	Method	Cell type	Reference
Decapoda	Portunidae	*Necora puber*	velvet swimming crab	15.17	FCM	MC	Bonnivard et al. (2009)
		Callinectes sapidus	blue crab	2.14	FD	WB	Rheinsmith et al. (1974)
		Scylla serrata	mangrove crab	2.1	FD	WB	Bachmann and Rheinsmith (1973)
		Thalamita edwardsi	crab	2	FD	WB	Bachmann and Rheinsmith (1973)
		Callinectes sapidus	blue crab	1.9	FD	WB	Bachmann and Rheinsmith (1973)
		Carcinus maenas	common shore crab	1.24	FCM	MC	Bonnivard et al. (2009)
		Carcinus maenas	green crab	1.07	FIA	HE	Gregory T R (unpublished data)
		Portunus trituberculatus	swimming crab	2.31	FCM	HE	this study
		Charybdis japonica	Japanese stone crab	2.33	FCM	HE	this study
		Scylla paramamosain	mangrove crab	1.64	FCM	HE	this study
	Grapsidae	*Sesarma cinereum*	wharf crab	4.4	FD	WB	Bachmann and Rheinsmith (1973)
		Sesarma cinereum	wharf crab	3.99	FD	WB	Rheinsmith et al. (1974)
		Unknown sp.	grapsid crab	3.02	FD	WB	Rheinsmith et al. (1974)
		Pachygrapsus crassipes	lined shore crab	2.8	FD	WB	Rheinsmith et al. (1974)
		Pachygrapsus crassipes	lined shore crab	2.8	FD	WB	Bachmann and Rheinsmith (1973)
		Metopograpsus messor	purple climber crab	2.4	FD	WB	Bachmann and Rheinsmith (1973)
		Plagusia depressa	cliff crab	1.8	FD	WB	Bachmann and Rheinsmith (1973)
		Plagusia depressa	cliff crab	1.49	FD	S	Musich and Skinner (1972)
		Grapsus grapsus	sally lightfoot crab	1.33	FD	WB	Rheinsmith et al. (1974)
		Eriocheir sinensis	Chinese mitten crab	2.29	FCM	HE	this study

Note: Each method was represented with a code: biochemical analysis (BCA), Feulgen densitometry (FD), flow cytometry (FCM). Cell type was represented with code as follows: muscle cells (MC), whole body (WB), haemocytes (HE), legs (L), and sperm (S).

coastal waters of south of China, while the other three species are more widely naturally distributed and cultured along the eastern coast of China from north to south. Evolution of the genome size of these four crab species may trend to fulfill various habitats, as selection factors favor larger genome size in species inhabiting multiplex environments (Rasch and Wyngaard, 2006).

There is considerable variation in nuclear DNA content among crustaceans, which makes it a worthy focus for studying of genome size. Genome size measurement for extreme environment crustacean species that live near hydrothermal vents, the Arctic or Antarctic, may become an interesting point in the future as most of these species have extremely large or small genome size (Rees et al., 2007; Bonnivard et al., 2009; Jeffery, 2012). On the other hand, scientists expect to discover useful genomic information responding to the extreme environments and use them for animal breeding programs. Overall, the genome size for four commercial crabs present here confirm that it is accurate and highly efficient to measure crustacean genome size using haemocytes cells by flow cytometry technology. More genome size measurements and genomic resources for Portunidae and Grapsidae crab species will be revealed by flow cytometry technology and whole genome sequencing in the future studies.

4 Conclusions

This study focused on the DNA content of four commercial crabs in China by flow cytometry which should contribute to the database of invertebrate genome size. The DNA content (C) in *P. trituberculatus*, *C. japonica*, *S. paramamosain*, and *E. sinensis* was (2.31±0.01) pg, (2.33±0.03) pg, (1.64±0.02) pg, and (2.29±0.03) pg, respectively. Such results sustained the hypothesis that the genome size of these crabs may be in accordance with their geographical habitat range. Our study proved that reliable

estimation of absolute DNA amounts in crabs using flow cytometry was achievable. The C-value enigma will remain an interesting and unsolved puzzle in the future unless its role at the crossroads of molecular biology, cell biology, anatomy, physiology, and genomics can be revealed.

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