CYTOGENETIC CHARACTERIZATION OF THE DWARF OYSTER OSTREA STENTINA (MOLLUSCA: BIVALVIA) AND COMPARATIVE KARYOLOGICAL ANALYSIS WITHIN OSTREINAE

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ABSTRACT Regardless of the high economic value and large geographical distribution of oysters, the current knowledge of oyster taxonomic relationships and systematics is still limited, particularly for flat oysters. In this study, the molecular cytogenetic characterization of mitotic chromosomes of the Provence flat oyster or dwarf oyster, Ostrea stentina, was performed through Giemsa staining, chromosome measurements, in situ restriction endonuclease banding, C-banding, fluorescence in situ hybridization with major ribosomal RNA genes (ITS1), and telomeric sequence (TTAGGG)n. The karyotype (2n = 20) consisted of 6 metacentric (1, 3, 4, 6, 8, and 10) and 4 submetacentric (2, 5, 7, and 9) chromosome pairs. Chromosome treatment with HaeIII produced specific banding patterns for all chromosomal pairs, confirming the efficiency of this restriction enzyme for chromosome banding in oysters. Results for C-banding revealed the presence of heterochromatin in the telomeric regions of the short arms: on a large metacentric chromosomal pair and on a submetacentric chromosomal pair. In situ hybridization with telomeric sequence revealed bright hybridization signals in the telomeres of all chromosomes. The location of the major ribosomal DNA (ITS1) displayed the presence of 2 signals: in the telomeric regions of the short arms of the largest metacentric chromosome and in a submetacentric chromosome. The cytogenetic data obtained was used to perform a comparative karyological analysis within the subfamily Ostreinae. It is also important to highlight that this type of work can provide new insights on major genomic changes at the chromosome level in the flat oysters.

KEY WORDS: Ostrea stentina, dwarf oyster, Ostreinae, karyotype, ribosomal genes, telomeric sequence, C-banding, FISH

INTRODUCTION

The Provence oyster, Ostrea stentina (Payradeau, 1826), also known as the dwarf oyster, is present along the Atlantic and Mediterranean coasts of Europe, and along the African Atlantic coast as far as South Africa (Lapegue et al. 2006). Studies on this flat oyster species are very limited, mainly because it is a species of low commercial value as a result of its small size.

Current knowledge of oyster systematics is still fragmentary, particularly in the subfamily Ostreinae, the flat oysters. According to the classification of Harry (1985) based on anatomy and shell morphology, the family Ostreidae includes three subfamilies: Lophaeinae, Crassostreinae, and Ostreinae. Within the subfamily Ostreinae, Harry (1985) subdivided them into four tribes and nine genera, and synonymize some previously described species. O. stentina was grouped with Ostreola equestris and Ostreola conchaphila, forming the genus Ostreola. However, the high morphological plasticity of oysters in the subfamily Ostreinae greatly complicates their identification and classification. Several molecular phylogenetic studies have questioned the validity of some of the tribes, genera, and synonymization proposed by Harry (1985). For instance, Lapegue et al. (2006), through sequence analysis of a 16S mitochondrial fragment, found that those three Ostreola species were nested in a clade with other flat oyster species (i.e., Ostrea puelchana, Ostrea denvelamella, Ostrea algoensis, and Ostrea chilensis) that were not included in the genus Ostreola per the synopsis by Harry (1985). Furthermore, Lapegue et al. (2006) also revealed that O. stentina, O. equestris and Ostreola aoporina were closely related. Moreover, the diagnostic morphological characters of the genus Ostreola have been questioned by Coan et al. (2000). Other similar studies (Jozelewicz & O’Foighil 1998, Kirkendale et al. 2004, Lapegue et al. 2006, Shilts et al. 2007) proposed the incorporation of Ostreola species (O. stentina, O. equestris, and O. conchaphila) in the genus Ostrea.

The identification of structural chromosomal features has proved useful in the clarification of systematic and taxonomic relationships in bivalves (see Leitão and Chaves (2008) for review). Among the flat brooding oyster species, the Ostreinae, 6 species have previously been investigated karyologically through karyotype description, AgNORs, and C-banding: Ostrea edulis (Thiriot-Quevrelux, 1984), O. denvelamella (Insua & Thiriot-Quevrelux 1991), O. puelchana (Insua & Thiriot-Quevrelux 1993), O. chilensis (Ladron de Guevara et al. 1996), Ostrea angasi (Li & Havenhand 1997) and O. conchaphila (Leitão et al. 2002).

