RESEARCH ARTICLE

Chromosomal organization of simple sequence repeats in the Pacific oyster (*Crassostrea gigas*): $(GGAT)_4$, $(GT)_7$ and $(TA)_{10}$ chromosome patterns

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Abstract

Chromosome identification is essential in oyster genomic research. Fluorescence *in situ* hybridization (FISH) offers new opportunities for the identification of oyster chromosomes. It has been used to locate satellite DNAs, telomeres or ribosomal DNA sequences. However, regarding chromosome identification, no study has been conducted with simple sequence repeats (SSRs). FISH was used to probe the physical organization of three particular SSRs, $(GGAT)_4$, $(GT)_7$ and $(TA)_{10}$ onto metaphase chromosomes of the Pacific oyster, *Crassostrea gigas*. Hybridization signals were observed in all the SSR probes, but the distribution and intensity of signals varied according to the oligonucleotide repeat. The intercalary, centromeric and telomeric bands were observed along the chromosomes, and for each particular repeat every chromosome pair presented a similar pattern, allowing karyotypic analysis with all the SSRs tested. Our study is the first in mollusks to show the application of SSR *in situ* hybridization for chromosome identification and karyotyping. This technique can be a useful tool for oyster comparative studies and to understand genome organization in different oyster taxa.

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Introduction

Studies on oyster cytogenetics have mainly been concerned with the data on chromosome number and gross morphology (Thiriot-Quiévreux 1984), with chromosome differences usually described by characteristic proportions of specific chromosome morphotypes. Differential staining techniques (G-, C- and NOR (nucleolus organizer regions)-banding techniques) have also been introduced to oyster cytogenetic studies (Insua and Thiriot-Quiévreux 1993; Ladron de Guevara *et al*. 1994). Chromosomes treated by these methods reveal more details, not only on interspecific differences, but also for interchromosomal differences, which can distinguish between morphologically similar chromosome pairs, and hence allows the construction of standardized karyotype. Recently, molecular techniques like fluorescent *in situ* hybridization (FISH) offer powerful tools for chromosome studies. FISH has several advantages over earlier used techniques. It is a relatively simple and rapid technique that also enables the simultaneous or successive localization of one or more DNA probes along chromosomes using different fluorescent systems for multi-probe analysis. FISH has been proven to be a reliable, reproducible and accurate method (Zhao *et al*. 1998). Another interest of the FISH technique is that restriction banding technique was shown to be compatible with FISH in mammals (Chaves *et al*. 2002).

Among oysters, the Pacific oyster, *Crassostrea gigas*, is an economically important mollusk species cultured

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throughout the world. This species has a haploid complement of 10 metacentric chromosomes (Thiriot-Quiévreux 1984). In *C. gigas*, several banding techniques were applied to chromosomes, in order to identify them individually and to establish a standardized karyotype. G-banding pattern (Rodríguez-Romero *et al*. 1979; Leitão *et al*. 1999, 2001) and chromosome banding with restriction enzymes (Leitão *et al*. 2004; Bouilly *et al*. 2005; Cross *et al*. 2005) have also been carried out in *Crassostrea* oysters. Recently, a molecular cytogenetics approach based on *in situ* hybridization of bacteriophage P1 clones was also used for chromosome-specific probes in *C. virginica* (Wang *et al*. 2005). FISH offers new opportunities for the identification of oyster chromosomes. By detecting hybridization signals produced by a specific DNA probe, FISH permits the direct mapping of genes or DNA sequences to specific chromosomes and/or subchromosomal regions. In animals, FISH has been used in a variety of applications including the characterization and identification of chromosomes (Wang *et al*. 2005), the detection of aneuploidy (Zudova *et al*. 2003), the physical mapping of genes (Insua and Méndez 1999), and comparative genome hybridization (Adega *et al*. 2006). Oyster tissue and embryo preparations of *C. gigas* have been shown to be suitable for FISH analysis using various molecular probes such as satellite DNAs, telomeres or ribosomal DNA sequences (Clabby *et al*. 1996; Guo and Allen 1997; Xu *et al*. 2001; Wang *et al*. 2001, 2004).

