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Molecular and cytogenetic characterization of *Crassostrea angulata* chromosomes

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Abstract

We have conducted a molecular cytogenetic study of *Crassostrea angulata*, by treating its mitotic chromosomes with C-banding, fluorochrome and oligopeptide staining, restriction enzyme banding and fluorescent in situ hybridization, using three repetitive or multicopy DNAs (the GATA sequence, the TTAGGG telomeric repeat and the 5S rDNA). Results on C-banding indicate the presence of heterochromatin in several chromosomes occupying telomeric positions. The staining with DAPI, DA/DAPI and AMD/DAPI allow concluding that large regions rich in AT in the chromosomes of *C. angulata* do not exist. Restriction banding has shown few restriction sites for *Alu*I (AGTC site) and a relative abundance of G-C in terminal and interstitial chromosome regions, inferred by the digestion with *Hae*III (GGCC site) and *Bam*HI (GGATCC site). In situ hybridization with GATA indicated that these repeated sequences are widely dispersed in the genome of this species, whereas hybridization with the telomeric repeat revealed small bright hybridization signals, uniform in size and intensity, on each telomere of all chromosomes but not in interstitial positions. Location of 5S rDNA displayed the presence of two 5S-bearing chromosome pairs, of large size, on the subterminal position of the karyotype of *C. angulata*, this location being different from the ones encoding the major ribosomal genes in chromosome pair 10 (described in a previous paper).

Keywords: *Crassostrea angulata*; C-banding; Restriction banding; DAPI; rDNA; FISH

1. Introduction

Crassostrea angulata is an oyster species whose main natural beds are at river mouths and estuaries in the southwest of the Iberian Peninsula (Michinina and Rebordinos, 1997). Its importance in aquaculture is due both to the commercial value and to the use as an environmental marker for the analysis of estuarine contamination (Rebordinos-González et al., 1999). Moreover, after decades of controversy, the taxonomic relationships among *C. angulata* and the Pacific oyster *C. gigas* have not yet been clarified. On one hand, some authors maintain that both of them are the same species and that the differences they show are

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only due to differences in their distribution (Menzel, 1974; Mathers et al., 1974; Buroker et al., 1979; Mattiucci and Villani, 1983; Huvet et al., 2002). On the other hand, analysis of the COI mitochondrial gene by sequencing and PCR-RFLP (O'Foighil et al., 1998; Boudry et al., 1998) enabled distinguishing populations of both species.

For this reason, additional data will be required in order to gain insight into the taxonomy of all genera. Since speciation is frequently promoted by chromosomal rearrangement, the analysis of chromatin organization in the oyster chromosomes could give valuable data in this context.

C. angulata possesses a common diploid number of $2n=20$ metacentric chromosomes of progressively decreasing length, which in most cases are difficult to identify individually. Banding techniques are very advantageous for the identification of individual chromosomes and also for particular regions of chromosomes. During the 1990s, conventional techniques were applied to several oyster species, such as *C. gigas*, *Ostrea edulis*, *O. denselamellosa*, *O. puelchana* and *Tiostrea chilensis* (Insua and Thiriot-Quiévreux, 1991, 1993; Thiriot-Quiévreux and Insua, 1992; ; Ladrón de Guevara et al., 1994) and the classical techniques were improved, by using others such as restriction banding and staining with fluorochromes. In addition to the use of C-banding, these allowed to analyze, among others, presence of heterochromatic sequences in the chromosomes of mussels (Martínez-Lage et al., 1994, 1995) and the oysters *O. denselamellosa* (Insua and Thiriot-Quiévreux, 1993) and *O. angasi* (Li and Havenhand, 1997).