In recent years, cytogenetic studies in bivalves have expanded as a result of the introduction of new molecular cytogenetic techniques such as in situ restriction endonuclease (RE) banding and fluorescence in situ hybridization (FISH). Three REs—ApaI, HaeIII, and PstI—were applied to the chromosomes of four oyster species (Crassostrea gigas, Crassostrea angulata, O. conchaphila, and O. edulis), and analysis of the banding pattern in the karyotypes revealed a greater similarity within the genera Ostrea and Crassostrea than between them (Leitao et al. 2004).

FISH is widely used for chromosome identification, gene mapping, and studies on chromosome rearrangement in a variety of organisms (Swiger & Tucker 1996, Nath & Johnson
1999, Xu et al. 2001). Repetitive DNA sequences are ideal to use as FISH probes because of their large target size. Telomere sequence (TTAGGG)\textsubscript{n} has been localized in the chromosomes of several species of oysters, clams, and mussels (for a review, see Leitao and Chaves (2008)). Ribosomal DNA (rDNA) has both fast- and slow-evolving regions, and is particularly useful in inferencing and phylogenetic relationships in bivalves (Mindell & Honeycutt 1990). Indeed, in recent years there has been an increase in attention to the structure and location of ribosomal genes in bivalve chromosomes, and in using these genes as a cytogenetic marker in taxonomic/evolutionary studies, including in the cupped oysters Crassostreinae (e.g., Xu et al. 2001, Wang & Guo 2004). However, until now, no FISH studies have been performed within Ostreinae.

In this study, we characterized for the first time the genome of O. stentina using cytogenetic approaches, such as C-banding, in situ RE banding, FISH with major ribosomal gene (ITS1), and telomere sequence (TTAGGG)\textsubscript{n}. Our cytogenetic data lend support to a recent proposal to reorganize the subfamily Ostreinae, specifically in what concerns the genus Ostrea and the genus Ostreola.

**MATERIALS AND METHODS**

**Biological Material and Chromosome Preparation**

The specimens used in this study were hatchery-produced juveniles, using as parents O. stentina collected in Ria Formosa Lagoon (Portugal). Chromosome metaphases were prepared from gill tissue. Twenty individuals (≈1.5 cm in length) were incubated for 8 h in a 0.005% solution of colchicine in seawater. The material was fixed in a freshly prepared mixture of absolute ethanol and acetic acid (3:1) with 3 changes of 20 min each. Fixed pieces of gill from each individual were dissociated in 50% acetic acid with distilled water. The suspension was dropped onto slides at 44 C and air-dried (Thriot-Quievreux & Ayraud 1982). The slides for in situ RE banding and FISH experiments were kept at −20 C until further use.

**Karyotyping**

For conventional karyotyping, gill preparations were stained with Giemsa (4%; pH, 6.8) for 10 min. Chromosome measurements of 10 suitable metaphases were made using the software ImageJ (version 1.32j). Relative length (100 \% chromosome length/total haploid length) and centromeric index on slides and covered with coverslips. Slides were then incubated in a humid chamber for 16 h at 37 C. Control slides were subjected to the same treatment as just described, but incubated without HaeIII. The slides were then washed in distilled water, air-dried, and stained with Giemsa (1% solution, diluted in phosphate buffer at pH 6.8).

**Constitutive Heterochromatin**

Constitutive heterochromatin regions (C-bands) were revealed using the method of Summer (1972), using propidium iodide as a counterstain.

**Probe Construction and Labeling**

Genomic DNA was extracted from ethanol-preserved adductor muscle as described by Pereira (2008). For major ribosomal gene mapping we used ITS1, a specific probe generated by PCR using the primers ITS1CA-CB (ITS1CA: 5'-GGTTTCGGATGTAACTTG-3', ITS1CB: 5'-CTCGTCTGATCTGAGGTCG-3') (Heath et al. 1995). Amplification reactions were performed in volumes of 25 μL; the reaction mixture contained 100 ng of genomic DNA, 200 nM of each dNTP, 1 nM of each primer, 0.5 U Taq polymerase (Fermentas, Life Sciences), and the buffer recommended by the supplier. The thermal cycler program consisted of an initial denaturation of 3 min at 94 C, 35 cycles at 94 C for 30 sec, 55 C for 30 sec, and 68 C for 45 min, and a final extension step at 72 C for 5 min. Labeling was obtained using a PCR procedure with the following dNTP concentrations: 200 nM dATP, 200 nM dCTP, 200 nM dGTP, 150 nM dTTP, and 50 nM dig-11-dUTP (Roche Molecular Biochemicals). Telomeric (TTAGGG)\textsubscript{n} probes were generated by PCR and labeled with digoxigenin in the absence of a template as described by Ijdo et al. (1991). PCR and labeling length products were evaluated by ethidium bromide (2% agarose gels. Each probe was precipitated separately with ethanol and then dissolved in hybridization solution.