Simple sequence repeats (SSRs) or microsatellites are candidate sequences that could produce chromosome-specific hybridization pattern in *C. gigas*. SSRs are a class of repetitive DNA sequences widespread in prokaryotic and eukaryotic genomes (Tautz and Renz 1984; Zane *et al*. 2002). SSRs consist of short motifs, 1–5-bp long, repeated in tandem arrays with identical, composite or degenerate motifs. SSRs are abundant within genomes and are present in both coding and noncoding regions. They are also usually characterized by a high degree of length polymorphism and, for this reason, they have proven to be extremely valuable tools for genome studies in many organisms and very powerful genetic markers that are often specific to single varieties or even individuals (Weising *et al*. 1989; Schmidt *et al*. 1993; Depeiges *et al*. 1995; Schuler *et al*. 1996; Knapik *et al*. 1998).

In the genus *Crassostrea*, the only report about using SSRs as chromosomal landmarks after *in situ* hybridization concerned *C. angulata* (Cross *et al*. 2005). In this work, numerous small hybridization signals generated by the oligonucleotide repeat GATA were observed throughout *C. angulata* chromosomes without any banding pattern, allowing the authors to conclude the dispersed distribution of the microsatellite throughout the genome.

In the present study, we applied the FISH technique with SSR probes for the individual identification of chromosomes in the Pacific oyster *C. gigas* and for better understanding the oyster genome organization.

Materials and methods

Biological material

Embryos from adult Pacific oysters, *C. gigas* bred at the IFREMER (Institut Français de Recherche pour l'Exploitation de la Mer) hatchery in La Tremblade (Charente-Maritime, France) were used. Gametes were collected by strip-spawning sexually mature animals. Fertilized gametes were cultured in seawater at 23°C in 150-l fibreglass larval.

Preparation of metaphase spreads

To block the mitosis in metaphase cells, 6-h-old embryos were incubated for 25 min in seawater containing 0.005% colchicine. The embryos were then treated for 10 min in 0.9% sodium citrate and fixed in a freshly prepared mixture of absolute ethanol: acetic acid (3:1). The fixed embryos were stored at 4°C. Slides were prepared following the air drying technique of Thiriot-Quiévreux and Ayraud (1982).

Probe labelling and FISH

The synthetic oligonucleotides $(GGAT)_4$, $(GT)_7$ and $(TA)_{10}$ were end-labelled with digoxigenin-11-dUTP (Roche Molecular Biochemicals, Penzberg, Germany) by terminal transferase (Roche Molecular Biochemicals, Penzberg, Germany) following the manufacturer's instructions.

Slides with well spread metaphase chromosomes were aged for 2 h at 65°C. They were rinsed twice in phosphate-buffered saline (PBS) for 5 min, and after incubation in 3% formaldehyde in PBS for 20 min at room temperature, they were washed twice in PBS for 5 min. These slides were then dehydrated in an ice-cold ethanol series of 70%, 90%, and 100%, 5 min each, and air-dried. The chromosomal DNA on the glass slide was then denaturated at 65°C in 70% formamide in 2 × SSC for 2 min, followed by dehydratation in 70%, 90% and 100% ethanol, for 3 min each at −20°C. The hybridization mixture contains 20 × SSPE (3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4), 50 × Denhardt's solution, 10% sodium dodecyl sulphate, 50 ng μl^{-1} denaturated *E. coli* DNA, 1–2 pmol labelled oligonucleotide probe and water to 30 μl. *In situ* hybridization was performed overnight at 37°C. Then, the slides were subjected to stringent washes in 6 × SSC at the respective duplex stability temperature (T_m) − 5°C for 1 min (Wallace *et al*. 1981). Washing temperature was 43°C for $(GGAT)_4$, 37°C for $(GT)_7$ and 35°C for $(TA)_{10}$. The blocking agent was 3% BSA, for 10 min at room temperature. The digoxigenin-labelled probes were detected with anti-digoxigenin-rhodamine fab fragments (Roche Molecular Biochemicals, Penzberg, Germany). The slides were counterstained with DAPI and mounted on Vectashield (Vector Laboratories, Burlingame, CA, USA).

