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Chromosomal mapping of rDNAs, core histone genes and telomeric sequences in *Perumytilus purpuratus* (Bivalvia: Mytilidae)

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ABSTRACT

The chromosomes of the mussel *Perumytilus purpuratus* were analyzed by means of 4',6-diamidino-2-phenylindole (DAPI)/propidium iodide (PI) and chromomycin A3 (CMA)/DAPI fluorescence staining and fluorescent in situ hybridization (FISH) with major rDNA, 5S rDNA, core histone gene and telomeric probes. The diploid chromosome number in this species is 32. The karyotype is composed by two metacentric, five submetacentric, one subtelo/submetacentric and eight subtelocentric chromosome pairs. The nucleolar organizing regions (NORs) detected by FISH using major rDNA probes were terminally located on the long arms of two pairs of chromosomes. NORs were associated with bright CMA fluorescence and dull DAPI fluorescence but not all the NORs showed bright CMA fluorescence in a given cell; intra- and inter-individual variability was found in this character. FISH detected a similar variability on the number of major rDNA signals. This species shows three 5S rDNA clusters on two chromosome pairs. Two of the clusters are terminal and intercalary located on one of the two NOR-bearing chromosome pairs; the third cluster is on the long arm of another chromosome pair. Core histone genes are clustered on a single locus near the centromere on the long arm of a different chromosome pair. This locus is not condensed in prophase I cells therefore suggesting a certain degree of histone gene expression during meiosis. As expected, *P. purpuratus* shows telomeric signals at both ends of every single chromosome but interstitial telomeric signals were also detected at centromeric positions on two chromosome pairs.

1. Introduction

Most karyological data on marine invertebrates comes from the study of species of the class Bivalvia. Chromosome numbers in bivalves range from 12 to 46 for diploid species (reviewed by Thiriot-Quiévreux, 1994, 2002) with modal values of $2n = 20$ for oysters, $2n = 28$ for mussels and $2n = 38$ for the other groups. In most families of this class, karyological data are limited to a few species and only in Pectinidae (scallops), Mytilidae (mussels), Ostreidae (oysters) and Veneridae (clams) the number of species studied is higher and allow to establish comparative chromosome analyses. Chromosome numbers are conserved in Ostreidae ($2n = 20$, 16 species; $2n = 18$, 1) and Veneridae ($2n = 38$, 12) but not in Pectinidae ($2n = 38$, 10; $2n = 32$, 5; $2n = 26$, 1) and Mytilidae ($2n = 22$, 1; $2n = 26$, 4; $2n = 28$, 14; $2n = 30$, 7; $2n = 32$, 5; and $2n = 34$, 1). Therefore, the family Mytilidae is an interesting taxon in which to study chromosome evolution in bivalves.

The great amount of karyological data obtained in bivalves using standard Giemsa staining is still not matched by molecular cytogenetic data. Although during the last years fluorescent in situ hybridization (FISH) has been used to map some repetitive sequences in bivalves (reviewed by Thiriot-Quiévreux, 2002; Guo et al., 2007; Leitão and Chaves, 2008) and molecular cytogenetic studies now represent approximately 77% of all recently published chromosomal works in bivalves (Leitão and Chaves, 2008), information on this field, of enormous importance on the analysis of chromosome evolution in other organisms, is still scarce. Ribosomal RNA genes in higher eukaryotes are highly conserved sequences organized in two independent tandemly repeated gene families, major (18 + 5.8 + 28S) and minor (5S) rRNA genes. Major rRNA genes (major rDNA) have been located in about 50 species of bivalves (Thiriot-Quiévreux, 2002; Guo et al., 2007; Fernández-Tajes et al., 2008 and references therein) including six species of the family Mytilidae (Martínez-Expósito et al., 1997; Insua and Méndez, 1998; Torreiro et al., 1999; González-Tizón et al., 2000; Vitturi et al., 2000; Insua et al., 2001). On the contrary, the chromosomal location of 5S rRNA genes is only known in 14 species, two of them belonging to the family Mytilidae (Insua et al., 2001). Histone genes are usually organized in tandem arrays clustered in one or more chromosomal positions in most eukaryotic genomes although in others are nontandemly clustered or dispersed (reviewed by Eirín-López et al., 2009). In bivalves, histone genes have been characterized in some species of Mytilidae (Drabent et al., 1999; Eirín-López et al., 2002; Albig et al., 2003; Eirín-López et al., 2004), Pectinidae (Li et al., 2006) and Veneridae (González-Romero et al., 2008) but their chromosomal locations are only known in the mussel *Mytilus galloprovincialis* (Eirín-López et al., 2004) and four species of

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Table 1
Combinations of primers used in the PCR amplification.