The Ag-NOR has been a useful chromosome marker, the polymorphisms (including number, location and size) of which are often species specific. With regard to oysters, Ag-NORs have been described in *O. angasi* (Li and Havenhand, 1997), *O. edulis*, *O. denselamellosa* (Insua and Thiriot-Quiévreux, 1991; Thiriot-Quiévreux and Insua, 1992), *O. conchaphila* (Leitao et al., 2002), *T. chilensis* (Ladrón de Guevara et al., 1994), *C. sikamea*, *C. virginica*, *C. ariakensis*, *C. angulata*, *C. gigas*, *C. gasar* and *Saccostrea commercialis* (Thiriot-Quiévreux and Insua, 1992; Leitao et al., 1999; Cross et al., 2003).

In the last decades, the cytogenetic studies made in marine molluscs are enjoying a very fast development due to the introduction on new molecular techniques, mainly the fluorescence in situ hybridization (FISH). In this sense, several sequences have displayed their utility to be used as probes for chromatin characterization. One of them is the highly repetitive GATA sequence which allow us to detect the presence of this minisatellite and hence the presence of A-T in the genome. It is phylogenetically conservative and described in a broad spectrum of eukaryotes, being sex-specifically arranged in some cases (Jones and Singh, 1985). Until now, this has not been investigated in any bivalve mollusc.

Another one is the sequence $(TTAGGG)n$ displayed in the telomeres of all vertebrates studied so far, but in invertebrates it is present in some species (Colomba et al., 2002) but lacking in others (Vitturi et al., 2000b). In bivalve molluscs, this vertebrate telomeric sequence has been mapped using FISH in oysters (Guo and Allen, 1997; Wang and Guo, 2001), clams (Wang and Guo, 2001) and mussels (Plohl et al., 2002).

The telomeric regions have also been described as the most gene-rich regions in the chromosomes due to its high content in G-C. The structural organization and function of telomeres are conserved among widely divergent taxonomic groups. However, telomeric DNA sequences are considered group-specific and appear variable between species (Blackburn, 2001).

Thus, the conventional techniques of chromosome banding have now been complemented with others of more precise characterization and chromosome location of sequences or regions. Nevertheless, in spite of this technical revolution, the study of species for which cytogenetic data are scarce, requires the essential use of all types of tools for a better approach to the chromosomal knowledge of these species.

The silver nitrate staining (Ag-NOR staining) only stains those NORs which have expressed themselves during the last interphase, because the silver binds to a complex of acidic protein associated with the nucleolus and nascent pre-RNA. The rDNA, corresponding to NORs, can be located on chromosomes by using in situ hybridization (ISH) with an rDNA probe, in particular FISH. A fluorescence staining with chromomycin A_3 (CMA_3) dyes detects chromosomal DNA sequences rich in GC. CMA_3-staining shows all the structural NORs on chromosomes in cold-blooded vertebrates and invertebrates. FISH in com-

bination with CMA_3-staining can allow us to precisely identify the NOR-bearing chromosomes. In a recent study, the use of silver staining, CMA_3-staining and FISH, allowed us to locate the gene encoding for the 18S-5.8S-28S rDNA in the telomeric region of the chromosome pair 10 of *C. angulata* (Cross et al., 2003). In oysters, NORs have been located using the FISH method in *C. virginica*, *C. gigas*, *C. rizhophorae*, *C. plicatula* and *C. ariakensis* (Zhang et al., 1999; Xu et al., 2001; Wang et al., 2004). On the other hand, there is presently a growing interest in the study of the arrangement and location of the other multigene family of ribosomal genes, the 5S rDNA. The 5S rRNA is codified by a gene grouped in tandem repetitions. Each repetition consists of a well conserved codifying region of 120 bp, and a nontranscribed spacer (NTS) in which the size and sequence are variable. In most organisms, the major genes which encode the 18S-5.8S-28S rRNA and the minor genes encoding the 5S rRNA are in different chromosomes (Drouin and Moniz de Sá, 1995). Nevertheless, RNA minor genes have also been described as related to the major genes (Drouin and Moniz de Sá, 1995; Mandrioli et al., 2000; Martins and Galetti, 2001). As a result of this characteristic, it will be interesting to make use of this gene as a cytogenetic marker in evolutionary studies of species. In marine organisms, most of the research has been carried out in fish (see review by Martins and Galetti, 2001), less in bivalve molluscs (Insua et al., 1998, 1999, 2001) and none at all in oysters.