C-banding

Slides were washed after hybridization. Air-dried slides were aged overnight at 65°C and then submitted to C-banding following the standard barium hydroxide procedure of Sumner (1972), with some modifications. Slides were immersed in 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate) for 1 h at 60°C. Then, slides were treated in 0.1 M HCl for 30 min at room temperature. Slides were incubated in 5% barium hydroxide solution for 10 min at room temperature. Finally, slides were immersed in 2 × SSC for 1 h at 60°C. Slides were washed with distilled water between each treatment and were stained using propidium iodide.

Chromosome observation

Digital images of chromosomes were captured using a 63X objective coupled to a Zeiss Axioplan 2 Imaging microscope (Zeiss, Gottingen, Germany) equipped with the appropriate filter sets for DAPI and rhodamine. Digitized images were prepared for printing in Adobe Photoshop version 5.0; contrast, overlaying, and colour optimization were the functions used, and all of them affected the whole of the image equally.

Results

Chromosome preparations were realized from embryos, thus more number of animals were examined in this study. We analysed around 40 *in situ* hybridization experiments for each SSR probe. Moreover, we also karyotyped 20 representative metaphases for each microsatellite. In general analysis, hybridization signals of different intensities were observed with all three probes on all metaphase chromosomes (figures 1,a,c&e), but always with a reproducible pattern. All probes presented intercalary, centromeric, and/or telomeric bands along the chromosomes.

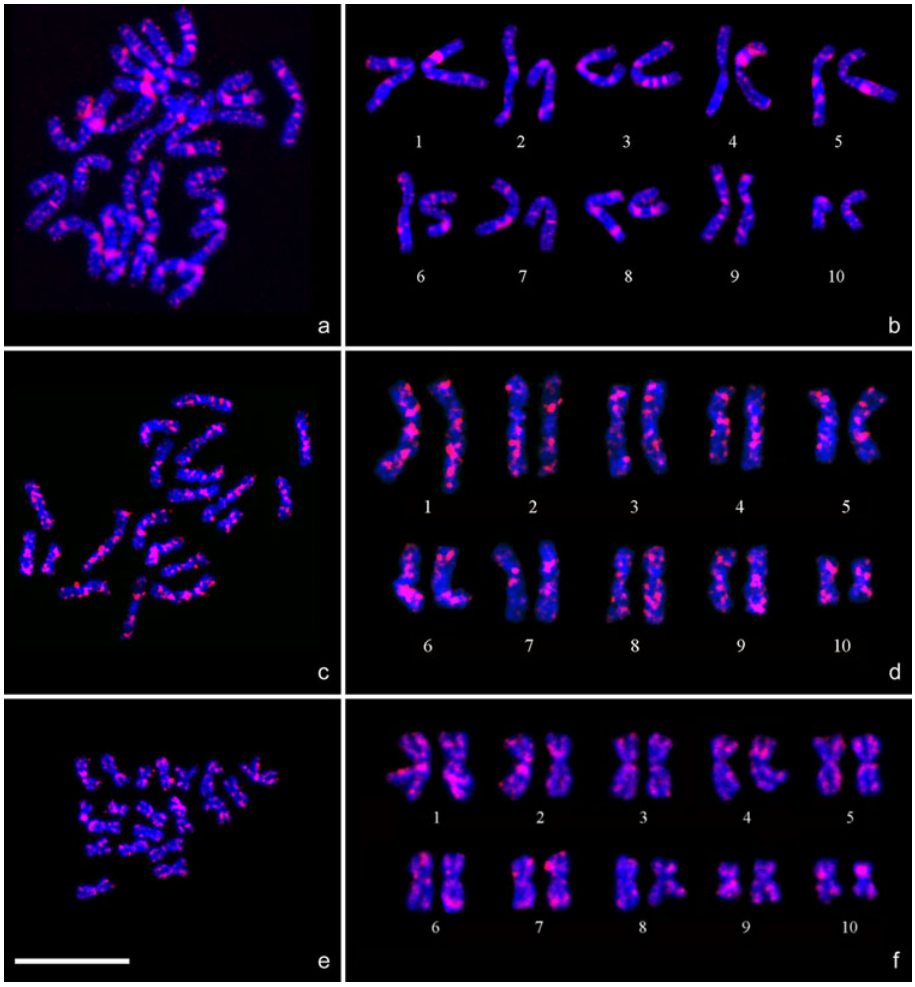

Figure 1. FISH with SSR probes applied on chromosomes of *Crassostrea gigas*. (*a*) A metaphase cell with $(GGAT)_4$ probe, and (*b*) its corresponding karyotype; (*c*) a metaphase cell with $(GT)_7$ probe, and (*d*) its corresponding karyotype; (*e*) a metaphase cell with $(TA)_{10}$ probe, and (*f*) its corresponding karyotype. Scale bar = 10 μm (only for the metaphase cell pictures).