Region	Primer sequence (5′–3′)	
ITS	*ITS5*	GGAAGTAAAAGTCGTAACAAGG
	ITS4	TCCTCCGCTTATTGATATGC
28S	*LR10R*	GACCCTGTTGAGCTTGA
	LR12	GACTTAGAGGCGTTCAG
5S	*5SD*	CAACGTGATATGGTCGTAGAC
	5SR	AACACCGGTTCTCGTCCGATC
H3	*H3D*	ATGGCTCGTACCAAGCAGACVGC
	H3R	ATATCCTTRGGCATRATRGTGAC
H2BA	*H2BAD*	CCCGAGATGTGATGGTAGA
	H2BAR	AGTACAGCCTGGATGTTTGGTAA

ITS: internal transcribed sequence of the major rDNA; 28S: 28S rRNA gene; 5S: 5S rRNA gene; H3: histone H3 gene; H2BA: H2B and H2A histone genes.

Table 2
Conditions used in the PCR amplification.

Region	Cycles	Denaturation	Annealing	Extension
ITS	30	95 °C, 30 s	48 °C, 30 s	72 °C, 30 s
28S	30	95 °C, 15 s	48 °C, 15 s	72 °C, 15 s
5S	30	95 °C, 20 s	48 °C, 20 s	72 °C, 30 s
H3	30	95 °C, 30 s	44 °C, 20 s	72 °C, 60 s
H2BA	30	95 °C, 30 s	45 °C, 30 s	72 °C, 40 s

ITS: internal transcribed sequence of the major rDNA; 28S: 28S rRNA gene; 5S: 5S rRNA gene; H3: histone H3 gene; H2BA: H2B and H2A histone genes.

Pectinidae (Zhang et al., 2007). Telomeric sequences have been characterized and located in a few species of bivalves including *Mytilus galloprovincialis* (Martínez-Expósito et al., 1997; Plohl et al., 2002). Although the presence of interstitial telomeric sequences is widespread in vertebrates (Lin and Yan, 2008), and has been described in other organisms as insects (López-Fernández et al., 2004), gastropods (Nomoto et al., 2001) and plants (Schubert, 2007), only Plohl et al. (2002) showed molecular data that points to the presence of these sequences at internal locations in the bivalve *M. galloprovincialis*.

Perumytilus (Brachidontes) purpuratus (Lamarck, 1819) is a marine bivalve naturally distributed from Ecuador to the Strait of Magellan on the Pacific coast of South America, and extending on the Atlantic coast up to Punta Valdés in Argentina. It is a small mussel species with an adult size of about 20–30 mm. The only cytogenetic study of this species (Álvarez-Sarret et al., 1991) reported a diploid chromosome number of $2n = 34$, the highest described in the family Mytilidae. In an effort to get insights on the chromosomal changes within the family Mytilidae, we analyzed the chromosomes of *P. purpuratus* by means of 4′,6-diamidino-2-phenylindole(DAPI)/propidium iodide (PI) and chromomycin A3 (CMA)/DAPI fluorescence staining and fluorescent in situ hybridization (FISH) with 18S + 28S rDNA, 5S rDNA, core histone genes and telomeric probes.

2. Materials and methods

2.1. Biological material

Juvenile specimens of *Perumytilus purpuratus* were collected from an intertidal population in Valparaíso (Chile). Animals were maintained in the laboratory in 5 L tanks of aerated, filtered seawater at 18 ± 1 °C and fed on a suspension of algal cells (*Tetraselmis suecica* and *Isochrysis galbana*).

2.2. DNA preparation, PCR amplification and sequencing

Total DNA was extracted according to Estoup et al. (1996) with minor modifications. About 3 mg of adductor muscle tissue was homogenized in 0.4 mL of 10% Chelex 100 (BioRad) and pronase (1.4 mg/mL) at 60 °C. After incubating for 1 h at 60 °C in agitation, the extracted DNA was stored at 4 °C.

