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ANIMAL SCIENCE

Cytogenetic characterization of the golden mussel (*Limnoperna fortunei*) reveals the absence of sex heteromorphic chromosomes.

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Abstract: The golden mussel (*Limnoperna fortunei*) is an aggressive invasive species in South America, where it endangers native species and freshwater ecosystems, in addition to causing extensive economic losses, mainly to the hydroelectric sector. Currently, there's no efficient control method available and the invasion has progressed across the continent. Its high reproduction rate is one of the key factors of the golden mussel's high invasive potential and, recently, efforts have been done in order to understand the reproduction and the sexual features of this species. However, its cytogenetics characterization is incipient and the possible occurrence of sex-specific cytogenetic features was never investigated. In this study, we aimed to characterize the chromosomal morphometry, the distribution profile of heterochromatin, and to detect possible sex-related epigenetic marks in the golden mussel. Results revealed that the karyotypic structure is similar in both sexes and no chromosome heteromorphism was observed between males and females specimens. The data increment the cytogenetic characterization of *Limnoperna fortunei* and contribute for future studies that aim to further investigate its reproduction and underlying sex determination processes.

Key words: Chromosome, DAPI/CMA banding, golden mussel, invasive species, *Limnoperna fortune*.

INTRODUCTION

Limnoperna fortunei (Dunker, 1758) (Bivalvia:Mytilidae), commonly known as golden mussel, is the most aggressive freshwater invasive mollusk in South America (Darrigran & Damborenea 2011). The invasion began in 1991 at the Rio de la Plata (Argentina), and since then the golden mussel has spread across the continent reaching four other countries (Uruguay, Brazil, Paraguay and Bolivia). Most recently, *L. fortunei* reached the São Francisco river and hydroelectric power plants in Brazil Northeast (Uliano-Silva et al. 2013, Barbosa et al. 2016). The golden mussel is present in 40% of the hydroelectric power plants in Brazil, causing a loss of revenue of ~USD 120 million per year only to this sector (M.F. Rebelo et al., unpublished data).

Besides the economic losses, the golden mussel causes extensive environmental impacts, physically altering the environment and affecting native species, for example, by reducing the availability of substrate and modifying water composition (Darrigran & Damborenea 2011). The Amazon basin is the highest freshwater biodiversity in the world (Jézéquel et al. 2020). It is still without records of *L. fortunei*, but according to predictive models, it is at a high risk of invasion (Uliano-Silva et al. 2013, Barbosa et al. 2018). The risk of invasion

can further increase by anthropogenic vectors, such as fish restocking programs, done without efficient control methods.

So far there are no effective methods to control the golden mussel, and it has been done mainly using chemical products in hydroelectric power plants. In 2018, the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) declared the golden mussel as a priority invasive species to be controlled until 2030, which is an ambitious goal without the development of an effective technology (M.F. Rebelo et al., unpublished data). A synthetic biology approach to control the golden mussel using a CRISPR-Cas9 gene drive sex-distortion strategy was recently proposed (M.F. Rebelo et al., unpublished data, de Paula et al. 2020). However, this technology requires solid knowledge in genome organization and sex determination and differentiation mechanisms of the target organism.

In the last decade, there were advances in areas regarding the mitochondrial genome (Uliano-Silva et al. 2016), somatic tissue-specific transcriptomes (Uliano-Silva et al. 2014), and the complete nuclear genome (Uliano-Silva et al. 2018). Recently, L.F. Afonso et al. (unpublished data) made progress in transcriptome of male and female gonads. However, there is still a knowledge gap regarding the cytogenetic characterization of this species and the existence of sex-specific features, such as sex chromosomes.

To the best of our knowledge, bivalves lack heteromorphic sex chromosomes, and little is known about sex determination in mollusks (L.F. Afonso et al., unpublished data, Breton et al. 2018). Only one Bivalvia, *Mulinia lateralis*, has been suggested to have a XX/XY sex-determination system (Guo & Allen 1994), and generally bivalve sex determination involves the influence of both genetic and environmental factors (Breton et al. 2018). Although the number of chromosomes (2n=30) has been previously determined for *L. fortunei* (Ieyama 1996), a thorough cytogenetic study for chromosome and heterochromatin characterization with differential staining techniques was never performed, and the question whether the golden mussel has heteromorphic sex chromosomes remains open. In addition, imunocytogenetic techniques for detecting DNA methylation and histone modifications also can be used to investigate sexual chromatin patterns between male and female (Piferrer 2013). Considering that abiotic conditions can alter the epigenetic patterns of sex-related loci, or chromatin sites (Kuroki et al. 2013, Kvist et al. 2020), a comparative characterization of these marks between the genders might add interesting information about differential gene expression in females and males. In this study we assessed the chromosome morphometry, heterochromatin distribution, and the epigenetic marks (acetylation and phosphorylation of H3 histone and DNA methylation) in the karyotype of male and female golden mussels from the state of São Paulo (Brazil) to investigate the presence of heteromorphic sexual chromosomes and other possible sex-biased characteristics.