As part of an effort to characterize the chromosomes of *C. angulata* and in order to identify markers which allow us to distinguish each chromosome pair, we have applied several techniques of chromosome banding with specific fluorochromes and restriction enzymes, and carried out FISH by using probes for the GATA and TTAGGG sequences, and for the 5S rDNA in order to determine its location and distribution in the genome of *C. angulata*.

2. Material and methods

2.1. Oyster samples

Samples of *C. angulata* of 2–3 cm in length were obtained from natural populations located in the mouth of the River Guadalquivir, in Sanlúcar de Barrameda (Cádiz). After feeding the oysters with phytoplankton for several days, they were placed for 8 h in colchicine. The gills were extracted and put under a hypotonic shock with KCl 0.4% for at least 1 h, and then transferred to fresh Carnoy for 1 h (Thiriot-Quiévreux, 1984).

2.2. Chromosome banding

The protocol of Sumner (1972) was used for the technique of C-banding. Restriction banding was carried out according to Martínez-Lage et al. (1995). The enzymes were loaded in a suitable buffer, and digestion was carried out by adding a drop of the enzymatic solution to the samples, which were later covered. The concentration of each used enzyme (*Alu*I, *Bam*HI and *Hae*III) varied between 0.3–1.0 U/μl. Slides were incubated at 37 °C for 6 h.

Techniques of staining by means of combined fluorochrome diamidino-2-phenylindole (DAPI) and base-specific peptide antibiotics like actinomycin D (AMD) and distamycin A (DA) were used because their different affinity by AT- and GC-rich regions can evidence different types of heterocromatin. These techniques were applied according to the protocol described by Schweizer et al. (1978).

2.3. PCR amplification

Telomeric (TTAGGG)*n* and (GATA)*n* probes were generated by PCR in the absence of a template (Ijdo et al., 1991), using $(TTAGGG)_5$ and $(CCCTAA)_5$, and $(GATA)_7$ and $(TATC)_7$, as primers, respectively. Nick translation labelling with digoxigenin was performed according to the manufacturer's instructions (Roche Molecular Biochemicals). A specific probe for oyster 5S rDNA was produced by PCR using contiguous primers A (5′-CGTCCGATCACCGAAGTTAA) and B (5′-ACCGGTGTTTTCAACGTGAT) with opposite orientation (Martínez-Lage, personal communication). Genomic DNA was extracted from 50–100 mg of mantle tissue using the NucleoSpin© Tissue kit (Macherey–Nagel). Thirty-five PCR amplification cycles were performed at an annealing temperature of 59 °C. Labelling was obtained using a PCR procedure with the following dNTP concentrations: 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 150 μM

dTTP and 50 μM dig-11-dUTP (Roche Molecular Biochemicals).

2.4. FISH techniques

Fluorescent in situ hybridization (FISH) of the three probes was made according to Cross et al. (2003), except for the post-hybridization washes of the GATA and telomeric sequences, in which the slides were washed two times (7.5 min each) with 2×SSC/50% formamide pH 7 at 37–38 °C $(GATA)_n$ and 42 °C $(TTAGGG)_n$; 2×5 min in 2×SSC at 38 °C; 1×5 min in 4×SSC/0.1% Tween 20 at room temperature (RT); 1×5min in 1×PBS/0.1% Tween 20/1% blocking reagent at RT and finally 30 min in 1×PBS/1% blocking reagent at RT. After immunocytochemical incubations, the chromosomes were counterstained with 20 μl of 500 ng/ml propidium iodide in Vectashield antifade (Vector). Photomicrographs were obtained using a fluorescence microscope (Zeiss Axioskop 2 plus) equipped with a digital camera.