**FISH**

Chromosome spreads were pretreated with 0.005% pepsin in 10 mM HCl for 5 min at 37 C. The slides were fixed with 1% formaldehyde for 20 min and 3 washes in 13 PBS solution for 5 min each at room temperature. Chromosome preparations were aged at 65 C overnight. The slides were then dehydrated in a graded ethanol series (70%, 90%, and 100%) and air-dried. The slides were denatured for 2 min at 65 C in 70% formamide in 23 SSC. Simultaneous denaturation of the DNA probe was performed at 65 C for 15 min and immediately put on ice for 5 min. Hybridization, posthybridization washes, and detection was (100 \% length of short arm/total chromosome length) were calculated. Terminology regarding centromere position followed that of Levan et al. (1964)

In situ RE Banding

Slides were aged for 6 h in a dry incubator at 65 C before digestion with the restriction enzyme HaeIII. The digestion was carried out as described by the manufacturer (Invitrogen, Life Technologies, Carlsbad, CA) using 30 U HaeIII in a total volume of 100 nL. The solution was placed carried out according to Chaves et al. (2002). Digoxigenin-labeled probes were detected with antidigoxigenin-rhodamine Fab fragments antibody (Roche Molecular Biochemicals).

**Microscopy and Image Processing**

Chromosomes were observed with a Zeiss Axioscope 2 Imaging microscope, coupled to an AxioCam digital camera with AxioVision software (version rel. 4.5; Zeiss). Digitized photos were prepared for printing in Adobe Photoshop (version 5.0); contrast and color optimization were the functions used, and they affected the whole of the image equally.
RESULTS

Analysis of 62 mitotic metaphase spreads from 20 individuals of O. stentina revealed a diploid chromosome number of 2n = 40. For karyotyping, the chromosomes of the 10 best spreads were used for chromosome measurements and classification (Table 1). The karyotype consists of 10 chromosome pairs. Pairs 1, 3, 4, 6, 8, and 10 are metacentric, and pairs 2, 5, 7, and 9 are submetacentric.

The application of the restriction enzyme HaeIII to chromosomes of O. stentina produced specific banding patterns, which allowed the precise organization of the karyotype of this species to be acquired (Fig. 1). The banding pattern obtained was indeed consistent between homologous chromosomes in each chromosomal pair. In the control slides, no banding pattern was induced in the chromosomes; all chromosomes showed a standard Giemsa staining.

Constitutive heterochromatin was analyzed with the C-banding technique (Fig. 2A). Telomeric C-bands were observed mostly in the telomeres of a metacentric chromosome and a submetacentric chromosome. Positive C-banding staining was also observed in the centromere of several chromosomal pairs (Fig. 2A). PCR amplification of ITS1 of the major ribosomal gene in O. stentina generated a single fragment of approximately 850 bp in length. FISH with the major ribosomal gene probe (ITS1) showed that this sequence is located on the telomeric regions of 2 chromosomes (Fig. 2B). Telomeric sequence (TTAGGG)n signals were located at both ends of all chromosomes. Although bright hybridization signals were present in all chromosomes, intensity varied among the different pairs. No hybridization signal was observed at any interstitial chromosomal site (Figs. 2C and 3). All data obtained in this work are assembled in Figure 3. Banding with the HaeIII RE produced specific banding patterns, allowing an accurate organization of the karyotype of O. stentina (Figs. 1 and 3). C-bands produced by barium hydroxide using propidium iodide were observed in telomeres of the short arms of a large metacentric chromosome (chromosome pair 3) and in the short arm of a submetacentric chromosome (chromosome pair 5). Furthermore, in chromosomes 1, 2, 7, and 8, positive C-banding staining in the centromere was also observed (Figs. 2A and 3). The major ribosomal DNA unit (ITS1), detected by FISH, was located at the telomeric regions of the short arm of 2 chromosomes: 3 and 7 (Figs. 2B and 3). Both chromosome pairs were polymorphic for this sequence.

DISCUSSION

This is the first report on karyotype and chromosome measurements of the dwarf oyster O. stentina (Table 1). The diploid chromosome number of 2n = 40 is characteristic of the genus Ostrea and is the most common throughout the Ostreacea (for a review, see Thirot-Quievreux (2002)).

The karyotype of O. stentina, with 6 metacentric and 4 submetacentric chromosome pairs, revealed a karyotypic formula very similar to that of O. conchaphila, presenting differences only in pairs 2 and 3, which are very similar in size. In 2002, Leitao et al. performed a karyotype comparison of 6 flat oyster species and highlighted the following issues: (1) a close similarity between O. edulis and O. angasi that had also been previously pointed out by Li and Havenhand (1997) and (2) the unique karyotype of O. puechiana resulting from the presence of a single telocentric chromosome pair. The three other studied flat oyster species—Ostrea denselamellosa, O. chilenis, and O. conchaphila—had related karyotypes. Ostreinae have karyotypes mostly with metacentric and submetacentric chromosome pairs, suggesting that oyster species might have diverged through pericentric inversions, reciprocal translocations, or centromere reposition.