MATERIALS AND METHODS

Histology and light microscopy

Forty animals were collected at the Chavantes reservoir, Paranapanema river (Chavantes, São Paulo, Brazil, 23°7'41.99"S, 49°43'59.02"O) in April 2019. For cytogenetic analysis, gills of 20 animals were used, 10 pre-identified (based on the color of the gonads) as female, and 10 as male. To confirm the sex of the animals used for metaphase preparations, fixed gonads samples were dehydrated by increasing concentrations of ethanol, clarified with xylene, and impregnated

in Paraplast Plus® (Sigma-Aldrich). Histological sections of 5 µm (thickness) were submitted to Hematoxylin Eosin (HE) staining and examined under a Pannoramic MIDI slide scanner microscope (3D Histech) for gender assessment according to Callil et al. (2012).

Metaphase spreads

Cytogenetic analyses were performed in *L. fortunei* males and females. Before dissection, the animals were subjected to treatment with 500 mL of colchicine 0.005% (w/v) (INLAB, cat. number 3265) diluted in freshwater, kept for 48 hours under constant aeration, as described in Ieyama (1996). After this treatment, gills were removed for the cytogenetic characterization, and gonads were stored in a fixation buffer [4% paraformaldehyde (w/v), Phosphate Buffer 0.1 M, pH 7.4] at 4°C until further histological processing for sex confirmation. Gills were subjected to hypotonic shock using distilled water for 1 hour under gentle agitation at room temperature, then washed three times in freshly prepared fixative solution (ethanol: glacial acetic acid, 3:1), during 20 minutes and stored in fixative solution at -20°C until use. For mitotic chromosome preparation, small gill fragments were dissociated on glass slides, with a drop of acetic acid 60% (v/v) using insulin needles. The slides were air-dried and transferred to a Coplin jar with acetic acid 45% (v/v) for 11 seconds, air-dried again and stored at room temperature until further use.

Chromosome morphometry

The length of chromosomes, their short and long arms, as well as the ratio between arms, were measured in 20 golden mussel specimens. Chromosome classification was done according to Levan et al. (1964). Ideograms were created to present chromosomes in decreasing order of size.

Chromosome banding

Chromosome banding was performed according to Schweizer (1976). After metaphase spread preparation, slides were aged for 7 days, stained with DAPI (4,6-diamidino-2-phenilindole) (Sigma-Aldrich, catalog number: D9542) (1 µg/mL in McIlvaine buffer pH 7.0), kept for 20 minutes in the dark, washed with distilled water and co-stained with CMA_3 (Chromomycin A3) (Sigma-Aldrich, catalog number: C2659) (0.1 mg/mL in McIlvaine buffer, pH 7.0 with 0.5 mM de $MgCl_2$) for 30 minutes. Slides were mounted in McIlvaine buffer pH 7.0 buffer-glycerol (1:1 v/v). In order to stabilize fluorescence, slides were stored in a refrigerator (4°C) for at least 24 hours before analysis. For this analysis, five metaphases from males and five metaphases from females were observed and captured using an Olympus DP72 digital camera. Chromosomes were observed using an epifluorescence microscope (Olympus BX 51) with an appropriate filter set.

Detection of the nucleolus on nuclei was done using silver staining (Ag-NOR) banding, according to Howell & Black (1980), with few modifications. A small drop of silver nitrate (50% w/v in water) was placed over the cell spreads on the slides and covered with a nylon screen, after which the slides were stored in a humid chamber at 60°C for 45 minutes. Next, the slides were analyzed and images captured under light microscopy using a CCD digital camera coupled to the Olympus BX51 microscope.