3. Results

3.1. Chromosome banding

The constitutive heterochromatin was analyzed in 15 metaphases in 5 animals. C-banding showed an abundant presence of heterochromatin in most of the ten chromosome pairs, occupying on the majority of the studied metaphases mainly telomeric but also some pericentric positions (Fig. 1A). Also observed, to a lesser extent, were secondary bands of minor size in some pairs.

In order to continue with the study of the chromosome regions and specifically to locate extensive regions rich in AT, three techniques were applied. These combined the use of fluorochromes and antibiotics: a) by simple staining with DAPI, that it has more affinity for AT regions and therefore these regions will fluoresce with greater intensity; b) by staining with DA/DAPI, which reveals the differences that could exist between different regions according to their content in AT; and c) by staining with AMD/DAPI, to increase contrast of the banding with DAPI. In all three cases, the result was a completely homogenous staining, showing the absence of these regions in *C. angulata*. That is to say, it cannot be demonstrated with the data collected from the applied technique that regions exist which are rich in AT, or if they do exist, they are not large enough to be detected at chromosomal level.

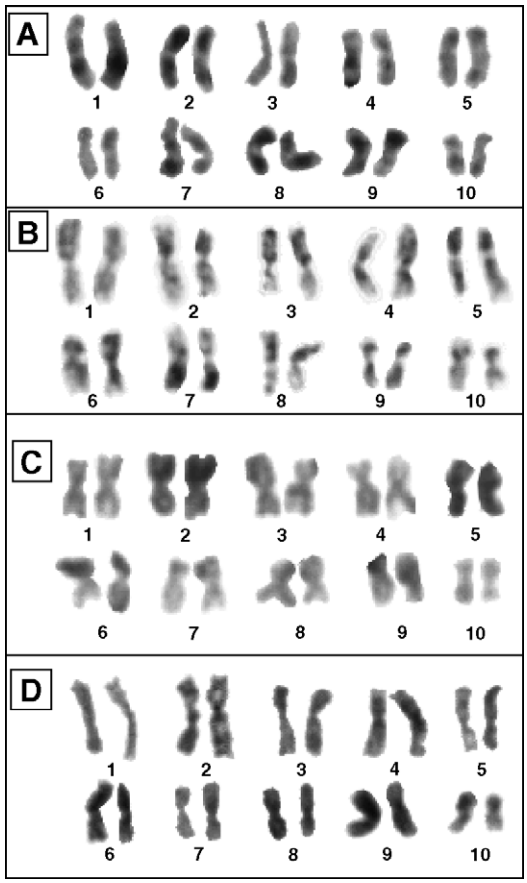

Fig. 1. Chromosome banding of *C. angulata*. (A) C-banding appeared mainly on the telomeric position on the chromosomes. (B) Restriction banding by using *Alu*I (AGCT) showed intercalary and terminal bands. (C) Restriction banding by using *Bam*HI (GGATCC) produced a homogenous digestion in all chromosomes with the exception of a metacentric chromosome of medium size and one arm of several chromosome pairs. (D) Restriction banding with *Hae*III (GGCC) digested the C-Bands of some chromosomes.

The digestion with *Alu*I (AGCT) enabled us to detect homogenous regions in composition of nucleotides. The results obtained showed that the digestion produced intercalary and terminal bands.

Some centromeres appeared digested and, in general, the telomeres were well defined. In addition, significant changes in the digestion of complete chromosomes were appraised (Fig. 1B). The heterochromatic regions of some chromosome pairs remained and the NOR regions in the chromosome pair of minor size (pair 10), were well defined (Leitao et al., 1999; Cross et al., 2003) and therefore these chromosome regions contain few or no restriction sites for *Alu*I.