The comparison of the HaeIII RE banding pattern of O. stentina in this study (Figs. 1 and 3) with those previously described for O. edulis and O. conchaphila (Leitao et al. 2004) revealed that although all three species showed a characteristic HaeIII RE banding pattern, there were larger similarities between O. stentina and O. conchaphila than between these two species and O. edulis, mainly in chromosome pairs 1, 9, and 10.

C-bands in O. stentina, were located in the telomeric regions of the short arms of chromosomes 3 and 5 (Figs. 2A and 3). A similar location was previously observed in O. conchaphila (Leitao et al. 2002) and in O. denselamellosa in which occasional telomeric C-bands bands were also observed in pairs 3 and 5 (Insua & Thirot-Quievreux 1991).

The ribosomal genes (ITS1) in O. stentina are located on chromosomes 3 and 7 (Figs. 2B and 3). We also detected polymorphisms on both chromosomes and variation in FISH signals, as already described for other bivalve species (e.g., Martinez et al. 1996, Wang & Guo 2004, Wang & Guo 2007).

Hybridization signals of the sequence (TTAGGG)n in O. stentina were located in the telomeres of all chromosomes, with no interstitial signals observed. The same pattern had been previously observed in the subfamily Crassostreinae, in C. angulata (Cross et al. 2005). On the other hand, in the mussel Mytilus galloprovincialis (Plohl et al. 2002), the telomeric sequence showed interstitial positions in the chromosomes beyond telomeric positions.

To infer cytotaxonomic relationships within Ostreinae, we constructed ideograms, based on relative length and centromeric index values, number and location of major ribosomal genes (either by FISH with ITS1 or AgNORs), and C-banding (Fig. 4).

The karyological data obtained in the current study of O. stentina highlighted a high resemblance between O. stentina, O. conchaphila, and O. denselamellosa, supporting the incorporation of the Ostreola genus into Ostrea, as suggested by Coan et al. (2000), Lappegue et al. (2006), and Shilts et al. (2007). The comparison of the number and location of the major ribosomal genes, some revealed by the silver nitrate technique, highlighted the similarity between O. stentina and O. denselamellosa. Moreover, the simultaneous presence of AgNORs/major ribosomal genes (ITS1) and C-band features observed in chromosome pair 3 in this study was previously observed in the subfamily Ostreinae in O. denselamellosa (Insua & Thirot-Quievreux 1991) and in O. conchaphila (Leitao et al. 2002), which might corroborate the close relationship between these two species, and in O. stentina as suggested by Lappegue et al. (2006) and Shilts et al. (2007) studies based on sequence data analysis of 16S mitochondrial fragment and internal transcribed spacer (ITS-1). Cytogenetic characterization of other Ostreinae species will certainly be useful and will help in the clarification of taxonomic relationships in this important group of flat oyster species.
This is the first study using FISH in chromosomes of Ostreinae species. The application of a large spectrum of probes to other species of this family will help us to clarify taxonomic issues and relationships within the family, and identify changes in chromosome number and structure to comprehend more fully the genome evolution of bivalves.

ACKNOWLEDGMENTS
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LITERATURE CITED


### TABLE 1.

Chromosome measurements and classification of *O. stentina*.

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<th>Chromosome Pair</th>
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<th>SD (μm)</th>
<th>Centromeric Length Mean (μm)</th>
<th>SD (μm)</th>
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<td>38.02</td>
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<td>42.08</td>
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m, metacentric; m-sm, metacentric-submetacentric; sm, submetacentric; sm-st, submetacentric-subtelocentric.

Figure 1. Example of a karyotype of *Ostrea stentina* banded with HaeIII.
Figure 2. Banding and FISH in chromosomes of O. stentina. (A) C-banding. (B) FISH with the rDNA probe (ITS1). (C) FISH with the telomeric probe (TTAGGG)$_n$. 
Figure 3. This figure presents a compilation of all the data obtained for banding of the O. stentina chromosomes: C-bands by barium hydroxide using propidium iodide (CBP), banding with the HaeIII restriction enzyme (RE), FISH with the telomeric probe (TEL), and FISH with the rDNA probe (ITS).

<table>
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Figure 4. Ideograms based on relative length; centromeric index values for O. stentina, O. conchaphila, O. puelchana, O. denselamellosa, O. edulis, O. angasi, and O. chilensis; number and location of major ribosomal genes (either by FISH with ITS1 and AgNORs); and C-banding.