DNA methylation

For the observation of methylated DNA regions, 50 µL of blocking solution [PBS containing 3% Bovine Serum Albumin (w/v) and 0.1% Triton x-10 (v/v)] were added to the slides, which were incubated in a humid chamber for 10 minutes at room temperature. Then, coverslips and the excess of blocking solution were removed. Each slide was covered with 15 µL of the primary

antibody diluted 1:200 in blocking solution [Mouse monoclonal (clone 33D3) Anti-5-Methylcytosine (Millipore)]. Slides were covered with parafilm coverslips and incubated overnight in a humid chamber at 4°C. Subsequently, slides were washed in PBST [phosphate-buffered saline with 0.05% Tween 20 (v/v)], three times (5 minutes each). Then, it was added 15 µL of the secondary anti-mouse IgG-FITC antibody (Sigma-Aldrich, catalog number: 057K6068), diluted 1:100 in PBST. Slides were kept in a humid chamber for 1 hour and 30 minutes, at 37°C. Finally, they were washed in PBST (three times, for 5 minutes each) and mounted in DAPI / Vectashield H-1000 (1:100).

Histone phosphorylation and acetylation

The immunodetection technique for labeling histone H3 phosphorylated on serine 28 (H3ser28phos) and H3 acetylated on lysine 27 (H3lys27ac) was performed following the method described by Guerra (2012), with few modifications. To avoid non-specific labeling, slides were incubated for 1 hour in 50 µL of blocking solution at room temperature. Then, each slide was covered with 25 µL of the primary antibody (Thermo Fisher H3ser28phos catalog number: 720099, and H3lys27ac catalog number: 720096), diluted 1:100 in blocking solution. The slides were kept in a humid chamber for at least 16 hours at 4°C. Next, slides were washed three times (5 minutes each) in 1x PBS and 25 µL of the secondary antibody (Rhodamine Thermo Fisher, catalog number: 31670 for H3lys27ac, and FITC Thermo Fisher, catalog number: A-11070 for H3ser28phos) diluted in blocking solution (1:100) was applied. Slides were kept in a humid chamber for 1 hour and 30 minutes in an oven at 37°C, in the dark. After incubation, slides were washed three times (5 minutes each) in 1x PBS buffer and counterstained in a solution of DAPI / Vectashield H-1000 (1:100). Further analysis was performed using an Olympus BX51 epifluorescence microscope with an appropriate filter set.

RESULTS

Histological sexing confirmed the pre-identified sex for all the specimens (Supplementary Material - Figs. S1, S2). Chromosome counts showed a diploid chromosome number 2n=30 for *L. fortunei* (Fig. 1), for both male and female specimens assessed here. We did not find differences between karyotypes when female and male individuals were assessed. The karyotype was composed of median, submedian and sub telocentric chromosomes, with karyotype formulae (KF) = 20m + 8sm + 2st for both sexes (Figs. 1a, c, Table I). Additionally, no structural or numerical variation was observed, all spread metaphases presented the same profile, regardless of the sex assessed. In general, the chromosomes presented a reduced length varying from 1.25 µm to 2.85 µm (absolute length) (Table I). Due to the reduced size of the chromosomes, it was not possible to observe the number of and the secondary constrictions sites, which would indicate the nucleolar organizing regions on the metaphase chromosomes. However, Ag-NOR banding results revealed a maximum number of four nucleoli per nucleus, for both males and females, suggesting four active nucleolar organizer regions (NORs) (Fig. S3).

Regarding DAPI/CMA banding, female and male mussels presented two GC-rich CMA_3^+ subterminal sites in the short arm of the chromosomes 6 and 12 (Figs. 1a, c). Differences in heterochromatin distribution between male and female karyotypes were not observed.

Immunodetection showed a dispersed profile for both acetylation and phosphorylation of H3 histones in both sexes. We did not observe