Restriction banding with *Bam*HI (GGATCC) did not show a clear pattern of bands, such as in the previous cases, and it produced a homogenous digestion in all the chromosomes, with the exception of a metacentric chromosome of medium size (pair 5), and one arm of chromosome pair 2 (Fig. 1C). Finally, banding with *Hae*III (GGCC) showed different digestion patterns between chromosomes and presence of intercalary and telomeric bands (Fig. 1D). The centromeres appeared in general well defined, and the C-bands of some chromosomes disappeared, pointing to these regions being rich in GC. However these regions do not give the appearance of DAPI-negative staining. Perhaps this it is due to the lower sensitivity of this technique for detecting variations in small chromosomal regions.

3.2. FISH with GATA and TTAGGG

The hybridization of repeated GATA sequences in *C. angulata*, by means of FISH, indicated a strong signal, and presented a dispersed distribution by all the genome. This was observed to be in form of small and numerous signals of hybridization throughout the chromosomes (Fig. 2A).

The hybridization with the telomeric probe (TTAGGG)*n* revealed bright hybridization signals, uniform in size and intensity, on each telomere of all chromosomes. These signals were located exclusively on terminal zones, and interstitial positions were not detected (Fig. 2B).

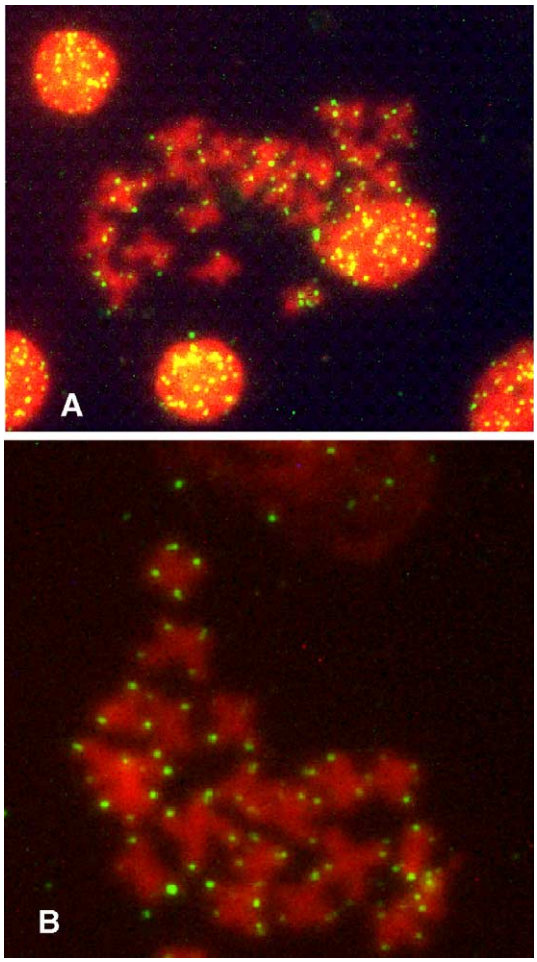

Fig. 2. FISH in chromosomes of *C. angulata* by using GATA and TTAGGG probes. (A) Hybridization with GATA probe produced a dispersed distribution by all the genome, in form of numerous signals of hybridization throughout the chromosomes. (B) The hybridization with the telomeric probe (TTAGGG) revealed bright hybridization signals, uniform in size and intensity, on each telomere of all chromosomes. These signals were only located on terminal zones.

3.3. 5S rDNA location

FISH experiments were carried out in order to identify the chromosomal location of the 5S rDNA genes in the *C. angulata* complement, in a total of 100 metaphase plates of four individuals. All metaphases showed signals of subtelomeric hybridization in two metacentric chromosome pairs of large size. (Fig. 3A–B). A FISH analysis showed that the metacentric chromosomes were different from those bearing the major rDNA genes described by Cross et al. (2003), because they displayed a telomeric unique position in the smaller chromosome pair of karyotypes of *C. angulata* (pair 10) (Fig. 3C). Hence both families of genes are in