The probe $(GGAT)_4$ provided a rich banding pattern along most chromosome arms (figure 1,a). Some signals were stronger and more extended than the others. The banding allowed the identification of most chromosomes of *C. gigas*. The organization of the karyotype was realized taking into account both the chromosome morphology and $(GGAT)_4$ hybridiztion pattern (figure 1,b). For instance, chromosomes of pair eight were easily recognized with their two large intercalary bands on the extremities of the long arms. The banding pattern on chromosomes of pair seven was characterized by the presence of only one major median band on the short arms, and also one large intercalary median band on the long arms. The banding pattern on chromosomes of pair nine was also very specific with two major bands at the extremities of the short arms.

The $(GT)_7$ probe produced the most intense hybridization signals within all SSR studied (figure 1,c). Signals were scattered on all the 20 chromosomes. Some bands were highly repetitive and characteristics of certain chromosome pairs. Homologous pairs were recognized, and thus, we used the $(GT)_7$ hybridization signals to construct a karyotype of Pacific oyster chromosomes using the chromosome arm length information and the $(GT)_7$ hybridization sites (figure 1,d). The homology between chromosomes was particularly evident in pair nine. In this pair, strong signals were observed near the telomeres of short and long arms, and pair nine was also characterized by the presence of two major bands in a centromeric position. Banding on pair seven was also very specific and characterized by the presence of two major bands near the centromere (one on the short arm, and one on the long arm). In comparison with the other chromosome pairs, pair seven exhibited the smallest number of hybridization signals suggesting that this chromosome pair could be poor in (GT) sequences. Pair eight was characterized by a rich banding pattern as pair one. Pair one presented two major bands in the short arms and three major bands in the long arms.

The $(TA)_{10}$ probe produced less hybridization signals (figure 1,e). However, some pairs showed a specific banding pattern allowing the identification of homologous pairs, and a karyotype was also produced (figure 1,f). For instance, the chromosomes of pair three were characterized by a telomeric band on the short arms, and two bands on the long arms, one near the centromere and the other near the telomeres. Pair four was characterized by an intense signal on the short arms. Signals on pair seven were specific with bands near the telomeres in both arms. Pairing in pairs five and 10 was supported by results on C-banding pattern (figures 2,a&b). A centromeric C-band was observed in chromosome pair five and strong telomeric C-bands were observed in chromosome pair 10.

Accurate karyotypes were produced and used to realize ideograms (figures 3,a–c). These figures show the haploid distribution of chromosome bands in *C. gigas* for each of the SSRs tested. For the construction of the ideograms, we described the number of bands, the intensity of the bands and each band's relative position. The ideograms summarize the results previously described.

Discussion

A few reports have described the physical organization of SSRs in plant chromosomes using *in situ* hybridization (Cuadrado and Schwarzacher 1998; Cuadrado *et al*. 2000). In mollusks, several studies were performed in gastropods with $(GATA)_n$ motif (Vitturi *et al*. 2000, 2002, 2005; Colomba *et al*. 2002; Gallardo-Escárate *et al*. 2005). They only reported the presence or absence of this repeated DNA sequence in the genome of different gastropod species. Until now, in bivalves, only Cross *et al*. (2005) investigated the hybridization of the same motif $(GATA)_n$ in the oyster *C. angulata*. They observed hybridization signals that allowed them to affirm the presence of $(GATA)_n$ motif in *C. angulata* genome. However, no specific signals were produced.