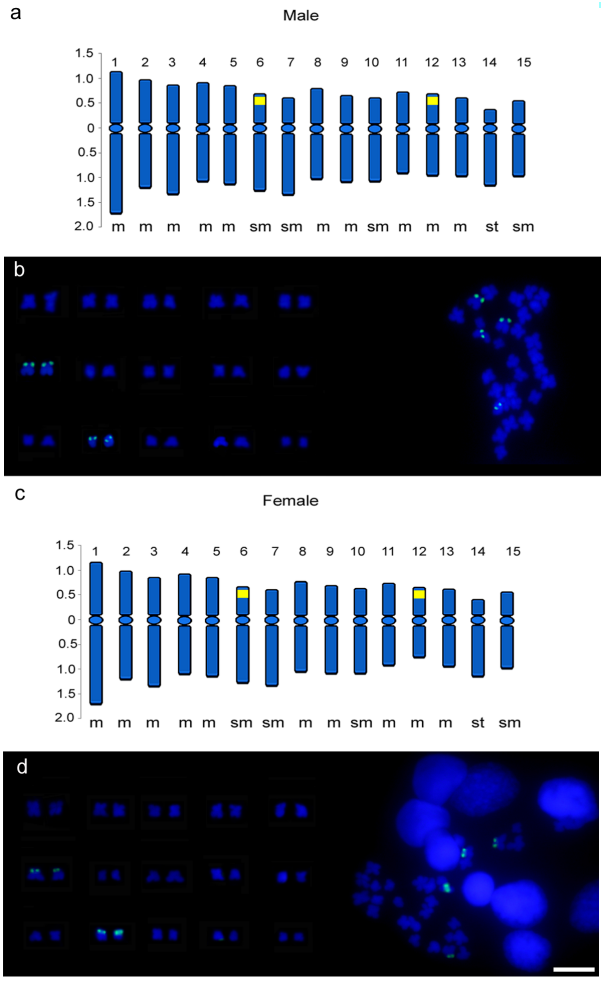

Figure 1. Representative ideograms and karyograms of DAPI$^-$/ CMA$_3^+$ marks in male (a,b) and female (c,d) of *Limnoperna fortunei*. CMA$_3^+$ bright bands are shown in chromosomes 6 and 12 in both males and females. Scale bar = 5 µm.

agglomerates or specific sites for epigenetic labeling of H3ser28phos and H3lys27ac. All nuclei presented the same pattern, with sites homogeneously spread. Immunostaining with anti-5-Methylcytosine did not demonstrate blocks or specific regions of DNA methylation (Fig. S3).

DISCUSSION

Karyological studies have been performed in a few mussels species within the Mytilidae family. Up to now, 35 out of approximately 250 species described were studied cytogenetically, with chromosome numbers ranging from 2n = 22 to 2n = 32 and similar size and morphology across species (Pérez-García et al. 2014, 2011, 2010a, b, Thiriot-Quiévreux 2002, Thiriot-Quiévreux & Insua 1992).

Chromosome number and karyotypic formulae of *L. fortunei* observed in this study were compatible with what was previously reported by Ieyama (1996) in Japan, with very similar chromosome sizes. The number of chromosomes was of 2n = 30 and comprises 20 medians, 8 submedian, and 2 sub telocentric chromosomes. The lack of numerical variation and rearrangements suggests a karyotypic stability for *L. fortunei*.

Besides a detailed description of the karyotype of *L. fortunei*, we aimed to search cytogenetic differences and chromosomal signatures between female and male individuals. With the tools used in this study, there were no differences observed in cytogenetic traits between females and males of *L. fortunei*. Morphometric data from both male and female karyotypes showed similar profiles with the same morphology and chromosomal classification. Heterochromatin distribution was evaluated by DNA staining with specific-base fluorochromes (DAPI/CMA$_3$), which allows the detection of blocks of heterochromatin rich in AT or GC, an useful approach for differentiating bands along chromosome arms in gonochoric organisms (Dutrillaux & Dutrillaux 2019). Our results with DAPI/CMA$_3$ banding, revealed two chromosome pairs containing DAPI$^-$ /CMA$_3^+$ sites, located in the same chromosomes for male and female, indicating once again a karyotypic homogeneity between the sexes in *L. fortunei*. Generally, blocks of CMA$_3^+$ heterochromatin are coincident to 28S DNAr sites, suggesting that the species presents four sites of ribosomal 28S genes. Several studies demonstrated the

Table I. Morphometry and classification of chromosomes of females and males of *Limnoperna fortunei*.

Chromosome	Short arm		Long arm		Absolute lenght		Ratio		Relative lenght		Classification	
	(F)	(M)	F	(M)	(F)	(M)	(F)	(M)	(F)	(M)	(F)	(M)
1	1.1	1.15	1.4	1.7	2.5	2.85	1.27	1.48	9.66	10.06	m	m
2	0.97	0.97	1.15	1.22	2.12	2.19	1.19	1.26	8.19	7.74	m	m
3	0.85	0.85	1.14	1.34	1.99	2.19	1.34	1.58	7.69	7.74	m	m
4	0.85	0.91	1.1	1.09	1.95	2.00	1.29	1.20	7.54	7.06	m	m
5	0.91	0.85	0.98	1.15	1.89	2.00	1.08	1.35	7.31	7.06	m	m
6	0.66	0.67	1.15	1.28	1.81	1.95	1.74	1.91	7.00	6.89	sm	sm
7	0.62	0.61	1.15	1.34	1.77	1.95	1.85	2.19	6.84	6.89	sm	sm
8	0.74	0.79	1.02	1.03	1.76	1.82	1.38	1.30	6.80	6.43	m	m
9	0.69	0.67	1.03	1.09	1.72	1.76	1.49	1.62	6.65	6.22	m	m
10	0.61	0.61	1.1	1.09	1.71	1.70	1.80	1.79	6.61	6.00	sm	sm
11	0.57	0.73	0.83	0.91	1.40	1.64	1.46	1.25	5.41	5.79	m	m
12	0.6	0.67	0.8	0.97	1.40	1.64	1.33	1.45	5.41	5.79	m	m
13	0.5	0.61	0.80	0.97	1.30	1.58	1.60	1.59	5.03	5.58	m	m
14	0.3	0.38	1.00	1.15	1.30	1.53	3.33	3.02	5.03	5.40	st	st
15	0.44	0.54	0.81	0.97	1.25	1.51	1.84	1.80	4.83	5.33	sm	sm