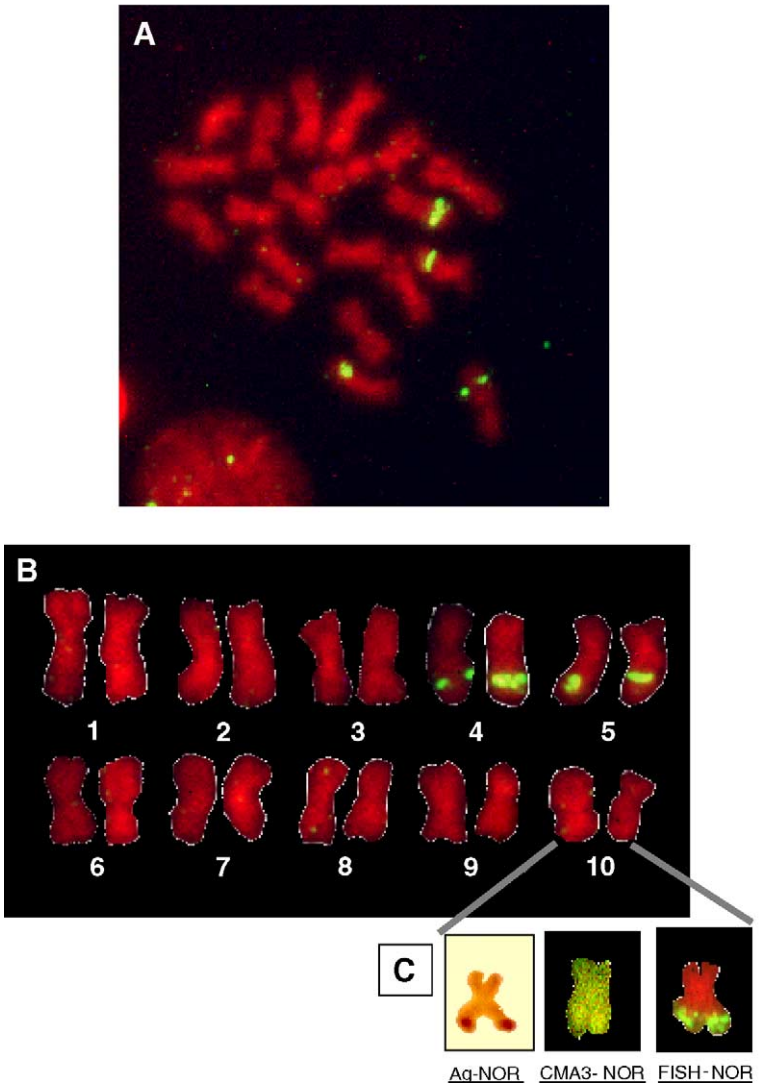

Fig. 3. Location of 5S rDNA by FISH. (A) A metaphase plate showing signals of subtelomeric hybridization in two metacentric chromosome pairs of large size. (B) Karyotype of *C. angulata* showing the location in two chromosome pairs of the 5S rDNA. (C) 18S-5.8S-28S rDNAs were located on chromosome pair 10.

different chromosomes and occupying different positions.

4. Discussion

The C-banding technique selectively stains the constitutive heterochromatin, and although the reason is not known exactly, it seems that the DNA contained in C-bands is very resistant to the extraction caused during the consecutive treatment with acids, bases and salts at the elevated temperatures of this technique (Sumner, 1972). It has been used less in molluscs than in fish. Thus in oysters, the chromosomes of *O. denselamellosa* (Insua and Thiriot-Quiévreux, 1991), *O. angasi* (Li and Havenhand, 1997) and *O. conchaphila* (Leitao et al., 2002) have been analyzed using C-banding. In *O. densela-*

mellosa and *O. angasi,* the C-bands are mainly located in centromeric position and the terminal heterochromatin shows variation in their response to the C-banding treatment. In *O. conchaphila* the C-bands are located in both the centromeric and telomeric positions. Instead, the C-bands appeared in some intermediate positions and in the telomeres, in the arms of certain chromosomes in the mussels *Mytilus edulis*, *Mytilus galloprovincialis* and *M. trossulus* (Martínez-Lage et al., 1994, 1995).