All probes used in the present study generated signals on all metaphase chromosomes. The strong hybridization signals observed at distinct locations along the chromosomes probably represent regions where SSRs are included within larger, tandemly repeat units or where SSRs are present as very large perfect or degenerate arrays. Thus, the

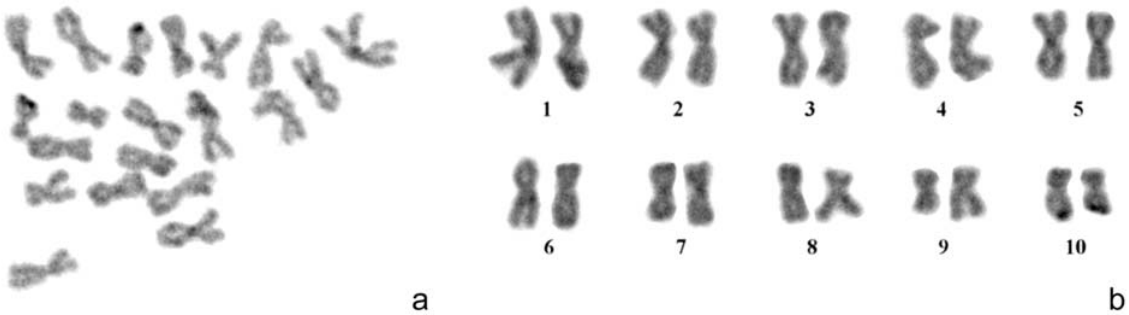

Figure 2. C-banding (CBP, C-banding with barium hydroxide and stained with propidium iodide) in chromosomes of *Crassostrea gigas*. (*a*) A metaphase cell (hybridized previously with $(TA)_{10}$ probe - figure 1,e), and (*b*) its corresponding karyotype. Scale bar = 7 μm (only for the metaphase cell picture).

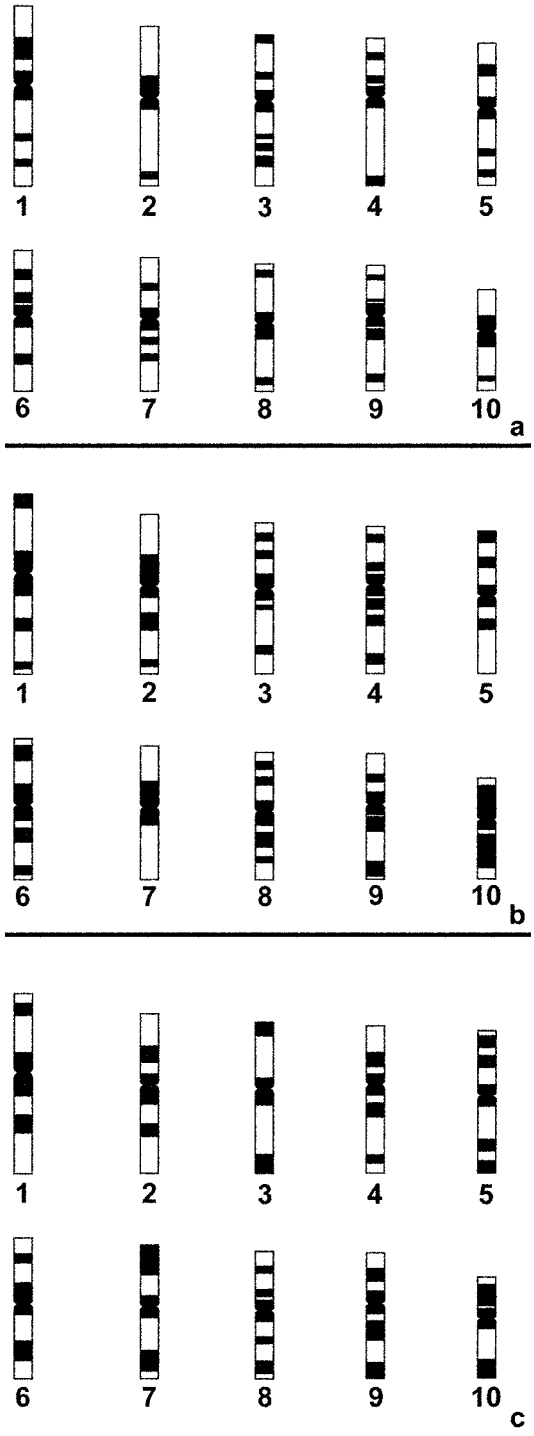

Figure 3. Schematic representation of the SSR banding patterns obtained for the three SSRs studied: (*a*) $(GGAT)_4$, (*b*) $(GT)_7$ and (*c*) $(TA)_{10}$ in *Crassostrea gigas*.