Footnote: Female (F); male (M); median (m); submedian (sm); subterminal (st).

relationship between GC-rich (CMA_3^+) regions with the number of NORs in Bivalvia and other mollusks (González-Tizón et al. 2000, Insua et al. 2001, Marti et al. 1997, Pérez-García et al. 2011). Consequently, four NORs seem to be present, as also shown by Ag-NOR staining results, which indicate four nucleoli in interphasic nuclei.

In addition to the morphometric and heterochromatin characterization, we investigated possible epigenetic marks in male and female specimens, to identify putative sex-related profiles. Generally, epigenetic marks are associated with environmental conditions, which seem to have a crucial role in the sex determination in bivalves (Breton et al. 2018). Among the most well known epigenetic modifications, DNA methylation comprises a mechanism that might be linked to sex determination (Piferrer 2013). In species with homomorphic sex chromosomes, for example, certain environmental parameters can alter the methylation patterns of sex-related *loci*, and determine the sex of individuals. Environmental conditions also can change the histone modification patterns and alter the expression of genes linked to sex-related *loci* (Piferrer 2013). Besides that, our imunocytogenetic data showed no differences in the male or female epigenetic patterns. We observed several fluorescent points spread throughout the genome, indicating presence of methyl groups, although not concentrated in specific genomic regions. The same profile was found for antibodies able to detect H3ser28phos and H3lys27ac, for which marks were dispersed in the nuclei, with no evident agglomerate of sites/

blocks related to the different sexes. There is a possibility that gills do not present conspicuous sites or evident alterations in the epigenetic profile, when females and males are compared. In order to verify this hypothesis, gonads and gametes should be assessed, to detect possible sex-specific epigenetic marks.

As pointed out for the bivalve species described so far (Breton et al. 2018), we observed that the golden mussel lacks heteromorphic pairs of sex chromosomes. Recently Yue et al. (2020), have scrutinized the entire genome of male and female *Crassostrea gigas* by using Restriction Site-associated DNA Sequencing, and did not find any sex-linked markers. Previous reports suggest that environmental stimulus can influence sex determination in this class, possibly through epigenetic mechanisms or an unusual Doubly Uniparental Inheritance system (DUI) of mitochondria (Ghiselli et al. 2012, 2013, Breton et al. 2018). The latter hypothesis has been excluded for *L. fortunei* (Ghabooli et al. 2013, Uliano-Silva et al. 2016).

Several genes known for their involvement in sex determination in model organims, such as *Sox* (Sry-type HMG box), *Fox* (Forkhead-box), and *Dmrt* (Doublesex and Mab-3-related transcription factor) family genes have been identified in bivalves, as in *Crassostrea gigas*, *Hyriopsis schlegelii* and *Crassostrea hongkongensis* (Santerre et al. 2014, Shi et al. 2015, Tong et al. 2015, Zhang et al. 2014). Members of these three gene families were also recently found in *L. fortunei* by L.F. Afonso et al. (unpublished data). The authors additionally identified 131 putative gene homologues which seems to be important for sex determination and differentiation in animals. Moreover, 15 of these genes have shown a sex-specific expression pattern in the gonads of adult golden mussels. Despite the absence of sex chromosomes observed here, the aforementioned data suggested that sexual determination and differentiation have a genetic component. Also, as hypothesized for other bivalves, these processes are likely to be orchestrated by gene-environment interactions.

The cytogenetic characterization performed in this study helped us better understand male and female karyotypes of *L. fortunei*. Through the approaches applied in this study to compare sexes, we could conclude that *L. fortunei* does not have heteromorphic sex chromosomes, as well as any other cytogenetic sex-specific features, such as epigenetic marks. Future investigations should focus on chromosome mapping, meiotic behavior and gonad epigenetic profiles to further clarify sex determination mechanisms in this species.

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