The results obtained in *C. angulata* showed that the distribution of the heterochromatin was not centromeric, and as in the case of *Mytilus*, it is located mainly in the telomeric regions of some chromosomes, although sometimes it appears in other inner positions within the chromosome arms. In order to explain the distribution of the heterochromatin, it has been proposed that the centromeres are the starting points for the transfer of heterochromatin through the telomeres, and hence the karyotypes with higher telomeric heterochromatin, must have an older phylogenetic status (Macgregor and Sessions, 1986; Martínez-Lage et al., 1995).

In relation to the study of chromosomes by means of the combined use of fluorochromes and oligopeptides, results showing a homogenous staining in all chromosomes indicated that sequences rich in AT are lacking in *C. angulata* chromosomes. *M. galloprovincialis* did not show significant differences between chromosome regions (Martínez-Lage et al., 1995) and the razor clam *Solen marginatus* showed a uniform fluorescence in all the chromosomes (Fernández-Tajes et al., 2003). Instead, in *Brachidontes rodriguezi*, a DAPI-negative staining has been observed in the positive regions of CMA_3, indicating that the regions are poor in AT. However, homogenous DAPI staining of the rest of the chromosomes was observed (Torreiro et al., 1999).

From the data collected from restriction banding it is possible to conclude that most of the regions of the repeated DNA of *C. angulata*, represented by C-bands, are poor in sites for *Alu*I, which digest regions with a homogenous distribution of bases (AGTC). Therefore, they must contain repeated regions rich in some specific bases. Thus, some regions that contain heterochromatin are abundant in GC, since they appear digested when treated with *Hae*III (GGCC). The analysis with restriction enzymes in *M. galloprovincialis* allowed to detect heterogeneity in the heterochromatin associated with C-bands and to divide the heterochromatin into several types, according to its behaviour after the treatment with restriction enzymes and C-banding (Martínez-Lage et al., 1994). In the Chilean Peruvian scallop, the *Hae*III treatment produced interstitial bands in two chromosomal pairs. With *Alu*I, telomeric and pericentromeric bands were viewed in a chromosomal pair (Gajardo et al., 2002).

It is known that the telomeric location of the repeated DNA is an important factor of evolution and that the heterochromatic areas which correspond to large amounts of C-bands are subject to greater reorganization and quantitative variation. This means that the species with large amount of C-bands could display greater plasticity, that is to say, could undergo mutations that favour the formation of new karyotypes and chromosomal rearrangements.

Our results with GATA showed the existence of signals with a wide and dispersed distribution by all chromosomes which allow us to conclude that a significant amount of DNA is formed by repeated DNA (minisatellites). In addition, the hybridization with (GATA)*n* confirms that this technique is more sensitive for the detection of these kinds of sequences of moderately repetitive and dispersed DNA than other techniques of chromosome banding (Vitturi et al., 2000a). The GATA repetitions displayed a dispersed distribution in the genome of a wide class of eukaryotes. Nevertheless, the repetitions have not been observed in ovine and bovine genome (Miklos et al., 1989) or yeasts (Singh et al., 1984). In relation to molluscs, in the gastropod *Melarhaphe neritoides* the study of the location and distribution of this sequence has revealed an absence of evident hybridization in its genome, suggesting the absence or shortage of GATA repetitions in this species (Colomba et al., 2002). Nevertheless, in the prosobranch *Fasciolaria lignaria* a strong signal of hybridization in all the chromosomes occurs, and like the results obtained in *C. angulata*, it has shown a disperse and abundant distribution within the genome (Vitturi et al., 2000a). These data reveal significant differences between the karyotypes of fairly closely related taxons, and point to the importance of such analysis in taxonomy.