Amplifications were performed in 20 μL of a solution containing 50 ng DNA, 1xPCR buffer, 0.5 mM each dNTP, 2.5 mM $MgCl_2$, 1 mM each primer and 1 U BioTaq DNA polymerase. Primer combinations are given in Table 1. Universal primers were used to amplify both the internal transcribed spacer (ITS) region and a fragment of the 28S gene of the major rDNA repeats (*ITS4*, *ITS5*, *LR10R*, *LR12*; White et al., 1990). For the 5S rDNA amplification, primers were designed from the sequence of the 5S rRNA of *Mytilus edulis* (Fang et al., 1982). The amplification of the H2A-H2B and the H3 histone genes was performed using primers designed from the histone genes of *Mytilus edulis* (Albig et al., 2003) and those described by Giribert and Distel (2003), respectively. After 5 min denaturation at 95 °C, 30 cycles of amplification were performed using the conditions that are shown in Table 2. A final extension step of 7 min at 72 °C was applied. All reactions were performed in a GeneAmp PCR system 9700 (Applied Biosystem) and PCR products were examined by electrophoresis on a 2% agarose gel. Single products were obtained after amplification using each set of primers. The amplified DNA was sequenced on an automated DNA sequencer (ABI PRISM 310, Applied Biosystems). Sequences were aligned and analyzed using Clustal W (Thompson et al., 1994). Nucleic acid sequences were subjected to BLAST 2 Sequences (Tatusova and Madden, 1999) at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast).

2.3. Chromosome preparation, fluorochrome staining and karyotype

Gill and mantle tissues excised from animals exposed to colchicine (0.005%) were immersed in diluted seawater and fixed in cold ethanol/acetic acid (Martínez-Expósito et al., 1997). Chromosome spreads were obtained by dissociating small pieces of tissue in acetic acid and dropping the cellular suspension onto clean slides. Some mitotic and meiotic preparations were stained for 8 min with a combination of DAPI (0.14 μg/mL) and PI (0.07 μg/mL) in 2xSSC. Other slides were stained with a combination of CMA (0.25 mg/mL) and DAPI. Chromosome counts were performed in 401 metaphases from 10 individuals. Karyotypes were constructed from ten complete metaphase plates and relative lengths and centromeric indices were calculated. Chromosome nomenclature follows Levan et al. (1964).

2.4. Fluorescent in situ hybridization (FISH)

Single and double FISH experiments using biotin and digoxigenin labeled major rDNA (ITS and 28S), 5S rDNA, and core histone (H2B/H2A and H3) gene probes were performed following previously

Fig. 1. Chromosomal location of major rDNA, 5S rDNA and histone genes on *Perumytilus purpuratus* chromosomes. Sequential staining of the same metaphase plate with DAPI/PI (a) and CMA (b) shows two GC-rich (DAPI dull/CMA bright) regions located at the ends of the long arms on two different chromosome pairs (arrows). Fluorescent in situ hybridization (FISH) using a 28S rDNA probe (biotin, fluorescein, green) on the same (c) and another metaphase (d) exemplifies the variability in the number of major rDNA signals detected. In the metaphase showing three signals (c), two of them are coincident with the DAPI dull/CMA bright positions but there is also an additional signal (arrowhead) showing homogeneous DAPI/CMA staining. The metaphase in (d) shows four major rDNA signals at terminal positions on two chromosome pairs. FISH using a 5S rDNA probe (biotin, fluorescein, green) demonstrates (e) the presence of three clusters of 5S rDNA on two chromosome pairs. FISH using a histone H3 gene probe (biotin, fluorescein, green) shows (f) a single cluster of histone genes near the centromere on the long arm of a chromosome pair. Double FISH experiments using a 5S rDNA probe (biotin, fluorescein, green) and a 28S rDNA probe (digoxigenin, rhodamine, red) demonstrate (g, h, i) that the chromosome bearing the double 5S signal also bears one of the major rDNA signals so allowing to easily analyze the variability in the chromosomal distribution of the major rDNA signals. The examples given are metaphases showing four (g) and three (h, i) major rDNA signals. Triple hybridization (j) using a biotin labeled 5S rDNA probe (green), a digoxigenin labeled 28S rDNA probe (red) and a double labeled histone H3 gene probe (yellow, as a result of the mixture of green and red) demonstrates that the histone gene cluster is independent of the rDNA clusters. This is clearly shown on the corresponding karyotype (k). Scale bars, 5 μm.