presence of large arrays of the oligonucleotides $(GGAT)_4$, $(GT)_7$ and $(TA)_{10}$ was demonstrated in *C. gigas* genome. The genomic distribution patterns of the different motifs varied considerably, thus being useful for mapping procedures. The SSRs variable distribution found in *C. gigas* genome could also have implications for amplification and dispersion mechanisms and hence be a valuable resource to study the evolution of these repeats. Why SSRs are found at particular regions is a question of importance for studies of genome organization. The oligonucleotide $(GT)_7$ seems to be the most abundant, and different SSRs showed specific and contrasting distribution patterns, suggesting that each motif is distributed independently. In *C. gigas*, a relevant DNA portion probably consists of (GT) regions.

SSRs can show a high levels of polymorphisms. In our study, the established karyotype for each SSR exhibited considerable uniformity between homologous chromosomes but some variability can be detected. Some chromosomes showed few additional minor changes probably because of intrachromosome polymorphism. For example, an additional band could appear in one of the homologues of one pair. That was the case for pair three (one chromosome showed two large bands and the other one showed three large bands on the long arms) with $(GGAT)_4$ motif.

In fish and mammals, *in situ* hybridization results showed that some simple repetitive DNA sequences are located in different chromosomal regions (e. g., heterochromatin on the sex chromosomes, NOR, and R-band sites), which are constrained considerably during evolution (Nanda *et al*. 1991). In *C. gigas*, it was shown that the major ribosomal rRNA genes, which correspond to NOR, are located in the long arms of chromosome 10 at the telomeric position (Xu *et al*. 2001; Wang *et al*. 2004). All the SSRs studied in the Pacific oyster are excluded from this localization. The SSR signals observed were also different from the major C-band positive heterochromatin. Although there are remarkable differences in the abundance of SSR motifs between different organisms (Lagercrantz *et al*. 1993), strong amplification of SSRs at specific chromosomal locations may be a general feature of eukaryotic genomes. SSRs have been considered as a cryptic source for genetic variability, hot spots for recombination or repetitive elements affecting chromatin structure (Schlötterer and Tautz 1992; Tautz *et al*. 1986; Löwenhaupt *et al*. 1989).

The chromosome localization of SSRs could have implications for genetic mapping. Hubert and Hedgecock (2004) built linkage maps of microsatellite DNA markers for *C. gigas*. By mapping microsatellite sequences that have also been used in linkage studies, links could be provided between the genetic and physical maps of the oyster genome. Microsatellites assigned to linkage groups could be assigned to individual chromosomes. Based on the data presented here, certain regions of the genome may be refractory to genetic mapping with SSRs because of their nonrandom distribution.

In conclusion, the distinctive hybridization patterns of the three SSRs presented here, showing chromosome-specific characteristics with many intercalary hybridization sites, and the simplicity of using them in multi-probe experiments will be of great importance in physical mapping projects, comparative interspecific studies and for the detection of any chromosomal rearrangement. Our study is the first one in

mollusks to open ways to such perspectives. Hybridization of the three studied SSRs has led to the identification of most of the chromosome pairs of *C. gigas*. Each SSR examined here has a specific *in situ* hybridization pattern, indicating that each SSR motif distributes independently. SSRs are major components of oyster genome and it is important to know the organization and abundance of these sequences in oyster genome. This study will form the basis for identification of not only the whole chromosomes, but also chromosome segments and be able to later describe chromosome rearrangements. The use of SSRs could also be applicable to other bivalve species with probably a small effort in finding the most informative motifs. This study also provides useful tools for oyster comparative studies, evolution and for understanding genome organization in oysters. Use of SSRs with other repetitive probes will result in a partial physical map of *C. gigas* genome and the possibility of analysing cytogenetically small sections of chromosomes. This will expand our ability to analyse and understand the genomes of oysters.

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