In vertebrates, the sequence (TTAGGG)*n* has been isolated from telomeres of chromosomes. The sequence has also been shown in the chromosomes of a wide variety of taxa, crustaceans (Pellicia et al., 1994) and annelids (Vitturi et al., 2000c), among others. In oysters, this sequence has been used as a probe in the study of chromosomes of the species *C. virginica* and others (Wang and Guo, 2001; Guo and Allen, 1997). In all cases it has been located in the telomeres of all the chromosomes, without any internal signals being produced. Moreover this sequence has also been located in the telomeres of the chromosomes of other bivalves such as clams (Wang and Guo, 2001; Plohl et al., 2002). In the mussel *M. galloprovincialis*, in addition to a telomeric position, the chromosomes have also shown some interstitial positions (Plohl et al., 2002).

The hybridization in the molluscs *M. neritoides* (Colomba et al., 2002) and *F. lignaria* (Vitturi et al., 2000a) also produced positive signals at the ends of the chromosomes. Nevertheless, it should be emphasized that the hybridization in *Oxynoe olivacea* (Opisthobranchia), did not produce any signal in its chromosomes, pointing to the phylogenetic distance of this group with respect to the rest of the molluscs (Vitturi et al., 2000b).

In relation to the location of 5S rDNA, results indicate that a) two genes are encoding the minor rDNA 5S, instead of one, as in the case of the major genes (Cross et al., 2003) and b) 5S rDNA genes are located in two pairs of chromosomes, but not in the pair 10, where the major genes are located (Cross et al., 2003). That means that we have found chromosome markers for another two pairs of chromosomes. The genetic control and location of major and minor genes encoding rDNAs have evidenced different evolutionary strategies. Indeed, several distributions have been described in molluscs, so that in *M. edulis* and *M. galloprovincialis* two signals of hybridization have been detected for 5S rDNA (a pericentric and another subtelomeric in a pair of metacentric chromosomes). Sometimes another signal of hybridization appears in an interstitial position in at least one member of another two metacentric pairs (Insua et al., 2001). In the European scallops, *Aequipecten opercularis* (Insua et al., 1998), a single chromosome hybridized with the 5S rDNA probe in two different subterminal positions within the chromosome. In the cockle *Cerastoderma edule*, five chromosomal pairs were identified which contained a 5S rDNA cluster in the telomere of the long arm whose location does not correspond to the major ribosomal gene (Insua et al., 1999). Again, this position is different from the one occupied by the 18S-5.8S-28S genes. Nevertheless in the caenogasteropod *M. neritoides* both families of genes are located together in the same chromosome (Colomba et al., 2002). In fish, the most commonly observed situation is that of the divergent location of both families of genes, that is to say, similar to what happens in *C. angulata*, both families of ribosomal genes do not share the position within the genome (Martins and Galetti, 2001).

It seems that in most taxa both kinds of genes are situated on different chromosomes as happens also in mammals, in which genes 5S rDNA are located generally in a single chromosomal pair, and the major genes are located in several chromosomes (Suzuki et al., 1996). Although in earthworm *Octodrilus complanatus*, the 18S-5.8S-28S genes and 5S also are located in the same chromosome pair (Vitturi et al., 2002).

In order to explain the independent location of both rDNAs, it has been suggested that the transcription of 18S-5.8S-28S genes with the RNA polymerase I and the transcription of 5S gene by the RNA polymerase III, could be favoured by a physical location different for both families of genes (Amarasinghe and Carlson, 1998). In addition, some evolutionary processes that act upon sequences repeated in tandem, such as unequal overcrossings and gene conversion, could produce non-desirable translocations of 5S in the interior of cluster 18S-5.8S-28S, providing an evolutionary advantage to those species that contain these genes in different chromosomes (Martins and Galetti, 2001).

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